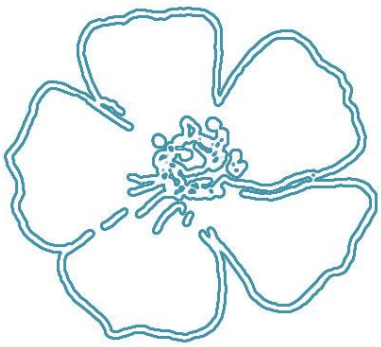
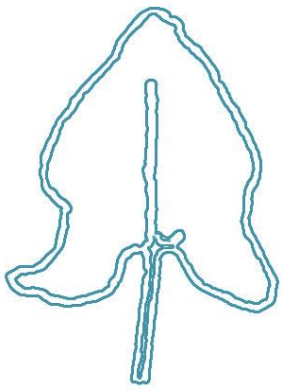
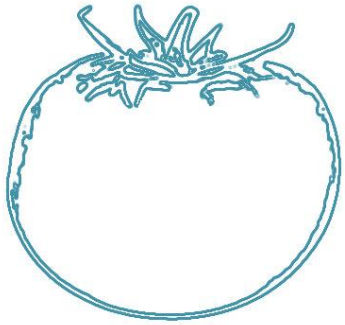

Retrieval of the sustainable use of traditional foods through innovative preservation and extraction methods

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Programa Doutoral em Química Sustentável
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“Saber más es ser más libre'”

César Vallejo

Aos meus Pais e Irmãos

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Abstract

Wild edible plants and local farmer' varieties are valuable sources of nourishment and bioactive compounds. After harvest, these traditional foods begin to lose quality due to its perishable nature through changes resulting from physical, chemical, enzymatic, or microbiological reactions. The commonly used chemical methods of sanitization and fumigation do not provide an efficient microbial reduction, besides being perceived negatively by the consumers, dangerous for human health, and harmful to the environment; and the conventional thermal treatments may negatively affect physical, nutritional and bioactive quality parameters of these foods. Today, alternative non-thermal technologies such as ionizing radiation, high hydrostatic pressure and innovative packaging systems, as well as combinations between them or with other preservation factors (hurdles) are being investigated. Some of these non-conventional technologies are also used to recover high added-value compounds from plant materials, since the conventionally used extraction methods are time-consuming and can lead to low extraction rates, large solvent consumption, and degradation of the target compounds. The use of sustainable preservation and extraction technologies can overcome these problems, meet the dominant trends of the "green chemistry" movement, and help to tackle societal challenges of the 21st century. At the same time, the use of traditional foods as matrices to be preserved and as sources of bioactive compounds may promote their retrieval and valorisation, since the nutritional value of these foods remains unknown, or they lack recognition as significant contributors to the human diet in developed countries.

This study aimed to promote the sustainable use of traditional foods through the application of non-conventional preservation (γ-ray irradiation and modified atmosphere packaging (MAP)) and extraction (high hydrostatic pressure (HHP) and microwave-assisted extraction (MAE)) technologies, and their nutritional, chemical and antioxidant characterization. Two medicinal plants (*Malva neglecta* Wallr. and *Tuberaria lignosa* (Sweet) Samp.) and two leafy vegetables (*Rumex induratus* Boiss. & Reut. and *Nasturtium officinale* R. Br.) were wild harvested in the North-eastern region of Portugal. Four tomato (*Lycopersicon esculentum* Mill.) farmers' varieties known as yellow (tomate amarelo), round (tomate redondo or batateiro), long (tomate comprido) and heart (tomate coração) tomatoes were gathered in local homegardens where they have been grown using extensive farming techniques.

In a first instance, the dried medicinal plants were subjected to a γ-ray irradiation treatment (at doses up to 10 kGy) to evaluate the suitability of the treatment in maintaining chemical and antioxidant parameters unchanged. The effects of the drying and oral preparation methods were investigated for *T. lignosa*, as well as the impact of irradiation on

the extraction kinetics of phenolic compounds. The CIE*L*a*b** colour values were measured using a colorimeter, phenolic compounds were analysed by high-performance liquid chromatography coupled to mass spectrometry (HPLC-DAD-ESI/MS) and organic acids by HPLC coupled to a photodiode array detector, and the *in vitro* antioxidant activity was evaluated via DPPH free-radical scavenging activity, reducing power, β-carotene bleaching inhibition and thiobarbituric acid reactive substances (TBARS) formation inhibition. For *T. lignosa*, the preparation method had higher influence on the phenolic profile and antioxidant activity than the irradiation treatment (being decoctions preferred over infusions); and colour was more sensitive to the drying method than to irradiation. In fact, these parameters were not significantly affected by the highest dose, while the 5 kGy dose promoted the extraction of ellagitannin derivatives. The study performed with *M. neglecta* allowed to deduce that the irradiation-induced modifications on colour depend not only on the applied dose but also on the plant material under investigation.

Then, irradiation (up to 6 kGy), vacuum-packaging and inert-gas (Ar and N_2) enriched atmospheres were tested for shelf-life extension of the leafy vegetables during storage at 4 ºC. Different quality parameters were evaluated, such as colour, proximate composition (moisture, proteins, fat, ash and carbohydrates), energy, free sugars (analysed by HPLC coupled to a refraction index detector), organic acids, tocopherols (analysed be HPLC coupled to a fluorescence detector), fatty acids (analysed by gas chromatography with flame ionization detection), and the *in vitro* antioxidant activity. In general, Ar was a suitable choice for preserving the overall postharvest quality of the selected vegetables during refrigerated storage. The adequacy of post-packaging irradiation treatments for shelf-life extension was also demonstrated. Nevertheless, MAP proved to be a more appropriate postharvest treatment than irradiation to preserve quality parameters. After characterization of the phenolic profile of *N. officinale*, the cold extraction of phenolic acids and flavonoids by HHP was optimized using the response surface methodology (RSM), combining the independent variables of processing time, pressure and ethanol concentration in a five-level central composite design. The developed theoretical models were successfully fitted to the experimental data and the process was characterized by requiring reduced extraction times, a green solvent, and being selective.

Regarding the nutritional composition and antioxidant properties of the four tomato farmers' varieties, the so-called round tomato proved to be the most powerful in antioxidant activity, total phenolics and carotenoids, while the variety known as yellow tomato revealed an interesting nutritional composition, characterized by higher levels of fructose, glucose, α-linolenic acid and total tocopherols. Quercetin pentosylrutinoside was the most abundant flavonoid in the four varieties. Then, the microwave-assisted extraction of hydrophilic and lipophilic antioxidants and phenolic compounds from tomato was optimized be RSM. A

central composite design was implemented considering the variables processing time, temperature, ethanol concentration and solid/liquid radio. The concentration-time response methods of crocin and β-carotene bleaching inhibition were used to measured hydrophilic and lipophilic antioxidant responses, respectively. The two major phenolic acids and flavonoids were set as dependent variables. The MAE process was found as a powerful and efficient extraction methodology that can be used in the valorisation and recycling of tomato wastes.

In conclusion, this study highlighted the potential of the tested preservation and extraction methods and contributes to the valorisation of the studied traditional plant foods, which may gain prominence in the minimally processed food sector and in contemporary diets due to its nutritional value, antioxidant properties and differentiated organoleptic properties. In further studies it will be interesting to investigate the effects of MAP and irradiation on other quality attributes and other food products, as well as the combined effects of these technologies. The recovery of heat sensitive compounds from tomato wastes using HHP should also be considered.

Keywords: traditional foods; valorisation; irradiation; modified atmosphere packaging; high hydrostatic pressure; microwave-assisted extraction; food analysis; postharvest quality; process optimization; antioxidants.

Resumo

As plantas silvestres comestíveis e as variedades locais são fontes valiosas de nutrientes e de compostos bioativos. Após colheita, esses alimentos tradicionais começam a perder qualidade devido à sua natureza perecível através de alterações resultantes de reações físicas, químicas, enzimáticas ou microbiológicas. Os métodos químicos de desinfeção e fumigação comummente usados não garantem uma redução microbiana eficiente, além de serem vistos negativamente pelo consumidor, perigosos para a saúde humana e prejudiciais ao meio ambiente; e os tratamentos térmicos convencionais podem afetar negativamente os parâmetros físicos, nutricionais e bioativos de qualidade destes alimentos. Atualmente estão a ser investigadas tecnologias alternativas não térmicas, tais como a radiação ionizante, a alta pressão hidrostática e sistemas de embalamento inovadores, bem como combinações entre elas ou com outros fatores de conservação (*hurdles*). Algumas dessas tecnologias não convencionais também são usadas para recuperar compostos de elevado valor acrescentado a partir de matrizes vegetais, uma vez que os métodos de extração convencionais são demorados e podem levar a baixos rendimentos de extração, a um grande consumo de solvente e à degradação dos compostos de interesse. O uso de tecnologias de conservação e extração sustentáveis pode ultrapassar estes problemas, atender às tendências do movimento da "química verde" e ajudar a superar os desafios societários do século XXI. Simultaneamente, a seleção de alimentos tradicionais como matrizes a serem conservadas e usadas como fonte de compostos bioativos promove a sua reutilização e valorização, uma vez que o valor nutricional destes alimentos permanece desconhecido ou não são reconhecidos como contribuintes significativos para a dieta humana em países desenvolvidos.

Este estudo teve como objetivo promover o uso sustentável de alimentos tradicionais através da aplicação de tecnologias não convencional de conservação (irradiação gama e embalamento em atmosfera modificada (MAP)) e extração (alta pressão hidrostática (HHP) e extração assistida por micro-ondas (MAE) e da sua caracterização nutricional, química e antioxidante. Duas plantas medicinais (*Malva neglecta* Wallr. e *Tuberaria lignosa* (Sweet) Samp.) e dois vegetais folhosos (*Rumex induratus* Boiss. & Reut. e *Nasturtium officinale* R. Br.) foram recolhidos na região Nordeste de Portugal. Quatro variedades tradicionais de tomate (*Lycopersicon esculentum* Mill.), conhecidas por tomate amarelo, tomate redondo, tomate comprido e tomate coração, foram colhidos em quintais da região onde são cultivados utilizando técnicas de agricultura extensiva.

Numa primeira fase, as plantas medicinais secas foram submetidas a um tratamento de irradiação (doses até 10 kGy) para avaliar a adequação do tratamento em manter parâmetros químicos e antioxidantes inalterados. Os efeitos dos métodos de secagem e de

preparação oral foram investigados para *T. lignosa*, bem como o impacto da irradiação na cinética de extração de compostos fenólicos. Os valores CIE*L*a*b** de cor foram medidos usando um colorímetro, os compostos fenólicos foram analisados por cromatografia líquida de alta eficiência acoplada a espectrometria de massa (HPLC-DAD-ESI/MS) e os ácidos orgânicos por HPLC acoplada a um detetor de fotodíodos, e a atividade antioxidante *in vitro* foi avaliada através da atividade sequestradora de radicais livres DPPH, do poder redutor, da inibição da descoloração do β-caroteno e da inibição da formação de substâncias reativas ao ácido tiobarbitúrico (TBARS). Para *T. lignosa*, o método de preparação teve uma maior influência no perfil fenólico e na atividade antioxidante do que o tratamento de irradiação (sendo as decocções preferíveis relativamente às infusões); e a cor foi mais sensível ao método de secagem do que à irradiação. De facto, estes parâmetros não foram significativamente afetados pela dose mais elevada, enquanto a dose de 5 kGy promoveu a extração dos derivados de elagitaninos. O estudo feito com *M. neglecta* permitiu deduzir que as alterações de cor provocadas pela irradiação dependem não só da dose aplicada, mas também do material vegetal sob investigação.

Seguidamente, a irradiação (doses até 6 kGy) e o embalamento a vácuo e sob atmosfera modificada enriquecida em gás inerte (Ar e N2) foram testados para alargar o tempo de vida útil dos vegetais folhosos durante o armazenamento a 4 °C. Neste ensaio avaliaram-se diferentes parâmetros de qualidade, tais como a cor, a composição centesimal (humidade, proteínas, gorduras, cinzas e hidratos de carbono), a energia, açúcares livres (analisados por HPLC com detetor de índice de refração), ácidos orgânicos, tocoferóis (analisados por HPLC com detetor de fluorescência), ácidos gordos (analisados por cromatografia gasosa com detetor de ionização por chama) e a atividade antioxidante *in vitro*. De um modo geral, Ar foi uma escolha adequada para conservar a qualidade global dos vegetais frescos durante o armazenamento refrigerado. Também foi demonstrada a adequação do tratamento de irradiação para aumentar o tempo de vida útil. Contudo, MAP foi um tratamento pós-colheita mais adequado do que a irradiação para conservar parâmetros de qualidade. Após a caracterização do perfil fenólico de *N. officinale*, a extração a frio de ácidos fenólicos e de flavonoides por HHP foi otimizada usando a metodologia de superfície de resposta (RSM), combinando as variáveis independentes tempo de processamento, pressão e concentração de etanol num desenho composto central de cinco níveis. Os modelos teóricos desenvolvidos foram ajustados com sucesso aos dados experimentais e o processo foi caracterizado por tempos de extração reduzidos, usar um solvente verde e ser seletivo.

Relativamente à composição nutricional e às propriedades antioxidantes das quatro variedades de tomate, o tomate redondo revelou a maior atividade antioxidante e teores elevados de fenóis totais e carotenoides, enquanto a variedade conhecida por tomate

amarelo apresentou uma composição nutricional interessante, caracterizada por teores elevados de frutose, glucose, ácido α-linolénico e tocoferóis totais. A quercetina pentosilrutinosido foi o flavonoide mais abundante nas quatro variedades. Posteriormente foi otimizada a extração assistida por micro-ondas de antioxidantes hidrofílicos e lipofílicos e de compostos fenólicos a partir de tomate utilizando RSM. Para isso foi implementado um desenho composto central considerando as variáveis tempo de processamento, temperatura, concentração de etanol e razão sólido/líquido. Os métodos de resposta dose-tempo de inibição da descoloração do crocin e do β-caroteno foram utilizados para medir os antioxidantes hidrofílicos e lipofílicos, respetivamente. Os dois ácidos fenólicos e flavonoides mais abundantes foram usados como variáveis dependentes. MAE demostrou ser um método de extração robusto e eficiente com elevado potencial para ser usado na valorização e reciclagem de resíduos de tomate.

Em conclusão, este trabalho destacou o potencial dos métodos de conservação e extração testados e contribui para a valorização dos alimentos tradicionais estudados, os quais apresentam potencial para ser incluídos no setor de alimentos minimamente processados e em dietas contemporâneas devido ao seu valor nutricional, ao seu potencial antioxidante e às suas propriedades organoléticas diferenciadas. Em estudos futuros será interessante avaliar os efeitos do embalamento MAP e da irradiação em outros parâmetros de qualidade e em outros produtos alimentares, bem como os efeitos combinados destas tecnologias. Também será interessante avaliar o potencial da tecnologia HHP para extrair compostos sensíveis ao calor a partir de resíduos de tomate.

Palavras-chave: alimentos tradicionais; valorização; irradiação; embalamento em atmosfera modificada; alta pressão hidrostática; extração assistida por micro-ondas; análise de alimentos; qualidade pós-colheita; otimização de processos; antioxidantes.

List of publications

Published works

Publications in ISI journals

16) José Pinela, M.A. Prieto, Lillian Barros, Ana Maria Carvalho, M. Beatriz P.P. Oliveira, Jorge A. Saraiva, Isabel C.F.R. Ferreira. Cold extraction of phenolic compounds from watercress by high hydrostatic pressure: Process modelling and optimization. Submitted to *Separation and Purification Technology*.
15) José Pinela, Lillian Barros, João C.M. Barreira, Ana Maria Carvalho, M. Beatriz P.P. Oliveira, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Postharvest changes in the phenolic profile of watercress induced by post-packaging irradiation and modified atmosphere packaging. Submitted to *Food Chemistry*.
14) José Pinela, Ana Maria Carvalho, Isabel C.F.R. Ferreira. Wild edible plants: Nutritional and toxicological characteristics, retrieval strategies and impact on today's society. Submitted to *Food and Chemical Toxicology.*
13) José Pinela, M.A. Prieto, Amilcar L. Antonio, Ana Maria Carvalho, M. Beatriz P.P. Oliveira, Lillian Barros, Isabel C.F.R. Ferreira (2017). Ellagitannin-rich bioactive extracts of *Tuberaria lignosa*: Insights into the radiation-induced effects in the recovery of high added-value compounds. *Food & Function*, 8, 2485-2499. DOI: 10.1039/c7fo00500h (IF: 3.247, Q1: Food Science & Technology).
12) José Pinela, M.A. Prieto, Maria Filomena Barreiro, Ana Maria Carvalho, M. Beatriz P.P. Oliveira, Thomas P. Curran, Isabel C.F.R. Ferreira (2017). Valorisation of tomato wastes for development of nutrient-rich antioxidant ingredients: a sustainable approach towards the needs of the today's society. *Innovative Food Science and Emerging Technologies*, 41, 160-171. DOI: 10.1016/j.ifset.2017.02.004 (IF: 2.997, Q1: Food Science & Technology).
11) José Pinela & Isabel C.F.R. Ferreira (2017). Nonthermal physical technologies to decontaminate and extend the shelf-life of fruits and vegetables: Trends aiming at quality and safety. *Critical Reviews in Food Science and Nutrition*. 57 (10), 2095-2111. DOI: 10.1080/10408398.2015.1046547 (IF: 6.077, Q1: Food Science & Technology).
10) José Pinela, M.A. Prieto, Ana Maria Carvalho, Maria Filomena Barreiro, M. Beatriz P.P. Oliveira, Lillian Barros, Isabel C.F.R. Ferreira (2016). Microwave-assisted extraction of phenolic acids and flavonoids and production of antioxidant ingredients from tomato: A nutraceutical-oriented optimization study. *Separation and Purification Technology*, 164, 114-124. DOI: 10.1016/j.seppur.2016.03.030 (IF: 3.299, Q1: Engineering, Chemical).

9) José Pinela, M.A. Prieto, Maria Filomena Barreiro, Ana Maria Carvalho, M. Beatriz P.P. Oliveira, J.A. Vázquez, Isabel C.F.R. Ferreira (2016). Optimization of microwave-assisted extraction of hydrophilic and lipophilic antioxidants from a surplus tomato crop by response surface methodology. *Food and Bioproducts Processing*, 98, 283-298. DOI: 10.1016/j.fbp.2016.02.002 (IF: 2.687, Q1: Food Science & Technology and Engineering, Chemical).

8) José Pinela, João C. M. Barreira, Lillian Barros, Sandra Cabo Verde, Amilcar L. Antonio, M. Beatriz P. P. Oliveira, Ana Maria Carvalho, Isabel C.F.R. Ferreira (2016). Modified atmosphere packaging and post-packaging irradiation of *Rumex induratus* leaves: a comparative study of postharvest quality changes. *Journal of Food Science and Technology - Mysore*, 53(7), 2943-2956. DOI: 10.1007/s13197-016-2260-6 (IF: 2.203; Q2: Food Science & Technology).

7) José Pinela, João C.M. Barreira, Lillian Barros, Sandra Cabo Verde, Amilcar L. Antonio, Ana Maria Carvalho, M. Beatriz P.P. Oliveira, Isabel C.F.R. Ferreira (2016). Suitability of gamma irradiation for preserving fresh-cut watercress quality during cold storage. *Food Chemistry*, 206, 50-58. DOI: 10.1016/j.foodchem.2016.03.050 (IF: 4.052, Q1: Food Science & Technology).

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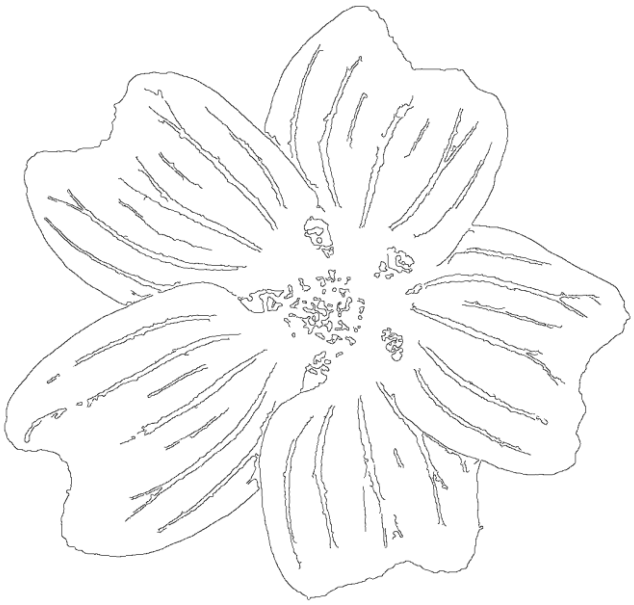
Abbreviations and symbols

Chromaticity on a green (-) to red (+) axis	*a**
Acetylcholinesterase	AChE
Alanine aminotransferase	ALT
Analysis of variance	ANOVA
Active packaging	AP
Area under the curve	AUC
Chromaticity on a blue (-) to yellow (+) axis	*b**
Constant coefficient	b_0
Coefficient of linear effect	b_i
Coefficients of quadratic effect	b_{ii}
Coefficient of interaction effect	b_{ij}
Cell adhesion molecules	CAMs
Catalase	CAT
Categorical principal components analysis	CATPCA
Carbon tetrachloride	CCl4
Cyclooxygenase-2	COX-2
Cold plasma	CP
Crocin	*Cr*
Irradiation dose	*D* and ID
Diode array detector	DAD
Deoxyribonucleic acid	DNA
Dense phase carbon dioxide	DP-CO_2
2,2-Diphenyl-1-picrylhydrazyl	DPPH
Dry weight	dw
Edible coating	EC
Effective concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay	EC_{50} value
Edible film	EF
Ethanol concentration	*Et*
Electrospray ionization	ESI
Fatty acids methyl ester	FAME
Food and Agriculture Organization	FAO
Food and Drug Administration	FDA
Flame ionizing detector	FID

Fresh weight	fw
Glutathione peroxidase	GPx
Glutathione	GSH
Hydrophilic	*H*
Low-density lipoprotein	HDL
High hydrostatic pressure	HHP
High-performance liquid chromatography	HPLC
High-voltage electrical discharges	HVED
International atomic energy agency	IAEA
International Consultative Group on Food Irradiation	ICGFI
Irradiation dose	ID and *D*
Isorhamnetin glycoside derivatives	*Igd*
Interleukin-6	IL-6
Inducible nitric oxide synthase	iNOS
Intelligent packaging	IP
I kappa B alpha	IκBα
c-Jun N-terminal kinase	JNK
Kaempferol glycoside derivatives	*Kgd*
Lipophilic	*L*
Lightness	*L**
Linear discriminant analysis	LDA
Low-density lipoprotein	LDL
Lipopolysaccharide	LPS
Microwave-assisted extraction	MAE
Modified atmosphere packaging	MAP
Mean absolute percentage error	MAPE
Malondialdehyde	MDA
Matrix metalloproteinase	MMP
Mean squared error	MSE
Monounsaturated fatty acids	MUFA
Nonalcoholic steatohepatitis	NASH
Nuclear factor kappa B	NF-κB
Nitric oxide	NO
Nitric oxide synthases	NOS
Protected substrate	$\tilde{P}$
Principal component analysis	PCA

Prostate cancer cells	PC-3
Photodiode array detector	PDA
Pulsed electric fields	PEF
Pulsed light	PL
Pressurized liquid extraction	PLE
Preparation methods	PM
Averaged maximum protected substrate	P_m
Polyunsaturated fatty acids	FUFA
Quercetin glycoside derivatives	*Qgd*
Adjusted coefficient of determination	R^2_{adj}
Root mean square of the error	RMSE
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Response surface methodology	RSM
Solid/liquid ratio	*S/L*
Saturated fatty acids	SFA
Supercritical fluid extraction	SFE
Superoxide dismutase	SOD
Processing or extraction time	*t*
Temperature	*T*
Thiobarbituric acid	TBA
Thiobarbituric acid reactive substances	TBARS
Tumour necrosis factors	TNF-α
Total phenolic compounds	TPC
Total soluble solids	TSS
Ultrasound-assisted extraction	UAE
Ultra-fast liquid chromatography	UFLC
Ultrasound	US
Ultraviolet	UV
Vascular cell adhesion molecule-1	VCAM-1
Average amount of protected molecules per g of extract	V_m
Wild edible plants	WEPs
World Health Organization	WHO
β-Carotene	*βC*

1. Motivation, Objectives and Thesis Structure



This **1st chapter** describes the motivation, the main objectives of this study and the structure of the thesis.

1.1. Motivation

In the North-eastern region of Portugal (known as Trás-os-Montes), wild edible plants (WEPs) have received high importance given their ability to provide nutrients and protection in scarcity times and their important role in complementing staple agricultural foods (Carvalho and Morales 2010, Carvalho and Barata 2016). These wild edibles were consumed raw, cooked, or prepared in recreational beverages; others were prepared in medicinal infusions and decoctions because of the perceived health-promoting effects (Carvalho 2010, Carvalho and Morales 2010). Different tomato farmers' varieties can also be found in homegardens of this region where they have been grown since a long time using extensive farming techniques. However, the nutritional composition of these farmer' varieties of tomato remains unknown and WEPs lack recognition as significant contributors to the human diet.

New trends have begun to highlight the importance of a wide range of hitherto underutilized WEPs and of local farmers' varieties of tomatoes (Carvalho and Morales 2010). Some wild species are re-emerging in gardens and kitchens around Europe due to their differentiated organoleptic properties, nutritional value and health-promoting effects, being increasingly found in farmers' markets, gourmet food shops, and restaurants (Tardío 2005, Łuczaj *et al.* 2012, Vasquez 2016). The cultivation and commercialization of certain food and non-food crops was already promoted in some regions of Europe (Łuczaj *et al.* 2012). Following this trend, and given the economic and genetic value of these natural resources threatened with extinction within different ecosystems due to multiple changes, retrieval strategies should be adopted to promote their sustainable use by different stakeholders.

In a first instance, these plant matrices should be characterized in terms of nutrients and bioactive non-nutrients in order to highlight the most promising ones for food purposes or to obtain high added-value compounds. Then, it becomes necessary to apply preservation technologies that allow extending the shelf-life of these perishable foods and obtaining safe and high quality products. This can be achieved using conventional (e.g., drying, packaging and refrigeration) or innovative (e.g., irradiation, modified atmosphere packaging (MAP)) preservation methods (Pinela and Ferreira 2017). Drying is the most commonly used method to preserve medicinal plants and can be complemented with packaging and irradiation in order to promote the preservation efficacy during storage. The former non-thermal technology is a sustainable alternative to the chemical agents commonly used to fumigate dried aromatic plants or sanitize fresh vegetables that leaves no residue on the treated food product (FAO/IAEA 2004, 2006, Lacroix 2014). MAP is another methodology used worldwide to preserve the quality and extend the shelf-life of minimally processed vegetables, but the

literature describing the suitability of non-conventional gases is still limited (Zhang *et al.* 2008, Artés *et al.* 2009, Char *et al.* 2012, Inestroza-Lizardo *et al.* 2016).

Wild plants and tomatoes, as well as by-products, are promising sources of high added value compounds (e.g., phenolic acids, flavonoids, ellagitannins) whose recovery should be performed using efficient and sustainable extraction methods such as microwave-assisted extraction (MAE) and high hydrostatic pressure (HHP). These methods are time- and solvent-saving and lead to higher yields when compared with conventional extraction methods (e.g., Soxhlet extraction) (Ameer *et al.* 2017, Antonio *et al.* 2017). Additionally, there is a growing demand by the food industry and consumers for foods containing natural functional ingredients instead of chemically synthesized molecules (Carocho *et al.* 2015). In this sense, the recovered and recycled of different molecules from plant material (including by-products and wastes) is a strategy that addresses the current challenges of the industrialized world.

Therefore, this work intends to recall and retrieve the sustainable use of traditional foods (medicinal plants, leafy vegetables and tomato farmers' varieties) through innovative preservation (irradiation and MAP) and extraction methods (MAE and HHP) in order to meet the growing demand for new foods and natural bioactive ingredients.

1.2. Overall objectives

The retrieval of traditional plant foods from the North-eastern region of Portugal through the application of non-conventional and innovative preservation and extraction methods was the main objective of this work. For this, two medicinal plants (dwarf mallow and perennial spotted rockrose), two leafy vegetables (buckler sorrel and watercress) and four tomato farmer' varieties (locally known as "amarelo", "redondo", "comprido" and "coração de boi") were selected, characterized in terms of nutrients, phytochemicals and antioxidant activity, and submitted to postharvest preservation treatments and/or to non-convention extraction methods as summarized in **Table 1**. The study was generally performed according to the tasks illustrated in **Figure 1** and originated the aforementioned scientific publications. These tasks were carried out in the following research centres:

- Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança;
- REQUIMTE/LAQV, Laboratório de Bromatologia e Hidrologia, Departamento de Ciências Químicas da Faculdade de Farmácia da Universidade do Porto;
- Dpto. Nutrición y Bromatología II, Facultad de Farmacia, Universidad Complutense de Madrid (UCM);
- Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca;

- Centro de Ciências e Tecnologias Nucleares (C2TN), IST, Universidade de Lisboa;
- Laboratory of Separation and Reaction Engineering (LSRE), Associate Laboratory LSRE/LCM, Instituto Politécnico de Bragança;
- Unidade de Investigação em Química Orgânica, Produtos Naturais e Agroalimentares (QOPNA), Departamento de Química, Universidade de Aveiro.

1.3. Structure of the thesis

This document is organized in six chapters covering all the proposed objectives. In this **1st chapter**, the motivation, the main objectives and the structure of the thesis are presented. The state of the art concerning the importance of WEPs for today' society, possible retrieval and conservation strategies, the health-promoting effects and industrial value of the tomato bioactive compounds, nonthermal physical techniques for preservation and shelf-life extension of fresh fruits and vegetables, and non-conventional methods for extraction of bioactive compounds from plant matrices is presented in the **2nd chapter**. The **3rd chapter** covers the work performed with the two medicinal plants; it describes the chemical composition and the antioxidant activity of these species, and the effects induced by the ionizing radiation treatment. The impact of this technology on the extraction kinetics of ellagitannin derivatives and other phytochemicals from *T. lignosa* is also reported. The **4th chapter** is dedicated to the leafy vegetables; the work related to their chemical and nutritional characterization, the preservation of quality parameters by the use of irradiation and MAP, and the optimization of the cold extraction of phenolic compounds from watercress using high pressures are presented. The **5th chapter** describes the nutritional value, chemical composition and antioxidant activity of the four farmers' varieties of tomatoes from North-eastern Portugal homegardens. The studies on the optimization of microwave-assisted extraction of antioxidants and phenolic compounds from these matrices using response surface methodology are also presented in this chapter. The **6th chapter** synthesizes the general conclusions of the study, highlights the potential of the studied traditional plant foods and of the applied technologies, and presents future perspectives.

Chapter 2 (state of the art) comprises material from a submitted review article and from the following publications: J. Pinela, M.B.P.P. Oliveira and I.C.F.R. Ferreira, in *Natural Bioactive Compounds from Fruits and Vegetables as Health Promoters,* eds. L.R. da Silva and B.M. Silva, Bentham Science Publishers, 2016, vol. 2, pp. 48–91; J. Pinela and I.C.F.R. Ferreira, *Crit. Rev. Food Sci. Nutr.*, 2017, 57, 2095–2111; and A.L. Antonio, E. Pereira, J. Pinela, *et al*., in *Food Safety: Innovative Analytical Tools for Safety Assessment*, eds. U.G. Spizzirri and G. Cirillo, Scrivener Publishing LLC, 2017, pp. 179–220.
Chapters 3, 4 and 5 are presented in the form of research article.

Table 1 Studied traditional foods, applied preservation/extraction technologies, evaluated physical/chemical/nutritional/antioxidant parameters, and specific objectives.

	Medicinal plants		**Leafy vegetables**		**Tomato farmer' varieties**
					
Scientific name	*Malva neglecta* Wallr.	*Tuberaria lignosa* (Sweet) Samp.	*Rumex induratus* Boiss. & Reut.	*Nasturtium officinale* R. Br.	*Lycopersicon esculentum* Mill.
Synonymy	-	*Xolantha tuberaria* (L.) M. J. Gallego et al.	*Rumex scutatus* subsp. *induratus* (Boiss. & Reut.) Nyman	*Rorippa nasturtium-aquaticum* (L.) Hayek	*Solanum lycopersicum* L.
Common name in English	Dwarf mallow	Perennial spotted rockrose	Buckler sorrel	Watercress	Yellow tomato, round tomato, long tomato and heart tomato
Common name in Portuguese	Malva branca	Alcária	Azedas	Agrião	Tomate amarelo, tomate redondo or batateiro, tomate comprido and tomate coração
Studied part	Leafy flowering stems	Flowering aerial part (basal leaves, stems and inflorescences)	Tender leaves	Aerial part (leaves and stems)	Fruit
Mode of consumption	Decoction and raw in salads	Infusion and decoction	Raw as a snack or in salads	Raw in salads and cooked	Raw and cooked
Evaluated parameters	Colour, organic acids, total phenolics and flavonoids and *in vitro* antioxidant activity	Colour, organic acids, free sugars, individual phenolic compounds and *in vitro* antioxidant activity	Colour, proximate composition, free sugars, organic acids, tocopherols, fatty acids, total phenolics and flavonoids and *in vitro* antioxidant activity	Colour, total soluble solids, pH, proximate composition, free sugars, organic acids, tocopherols, fatty acids, individual phenolic compounds, total phenolics and flavonoids and *in vitro* antioxidant activity	Proximate composition, free sugars, tocopherols, fatty acids, vitamin C, carotenoids, individual phenolic compounds, total phenolics, flavanols and anthocyanins, and *in vitro* antioxidant activity
Applied technologies	Irradiation	Drying and irradiation	Irradiation and MAP	Irradiation, MAP and HHP	MAE
Main objective	Chemical characterization and preservation of quality attributes	Chemical characterization, preservation of quality attributes and extraction of phenolic compounds	Nutritional and chemical characterization, quality preservation and shelf-life extension	Nutritional and chemical characterization, quality preservation, shelf-life extension and extraction of phenolic compounds	Nutritional and chemical characterization and extraction of antioxidants and phenolic compounds

Photos: Ana Maria Carvalho

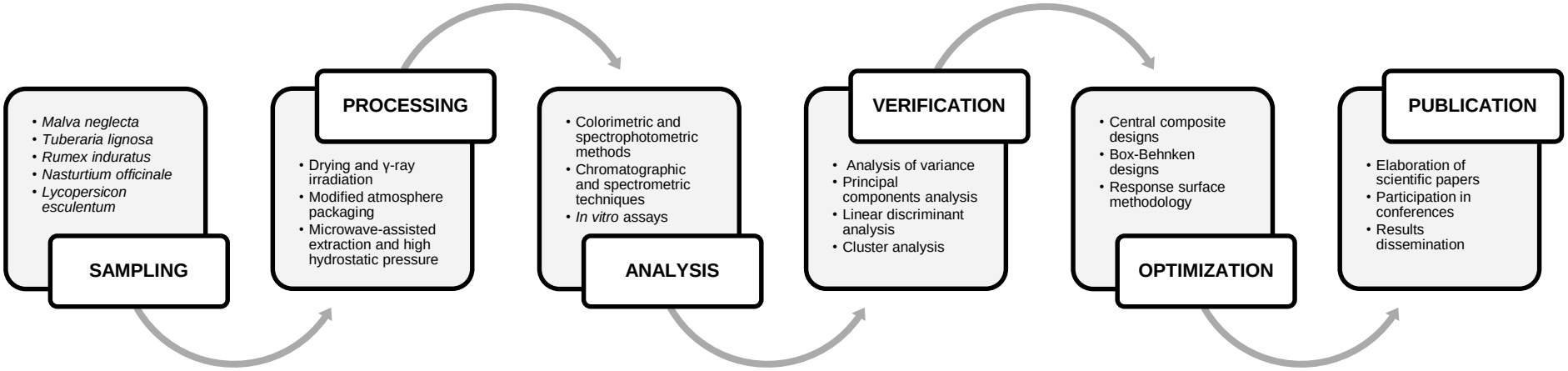


Figure 1 Schematic illustration of the tasks performed in this study.

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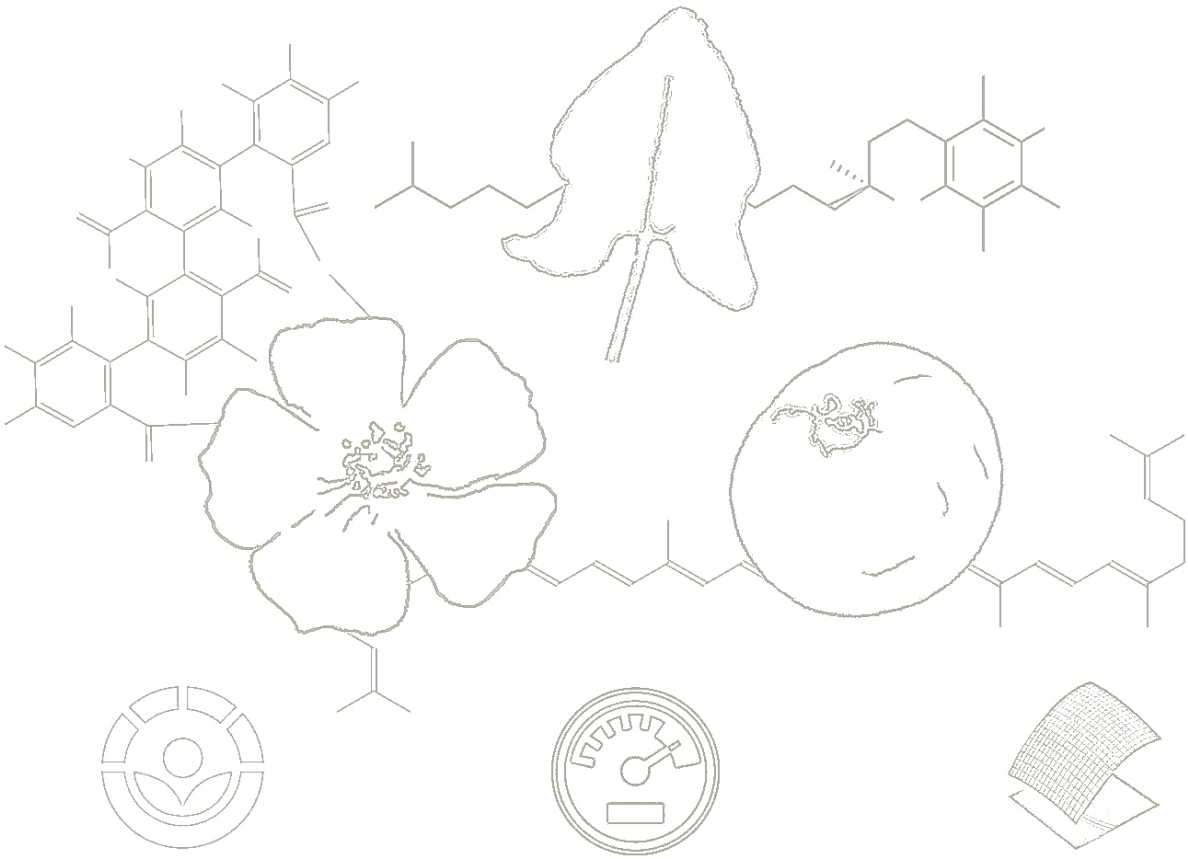
2. State of the Art



This **2nd chapter** presents the state of the art concerning the importance of traditional plant foods for today' society, possible retrieval strategies for these food matrices, the health-promoting effects and industrial value of tomato bioactive compounds, innovative nonthermal technologies for preservation and shelf-life extension of fresh fruits and vegetables, and non-conventional methods for extraction of bioactive compounds from plant materials.

2.1. Wild edible plants: Nutritional and toxicological aspects, retrieval strategies and importance for today's society

2.1.1. Wild edible plants

Wild plants have received great importance at different places and times of the human history given their ability to provide nutrients during scarcity periods and protection for minor health conditions (Torija-Isasa and Matallana-González 2016). Their popularity comes from the need for nourishment in regions experiencing food shortages, where wild edible plants (WEPs) have played an important role in complementing staple agricultural foods and poverty alleviation, and from the perceived health-promoting effects and cultural trends (Sõukand 2016). Moreover, the long use of WEPs is associated with particular wisdom and practices, and above all great creativity pulsing with life, and with each user, household, region or country.

The current underutilization of WEPs was promoted by industrial revolution, lifestyle changes, large-scale cultivation of a limited number of crops (such as wheat, maize and rice), and less contact with nature, among other reasons (Łuczaj *et al.* 2012). In fact, in developed countries, cultivated plant-based foods purchased at the supermarket are placed on the table with relatively less effort than if collected from the wild. Nevertheless, many people around the world continue to rely on WEPs to meet at least part of their daily nutritional needs (Turner *et al.* 2011). Moreover, a new phenomenon associated with the use of WEPs is emerging in modern societies (Carvalho and Barata 2016).

Several studies carried out in the last years revealed the important role of WEPs as sources of nutrients and contributors to human dietary requirements (Sánchez-Mata *et al.* 2016). Some WEPs have also been described as being functional foods, because they contain physiologically active ingredients capable of providing health benefits beyond basic nutrition (Pinela, Carocho, *et al.* 2016); while others may contain potentially toxic compounds to humans (EFSA 2009, 2012). Therefore, this article reviews the nutritional composition of WEPs from the North-eastern region of Portugal and safety issues related to their use. These species were selected based on ethnobotanical data that have recalled from memory such traditional knowledge and practices regarding the use of WEPs (Tardío *et al.* 2006, Carvalho 2010, Carvalho and Morales 2010, Tuttolomondo *et al.* 2014). Strategies for retrieval of these species are also herein discussed, highlighting their importance for today's society. Moreover, these approaches might contribute to local sustainable development and innovative options for rural and marginalized areas, meeting the main goals of United Nations, such as promote inclusive and sustainable socioeconomic growth and provide wellbeing and healthy lives for all (European Commission 2016).

2.1.2. Wild edible plants from North-eastern Portugal

WEPs traditionally consumed in the North-eastern region of Portugal are presented in **Table 2**. Species belonging to 17 families including Asteraceae, Lamiaceae, Polygonaceae, Amaranthaceae, Apiaceae, Boraginaceae, Portulacaceae, Asparagaceae, Cannabaceae, Caryophyllaceae, Dioscoreaceae, Malvaceae, and Papaveraceae were reviewed, as well as others of economically important botanical families or locally highly prized such as Brassicaceae, Cucurbitaceae, Fabaceae, and Rosaceae. These species are usually gathered and consumed as wild vegetables, spices/condiments, snacks, recreational beverages and fruits, but are also prepared in medicinal infusions and decoctions for the treatment and prevention of various diseases and health conditions. Most of the times, these WEPs are collected from the wild where they grow spontaneously; but some wild forms are occasionally brought to the garden and cultivated directly for food purposes, such as *Origanum vulgare* L., *Scolymus hispanicus* L. (**Figure 2**), *Silene vulgaris* (Moench) Garcke and *Rumex* sp. pl., with the aim of obtaining a higher yield, an immediate availability, and overcome a possible lack of such species in the region.

2.1.2.1. Wild plants as sustainable foods

Wild vegetables have been the mainstay of human diets for centuries and people still continue reserving some time for collecting these foods, which are rich in micronutrients and highly appreciated raw in salads or cooked in traditional recipes. As shown in **Table 2**, the edible parts harvested and consumed are different depending on species and growth stage. Some examples of the most common plant parts used are basal leaves (including the ones of *Sonchus oleraceus* L., *Papaver rhoeas* L., *Cichorium intybus* L., *Chondrilla juncea* L., *Beta maritima* L., *Rumex papillaris* Boiss. & Reut., *Rumex pulcher* L. and *Anchusa azurea* Mill.), or just the midribs (as in the case of *Scolymus hispanicus* L. and *Silybum marianum* (L.) Gaertn.), young stems with leaves (like the four widespread species of wild asparagus *Asparagus acutifolius* L., *Bryonia dioica* Jacq., *Humulus lupulus* L. and *Tamus communis* L.; **Figure 2**), and other organs such as the edible bulb and pseudostem (lower part of the leaves) of *Allium ampeloprasum* L. (wild leek). These vegetables are consumed both raw and cooked, although others are more often eaten cooked (such as *Rumex* species, *Borago officinalis* L. and *Silene vulgaris*.

Table 2 Food and medicinal uses of WEPs traditionally used in the North-eastern region of Portugal. Scientific nomenclature and synonymy according to The Plant List (2013).

Wild edible plants			**Traditional cuisine**		**Folk medicine**		
Scientific name	**Vernacular name**	**Family**	**Edible part (Ep)**	**Mode of consumption**	**Used part (Up)**	**Preparation method**	**Indications/attributed properties**
Allium ampeloprasum L.	Wild leek	Amaryllidaceae	Bulb and pseudostem (Ep1)	Raw in salads, sautéed or cooked			Diarrhea. Diuretic. Drunkenness. Hoarseness.
Anchusa azurea Mill.	Vurraine	Boraginaceae	Basal leaves (Ep1), flowers	Raw in salads or cooked			Anti-inflammatory, expectorant. Gastralgia. Cold. Kidney stones. Skin problems
Apium nodiflorum (L.) Lag.	Fool's watercress	Apiaceae	Young stems with leaves (Ep1)	Raw in salads			
Asparagus acutifolius L.	Wild asparagus	Asparagaceae	Young shoots (Ep1)	Raw in salads, sautéed with eggs or cooked	Turion	Decoction	Diuretic. Vermifuge
Beta maritima L. syn. *Beta vulgaris* L.	Sea beet	Amaranthaceae	Basal leaves (Ep1)	Cooked			Digestive disorders, burn and throat pains and anaemia
Borago officinalis L.	Borage	Boraginaceae	Young basal leaves (Ep1)	Cooked and sautéed	Aerial part, leaves	Decoction, broth	Digestive system, intestinal regulator, laxative. Dysmenorrhea. Vulnerary. Tonic
Bryonia dioica Jacq.	White bryony	Cucurbitaceae	Young shoots with tendrils and immature leaves (Ep1)	Cooked and sautéed with eggs	Root (Up1). Non edible, immature and ripen fruits (Up2)	Alcoholic maceration, direct application (Up2)	Abortive (Up1). Antispasmodic and antirheumatics (Up2).
Cichorium intybus L.	Common chicory	Asteraceae	Basal leaves (Ep1)	Raw in salads or cooked. Coffee substitute	Root and aerial par	Broth and decoction	Constipation. Digestive disorders, hypoglycaemic, depurative, disinfectant of urinary tract, hepatoprotective, and antirheumatics
Chenopodium ambrosioides L. syn. *Dysphania ambrosioides* (L.) Mosyakin & Clemants	Epazote, wormseed, Jesuit's tea, Mexican tea	Amaranthaceae	Tender leaves, inflorescence and upper leaves (Ep1)	Cooked. Tisane	Leaves and flowering stems	Infusion	Digestive system, respiratory system, stomachic, vermifuge and emetic.
Chondrilla juncea L.	Rush skeleton weed, gum succory, devil's grass	Asteraceae	Basal leaves (Ep1)	Raw in salads or stewed	Sap	Direct application	Skin injuries. Wound healing
Foeniculum vulgare Mill.	Fennel	Apiaceae	Young shoots with leaves (Ep1), shoots (Ep2), leaves (Ep3), stems (Ep4), inflorescences (Ep5)	Raw as a snack or in salads, stewed or as condiment	Aerial part	Infusion and decoction	Digestive. Gastrointestinal disorders. Diuretic. Carminative. Emmenagogue. Abdominal swelling. Asthma
Glechoma hederacea L.	Ground ivy	Lamiaceae	Leave and stems (Ep1)	Condiment/spices flavouring and seasoning traditional dishes. Cooked in soups and stews. Restorative bouillon. Tisane	Leaves	Infusion (P1), decoction (P2) and broth	Respiratory and gastrointestinal systems. For relieving colds, coughs, throat irritations and abdominal pains (P1). External inflammations and skin diseases (P2)
Helichrysum stoechas (L.) Moench.	Shrubby everlasting	Asteraceae	Aerial part (inflorescences and leafy flowering stems) (Ep1)	Condiment	Aerial part	Infusion	Respiratory system. Cold, bronchitis. Febrifuge

Wild edible plants			**Traditional cuisine**		**Folk medicine**		
Scientific name	**Vernacular name**	**Family**	**Edible part (Ep)**	**Mode of consumption**	**Used part (Up)**	**Preparation method**	**Indications/attributed properties**
Humulus lupulus L.	Wild hop	Cannabaceae	Young shoots with unexpanded leaves (Ep1), inflorescences	Raw in salads or cooked. Liqueur	Inflorescence	Infusion and maceration	Digestive, appetizer. Stomachache.
Malva neglecta Wallr.	Dwarf mallow	Malvaceae	Leaves (Ep1)	Raw in salads	Leaves	Decoctions	Disinfectant and anti-inflammatory
Malva sylvestris L.	Common mallow	Malvaceae	Leaves (Ep1), flowers (Ep2), immature fruits (Ep3), leafy flowering stems (Ep4)	Raw in salads or cooked. Raw as a snacks or in salads	Leaves (Up1), flower (Up2)	Decoction, warmed leaves (poultices) (Up1). Infusion (Up2)	Toothache. Genital tract, dermatitis, acne, skin injuries, burns. Stomachic. Diarrhea. Rheumatism. Eyes. Cough.
Mentha pulegium L.	Pennyroyal	Lamiaceae	Flowering aerial part (Ep1)	Liqueur making, tisane. Condiment/spices flavouring and seasoning traditional dishes	Aerial part	Infusion	Digestive. Carminative. Bronchitis. Liver conditions. Headache. Respiratory system. Cholesterol.
Montia fontana L.	Water-blinks	Portulacaceae	Aerial part (Ep1)	Raw in salads or cooked			Excretory system
Nasturtium officinale R. Br.	Watercress	Brassicaceae	Aerial part (Ep1)	Raw in salads or cooked	Whole plant	Infusion, decoction and maceration	Respiratory and digestive systems. Amenorrhoea. Kidney problems. Dermatitis. Acne. Tonic. Diuretic. Hypertensive. Cough. Laxative. Anaemia. Liver conditions
Origanum vulgare subsp. *virens* (Hoffmanns. & Link) Ietswaart	Oregano	Lamiaceae	Inflorescences (Ep1)	Condiment/spices flavouring and seasoning traditional dishes and sausages. Summer salads. Food preservative, specially olives	Aerial part	Infusion, decoction and syrup	Toothache. Against cold and flu, antitussive, anti-inflammatory
Papaver rhoeas L.	Common poppy	Papaveraceae	Basal leaves (Ep1)	Cooked and sometimes raw in salads	Fruit, seeds	Infusion	Nervousness, insomnia, sedative, digestive and respiratory disorders, cough, baldness, eye infection
Portulaca oleracea L.	Common purslane, verdolaga	Portulacaceae	Leaves (Ep1)	Raw in salads or cooked			Diuretic. Gastrointestinal pain. Inflamed haemorrhoids. Laxative. Urinary affections
Pterospartum tridentatum (L.) Willk. syn. *Genista tridentata* L.	Unknown; pt: carqueja	Fabaceae	Flowers (Ep1)	Condiment spices flavouring and seasoning traditional dishes	Root, flower	Infusion, decoction	Respiratory and gastrointestinal systems. Diabetes. Excretory system, Hypotensive. Nervine. Depurative. Skin injuries
Rubus ulmifolius Schott	Elm-leaved blackberry	Rosaceae	Flower buds (Ep1), fully opened flowers (Ep2), fruits	Raw in salads, sautéed with eggs or cooked. Jam and liqueur.	Fruit, leaves, flower buds	Infusion, decoction and direct application	Digestive system, stomachache, abdominal pain, diarrhea. Wounds. Haemostatic
Rumex acetosella L.	Sheep sorrel	Polygonaceae	Shoots (Ep1)	Raw as a snack or in salads	Leaves	Decoction and ointments	Skin inflammations
Rumex induratus Boiss. & Reut.	Buckler sorrel	Polygonaceae	Tender leaves (Ep1)	Raw as a snack or in salads			
Rumex obtusifolius L.	Round-leaved dock, bitter dock	Polygonaceae	Basal young leaves. Dried shoots and inflorescences	Raw in salads or cooked	Inflorescences	Infusion and decoction	Digestive system. Diarrhea. Vulnerary. Skin injuries
Rumex papillaris Boiss. & Reut.	Docks	Polygonaceae	Basal leaves (Ep1)	Raw as a snack or in salads			

Wild edible plants			**Traditional cuisine**		**Folk medicine**		
Scientific name	**Vernacular name**	**Family**	**Edible part (Ep)**	**Mode of consumption**	**Used part (Up)**	**Preparation method**	**Indications/attributed properties**
Rumex pulcher L.	Fiddle dock	Polygonaceae	Basal leaves (Ep1)	Raw as a snack or in salads			
Scolymus hispanicus L.	Common golden thistle, Spanish oyster thistle	Asteraceae	Midribs of basal leaves (Ep1)	Cooked, sautéed and sometimes raw in salads. Lowers used as a saffron substitute	Leaves, flower and root	Infusion	Digestive disorders as gastralgia, in Malta fever and in eye infection
Silene vulgaris (Moench) Garcke	Bladder campion	Caryophyllaceae	Tender stems with leaves (Ep1)	Cooked or sometimes raw in salads	Flower	Decoction	Antianaemic. Liver conditions
Silybum marianum (L.) Gaertn.	Milk thistle	Asteraceae	Midribs of basal leaves (Ep1)	Cooked and sometimes raw in salads	Aerial part	Direct application	Liver conditions. Stomach pains. Gall-blander infection and haemorrhoids
Sonchus oleraceus L.	Sow thistle	Asteraceae	Basal leaves (Ep1)	Raw in salads or cooked	Aerial part	Broth	Depurative and diuretic. Contusions and burns. Haemorrhoids prevention. Digestive
Tamus communis L.	Black bryony	Dioscoreaceae	Young shoots with unexpanded leaves (Ep1)	Cooked and sautéed with eggs	Leaves and stems (Up1). Non edible, immature and ripen fruits (Up2)	Infusion (Up1). Alcoholic maceration, direct application, ointments (Up2)	Digestive, laxative (Up1). Antispasmodic and antirheumatics (Up2)
Taraxacum obovatum (Willd.) DC.	Pissenlit obovale, dandelion	Asteraceae	Young basal leaves (Ep1)	Raw in salads			Hepatoprotective. Digestive system
Thymus mastichina L.	Mastic thyme	Lamiaceae	Flowering aerial part (Ep1)	Condiment/spices flavouring and seasoning traditional dishes and salads. To preserve olives. Used instead of salt	Flowering aerial part	Infusion	Digestive system
Thymus pulegioides L.	Broad-leaved thyme, lemon thyme	Lamiaceae	Flowering aerial part (Ep1)	Condiment/spices flavouring and seasoning	Aerial part	Infusion	Respiratory system. Against cold and flu. Antitussive

Sources: Carvalho (2010), Carvalho and Morales (2013), Łuczaj *et al.* (2012), Menendez-Baceta *et al.* (2012), Pardo-de-Santayana *et al.* (2007), Sõukand *et al.* (2013), Tardío (2005), Tardío *et al.* (2006), and Tuttolomondo *et al.* (2014).

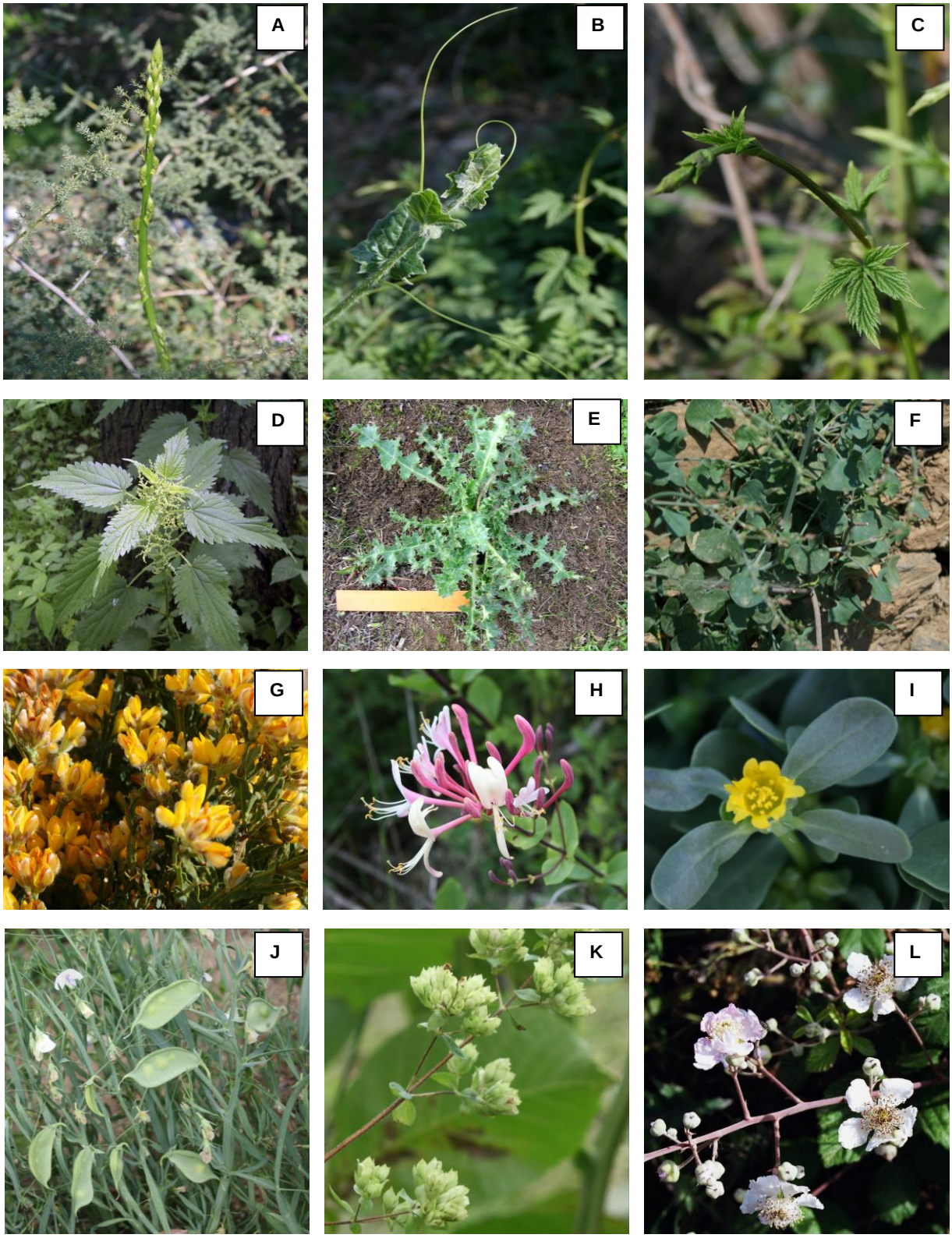


Figure 2 Some examples of wild species and plant materials traditionally used in the North-eastern region of Portugal as food and/or medicine. A: *Asparagus acutifolius* L.; B: *Bryonia dioica* Jacq.; C: *Humulus lupulus* L.; D: *Urtica dioica* L.; E: *Scolymus hispanicus* L.; F: *Rumex induratus* Boiss. & Reut.; G: *Genista tridentata* L.; H*: Lonicera etrusca* Santi; I: *Portulaca oleracea* L.; J: *Lathyrus sativus* L.; K: *Origanum vulgare* L.; and L: *Rubus ulmifolius* Schott. Nomenclature according to The Plant List (2013).

In times of scarcity, some WEPs were eaten in the field as snacks without any preparation, or chewed and spitted for entertainment, as hunger or thirst quencher, or to enjoy its flavour. These snacks consisted mostly of flowers that were sucked because of the sweet taste of the nectar (*e.g., Malva sylvestris* L. and *Lamium purpureum* L.), but also in the tasty and interesting-looking immature fruits of Fabaceae such as *Astragalus* and *Vicia* species, *Foeniculum vulgare* Mill., *Malva sylvestris* and *Capsella bursa-pastoris* (L.) Medik. (Tardío 2005, Tardío *et al.* 2006, Carvalho 2010). The peeled young shoots of *Rubus ulmifolius* Schott, as well as of wild roses and the young shoots and leaves of *Rumex* sp.pl., were eaten raw in spring. *Foeniculum vulgare* was used as thirst quencher or breath refreshment (Tardio *et al.* 2002). These snacks were eaten occasionally (mostly by children and shepherds) and not as regular food, although some nutritional value was perceived (Menendez-Baceta *et al.* 2012).

Aromatic plants are very important in the gastronomy of the North-eastern Portugal. Flowering parts of *Foeniculum vulgare Origanum vulgare*, *Mentha pulegium* L., *Thymus pulegioides* L., *Thymus mastichina* L., *Genista tridentata* L. (syn. *Pterospartum tridentatum* (L.) Willk.; **Figure 2**), *Helichrysum stoechas* (L.) Moench. and *Glechoma hederacea* L. are traditionally used as condiments/spices for flavouring and seasoning of traditional dishes and salads (**Table 2**). *T. mastichina* is also used as a salt substitute and olives preservative. Some of these aromatic plants were used for seasoning soups and purees in order to diversify the monotonous diet (Pardo-de-Santayana *et al.* 2007, Carvalho and Morales 2010).

Other WEPs were prepared in recreational beverages, both alcoholic and non-alcoholic, such as tisanes (herbal teas) and liqueurs. These herbal teas are consumed in a food context for their social and/or recreational value or their attributes of healthy drinks, not including infusions/decoctions taken only for specific medicinal purposes (Sõukand *et al.* 2013). While medicinal beverages are purposely taken for a limited number of days to treat a certain health condition, there is no limit to the duration that recreational teas can be consumed. In addition, herbal teas are drunk hot or cold according to the season. As shown in **Table 2**, *M. pulegium*, *G. hederacea* and *Dysphania ambrosioides* (L.) Mosyakin & Clemants (syn. *Chenopodium ambrosioides* L.) were prepared in tisanes. In turn, *M. pulegium* and *Humulus lupulus* inflorescences and the fruits of *Rubus ulmifolius* and *Prunus spinosa* L. were used to make liqueurs (Carvalho 2010, Menendez-Baceta *et al.* 2012).

2.1.2.2. Wild plants as folk medicines

The majority of the species presented in **Table 2** were perceived as having medicinal properties. They were used in local folk medicine, prepared mainly in medicinal infusions and decoctions to treat several health conditions, but also in macerations, syrups, poultices,

broths, or applied directly. Medicinal beverages were taken to treat mainly digestive disorders (such as those prepared from *Beta maritima*, *Thymus mastichina*, *Borago officinalis*, *Rubus ulmifolius*, *Humulus lupulus* and *Foeniculum vulgare*) and respiratory system problems (including the ones prepared from *Mentha pulegium*, *Genista tridentata*, *Helichrysum stoechas* and *Papaver rhoeas*). Others were used for their disinfectant or anti-inflammatory effects (namely *Origanum vulgare*, *Anchusa azurea*, *Chichorium intybus*, *Glecoma hederacea* and *Rumex acetosella* L.). Liver conditions, urinary affections, skin problems, toothache, diabetes, cold, flu and bronchitis, among other problems, were also treated using WEPs (**Table 2**). However, the traditional knowledge and local healers assume that the oral preparations from some of these plants can lead to some risks, especially due to excessive doses and long-term treatments, thus recommending specific dosages and controlled periods of intake with ritual healing practices (Carvalho 2010).

Other examples of edible species with medicinal applications are as follows: the fruits of *Bryonia dioica* and *Tammus communis* used to be macerated in alcohol or crushed and applied topically as an ointment to treat rheumatic and muscular pains. The sap of *Chondrilla juncea* was applied to treat and heal skin injuries. Dermatitis, burns and skin injuries were also treated with warmed leaves of *Malva sylvestris* as poultices. Syrup against cold and flu used to be prepared from *Origanum vulgare* aerial parts. *Borago officinalis*, *Cichorium intybus* and *Sonchus oleraceus* were also consumed in medicinal broths.

2.1.2.3. Crop wild relatives and landrace diversity

Crop wild relatives are wild plant species closely related to cultivated plants (Maxted *et al.* 2006). These species are components of both natural habitats and agro-ecosystems and constitute an important element of plant genetic heritage that needs to be conserved (Castañeda-Álvarez *et al.* 2016). An inventory performed by Magos Brehm *et al.* (2008) reported the existence of 2262 taxa (including subspecies and varieties) of crop wild relatives in the Portuguese flora, representing ~9.6% of the European and Mediterranean crop wild relatives' flora. Examples of crop wild relatives that used to be eaten in north-eastern Portugal include:

- *In the Fabaceae family:* The immature pods of wild pea (*Lathyrus cicera* L.), flat pea (*Lathyrus sylvestris* L.), milkvetches (*Astragalus cymbaecarpos* Brot. and *A. pelecinus* (L.) Barneby), narrow-leaved vetch (*Vicia angustifolia* L.), hairy vetch (*Vicia villosa* Roth) and yellow vetch (*Vicia lutea* L.) were eaten raw. Single-flower vetch (*Vicia articulata* Hornem.) seeds were consumed due to its resemblance with those of small-seeded lentil cultivars.
- *In the Brassicaceae family:* Wild arugula (*Eruca vesicaria* (L.) Cav.), garlic mustard (*Alliaria petiolata* (M. Bieb.) Cavara & Grande), white wall-rocket (*Diplotaxis erucoides*

(L.) DC.), wild mustard (*Sinapis arvenses* L.), wild watercress (*Nasturtium officinale* R. Br.) and white mustard (*Sinapis alba* L.). Their basal leaves and young shoots were eaten raw in salads or cooked and consumed as greens or in soups. The seeds of white mustard were used as a mild flavour for pickling or grilled meat.

- *In the Rosaceae family:* Wild strawberry (*Fragaria vesca* L.), common hawthorn (*Crataegus monogyna* Jacq.), blackthorn (*Prunus spinosa* L.), European crab apple (*Malus sylvestris* (L.) Mill.) and elm-leaved blackberry (*Rubus ulmifolius*) (**Figure 2**). From all these species the bletted fruits, that are sweeter, were eaten raw as snacks. When ripen they were also cooked to be edible and used to prepare marmalades. The young shoots (pointed ends) of elm-leaved blackberry were consume like asparagus and served as a garnish.
- *In the Asteraceae family:* Wild lettuces (*Lactuca serriola* L., *L. virosa* L., and *Taraxacum* species)
- *In the Apiaceae family:* Wild carrots (*Daucus carota* L.) and *Angelica sylvestris* L. and *A. major* Lag. *D. carota* tuberous root is edible while young and was consumed either raw or cooked. The leaves, tender shoots and stems of Angelica species were prepared as vegetables. Sometimes could be boiled to a stew. They were also used raw, as an aromatic addition to salads.

Some of these wild foods (*e.g.,* the edible fruits of the four Rosaceae species *C. monogyna*, *P. spinosa*, *R. ulmifolius* and *F. vesca*) have been already characterized in terms of nutrients and bioactive properties in order to highlight their potential for human nutrition (Barros, Carvalho, Morais, *et al.* 2010, Morales *et al.* 2013, Dias *et al.* 2016). However, despite the importance of these crops' wild relatives, this review is focused on the wild harvested plants presented in **Table 2**.

2.1.3. Nutritional composition

The nutritional composition of the selected WEPs is discussed below, as well as its contribution to the recommended dietary allowances (RDA) of certain nutrients for male and female adults (31 through 50 years), *i.e.,* the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all healthy individuals in this age-specific group (Otten *et al.* 2006). Additionally, a plant part was considered as a *source* of a certain nutrient when a 100-g portion contributes in more than 15% for the RDA and as having a *high content* when the same 100-g portion contributes to more than 30% (**Annexe 1**).

2.1.3.1. Proximate composition

The proximate composition comprising the major nutritional value indicators and the energetic value of the selected WEPs is presented in **Table 3**. The midribs of basal leaves of *Silybum marianum*, the aerial parts of the semi-aquatic plants *Montia fontana* L. and *Nasturtium officinale*, the young stems with leaves of *Apium nodiflorum* (L.) Lag. and the leaves of *Portulaca oleracea* L. have the highest moisture content (ranging from 91.8 to 93.4 g/100 g) (Cowan *et al.* 1963, Oliveira *et al.* 2009, Pereira *et al.* 2011, Tardío *et al.* 2011, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Pinela, Barreira, Barros, Antonio, *et al.* 2016). These plants are usually consumed raw in salads, sautéed or cooked (**Table 2**). In turn, the immature fruits of *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a) and plants used as a condiment or spice (mainly flowering parts) reveal the lowest moisture content (< 62 g/100 g). This is the case of *Thymus pulegioides*, *T. mastichina*, *Mentha pulegium*, *Origanum vulgare*, *Pterospartum tridentatum* and *Helichrysum stoechas* (Barros *et al.* 2010, 2011, Fernandes *et al.* 2010, Pinela *et al.* 2011).

The protein levels of the selected WEPs range from 0.6 to 6.2 g/100 g (**Table 3**). The flowers of *P. tridentatum* (Pinela *et al.* 2011) and the young aerial parts of *Bryonia dioica* (**Figure 2**) (Martins *et al.* 2011, García Herrera 2014) present the highest amounts. However, a 100-g portion of these plants only contributes in 11.1/13.5 and 10.0/12.2% for the RDA for male/female adults, respectively (**Annexe 1**). Thus, the selected edible plants are not good sources of proteins. These gross constituents are generally calculated based on the amount of total Kjeldahl nitrogen that is multiplied by the conversion factor 6.25 (Regulation (EU) No 1169/2011, AOAC 2016).

The fat levels are ≤ 4.87 g/100 g and the higher amounts are presented by the immature fruits of *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a) and the flowering aerial parts of the aromatic plants *T. mastichina* and *O. vulgare* (Barros *et al.* 2011). Taking into consideration the reference intake of 70 g/day for total fat for adults (Regulation (EU) No 1169/2011), it can be concluded that all selected plants are low-fat foods (contributions ≤ 7%).

The immature fruits of *M. sylvestris* and the flowering aerial parts of *T. pulegioides* and *O. vulgare* were those to present the highest carbohydrates content (Barros, Carvalho, and Ferreira 2010a, Fernandes *et al.* 2010, Barros *et al.* 2011), which provide 31 – 34% of the RDA (130 g/day) for adults. Other seven pants, including the flowering aerial parts of *T. mastichina* (36.6 g/100 g) (Barros *et al.* 2011) and *M. pulegium* (34.4 g/100 g) (Fernandes *et al.* 2010) were also reported as a rich source of carbohydrates. These macronutrients are important short-term energy-storage compounds.

Table 3 Proximate composition (g/100 g) and energetic value (kcal/100 g) of the selected WEPs. It is presented the mean value and, in parentheses, the range of variability of the literature data.

Plant species	Ep[a]	Moisture	Proteins	Fat	Carbohydrates	Dietary fibre	Ash	Energy[b]	References
A. ampeloprasum	Ep1	78.3 (76.0-81.5)	1.7 (1.2-2.0)	0.18 (0.12-0.23)	16.6 (12.0-20.9)	4.2 (3.6-4.7)	0.8 (0.5-1.0)	79	García-Herrera *et al.* (2013), García-Herrera, Morales, *et al.* (2014)
A. azurea	Ep1	91.2 (88.9-92.7)	1.9 (1.1-2.8)	0.15 (0.07-0.23)	1.3 (0.9-1.8)	3.9 (3.5-4.4)	1.9 (0.1.8-2.1)	18	García Herrera (2014)
A. nodiflorum	Ep1	92.0 (90.0-94.0)	1.6 (1.1-2.1)	0.10 (0.07-0.14)	1.2 (0.7-2.1)	2.7 (1.9-3.4)	1.7 (1.0-3.3)	15	García Herrera (2014)
A. acutifolius	Ep1	84.8 (80.1-87.2)	3.2 (2.9-3.4)	0.29 (0.12-0.61)	5.4 (2.1-9.4)	4.3 (3.3-5.9)	1.4 (1.1-1.9)	41	Martins *et al.* (2011), García Herrera (2014)
B. maritima	Ep1	84.5 (75.4-89.1)	2.6 (1.8-3.6)	0.24 (0.16-0.40)	3.6 (2.9-4.3)	5.9 (3.9-9.5)	3.4 (2.0-5.6)	33	García Herrera (2014)
B. officinalis	Ep1	86.9 (86.5-87.3)	1.2 (1.0-1.4)	0.16 (0.13-0.19)	9.5 (9.2-9.7)	-	2.4 (2.2-2.5)	44	Pereira *et al.* (2011)
B. dioica	Ep1	84.0 (70.9-89.8)	5.6 (1.0-11.9)	0.69 (0.17-2.58)	3.3 (0.8-10.2)	5.6 (3.4-11.7)	1.9 (1.1-3.3)	47	Martins *et al.* (2011), García Herrera (2014)
C. intybus	Ep1	86.4 (84.8-87.9)	2.9 (1.5-4.3)	0.13 (tr-0.25)	3.5 (1.8-4.7)	6.1 (5.1-6.7)	1.8 (1.7-2.1)	33	García-Herrera, Sánchez-Mata, *et al.* (2014)
C. ambrosioides	Ep1	72.61	-	-	-	-	-	-	Barros *et al.* (2013)
C. juncea	Ep1	84.6 (65.9-89.7)	2.1 (1.9-6.1)	0.35 (0.09-0.79)	2.7 (1.5-9.7)	7.8 (4.1-13.4)	2.3 (1.4-4.4)	30	García-Herrera, Sánchez-Mata, *et al.* (2014), Ranfa *et al.* (2014)
F. vulgare	Ep1	86.7 (85.1-90.1)	2.1 (0.6-3.8)	0.17 (0.08-0.23)	3.1 (1.4-4.9)	5.1 (3.5-6.2)	1.9 (1.7-2.3)	27	Trichopoulou *et al.* (2000), García Herrera (2014)
	Ep2	73.9 (73.1-74.7)	1.3 (1.3-1.4)	0.49 (0.44-0.54)	21.9 (21.4-22.5)	-	2.4	84	Barros, Carvalho, and Ferreira (2010b)
	Ep3	76.4 (76.0-76.7)	1.2 (1.1-1.2)	0.61 (0.45-0.77)	18.4 (18.4-18.5)	-	3.4 (3.4-3.5)	86	Barros, Carvalho, and Ferreira (2010b)
	Ep4	77.5 (76.4-78.5)	1.1	0.45 (0.38-0.52)	19.4 (18.7-20.0)	-	1.6 (1.5-1.7)	97	Barros, Carvalho, and Ferreira (2010b)
	Ep5	71.3 (67.3-75.3)	1.4 (1.3-1.4)	1.28 (1.00-1.56)	22.8 (19.8-25.9)	-	3.2 (3.2-3.3)	108	Barros, Carvalho, and Ferreira (2010b)
G. hederacea	Ep1	73.0 (65.0-81.1)	1.3	1.18 (0.95-1.41)	21.0 (20.8-21.2)	-	3.47	100	Barros *et al.* (2011)
H. stoechas	Ep1	61.85	-	-	-	-	-	-	Barros *et al.* (2010)
H. lupulus	Ep1	85.5 (85.2-93.2)	4.3 (3.1-5.1)	0.20 (0.11-0.26)	1.6 (1.4-1.8)	5.2 (4.3-6.4)	1.4 (0.9-2.0)	31	García Herrera (2014)
M. sylvestris	Ep1	76.3 (75.8-76.8)	2.9 (2.7-3.1)	0.65 (0.56-0.74)	16.9 (16.8-17.1)	-	3.2 (3.1-3.3)	85	Barros, Carvalho, and Ferreira (2010a)
	Ep2	72.5 (70.6-74.4)	2.3 (2.2-2.5)	0.78 (0.68-0.88)	21.5 (21.4-21.6)	-	2.9 (2.8-3.0)	85	Barros, Carvalho, and Ferreira (2010a)
	Ep3	45.6 (44.6-46.6)	1.8 (1.6-1.9)	4.87 (4.75-4.99)	40.8 (40.2-41.4)	-	7.0 (6.6-7.4)	102	Barros, Carvalho, and Ferreira (2010a)
	Ep4	77.3 (75.9-78.6)	3.2 (3.1-3.3)	0.70 (0.64-0.76)	16.4 (16.3-16.4)	-	2.5 (2.4-2.5)	214	Barros, Carvalho, and Ferreira (2010a)
M. pulegium	Ep1	59.5 (50.3-68.7)	2.9 (2.7-3.1)	0.90 (0.81-0.99)	34.4 (34.1-34.6)	-	2.4 (2.4-2.5)	157	Fernandes *et al.* (2010)
M. fontana	Ep1	92.2 (88.9-95.2)	1.6 (0.6-2.0)	1.66 (0.15-2.15)	2.0 (0.5-3.3)	4.4 (4.0-5.4)	1.1 (0.7-1.5)	34	Pereira *et al.* (2011), Tardío *et al.* (2011), García Herrera (2014)

Plant species	Ep[a]	Moisture	Proteins	Fat	Carbohydrates	Dietary fibre	Ash	Energy[b]	References
N. officinale	Ep1	92.2 (91.1-93.2)	1.6 (0.9-2.2)	0.17 (0.14-0.20)	4.1 (3.6-4.5)	1.8 (1.5-2.0)	1.0 (0.9-1.1)	24	Souci *et al.* (2008), Pereira *et al.* (2011), Pinela, Barreira, Barros, Antonio, *et al.* (2016)
O. vulgare	Ep1	51.8 (46.7-56.9)	2.3 (2.2-2.3)	2.81 (2.48-3.14)	40.2 (39.9-40.5)	-	2.9 (2.8-2.9)	195	Barros *et al.* (2011)
P. rhoeas	Ep1	88.1 (68.5-91.0)	3.7 (1.5-5.9)	0.25 (0.15-0.38)	3.5 (2.9-5.3)	5.5 (2.7-11.1)	3.1 (1.9-5.2)	37	Trichopoulou *et al.* (2000), García Herrera (2014)
P. oleracea	Ep1	91.8 (91.1-92.3)	2.5	0.39 (0.30-0.44)	2.7	0.9	1.8	25	Cowan *et al.* (1963), Oliveira *et al.* (2009)
P. tridentatum	Ep1	60.8 (60.6-61.0)	6.2 (6.0-6.5)	1.05 (0.85-1.25)	31.0 (30.7-31.3)	-	0.93	158	Pinela *et al.* (2011)
R. ulmifolius	Ep1	49.19	-	-	-	-	-	-	Barros *et al.* (2010)
	Ep2	82.89	-	-	-	-	-	-	Barros *et al.* (2010)
R. acetosella	Ep1	89.1 (88.1-90.1)	0.9 (0.7-1.1)	0.26 (0.23-0.29)	8.6 (8.4-8.8)	-	1.2 (1.1-1.3)	40	Pereira *et al.* (2011)
R. induratus	Ep1	90.1 (90.0-90.3)	2.1 (1.3-2.9)	0.39 (0.37-0.41)	6.2 (5.5-6.9)	-	1.0 (0.9-1.1)	37	Pereira *et al.* (2011), Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* (2016)
R. papillaris	Ep1	89.1 (87.8-90.7)	2.4 (1.6-3.5)	0.22 (0.26-0.28)	2.0 (1.6-2.7)	4.4 (4.0-5.0)	1.0 (0.4-1.3)	24	García Herrera (2014)
R. pulcher	Ep1	86.6 (87.4-89.2)	3.2 (1.9-5.5)	0.20 (0.10-0.32)	3.3 (1.5-4.5)	4.7 (4-5.2)	1.9 (1.1-3.1)	33	García Herrera (2014)
S. hispanicus	Ep1	84.1 (81.8-92.7)	1.8 (0.3-5.3)	0.09 (0.08-0.11)	3.4 (1.1-9.2)	7.0 (3.1-12.)	3.19 (1.7-5.2)	29	García-Herrera, Sánchez-Mata, *et al.* (2014)
S. vulgaris	Ep1	87.1 (80.4-88.5)	3.1 (1.9-3.6)	0.63 (0.35-0.80)	2.9 (1.0-3.9)	3.6 (2.6-7.0)	0.8 (0.3-3.8)	33	Alarcón *et al.* 2006, García Herrera (2014)
S. marianum	Ep1	93.4 (92.9-93.8)	0.6 (0.5-0.8)	0.01 (tr-0.03)	1.1 (0.5-1.7)	2.6 (2.3-2.9)	1.5 (1.0-1.9)	10	García-Herrera, Sánchez-Mata, *et al.* (2014)
S. oleraceus	Ep1	88.7 (83.2-91.9)	2.3 (1.3-3.5)	0.39 (0.20-0.75)	2.5 (0.9-4.2)	3.9 (2.6-5.6)	2.2 (1.6-3.0)	27	Guil-Guerrero *et al.* (1998), Trichopoulou *et al.* (2000), García-Herrera, Sánchez-Mata, *et al.* (2014)
T. communis	Ep1	86.2 (83.3-89.0)	3.2 (2.5-3.8)	0.24 (0.10-0.51)	4.1 (1.9-11.6)	4.7 (3.5-6.0)	1.4 (0.9-2.4)	36	Martins *et al.* (2011), García Herrera (2014)
T. obovatum	Ep1	83.3 (79.2-86.7)	1.6 (1.0-2.1)	0.22 (0.19-0.27)	3.3 (1.6-5.4)	7.0 (5.4-8.7)	2.1 (1.8-2.5)	29	García-Herrera, Sánchez-Mata, *et al.* (2014)
T. mastichina	Ep1	54.7 (47.6-61.7)	2.2 (2.2-2.3)	3.80 (3.70-3.90)	36.6 (36.6-36.7)	-	2.7 (2.6-2.8)	189	Barros *et al.* (2011)
T. pulegioides	Ep1	47.7 (35.1-60.3)	3.7 (3.5-4.0)	1.16 (1.04-1.28)	44.4 (44.0-44.7)	-	3.1 (3.1-3.2)	203	Fernandes *et al.* (2010)

[a] Edible part (Ep) is presented in **Table 2**.

[b] Energy value was calculated according to the Regulation (EU) No 1169/2011 of the European Parliament and of the Council using mean values.

Some values were converted from a dry weight basis or other units.

As shown in **Table 3**, information on dietary fibre content in the selected WEPs is limited. The RDA of this food component for male and female adults is 38 and 25 g/day, respectively (Otten *et al.* 2006). The basal leaves of *C. juncea*, *Taraxacum obovatum* (Willd.) DC., *C. intybus* and *B. maritima* and the midribs of basal leaves of *S. hispanicus* are those that contribute the most (15.5 – 20.5%) to the daily intakes established for male adults (**Annexe 1**). For female adults, other plants arise as interesting sources of dietary fibre (with levels ranging from 3.9 to 5.6 g/100 g), namely the four species of wild asparagus (*A. acutifolius*, *H. lupulus*, *T. communis* and *B. dioica*) (Martins *et al.* 2011, García Herrera 2014), the basal leaves of *P. rhoeas*, *R. pulcher*, *R. papillaris*, *S. oleraceus* and *A. azurea* (Guil-Guerrero *et al.* 1998, Trichopoulou *et al.* 2000, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014), the young shoots with leaves of *F. vulgare* (Trichopoulou *et al.* 2000, García Herrera 2014), the bulb and pseudostem of *A. ampeloprasum* (García-Herrera *et al.* 2013, García-Herrera, Morales, *et al.* 2014), and the aerial parts of *M. fontana* (Pereira *et al.* 2011, Tardío *et al.* 2011, García Herrera 2014). Thus, the inclusion of these plants in contemporary diets can potentially increase the dietary fibre intake.

The ash content corresponds to the total amount of minerals present within a food. In addition to high levels of fat and carbohydrates, the immature fruits of *M. sylvestris* also present the higher ash content (7.0 g/100 g) (Barros, Carvalho, and Ferreira 2010b). With high levels also arise the leaves and stems of *G. hederacea* (Barros *et al.* 2011), the leaves of *F. vulgare* and *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a, 2010b), the basal leaves of *B. maritima* and *P. rhoeas* (García Herrera 2014), the inflorescences *F. vulgare* (Barros, Carvalho, and Ferreira 2010b) and the flowering aerial parts of *T. pulegioides* (Fernandes *et al.* 2010).

Based on the amount (mean value) and calorific contribution from proteins (4 kcal/100 g), carbohydrates (4 kcal/100 g), fat (9 kcal/100 g) and dietary fibre (2 kcal/100 g) (Regulation (EU) No 1169/2011), it was possible to calculate the energetic value for each edible plant (**Table 3**). The plant parts with the highest energetic value are the flowering stems of *M. sylvestris* and the inflorescences of *T. pulegioides* (214 and 203 kcal/100 g, respectively). Based on the reference intakes for energy (2000 kcal/day, for adults) (Regulation (EU) No 1169/2011), a 100-g portion of these plants only contributes in ~10 – 11%. However, it is a daily amount difficult to reach, since the flowering stems of *M. sylvestris* are usually consumed as infusions to relieve stomach pain and to regulate the intestine, while the inflorescences of *T. pulegioides* are used as a condiment or spice and in tisanes (Carvalho 2010). The contribution of all the other plants is lower than 10%. Therefore, all these WEPs are appropriate foods for low-calorie diets.

2.1.3.2. Individual sugars

The composition in individual sugar molecules of the selected WEPs is presented in **Table 4**. These compounds are included within the carbohydrates fraction and can be classified by the number of sugar units into monosaccharides (such as fructose and glucose), disaccharides (such as sucrose and trehalose) and oligosaccharides (such as raffinose). *F. vulgare* leaves, stems and inflorescences present a total sugar content of 6570, 4920 and 4070 mg/100 g, respectively (Barros, Carvalho, and Ferreira 2010b). The sugars fraction of these *F. vulgare* edible parts is composed by 70-72% fructose, 23-30% glucose and 0-5% sucrose. This plant has important culinary and medicinal uses (**Table 2**). Shoots, tender leaves and stems are chewed and sucked due to its exquisite aniseed flavour and used as vegetables (raw as a snack or in salads, cooked), seasonings, and food preservatives. The stems are traditionally used in the brines prepared for the olives' cure. Herbal beverages prepared with fresh tender or dried flowering stems are drunk chilled or hot, depending on the season (Tardío *et al.* 2006, Carvalho 2010). In fact, the culinary value of this plant might be related to its organoleptic properties and high levels of sugars.

In general, while the highest sugars content is presented in the flowering parts of the selected wild plants, the lowest amounts are found in leafy vegetables. The flowering parts of *Malva sylvestris* (Barros, Carvalho, and Ferreira 2010a), *M. pulegium* (Fernandes *et al.* 2010), *R. ulmifolius* (Barros *et al.* 2010), *F. vulgare* (Barros, Carvalho, and Ferreira 2010b) and *P. tridentatum* (Pinela *et al.* 2011) were reported as having a total sugars content ranging from 2066 mg/100 (in *P. tridentatum*) to 5508 mg/100 g (in *M. sylvestris*). Some of these plant parts have been used as condiment and/or in herbal beverages to treat a number of health conditions (**Table 2**). In turn, amounts lower than 700 mg/100 g were detected in *P. oleracea* (Petropoulos *et al.* 2015), *N. officinale*, *R. induratus* (Pereira *et al.* 2011, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016), *R. acetosella*, *B. officinalis* and *M. fontana* (Pereira *et al.* 2011), wild greens commonly eaten raw in salads or cooked (**Table 2**).

Table 4 Composition in individual sugars (mg/100 g) of the selected WEPs. It is presented the mean value and, in parentheses, the range of variability of the literature data.

Plant species	Ep[a]	Fructose	Glucose	Sucrose	Total sugars	References
A. acutifolius	Ep1	384 (363-404)	305 (299-311)	658 (639-676)	1423 (1380-1466)	Martins *et al.* (2011)
B. officinalis	Ep1	18 (15-22)	76 (68-84)	200 (183-217)	324 (302-345)	Pereira *et al.* (2011)
B. dioica	Ep1	590 (576-604)	508 (493-523)	98 (93-100)	1248 (1216-1281)	Martins *et al.* (2011)
C. ambrosioides	Ep1	66 (64-69)	126 (123-129)	392 (359-425)	833 (813-852)	Barros *et al.* (2013)
F. vulgare	Ep2	1510 (1450-1570)	4710 (4560-4860)	350 (290-410)	6570 (6400-6740)	Barros, Carvalho, and Ferreira (2010b)
	Ep3	490 (440-540)	760 (640-880)	40 (39-41)	1290 (1090-1490)	
	Ep4	1490 (1450-1530)	3430 (3230-3630)	nd	4920 (4690-5150)	
	Ep5	1100 (1060-1140)	2940 (2830-3050)	30 (29-31)	4070 (3910-4230)	
G. hederacea	Ep1	150 (140-160)	80 (60-100)	400 (340-460)	1040 (970-1110)	Barros *et al.* (2011)
H. stoechas	Ep1	389 (374-404)	225 (217-233)	702 (668-736)	1476 (1449-1503)	Barros *et al.* (2010)
M. sylvestris	Ep1	431 (377-486)	747 (645-848)	941 (934-948)	2752 (2631-2872)	Barros, Carvalho, and Ferreira (2010a)
	Ep2	2399 (2360-2437)	2025 (1987-2061)	680 (666-693)	5508 (5436-5579)	
	Ep3	218 (201-234)	827 (789-865)	60 (44-76)	1251 (1197-1306)	
	Ep4	803 (762-844)	1078 (1037-1119)	750 (728-773)	3336 (3225-3447)	
M. pulegium	Ep1	969 (924-1013)	1366 (1277-1455)	1872 (1759-1986)	4576 (4329-4823)	Fernandes *et al.* (2010)
M. fontana	Ep1	36 (28-44)	48 (47-49)	21 (19-23)	137 (121-152)	Pereira *et al.* (2011)
N. officinale	Ep1	85 (59-113)	63 (50-72)	45 (18-79)	206 (192-228)	Pereira *et al.* (2011), Pinela, Barreira, Barros, Antonio, *et al.* (2016)
O. vulgare	Ep1	190 (180-200)	580 (570-590)	300 (299-301)	1120 (1100-1140)	Barros *et al.* (2011)
P. oleracea	Ep1	259 (118–352)	86 (52-138)	151 (75-271)	549 (293-785)	Petropoulos *et al.* (2015)
P. tridentatum	Ep1	1368 (1325-1411)	466 (447-486)	227 (216-239)	2066 (2031-2101)	Pinela *et al.* (2011)
R. ulmifolius	Ep1	1692 (1677-1707)	1885 (1870-1900)	432 (427-437)	4487 (4477-4497)	Barros *et al.* (2010)
	Ep2	284 (248-320)	382 (349-414)	229 (204-255)	1032 (931-1133)	
R. acetosella	Ep1	65 (65-66)	80 (79-81)	23 (15-31)	250 (229-271)	Pereira *et al.* (2011)

Plant species	Ep[a]	Fructose	Glucose	Sucrose	Total sugars	References
R. induratus	Ep1	283 (157-418)	254 (103-402)	94 (62-151)	697 (452-945)	Pereira *et al.* (2011), Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* (2016)
T. communis	Ep1	640 (618-661)	301 (277-324)	116 (108-124)	1279 (1258-1301)	Martins *et al.* (2011)
T. mastichina	Ep1	450 (440-460)	970 (860-1080)	20 (19-21)	1440 (1330-1550)	Barros *et al.* (2011)
T. pulegioides	Ep1	115 (115-116)	173 (157-188)	555 (544-565)	1251 (1183-1319)	Fernandes *et al.* (2010)

[a] Edible part (Ep) is presented in **Table 2**.

Some values were converted from a dry weight basis or other units.

nd: not detected

As presented in **Table 4**, fructose and glucose are the most abundant sugars in almost all plants. These molecules are absorbed by the human body and transported to the cells as a source of energy. High levels of these reducing sugars were reported in flowers of *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a), flower buds of *R. ulmifolius* (Barros *et al.* 2010) and leaves, stems and inflorescences of *F. vulgare* (Barros, Carvalho, and Ferreira 2010b). In turn, the flowering parts of *M. pulegium* (Fernandes *et al.* 2010), the flowering stems and leaves of *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a) and the inflorescences and leafy flowering stems of *H. stoechas* (Barros *et al.* 2010) were those with the highest sucrose content (702 to 1872 mg/100 g). Nevertheless, if considering the relative percentage of each sugar, it is possible to verify that the sugars fraction of *P. tridentatum*, *T. communis*, *B. dioica* and *P. oleracea* is mainly formed by fructose (> 47%), while glucose predominates in *F. vulgare*, *T. mastichina*, *M. sylvestris* and *O. vulgare* (> 50%). Despite low in sugars, sucrose was the most abundant sugar molecule in *B. officinalis* (62%) and *C. ambrosioides* (47%).

According to the Regulation (EU) No 1169/2011 of the European Parliament and of the Council, the reference intake for sugars is 90 g for adults. A 100-g portion of the selected wild plants contributes to less than 7.5% for the intake of these macronutrients. Sugars make up about 38% of the carbohydrates fraction of *B. dioica* and 30-31% of the carbohydrates fraction of the *F. vulgare* shoots and *T. communis*. The three plants reported as a source of carbohydrates (*M. sylvestris* immature fruits, *T. pulegioides* and *O. vulgare*) (Barros, Carvalho, and Ferreira 2010a, Fernandes *et al.* 2010, Barros *et al.* 2011) present only 3% of individual sugars in the composition of these nutritional value indicator. This means that other constituents (such as starch and polyols or dietary fibre) are present in greater quantity in this fraction (Smolin and Grosvenor 2016). In fact, when the dietary fibre content is not analysed, it is included in the carbohydrates fraction that can be calculated by difference (AOAC 2016). In this case, the result is expressed in total carbohydrates content. On the other hand, the result is expressed in available carbohydrates content when the dietary fibre is not included.

2.1.3.3. Fatty acids

The relative percentage of palmitic (C16:0), oleic (C18:1*n*-9), linoleic (C18:2*n*-6) and α-linolenic (C18:3*n*-3) acids and of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in the selected edible wild species is presented in **Table 5**. In many cases, the crude fat is composed by more than 90% of these four fatty acids. C16:0 predominates in the midribs of basal leaves of *S. marianum* (28.7%) (Morales, Ferreira, *et al.* 2012), in the bulb and pseudostem of *A. ampeloprasum* (26.4%) (Morales, Ferreira, *et al.* 2012, García-Herrera, Morales, *et al.* 2014) and in stems of *F.*

vulgare (25.4%) (Barros, Carvalho, and Ferreira 2010b). However, the higher levels of SFA (> 51%) were reported in *B. officinalis* (Pereira *et al.* 2011), mainly due to the contribution of C16:0 (12%), C20:0 (11.9%) and C22:0 (12%). The relative percentage of C18:1*n*-9 was reported to be particularly high in leaves and stems of *G. hederacea* (35.1%) (Barros *et al.* 2011), but also in the edible parts of *P. oleracea* (12.4%) (Petropoulos *et al.* 2015) and *T. pulegioides* (11.4%) (Fernandes *et al.* 2010). These species also show the highest MUFA content (36.2, 15.1 and 12.8%, respectively).

Regarding PUFA, levels above 80% were reported in young shoots with leaves of *O. vulgare*, in basal leaves of *C. intybus*, *P. rhoeas*, *R. pulcher*, *A. azurea* and *S. oleraceus*, in aerial parts of *N. officinale*, and in leaves of *M. sylvestris* (Barros *et al.* 2011, Pereira *et al.* 2011, Morales, Ferreira, *et al.* 2012). These species have the highest percentage of C18:3*n*-3 (ranging from 60.5 to 68.4%; **Table 5**); except for *S. oleraceus* where this fatty acid was not detected. In this plant, γ-linolenic acid (C18:3*n*-6) was quantified as the most abundant one (66.3%) (Morales, Ferreira, *et al.* 2012), which was also present in high amounts in *B. officinalis* (10.9%) (Pereira *et al.* 2011). C18:3*n*-3 is precursor of the long-chain *n*-3 PUFA eicosapentaenoic (C20:5*n*-3) and docosahexaenoic (C22:6*n*-3) acids. However, humans have a limited capacity for the conversion of C18:3*n*-3 to C20:5*n*-3 and C22:6*n*-3 (Baker *et al.* 2016). The PUFA C18:2*n*-6 constitutes 40 to ~54% of the crude fat of *A. ampeloprasum*, *M. sylvestris* (immature fruits), *A. acutifolius*, *T. communis* and *F. vulgare* (shoots) (Barros, Carvalho, and Ferreira 2010a, 2010b, Martins *et al.* 2011, Morales, Ferreira, *et al.* 2012, García-Herrera, Morales, *et al.* 2014). The *n*-6 PUFA C18:2*n*-6 and the *n*-3 PUFA C18:3*n*-3 are essential fatty acids that must be obtained from dietary sources (Baker *et al.* 2016). A daily intake of 17/12 g of C18:2*n*-6 and 1.6/1.1 g of C18:3*n*-3 is recommended for male/female adults to prevent a number of diseases (Otten *et al.* 2006).

In general, the WEPs under review have a healthy fatty acids profile, with PUFA/SFA ratios higher than 0.45. Its inclusion in the human diet may be beneficial, since the intake of essential fatty acids have been linked with optimal brain and vision function, reduction of cardiovascular, inflammatory and autoimmune diseases, and cancer prevention (Simopoulos 2002, Kaur *et al.* 2014, Baker *et al.* 2016). These beneficial effects may be related to different mechanisms, including alteration in cell membrane composition, gene expression or eicosanoid production (Kaur *et al.* 2014).

Table 5 Composition in main fatty acids and categories (relative percentage) of the selected WEPs. It is presented the mean value and, in parentheses, the range of variability of the literature data.

Plant species	Ep[a]	Individual fatty acids				Categories			References
		Palmitic acid	Oleic acid	Linoleic acid	α-Linolenic acid	SFA	MUFA	PUFA	
A. ampeloprasum	Ep1	26.4 (26.1-26.7)	7.4 (7.0-7.8)	53.5 (53.2-53.7)	nd	38.2 (37.6-38.9)	7.6 (7.2-8.1)	54.2 (53.9-54.5)	Morales, Ferreira, *et al.* (2012), García-Herrera, Morales, *et al.* (2014)
A. azurea	Ep1	10.5 (9.8-11.1)	2.2 (-)	12.2 (12.1-12.3)	64.7 (64.5-65.0)	16.6 (15.8-17.4)	3.2 (3.1-3.2)	80.3 (79.4-81.1)	Morales, Ferreira, *et al.* (2012)
A. nodiflorum	Ep1	16.3 (15.3-17.3)	3.3 (3.3-3.4)	24.6 (23.8-25.4)	43.5 (43.4-43.5)	23.9 (22.4-25.5)	5.4 (4.9-5.8)	70.7 (68.7-72.7)	Morales, Ferreira, *et al.* (2012)
A. acutifolius	Ep1	23.1 (17.5-28.7)	4.9 (4.8-5.0)	43.4 (42.3-44.5)	18.9 (14.0-23.7)	32.5 (26.2-38.8)	5.1 (4.8-5.3)	62.4 (56.3-68.5)	Martins *et al.* (2011), Morales, Ferreira, *et al.* (2012)
B. maritima	Ep1	11.0 (10.9-11.2)	3.5 (-)	21.3 (21.2-21.3)	57.8 (-)	16.7 (-)	4.0 (-)	79.3 (-)	Morales, Ferreira, *et al.* (2012)
B. officinalis	Ep1	12.0 (11.3-12.7)	2.1 (1.9-2.3)	9.5 (8.3-10.8)	12.3 (12.1-12.5)	51.3 (50.4-52.3)	3.3 (2.8-3.8)	45.4 (43.5-47.3)	Pereira *et al.* (2011)
B. dioica	Ep1	15.3 (13.5-17.0)	1.4 (1.2-1.6)	6.4 (6.2-6.6)	69.0 (67.8-70.3)	22.7 (21.4-24.0)	1.7 (1.6-1.8)	75.6 (74.4-77.0)	Martins *et al.* (2011), Morales, Ferreira, *et al.* (2012)
C. intybus	Ep1	10.6 (10.0-11.3)	1.6 (1.6-1.7)	21.1 (21.1-21.2)	60.5 (60.0-60.9)	16.0 (15.4-16.5)	2.0 (-)	82.1 (82.0-82.2)	Morales, Ferreira, *et al.* (2012)
C. ambrosioides	Ep1	14.2 (14.1-14.2)	6.9 (6.8-7.0)	19.2 (19.1-19.4)	48.5 (48.4-48.7)	23.9 (23.8-24.0)	7.7 (7.5-7.9)	68.4 (68.4-68.5)	Barros *et al.* (2013)
C. juncea	Ep1	13.0 (12.5-13.4)	1.9 (-)	19.9 (19.8-20.1)	56.3 (56.1-56.4)	21.4 (-)	2.1 (-)	76.5 (76.5-76.6)	Morales, Ferreira, *et al.* (2012)
F. vulgare	Ep1	17.4 (17.1-17.7)	2.1 (2.1-2.2)	37.0 (36.9-37.1)	35.5 (35.0-36.1)	24.0 (23.7-24.2)	3.1 (3.0-3.2)	73.0 (72.6-73.3)	Morales, Ferreira, *et al.* (2012)
	Ep2	12.8 (12.7-12.9)	2.6 (2.2-2.9)	40.0 (39.3-40.7)	36.8 (36.3-37.4)	20.0 (19.8-20.1)	2.7 (2.4-3.1)	77.3 (77.1-77.6)	Barros, Carvalho, and Ferreira (2010b)
	Ep3	20.2 (20.1-20.2)	4.4 (4.0-4.7)	23.3 (23.2-23.3)	43.6 (43.2-44.0)	28.0 (-)	5.0 (4.6-5.4)	67.1 (66.6-67.5)	Barros, Carvalho, and Ferreira (2010b)
	Ep4	25.4 (-)	4.4 (3.8-4.9)	38.2 (37.5-39.2)	22.9 (21.6-24.2)	33.8 (33.8-33.9)	4.8 (4.2-5.4)	61.4 (60.8-62.0)	Barros, Carvalho, and Ferreira (2010b)
	Ep5	23.9 (23.8-24.0)	5.1 (-)	38.9 (38.7-39.2)	17.6 (-)	37.4 (37.2-37.7)	5.6 (5.5-5.7)	56.9 (56.8-57.1)	Barros, Carvalho, and Ferreira (2010b)
G. hederacea	Ep1	12.2 (12.0-12.5)	35.1 (34.9-35.4)	8.2 (8.1-8.2)	27.9 (27.7-28.1)	26.8 (26.3-27.3)	36.2 (35.9-36.4)	37.1 (36.8-37.3)	Barros *et al.* (2011)
H. stoechas	Ep1	13.2 (13.1-13.4)	6.2 (2.4-3.9)	25.7 (25.6-25.8)	22.8 (20.9-24.7)	44.0 (43.2-44.9)	7.0 (6.1-7.8)	48.7 (46.9-50.5)	Barros *et al.* (2010)
H. lupulus	Ep1	19.5 (18.9-20.1)	1.9 (1.8-2.0)	29.7 (28.9-30.6)	38.2 (38.1-38.2)	37.5 (37.2-37.7)	5.6 (5.5-5.7)	69.0 (68.2-69.9)	Morales, Ferreira, *et al.* (2012)
M. sylvestris	Ep1	9.8 (8.7-10.9)	3.3 (2.9-3.7)	12.0 (11.5-12.4)	67.8 (66.8-68.8)	16.3 (15.4-17.2)	3.7 (3.3-4.0)	80.0 (79.5-80.5)	Barros, Carvalho, and Ferreira (2010a)
	Ep2	17.2 (17.1-17.2)	6.1 (-)	23.5 (23.4-23.7)	33.5 (33.4-33.6)	35.8 (35.6-36.1)	6.8 (6.6-7.1)	57.3 (-)	Barros, Carvalho, and Ferreira (2010a)
	Ep3	19.8 (19.3-20.2)	6.2 (6.1-6.2)	46.0 (45.9-46.2)	10.3 (10.3-10.4)	36.8 (-)	6.5 (6.5-6.6)	56.7 (56.6-57.3)	Barros, Carvalho, and Ferreira (2010a)
	Ep4	12.9 (12.7-13.0)	3.2 (3.1-3.2)	15.7 (15.0-16.4)	53.1 (52.5-53.6)	24.6 (24.5-24.8)	3.5 (-)	71.9 (71.7-72.1)	Barros, Carvalho, and Ferreira (2010a)

Plant species	Ep[a]	Individual fatty acids				Categories			References
		Palmitic acid	Oleic acid	Linoleic acid	α-Linolenic acid	SFA	MUFA	PUFA	
M. pulegium	Ep1	14.8 (14.7-14.9)	5.8 (5.6-6.0)	16.3 (15.9-16.6)	37.0 (36.7-37.4)	37.6 (36.8-38.5)	6.8 (6.6-7.0)	55.5 (55.0-56.0)	Fernandes *et al.* (2010)
M. fontana	Ep1	14.3 (11.3-17.2)	4.2 (2.4-6.1)	18.5 (18.2-18.7)	51.6 (47.6-55.6)	23.1 (20.8-25.4)	5.7 (4.0-7.5)	71.1 (67.1-75.2)	Pereira *et al.* (2011), Morales, Ferreira, *et al.* (2012)
N. officinale	Ep1	13.2 (12.9-13.5)	0.7 (-)	11.8 (11.7-12.0)	68.4 (68.2-68.7)	16.7 (16.2-17.2)	2.1 (-)	81.2 (80.7-81.7)	Pereira *et al.* (2011)
O. vulgare	Ep1	5.0 (4.9-5.1)	5.1 (-)	23.2 (23.1-23.4)	62.3 (62.3-62.4)	8.9 (8.8-9.1)	5.3 (-)	85.8 (85.6-86.0)	Barros *et al.* (2011)
P. rhoeas	Ep1	9.7 (9.3-10.1)	1.4 (-)	16.5 (-)	65.0 (64.9-65.1)	16.4 (16.2-16.6)	1.8 (-)	81.9 (81.6-82.1)	Morales, Ferreira, *et al.* (2012)
P. oleracea	Ep1	24.7 (23.4-26.9)	12.4 (9.7-15.1)	28.8 (25.1-32.9)	23.6 (17.9-28.4)	32.5 (29.3-37.1)	15.1 (14.1-16.3)	52.4 (48.6-56.1)	Petropoulos *et al.* (2015)
P. tridentatum	Ep1	14.8 (14.0-15.7)	9.2 (8.1-10.3)	19.6 (18.9-20.3)	29.5 (27.5-31.5)	40.4 (38.8-41.9)	10.0 (8.8-11.2)	49.6 (46.9-52.4)	Pinela *et al.* (2011)
R. ulmifolius	Ep1	12.0 (-)	4.3 (-)	16.0 (16.0-16.1)	39.6 (39.5-39.6)	39.2 (39.0-39.3)	4.6 (-)	56.1 (56.0-56.3)	Barros *et al.* (2010)
	Ep2	12.1 (11.5-12.6)	3.0 (3.0-3.1)	15.0 (14.9-15.1)	38.0 (38.0-38.1)	43.0 (42.3-43.7)	3.3 (2.4-4.1)	53.6 (53.4-53.7)	
R. acetosella	Ep1	11.2 (10.5-12.0)	3.4 (3.1-3.8)	20.2 (19.7-20.7)	51.3 (49.9-52.8)	19.5 (18.3-20.6)	8.0 (7.9-8.0)	72.6 (71.5-73.7)	Pereira *et al.* (2011)
R. induratus	Ep1	14.4 (9.4-19.5)	2.7 (2.2-3.1)	14.2 (14.7-13.8)	48.8 (38.7-58.8)	30.2 (19.8-40.6)	5.3 (3.4-7.2)	64.5 (56.0-73.0)	Pereira *et al.* (2011), Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* (2016)
R. papillaris	Ep1	11.2 (10.9-11.5)	5.8 (5.7-5.9)	22.8 (22.6-23.0)	51.8 (51.6-51.9)	15.9 (15.8-16.0)	9.0 (8.7-9.3)	75.1 (74.8-75.3)	Morales, Ferreira, *et al.* (2012)
R. pulcher	Ep1	9.3 (9.2-9.4)	4.2 (-)	17.0 (16.9-17.2)	63.0 (62.9-63.0)	14.8 (14.7-14.9)	4.8 (4.7-5.0)	80.4 (80.3-80.5)	Morales, Ferreira, *et al.* (2012)
S. hispanicus	Ep1	20.7 (19.8-21.5)	6.4 (6.3-6.5)	26.4 (26.2-26.7)	30.6 (30.3-30.8)	34.2 (33.6-34.7)	8.2 (-)	57.7 (57.1-58.2)	Morales, Ferreira, *et al.* (2012)
S. vulgaris	Ep1	15.8 (13.4-18.2)	2.6 (2.4-2.7)	22.3 (21.9-24.1)	49.6 (44.6-54.5)	28.2 (27.3-29.0)	4.4 (4.2-4.5)	67.5 (66.5-68.5)	Alarcón *et al.* 2006, Morales, Ferreira, *et al.* (2012)
S. marianum	Ep1	28.7 (27.1-30.3)	3.7 (3.8-4.0)	31.0 (30.4-31.6)	21.6 (21.4-21.9)	43.5 (42.6-44.5)	3.9 (3.8-4.0)	52.6 (51.7-53.5)	Morales, Ferreira, *et al.* (2012)
S. oleraceus	Ep1	10.4 (9.7-11.1)	0.9 (0.8-1.0)	13.8 (13.2-14.4)	nd	18.9 (18.1-19.6)	0.9 (0.8-1.0)	80.2 (79.6-80.9)	Morales, Ferreira, *et al.* (2012)
T. communis	Ep1	16.0 (14.9-17.0)	6.0 (4.6-7.5)	42.2 (41.7-42.3)	29.4 (27.5-31.3)	20.4 (18.8-22.0)	4.5 (0.9-8.2)	75.1 (69.9-80.2)	Martins *et al.* (2011), Morales, Ferreira, *et al.* (2012)
T. obovatum	Ep1	11.8 (11.7-11.9)	3.2 (3.2-3.3)	17.6 (17.6-17.7)	58.5 (58.3-58.8)	19.6 (19.3-19.9)	3.7 (3.6-3.7)	76.6 (76.2-76.9)	Morales, Ferreira, *et al.* (2012)
T. mastichina	Ep1	10.2 (10.0-10.4)	9.8 (9.6-10.0)	11.8 (11.8-11.9)	45.7 (45.1-46.2)	29.3 (29.2-29.4)	10.7 (10.4-11.0)	60.0 (59.6-60.4)	Barros *et al.* (2011)
T. pulegioides	Ep1	16.7 (14.5-16.9)	11.4 (11.3-11.5)	13.0 (12.5-13.5)	36.7 (36.0-36.5)	37.5 (36.7-38.3)	12.8 (12.8-12.9)	49.7 (48.9-50.4)	Fernandes *et al.* (2010)

[a] Edible part (Ep) is presented in **Table 2**.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3).

nd: not detected.

2.1.3.4. Mineral elements

Minerals are essential nutrients for the proper functioning of the human body (Otten *et al.* 2006, Gupta and Gupta 2014, Quintaes and Diez-Garcia 2015). They can be grouped into macrominerals (needed in higher amounts by the human body, such as sodium (Na), potassium (K), calcium (Ca) and magnesium (Mg)) and trace elements (required in lower amounts, such as copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn)). The composition in macrominerals and trace elements of the selected WEPs is presented in **Table 6**. The range of Na in the selected edible plants is between 12 mg/100 g of edible portion in *N. officinale* (Souci *et al.* 2008) and 244 mg/100 g in the young stems with leaves of *Apium nodiflorum* (García Herrera 2014), while that of K is between 165 mg/100 g in the last plant and 1223 mg/100 g in the basal leaves of *B. maritima* (Guil-Guerrero *et al.* 1999, García Herrera 2014). In fact, only *A. nodiflorum* stood out as a source of Na (a 100-g portion contributes in 16.3% for the RDA; **Annexe 1**). The contribution of all other plants (except for *B. maritima*) is lower than 9%. Besides *B. maritima*, the edible parts of *S. hispanicus*, *S. vulgaris*, *C. juncea*, *S. marianum*, *P. rhoeas* are also sources of K, with levels exceeding 711 mg/100 g (Guil-Guerrero *et al.* 1999, Trichopoulou *et al.* 2000, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Ranfa *et al.* 2014, Renna *et al.* 2015). The RDA for K is 4700 mg/day for individuals with 14 or more years old (Otten *et al.* 2006). This nutrient is easily absorbed by the body and essential to the cells. Its deficiency, despite uncommon, can cause muscle weakness, cardiac arrhythmias, high blood pressure (hypertension) and risk of cardiovascular diseases (Stein 2010).

Ca is the most abundant element in the human body. It promotes the bone health, reduces the risk of osteoporosis and helps to prevent cardiovascular diseases (Gupta and Gupta 2014). Eleven wild plant parts were found to be interesting sources of Ca (which 100-g portion provides more than 15% of the RDA for adults; **Annexe 1**), namely the basal leaves of *C. juncea*, *B. officinalis*, *P. rhoeas*, *A. azurea* and *S. oleraceus*, the midribs of basal leaves of *S. hispanicus*, the leaves of *M. sylvestris* and *P. oleracea*, the aerial parts of *N. officinale*, and the young stems with leaves of *F. vulgare* and *A. nodiflorum* (Bianco *et al.* 1998, Guil-Guerrero *et al.* 1999, Trichopoulou *et al.* 2000, Souci *et al.* 2008, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Ranfa *et al.* 2014, Petropoulos *et al.* 2015, Renna *et al.* 2015). These wild plants are traditionally consumed raw in salads, cooked or sautéed (**Table 2**).

Table 6 Composition in mineral elements (mg/100 g) of the selected WEPs. It is presented the mean value and, in parentheses, the range of variability of the literature data.

Plant species	Ep[a]	Macrominerals				Trace elements				References
		Na	K	Ca	Mg	Cu	Fe	Mn	Zn	
A. ampeloprasum	Ep1	55 (44-67)	439 (147-533)	70 (30-82)	14 (9-16)	0.11 (0.05-0.22)	0.60 (0.20-0.92)	0.11 (0.06-0.15)	0.75 (0.03-1.67)	García-Herrera *et al.* (2013), García-Herrera, Morales, *et al.* (2014)
A. azurea	Ep1	29 (14-37)	563 (488-1172)	158 (126-219)	25 (18-35)	0.13 (0.09-0.28)	1.90 (0.60-2.70)	0.24 (0.15-0.41)	0.43 (0.32-0.86)	García Herrera (2014)
A. nodiflorum	Ep1	244 (137-379)	165 (105-225)	152 (64-246)	28 (16-45)	0.08 (0.04-0.15)	1.80 (0.80-3.10)	0.29 (0.17-0.34)	0.50 (0.42-0.70)	García Herrera (2014)
A. acutifolius	Ep1	20 (9-29)	438 (318-816)	24 (6-47)	13 (2-19)	0.17 (0.13-0.24)	0.49 (0.40-0.59)	0.17 (0.08-0.47)	0.96 (0.75-1.35)	García Herrera (2014)
B. maritima	Ep1	207 (45-288)	1223 (597-2356)	96 (19-250)	73 (13-136)	0.21 (0.09-0.35)	2.24 (1.42-3.97)	0.82 (0.57-1.23)	0.88 (0.64-1.26)	Guil-Guerrero *et al.* (1999), García Herrera (2014)
B. officinalis	Ep1	88 (66-109)	214 (198-230)	232 (219-245)	20 (17-23)	0.05 (0.04-0.05)	2.79 (2.14-3.45)	1.62 (1.40-1.84)	0.35 (0.35-0.36)	Renna *et al.* (2015)
B. dioica	Ep1	33 (12-57)	623 (385-1029)	75 (39-142)	43 (25-71)	0.18 (0.01-0.46)	1.00 (0.68-1.78)	0.37 (0.11-0.61)	1.17 (0.27-2.63)	García Herrera (2014)
C. intybus	Ep1	76 (37-170)	481 (104-1085)	121 (45-168)	24 (10-34)	0.11 (0.06-0.21)	1.03 (0.41-2.00)	0.21 (0.09-0.47)	0.36 (0.08-0.51)	Bianco *et al.* (1998), García-Herrera, Sánchez-Mata, *et al.* (2014), Renna *et al.* (2015)
C. ambrosioides	Ep1									
C. juncea	Ep1	24 (4-58)	859 (433-1277)	273 (22-472)	53 (3-100)	0.43 (0.12-0.90)	3.97 (1.47-6.57)	0.97 (0.57-1.48)	1.63 (0.53-3.81)	García-Herrera, Sánchez-Mata, *et al.* (2014), Ranfa *et al.* (2014)
F. vulgare	Ep1	76 (34-126)	402 (266-619)	227 (153-341)	36 (25-54)	0.05 (0.01-0.11)	1.04 (0.07-2.35)	0.62 (0.03-0.97)	0.36 (0.25-0.57)	Trichopoulou *et al.* (2000), García Herrera (2014), Renna *et al.* (2015)
H. lupulus	Ep1	29 (25-32)	517 (314-675)	89 (50-134)	36 (29-48)	0.14 (0.10-0.17)	0.91 (0.37-1.32)	0.36 (0.18-0.55)	1.13 (0.71-1.51)	García Herrera (2014)
M. sylvestris	Ep1	93 (68-128)	652 (547-836)	240 (165-301)	283 (30-368)	0.21 (0.10-0.33)	3.61 (0.76-6.29)	0.49 (0.20-0.76)	1.58 (0.04-2.67)	Bianco *et al.* (1998), Guil-Guerrero *et al.* (1999), Sánchez-Mata and Tardío (2016)
M. fontana	Ep1	75 (61-88)	357 (265-310)	31 (24-48)	32 (26-40)	0.05 (0.04-0.06)	1.30 (0.60-1.68)	1.07 (0.66-1.93)	0.38 (0.27-0.56)	Tardío *et al.* (2011)
N. officinale	Ep1	12 (-)	276 (-)	175 (170-180)	25 (15-34)		1.65 (2.2-3.1)		0.09 (-)	Souci *et al.* (2008)
P. rhoeas	Ep1	42 (3-77)	711 (188-1673)	200 (50-545)	33 (9-74)	0.34 (0.09-1.07)	5.52 (0.90-16.33)	0.67 (0.39-1.06)	1.43 (0.20-3.19)	Trichopoulou *et al.* (2000), García Herrera (2014), Renna *et al.* (2015)

Plant species	Ep[a]	Macrominerals				Trace elements				References
		Na	K	Ca	Mg	Cu	Fe	Mn	Zn	
P. oleracea	Ep1	21 (7-42)	540 (298-705)	160 (51-234)	143 (56-276)	0.27 (0.16-0.39)	2.16 (0.16-6.82)	0.50 (0.29-0.63)	0.46 (0.21-0.88)	Bianco *et al.* (1998), Guil-Guerrero *et al.* (1999), Petropoulos *et al.* (2015), Renna *et al.* (2015)
R. papillaris	Ep1	26 (16-37.9)	351 (255-468)	60 (37-90)	45 (34-51)	0.08 (0.03-0.15)	1.00 (0.40-1.20)	0.75 (0.41-0.74)	0.36 (0.24-0.42)	García Herrera (2014)
R. pulcher	Ep1	80 (33-123)	663 (382-955)	50 (2.3-124)	31 (2.2-62)	0.12 (0.05-0.24)	1.85 (0.82-2.70)	0.33 (0.20-0.40)	0.77 (0.44-1.61)	García Herrera (2014)
S. hispanicus	Ep1	39 (11-65)	1040 (559-1772)	235 (124-410)	94 (18-210)	0.09 (0.05-0.13)	2.36 (1.39-3.11)	0.37 (0.16-0.57)	0.50 (0.34-0.92)	García-Herrera, Sánchez-Mata, *et al.* (2014)
S. vulgaris	Ep1	29 (18-53)	986 (619-1583)	93 (22-144)	68 (13-107)	0.07 (0.04-0.11)	0.80 (0.50-1.10)	0.75 (0.59-0.92)	0.57 (0.30-1.21)	García Herrera (2014)
S. marianum	Ep1	81 (25-128)	718 (432-1300)	132 (42-171)	17 (10-23)	0.08 (0.01-0.17)	0.50 (0.47-0.55)	0.10 (0.03-0.21)	0.26 (0.21-0.35)	García-Herrera, Sánchez-Mata, *et al.* (2014)
S. oleraceus	Ep1	125 (44-269)	507 (319-790)	156 (127-230)	31 (25-48)	0.05 (0.02-0.10)	0.63 (0.01-1.19)	0.46 (0.37-0.63)	0.57 (0.44-0.84)	Bianco *et al.* (1998), Trichopoulou *et al.* (2000), García-Herrera, Sánchez-Mata, *et al.* (2014)
T. communis	Ep1	21 (12-32)	435 (337-562)	52 (21-94)	26 (15-42)	0.12 (0.06-0.17)	0.83 (0.44-1.36)	0.22 (0.13-0.34)	0.85 (0.74-0.99)	García Herrera (2014)
T. obovatum	Ep1	35 (5-62)	566 (375-685)	117 (16-269)	18 (2-35)	0.15 (0.08-0.22)	3.57 (2.58-4.18)	0.33 (0.15-0.53)	0.50 (0.22-0.90)	García-Herrera, Sánchez-Mata, *et al.* (2014)

[a] Edible part (Ep) is presented in **Table 2**.

Some values were converted from a dry weight basis or other units.

Mg is the fourth most abundant cation in the body and plays an important role in many physiological processes (Swaminathan 2003). The leaves of *M. sylvestris* and *P. oleracea* present the highest levels of Mg (283 and 143 mg/100 g, respectively; **Table 6**) (Bianco *et al.* 1998, Guil-Guerrero *et al.* 1999, Petropoulos *et al.* 2015). A 100-g portion of these vegetables provides about 67/88% and 34/45% of the RDA of Mg established for male/female adults, respectively (**Annexe 1**). The edible parts of *S. hispanicus*, *B. maritima*, *S. vulgaris* and *C. juncea* also arise as sources of this macromineral, with amounts between 53 and 94 mg/100 g (Guil-Guerrero *et al.* 1999, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Ranfa *et al.* 2014).

The basal leaves of *C. juncea* and *P. rhoeas* and the leaves of *P. oleracea* present a high content of Cu (ranging from 0.27 mg/100 g in *P. oleracea* to 0.43 mg/100 g in *C. juncea*) (**Table 6**) (Bianco *et al.* 1998, Guil-Guerrero *et al.* 1999, Trichopoulou *et al.* 2000, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Ranfa *et al.* 2014, Petropoulos *et al.* 2015, Renna *et al.* 2015). Its contribution for the RDA of 900 µg/day established for Cu was higher than 30% (**Annexe 1**). The edible parts of *M. sylvestris* (leaves), *B. maritima*, *B. dioica*, *A. acutifolius*, *T. obovatum* and *H. lupulus* were also interesting dietary sources of this trace element (Bianco *et al.* 1998, Guil-Guerrero *et al.* 1999, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014). Regarding Fe, a 100-g portion of basal leaves of *P. rhoeas*, *C. juncea*, *T. obovatum* and *B. officinalis* and of *M. sylvestris* leaves is enough to provide more than 30% of the RDA for male adults (8 mg/day), but only the first plant provides the same RDA for female adults (18 mg/day). As shown in **Annexe 1**, other plants can be interesting sources of Fe. A deficiency in Fe can cause anaemia (Quintaes and Diez-Garcia 2015). Additionally, its levels can be decreased in some tissues by a deficiency in dietary Cu, once Cu integrates enzymes involved in its absorption and metabolism (Gupta and Gupta 2014, Quintaes and Diez-Garcia 2015).

Mn is a trace element required for blood sugar regulation and a healthy immune system (Gupta and Gupta 2014). Among the plants under review, the higher levels of Mn are presented by the basal leaves of *B. officinalis*, *C. juncea*, *B. maritima* and *R. papillaris*, the aerial parts of *M. fontana*, and by the tender stems with leaves of *S. vulgaris* (**Table 6**) (Guil-Guerrero *et al.* 1999, Tardío *et al.* 2011, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Ranfa *et al.* 2014, Renna *et al.* 2015). The highest contribution for the RDA of Mn (~70/90% for male/female adults) is provided by *B. officinalis*, whose content reaches 1.62 mg/100 g (Renna *et al.* 2015). As shown in **Annexe 1**, other plants arise as interesting sources of this trace element (RDA > 15%). The RDA of Zn was calculated in 11 and 8 mg/day for male and female adults with 19 or more years old, respectively (Otten *et al.* 2006). This micronutrient is essential for cell division and critical in wound healing (Gupta and Gupta 2014). The basal leaves of *C. juncea* and *P. rhoeas* and the leaves of *M.*

sylvestris present the highest Zn contents (Bianco *et al.* 1998, Guil-Guerrero *et al.* 1999, Trichopoulou *et al.* 2000, García Herrera 2014, García-Herrera, Sánchez-Mata, *et al.* 2014, Ranfa *et al.* 2014, Renna *et al.* 2015). However, its contribution to the RDA for this trace element was higher than 15% just for female adults (**Annexe 1**). In general, the edible plants under review are not good sources of Zn.

It is interesting to note that any 100-g portion of the different edible plants exceeds the tolerable upper intake levels (UL) for minerals. In fact, an excessive intake of certain minerals can be toxic and cause health problems (Otten *et al.* 2006, Stein 2010).

2.1.3.5. Vitamins

Vitamins are micronutrients involved in various physiological processes and most of them must be acquired through dietary sources. In general, plants are interesting sources of hydrophilic vitamins. However, the content in lipophilic vitamins may be low, since these micronutrients are associated to the lipid fraction of the tissues and plants are fat-poor foods (Sánchez-Mata and Tardío 2016). **Table 7** presents the composition in hydrophilic (vitamin B_9 and vitamin C) and lipophilic (vitamins E) vitamins of the selected WEPs. There are several wild edible species whose vitamin B_9 (total folates/folic acid) content remains unknown. *R. pulcher* appears at the top of the table with the highest total folates content 506.5 µg/100 g (Morales *et al.* 2015), a leafy vegetable whose 100-g portion provides ~127% of the RDA for adults (**Annexe 1**). Other 12 plants stand out for their high content and 4 plants as a source of this vitamin. A 100-g portion of basal leaves of *B. maritima*, *A. azurea* and *C. intybus* and of young shoots (sometimes with leaves) of *F. vulgare*, *S. vulgaris* and *A. acutifolius* provides more than 50% of the RDA of vitamin B_9 (**Annexe 1**). However, the total folate content in plant foods decreases during boiling; and this should be taken into account as some of these species are cooked before consumption (**Table 2**). In these species (vascular plants), methyl and formyl folates are the predominant ones. The methyl folate derivatives arise in the soluble fraction (vacuoles and cytosol), while the major fraction of the formyl derivatives is found in cellular organelles (Saini *et al.* 2016). After ingestion, dietary folates have to be cleaved from protein carriers and polyglutamate hydrolysed to monoglutamates in order to be absorbed in the intestine. Folates are essential for various physiological and developmental functions in the human body (Saini *et al.* 2016). They are critical for the embryonic development (Kim *et al.* 2009) and inadequate intakes may result in higher risk of certain cancers (Mason and Tang 201AD). Therefore, since humans cannot synthesize this vitamin, its intake needs to be ensured from dietary sources. In addition, the UL for folates (1000 µg/day for individuals with 19 or more years old) is only applied to the synthetic forms obtained from dietary supplements and/or fortified foods (Otten *et al.* 2006).

Table 7 Composition in folates (µg/100 g), ascorbic acid (mg/100 g), tocopherols (mg/100 g), and total phenolics (mg GAE/g methanolic extract) and flavonoids (mg CE/g methanolic extract) of the selected WEPs. It is presented the mean value and, in parentheses, the range of variability of the literature data.

Plant species	Ep[a]	Vitamin B_9	Vitamin C	Vitamin E		Bioactive non-nutrients		References
		Total folates	Ascorbic acid	α-Tocopherol	Total tocopherols	Total phenolics	Total flavonoids	
A. ampeloprasum	Ep1	145.1 (80.0-170.2)	4.15 (1.58-7.89)	0.03 (0.02-0.04)	0.05 (0.04-0.06)	5.70 (5.08-6.32)	0.86 (0.81-0.91)	Sánchez-Mata *et al.* (2012), García-Herrera *et al.* (2013), García-Herrera, Morales, *et al.* (2014), Morales *et al.* (2015)
A. azurea	Ep1	278.2 (256.6-299.7)	0.67 (0.53-0.81)	0.36 (0.28-0.44)	0.48 (0.39-0.57)	148.62 (146.62-150.62)	84.81 (80.78-88.84)	Morales *et al.* (2014, 2015)
A. nodiflorum	Ep1	125.2 (97.0-153.4)	8.90 (4.91-12.89)	0.23 (-)	0.27 (-)	80.47 (76.06-84.88)	45.48 (43.87-47.09)	Morales (2011), Morales, Carvalho, *et al.* (2012), Morales *et al.* (2015)
A. acutifolius	Ep1	217.3 (194.9-239.8)	25.56 (25.54-25.58)	7.50 (0.28-14.72)	10.60 (0.41-20.79)	320.80 (17.60-624.00)	31.95 (6.09-57.80)	Martins *et al.* (2011), Morales, Carvalho, *et al.* (2012), Pereira *et al.* (2013), Morales *et al.* (2015)
B. maritima	Ep1	302.1 (249.8-354.4)	10.13 (8.37-11.63)	0.51 (0.49-0.53)	0.63 (0.48-0.78)	61.91 (54.4-69.42)	21.55 (20.68-22.42)	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014, 2015)
B. officinalis	Ep1	-	1.26 (0-2.74)	1.15 (1.08-1.22)	1.51 (1.42-1.60)	113.58 (112.66-114.5)	88.17 (85.44-90.9)	Pereira *et al.* (2011, 2013)
B. dioica	Ep1	43.2 (39.3-47.1)	13.24 (4.28-20.27)	3.26 (0.10-7.33)	5.04 (0.21-10.88)	146.55 (32.67-280.00)	17.21 (15.61-19.3)	Martins *et al.* (2011), Morales, Carvalho, *et al.* (2012), Sánchez-Mata *et al.* (2012), Pereira *et al.* (2013), Morales *et al.* (2015)
C. intybus	Ep1	253.5 (244.5-262.5)	4.41 (0.92-7.28)	0.99 (0.88-1.10)	2.98 (2.79-3.17)	73.68 (73.02-74.34)	31.35 (30.35-32.35)	Sánchez-Mata *et al.* (2012, Morales *et al.* (2014, 2015)
C. ambrosioides	Ep1		5.48 (5.20-5.75)	54.61 (53.26-55.96)	55.42 (54.05-56.79)	8.22 (8.10-8.34) **	7.68 (7.57-7.79) **	Barros *et al.* (2013)
C. juncea	Ep1	90.2 (74.5-106.0)	1.96 (0.56-3.75)	0.57 (0.39-0.75)	0.74 (0.53-0.95)	37.66 (35.26-40.06)	7.43 (7.151-7.71)	Sánchez-Mata *et al.* (2012, Morales *et al.* (2014, 2015)
F. vulgare	Ep1	271.6 (256.2-287.0)	13.81 (10.49-17.30)	0.43 (0.30-0.56)	0.57 (0.44-0.70)	42.16 (41.18-43.14)	9.72 (9.02-10.42)	Morales, Carvalho, *et al.* (2012), Sánchez-Mata *et al.* (2012), Morales *et al.* (2015)
	Ep2	-	14.91 (-)	0.74 (0.71-0.77)	0.90 (0.87-0.93)	65.85 (65.11-66.59)	18.64 (17.74-19.54)	Barros *et al.* (2009)
	Ep3	-	8.52 (8.51-8.53)	1.19 (1.16-1.22)	1.32 (1.28-1.36)	39.49 (38.87-40.11)	nd	Barros *et al.* (2009)
	Ep4	-	4.10 (4.09-4.11)	0.02 (-)	0.07 (0.06-0.08)	8.61 (8.52-8.70)	nd	Barros *et al.* (2009)
	Ep5	-	4.47 (0-8.93)	0.14 (0.13-0.15)	0.27 (0.26-0.28)	34.68 (33.94-35.42)	nd	Barros *et al.* (2009), Pereira *et al.* (2013)
G. hederacea	Ep1		4.55 (4.49-4.61)	73.53 (71.59-75.47)	99.64 (98.10-101.18)	196.61 (190.61-202.61)	95.02 (92.29-97.75)	Barros *et al.* (2010)
H. stoechas	Ep1		30.27 (0.62-63.92)	25.34 (23.96-26.72)	28.08 (26.61-29.55)	184.42 (184.07-184.77)	34.75 (33.92-35.58)	Barros *et al.* (2010), Pereira *et al.* (2013)
H. lupulus	Ep1	143.9 (108.4-179.4)	12.79 (10.04-16.15)	0.58 (0.52-0.64)	1.83 (1.76-1.90)	55.83 (54.49-57.17)	9.56 (8.91-10.21)	Morales, Carvalho, *et al.* (2012), Sánchez-Mata *et al.* (2012), Morales *et al.* (2015)

Plant species	Ep[a]	Vitamin B_9	Vitamin C	Vitamin E		Bioactive non-nutrients		References
		Total folates	Ascorbic acid	α-Tocopherol	Total tocopherols	Total phenolics	Total flavonoids	
M. sylvestris	Ep1		4.03 (2.84-5.22)	19.84 (19.37-20.31)	25.24 (24.51-25.97)	386.45 (377.91-394.99)	210.81 (202.82-218.80)	Barros, Carvalho, and Ferreira (2010a)
	Ep2		26.31 (24.65-37.97)	3.86 (3.66-4.06)	4.78 (4.50-5.06)	258.65 (232.61-284.69)	46.55 (41.29-51.81)	Barros, Carvalho, and Ferreira (2010a)
	Ep3		14.69 (14.47-14.91)	1.13 (1.12-1.14)	1.42 (1.41-1.43)	56.76 (54.75-58.77)	25.35 (22.63-28.07)	Barros, Carvalho, and Ferreira (2010a)
	Ep4		8.74 (6.57-13.06)	6.46 (6.40-6.52)	7.94 (7.93-7.95)	317.93 (315.32-320.54)	143.40 (135.54-151.26)	Barros, Carvalho, and Ferreira (2010a), Pereira *et al.* (2013)
M. pulegium	Ep1		3.20 (3.13-3.27)	28.18 (23.54-32.82)	36.36 (30.37-42.35)	331.69 (315.06-348.32)	139.85 (138.58-141.12)	Fernandes *et al.* (2010)
M. fontana	Ep1	41.8 (41.7-41.9)	15.43 (0-28.96)	1.45 (0.72-2.43)	1.85 (0.97-3.06)	61.50 (45.85-82.58)	21.28 (16.05-26.89)	Pereira *et al.* (2011, 2013), Tardío *et al.* 2011, Morales, Carvalho, *et al.* 2012, Morales *et al.* 2015)
N. officinale	Ep1	127.9 (117.1-138.7)	0.59 (0-1.17)	1.01 (0.47-1.52)	1.17 (0.51-1.82)	50.42 (47.65-53.19)	35.17 (31.81-38.53)	Pereira *et al.* (2011, 2013), Fajardo *et al.* (2015), Pinela, Barreira, Barros, Antonio, *et al.* (2016)
O. vulgare	Ep1		4.11 (0-8.48)	4.90 (4.69-5.11)	6.10 (5.85-6.36)	368.58 (350.40-386.76)	224.15 (223.19-225.11)	Barros *et al.* (2010), Pereira *et al.* (2013)
P. rhoeas	Ep1	152.3 (151.0-153.6)	13.71 (11.86-16.36)	1.13 (1.07-1.19)	1.87 (1.68-2.06)	25.86 (22.34-29.38)	12.00 (11.54-12.46)	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014, 2015)
P. oleracea	Ep1	12 (-)	26.6 (-)	12.2 (-)	-	12.89 (7.65-20.1)*	1.76 (0.12-5.30)*	Petropoulos *et al.* (2015, 2016)
P. tridentatum	Ep1		15.68 (0-35.28)	3.58 (3.39-3.77)	3.78 (3.58-3.98)	523.42 (487.33-559.51)**	58.12 (52.34-63.90)	Pinela *et al.* (2011), Pereira *et al.* (2013)
R. ulmifolius	Ep1		87.85 (86.89-88.81)	3.03 (2.93-3.13)	6.24 (6.13-6.35)	257.89 (254.61-261.17)	172.45 (169.03-175.87)	Barros *et al.* (2010)
	Ep2		30.30 (0.17-61.62)	1.00 (-)	1.69 (1.68-1.70)	715.94 (687.39-744.49)	624.76 (623.58-625.94)	Barros *et al.* (2010), Pereira *et al.* (2013)
R. acetosella	Ep1		45.91 (19.21-72.23)	5.70 (4.70-6.70)	10.77 (8.93-12.61)	141.58 (137.91-145.25)	37.91 (34.55-41.27)	Pereira *et al.* (2011, 2013)
R. induratus	Ep1		51.69 (0.02-103.36)	4.94 (3.60-6.23)	6.99 (5.50-8.24)	117.08 (114.54-119.62)	89.78 (86.97-92.59)	Pereira *et al.* (2011), Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* (2016)
R. papillaris	Ep1	187.3 (117.3-197.3)	38.90 (7.31-87.88)	1.29 (0.85-1.73)	1.67 (1.09-2.25)	104.18 (100.01-108.35)	39.49 (36.23-42.75)	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014, 2015)
R. pulcher	Ep1	506.5 (482.6-530.5)	20.37 (14.81-24.88)	0.44 (0.42-0.46)	0.54 (0.52-0.56)	73.44 (68.12-78.76)	26.14 (25.27-27.01)	Sánchez-Mata *et al.* (2012, Morales *et al.* (2014, 2015)
S. hispanicus	Ep1	103.4 (96.2-110.7)	0.88 (0.41-1.29)	0.02 (-)	0.05 (-)	21.51 (20.00-23.02)	8.39 (7.27-9.51)	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014, 2015)
S. vulgaris	Ep1	267.7 (214.4-320.9)	22.02 (16.05-29.44)	1.48 (1.31-1.65)	1.70 (1.51-1.89)	26.72 (25.09-2835)	21.65 (16.12-27.18)	Morales, Carvalho, *et al.* (2012), Sánchez-Mata *et al.* (2012), Morales *et al.* (2015)
S. marianum	Ep1	41.7 (11.7-71.6)	0.47 (0-1.23)	0.04 (-)	0.15 (-)	3.72 (3.36-4.08)	1.13 (0.86-1.40)	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014, 2015)
S. oleraceus	Ep1	85.9 (70.2-101.5)	2.80 (1.56-4.04)	1.70 (1.65-1.75)	2.22 (2.16-2.28)	51.33 (49.58-53.08)	14.83 (13.85-15.81)	Morales *et al.* (2014, 2015)

Plant species	Ep[a]	Vitamin B_9	Vitamin C	Vitamin E		Bioactive non-nutrients		References
		Total folates	**Ascorbic acid**	**α-Tocopherol**	**Total tocopherols**	**Total phenolics**	**Total flavonoids**	
T. communis	Ep1	38.1 (37.2-39.0)	42.33 (23.55-53.36)	2.63 (0.16-5.20)	4.57 (0.45-8.80)	404.26 (45.44-788.00)	79.67 (7.89-162.00)	Martins *et al.* (2011), Morales, Carvalho, *et al.* (2012), Sánchez-Mata *et al.* (2012), Pereira *et al.* (2013), Morales *et al.* (2015)
T. obovatum	Ep1	110.7 (91.0-130.4)	1.76 (1.06-2.83)	0.51 (0.49-0.56)	0.60 (0.59-0.61)	58.26 (57.36-59.16)	30.03 (29.64-30.96)	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014, 2015)
T. mastichina	Ep1		2.92 (tr-5.93)	0.16 (0.13-0.19)	1.88 (1.79-1.97)	165.29 (164.18-166.40)	83.85 (82.43-85.27)	Barros *et al.* (2010), Pereira *et al.* (2013)
T. pulegioides	Ep1		3.11 (3.05-3.17)	6.61 (6.18-7.04)	7.06 (6.58-7.54)	210.49 (189.33-231.65)	128.24 (122.24-134.24)	Fernandes *et al.* (2010)

[a] Edible part (Ep) is presented in **Table 2**.

* Methanol:water (80:20, v/v) extracts

** Quantification by HPLC-DAD-ESI/MS

Some values were converted from a dry weight basis or other units.

nd: not detected; tr: traces

Vitamin C is mainly ingested as ascorbic acid (the major form in plants) but also as dehydroascorbic acid (oxidized form). The four Polygonaceae species *R. ulmifolius* (Barros *et al.* 2010, Pereira *et al.* 2013), *R. induratus* (Pereira *et al.* 2011, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016), *R. acetosella* (Pereira *et al.* 2011, 2013) and *R. papillaris* (Sánchez-Mata *et al.* 2012, Morales *et al.* 2014), the wild asparagus *T. communis* and *A. acutifolius* (Martins *et al.* 2011, Sánchez-Mata *et al.* 2012, Pereira *et al.* 2013), *P. oleracea* (Petropoulos *et al.* 2015, 2016), *H. stoechas* (Barros *et al.* 2010, Pereira *et al.* 2013) and *M. sylvestris* flowers (Barros, Carvalho, and Ferreira 2010a) were reported as having a high content of ascorbic acid (**Table 7**). Based on the mean values of this vitamin, it is possible to conclude that a 100-g portion of these WEPs is sufficient to provide more than 30% of the RDA of vitamin C for male and/or female individuals with 19 or more year old (**Annexe 1**). However, the ascorbic acid values described for some of the species present a high range, which may be related, in part, to the analytical method used. In fact, while some authors (Tardío *et al.* 2011, Sánchez-Mata *et al.* 2012, Barros *et al.* 2013, Pereira *et al.* 2013, Morales *et al.* 2014, Pinela, Barreira, Barros, Antonio, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016) have analysed the ascorbic acid by high-performance liquid chromatography (HPLC) or ultra-fast liquid chromatography (UFLC), others have used spectrophotometric assays (2,6-dichloroindophenol titrimetric method) (Barros *et al.* 2009, 2010, 2010, Barros, Carvalho, and Ferreira 2010a, Martins *et al.* 2011, Pereira *et al.* 2011, Pinela *et al.* 2011).

With average values between 12.79 and 22.02 mg/100 g of ascorbic acid, *S. vulgaris*, *R. pulcher* (Sánchez-Mata *et al.* 2012), *P. tridentatum* (Pinela *et al.* 2011), *M. fontana* (Pereira *et al.* 2011, Tardío *et al.* 2011), *M. sylvestris* immature fruits (Barros, Carvalho, and Ferreira 2010a), *F. vulgare* shoots (Barros *et al.* 2009) and young shoots with leaves (Sánchez-Mata *et al.* 2012), *P. rhoeas* (Sánchez-Mata *et al.* 2012, Morales *et al.* 2014), *B. dioica* (Martins *et al.* 2011, Sánchez-Mata *et al.* 2012, Pereira *et al.* 2013) and *H. lupulus* (Sánchez-Mata *et al.* 2012) stand out as a source of this vitamin (a 100-g portion provides more than 15% of the RDA; **Annexe 1**). Despite the high mean vales of ascorbic acid in *R. induratus* (51.69 mg/100 g), *R. ulmifolius* (Ep2, 30.30 mg/100 g), *H. stoechas* (30.27 mg/100 g), *P. tridentatum* (15.68 mg/100 g) and *M. fontana* (15.43 mg/100 g) (**Table 7**), this vitamin was not detected or detected in small quantity by some authors (Pereira *et al.* 2013, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016) in some of the samples.

Ascorbic acid has the ability to scavenge aqueous radicals and regenerate other antioxidants from their radical form (Niki *et al.* 1995, Lee *et al.* 2004, Niki 2014). This powerful hydrophilic antioxidant can be oxidized to dehydroascorbic acid due to its involvement in the oxidative stress and then regenerated to its reduced form (Niki *et al.* 1995). It also facilitates the absorption of soluble non-heme Fe (Otten *et al.* 2006). Ascorbic

acid can, however, behave as a prooxidant (Carr and Frei 1999). It is also important to note that this vitamin is greatly affected by processing (Ottaway 2010, Vinha *et al.* 2015). A study of Vinha *et al.* (2015) demonstrated that boiling *N. officinale* for 10 min promotes a significant loss of vitamin C and antioxidant activity. However, the phenolics content was preserved.

The amounts of α-tocopherol and total tocopherols reported in the selected WEPs are presented in **Table 7**. A 100 g-portion of *C. ambrosioides*, *H. stoechas*, *M. sylvestris* leaves, and of the two Lamiaceae *G. hederacea* and *M. pulegium* provide more than 100% of the RDA established for vitamin E (15 mg/day for individuals with 14 or more years old (Otten *et al.* 2006)) (**Annexe 1**). The dietary reference intakes for vitamin E are calculated based on the amounts of α-tocopherol (including the *RRR*-α-tocopherol that occurs naturally in foods and the 2*R*-stereoisomeric forms of α-tocopherol that occur in fortified foods and supplements) (Otten *et al.* 2006). Very high levels of α-tocopherol (71.59–75.47 mg/100 g) were reported in *G. hederacea* (Barros *et al.* 2010), corresponding to approximately 74% of the total tocopherols content. Leaves of this perennial medicinal plant are recommended to the respiratory and gastrointestinal systems. Infusions prepared from the dried plant are useful for relieving colds, coughs, throat irritations and abdominal pains. Decoctions of the leaves are used for external inflammations and skin diseases (**Table 2**) due to its claimed anti-inflammatory and antiseptic properties (Carvalho 2010). A high content was also reported in *P. oleracea* (Petropoulos *et al.* 2016), *A. acutifolius* (García-Herrera *et al.* 2013, García-Herrera, Morales, *et al.* 2014), *T. pulegioides* (Fernandes *et al.* 2010), *M. sylvestris* leafy flowering stems (Barros, Carvalho, and Ferreira 2010a), *R. acetosella* (Pereira *et al.* 2011), *R. induratus* (Pereira *et al.* 2011, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016) and *O. vulgare* (Barros *et al.* 2010). A 100-g portion of these plants contributes in more than 30% for the RDA of vitamin E. Other plants stand out as a source of these lipophilic antioxidants. With lower levels, but still being interesting sources of this lipophilic vitamin, arise the flowering parts of *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a), *P. tridentatum* (Pinela *et al.* 2011) and *R. ulmifolius* (Barros *et al.* 2010), and the wild asparagus *B. dioica* and *T. communis* (Martins *et al.* 2011, Morales, Carvalho, *et al.* 2012).

Apart from α-tocopherol, the isoforms β, γ, δ can also be detected in plant species. The isoform α comprises less than 30% of the total fraction of tocopherols in *F. vulgare* stems, *S. marianum* and *T. mastichina*. γ-Tocopherol was detected as the major vitamer in the last plant (1.70 mg/100 g) (Barros *et al.* 2010) and also in *C. intybus* (1.88 mg/100 g) (Morales *et al.* 2014). In the North-eastern region of Portugal, *T. mastichina* is used as condiment/spice for flavouring and seasoning traditional dishes and salads. It is also used to preserve olives and as a salt substituent (**Table 2**).

Tocopherols are amphipathic molecules in which the hydrophobic phytyl side chain is embedded within the cell membrane bilayer and the polar chromanol head is exposed to the

membrane surface. This ingenious arrangement facilitates the regeneration of α-tocopherol from the radical form (α-tocopheroxyl radical) by the ascorbic acid and other hydrophilic reducing agents (Lee *et al.* 2004). These strong antioxidants halt the lipid peroxidation chain reaction through donation of the chromanol ring phenolic hydrogen, thus maintain the integrity of the long-chain PUFA of the cell membranes (Niki 2014). These antioxidants are involved in the non-enzymatic plant protection mechanism against the abiotic stress-induced oxidative damage, being synthesized in response to these adverse conditions (Abbasi *et al.* 2007, Pinela, Barreira, Barros, Antonio, *et al.* 2016). In humans, vitamin E plays a vital role in the fight against various diseases and health conditions such as oxidative stress, inflammatory and degenerative processes, cardiovascular disease and different types of cancer, mainly due to its antioxidant capacity (Niki 2014, Galli *et al.* 2017). The involvement of physiological metabolites of vitamin E in gene regulation and homeostasis in experimental models of inflammatory, neuronal and hepatic cells, and *in vivo* in animal models of acute inflammation has been demonstrated (Galli *et al.* 2017). The UL for vitamin E (1000 mg/day for adults with 19 or more old) is only applied to the synthetic forms obtained from dietary supplements and/or fortified foods (Otten *et al.* 2006).

The inclusion of WEPs in human contemporary diets can be seen as a strategy to help achieving adequate intake levels of vitamin B_9, vitamin C and vitamin E.

2.1.4. Bioactive non-nutrients

Phenolic compounds are secondary metabolites synthesized via shikimate-phenylpropanoids-flavonoids pathways. They can be grouped in different classes according to their basic skeleton, including phenolic acids (C_6-C_1), hydroxycinnamic acids and coumarins (C_6-C_2), naphthoquinones (C_6-C_4), xanthones (C_6-C_1-C_6), stilbenes and anthraquinones (C_6-C_2-C_6), flavonoids and isoflavonoides (C_6-C_3-C_6), lignans (C_6-C_3)$_2$), biflavonoids (C_6-C_3-C_6)$_2$), lignins (C_6-C_3)$_n$) and condensed tannins (C_6-C_3-C_6)$_n$) (Vermerris and Nicholson 2008a). In plants, these compounds are involved in growth, reproduction and defence against ultraviolet radiation or pathogens (Pandey and Rizvi 2009). The interest of phenolic compounds comes from its health-promoting effects in humans that are conferred by antioxidant, anti-inflammatory, antitumor and antimicrobial properties, among other effects (Vermerris and Nicholson 2008b). Therefore, these bioactive molecules affect the nutritional quality of foods (Smolin and Grosvenor 2016) since they make the food to be functional.

The content in total phenolics and flavonoids of the selected WEPs is presented in **Table 7**. The fully opened flowers of *R. ulmifolius* (**Figure 2**) revealed the highest levels of total phenolics (715.94 mg GAE/g extract) and flavonoids (624.76 mg CE/g extract) (Barros *et al.* 2010). High levels of phenolics (> 300 mg GAE/g extract) were also measured in extracts prepared from the flowering parts of *P. tridentatum* (Pinela *et al.* 2011), *O. vulgare*

(Barros *et al.* 2010), *M. pulegium* (Fernandes *et al.* 2010), *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a), young shots of *T. communis* and *A. acutifolius* (Martins *et al.* 2011, Morales, Carvalho, *et al.* 2012), and leaves of *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a). High levels of flavonoids (> 128 mg GAE/g extract) were reported in the flowering parts of *R. ulmifolius* (Barros *et al.* 2010), *O. vulgare* (Barros *et al.* 2010), *M. pulegium*, *T. pulegioides* (Fernandes *et al.* 2010) and *M. sylvestris* (Barros, Carvalho, and Ferreira 2010a). A high content (210.81 mg GAE/g extract) was also detected in the leaves of the last plant. In turn, the lowest amounts of total phenolics (< 13 mg GAE/g extract) were detected in extracts of the midribs of basal leaves of *S. marianum* (Morales *et al.* 2014), bulb and pseudostem of *A. ampeloprasum* (García-Herrera *et al.* 2013, García-Herrera, Morales, *et al.* 2014), inflorescence and upper leaves of *C. ambrosioides* (Barros *et al.* 2013), stems of *F. vulgare* (Barros *et al.* 2009) and leaves of *P. oleracea* (Petropoulos *et al.* 2015).

The Folin-Ciocalteu method is commonly used to measure the total phenolic content of plant matrices. This analysis is based on the formation of a blue-coloured complex between molybdenum and tungsten present in the Folin-Ciocalteu's phenol reagent upon reaction with reducing agents, which is measured spectrophotometrically at 765 nm. As presented in **Table 7**, the results were expressed by the authors in mg of gallic acid equivalents (GAE) per g of extract. The aluminium chloride colorimetric method is a simple way to measure the total flavonoid content. This assay is based on the formation of a flavonoid-aluminium complex that absorbs at 510 nm. The results for total flavonoids were expressed in mg of catechin equivalents (CE) per g of extract. However, advanced chromatographic and spectrometric techniques are used to identify and quantify individual phenolic compound. The phenolic profile of *C. ambrosioides* was characterized by Barros *et al.* (2013) using an HPLC system coupled to a diode array detector (DAD). Double online detection was carried out in the DAD and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. It was found that flavonoids (768.27 mg/100 g of dry weight) predominate over phenolic acids (54.07 mg/100 g of dry weight) in this plant. Quercetin-3-*O*-rutinoside, kaempferol dirhamnoside-*O*-pentoside and kaempferol 3-*O*-rutinoside were identified as the most abundant phenolic compounds in this plant (Barros *et al.* 2013).

Due to the health-promoting effects and high potential of phenolic compounds as nutraceuticals, phenolic-rich extracts have been incorporated in different food and cosmeceutical formulations. Martins *et al.* (2014) developed a new yogurt with a high antioxidant activity through the incorporation of free and microencapsulated extracts of *R. ulmifolius* flower buds. The phenolic extract was rich in ellagitannin derivatives, namely a sanguiin H-10 isomer and lambertianin C. In another study, yogurt and cottage cheese were functionalized by the incorporation of an extract of *F. vulgare* aerial parts (Caleja *et al.* 2015,

Caleja, Barros, *et al.* 2016, Caleja, Ribeiro, *et al.* 2016) rich in phenolic acids and flavonoids (mostly 5-*O*-caffeolyquinic acid and quercetin-3-*O*-glucuronide, respectively), which conferred bioactive properties to these food products. The antioxidant potential of *H. stoechas* phenolic compounds was explored for cosmetic applications (Barroso *et al.* 2014). The authors successfully incorporated polycaprolactone-based microspheres containing the *H. stoechas* extract rich in 3,5-*O*-dicaffeoylquinic acid and myricetin *O*-acetylhexoside into a moisturizer. These studies open new perspectives for the exploitation of wild plants as interesting sources of bioactive/nutraceutical ingredients with applications in the food and cosmeceutical industries.

2.1.5. Antinutritional and potentially toxic compounds

2.1.5.1. Oxalic acid

In nature, oxalic acid occurs commonly as a water-soluble (Na and K) or water-insoluble (Ca, Mg, Fe and Zn) salt. This compound has a quite low toxicity, but it can reduce the bioavailability of dietary Ca by the formation of an insoluble complex of calcium oxalate (Kristanc and Kreft 2016a). Known as raphides in plants, this calcium salt is the primary constituent of the most common kind of human kidney stones (Amalraj and Pius 2015). For this reason, the ingestion of foods with high levels of this antinutritional compound is not desirable. Some authors (Guil *et al.* 1996) recommend an oxalic acid/Ca ratio not higher than 2.5 to avoid this negative effect. Among the selected plants, this limit is greatly exceeded by *M. fontana* (~11.8) but also by other species, namely *B. maritima*, *C. ambrosioides*, *S. marianum*, *R. pulcher*, *A. nodiflorum*, *A. acutifolius*, *S. oleraceus*, *P. oleracea*, *N. officinale* and *R. papillaris* (**Figure 3** and **Annexe 2**).

Oxalic acid levels ranging from 0.7 mg/100 g in *R. induratus* (Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016) to 1545 mg/100 g in *C. ambrosioides* (Barros *et al.* 2013) have been reported (**Figure 4** and **Annexe 2**). Therefore, the consumption of 324 mg of *C. ambrosioides* can provide the amount of oxalic acid (5 g) set as the minimal lethal dose for an adult (a level difficult to reach through the diet). However, it should be noted that the bioavailability of ingested oxalate is estimated to be less than 15% and is influenced by a number of food composition and physiological related factors (Brogren and Savage 2003, Liebman and Al-Wahsh 2011, Kristanc and Kreft 2016a).

Wild plants of the genus *Oxalis*, *Rumex*, *Amaranthus* and *Chenopodium* have been referred as having significant levels of oxalic acid (Guil *et al.* 1996, Vizgirdas and Rey-Vizgirdas 2006). Amounts between 137 to 256 mg/100 g were described for *R. pulcher*, *R. papillaris* (Sánchez-Mata *et al.* 2012, Morales *et al.* 2014) and *R. acetosella* (Pereira *et al.* 2013); but a small amount of 0.7 mg/100 g was reported in tender leaves of *R. induratus* (Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016). Contrariwise, a high

content in oxalic acid was found by Gerra *et al.* (2008) in an aqueous extract of the same plant. The authors also verified that the oxalic acid content vary depending on the development stage of the plant and environmental conditions. In another study, it was demonstrated that the harvest time and the nitrate to ammonium ratios affect the oxalic acid content in hydroponically grown *P. oleracea* plants (Palaniswamy *et al.* 2004). Levels ranging from 371 to 753 mg/100 g were reported by Petropoulos *et al.* (2015) in this plant. These observations can also be deduced from the range of values reported for oxalic acid in *S. hispanicus*, *T. obovatum*, *T. communis*, *S. marianum*, *A. ampeloprasum*, *C. juncea* and *R. pulcher* (**Figure 4** and **Annexe 2**), in which the variation exceeds 100%.

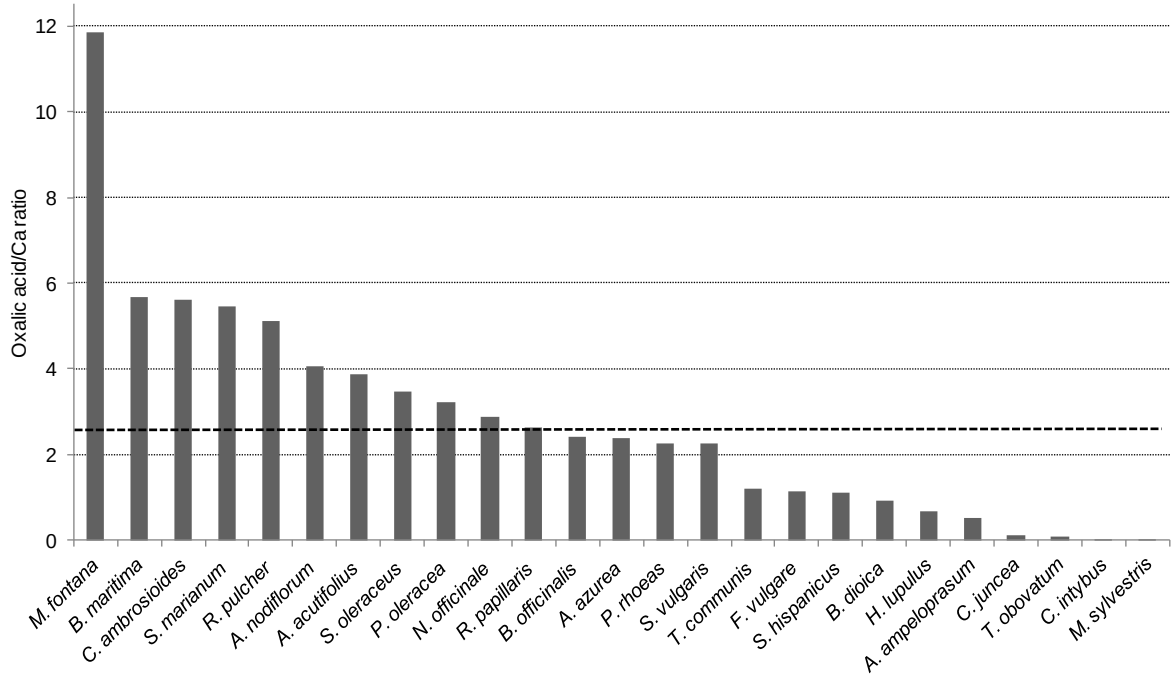


Figure 3 Oxalic acid/Ca ratios of the selected WEPs. The values above the dashed line are greater than 2.5. Ep1 for all plant parts except for *M. sylvestris* (Ep4). For *C. ambrosioides*, the oxalic acid/Ca ratio was calculated based on the Ca levels of the USDA tables. The represented values are shown in **Annexe 2**.

So far, it is not clearly known whether the oxalic acid is harmful by itself or only when it is in the form of calcium oxalate (Kristanc and Kreft 2016a). Despite this, people who easily form kidney stones should give preference to plant foods with a low oxalic acid content, like the basal leaves of *C. intybus*, *T. obovatum* and *C. juncea* (Sánchez-Mata *et al.* 2012, Morales *et al.* 2014), the wild asparagus *T. communis*, *H. lupulus* and *B. dioica* (Sánchez-Mata *et al.* 2012, Pereira *et al.* 2013), the bulb and pseudostem of *A. ampeloprasum* (Sánchez-Mata *et al.* 2012, García-Herrera, Morales, *et al.* 2014), or the flowering parts of *M. sylvestris* and *P. tridentatum* (Barros *et al.* 2013, Pereira *et al.* 2013), which provide less than

70 mg/100 g of oxalic acid (**Annexe 2**) and have an oxalic acid/Ca ration lower than 2.5 (**Figure 3**).

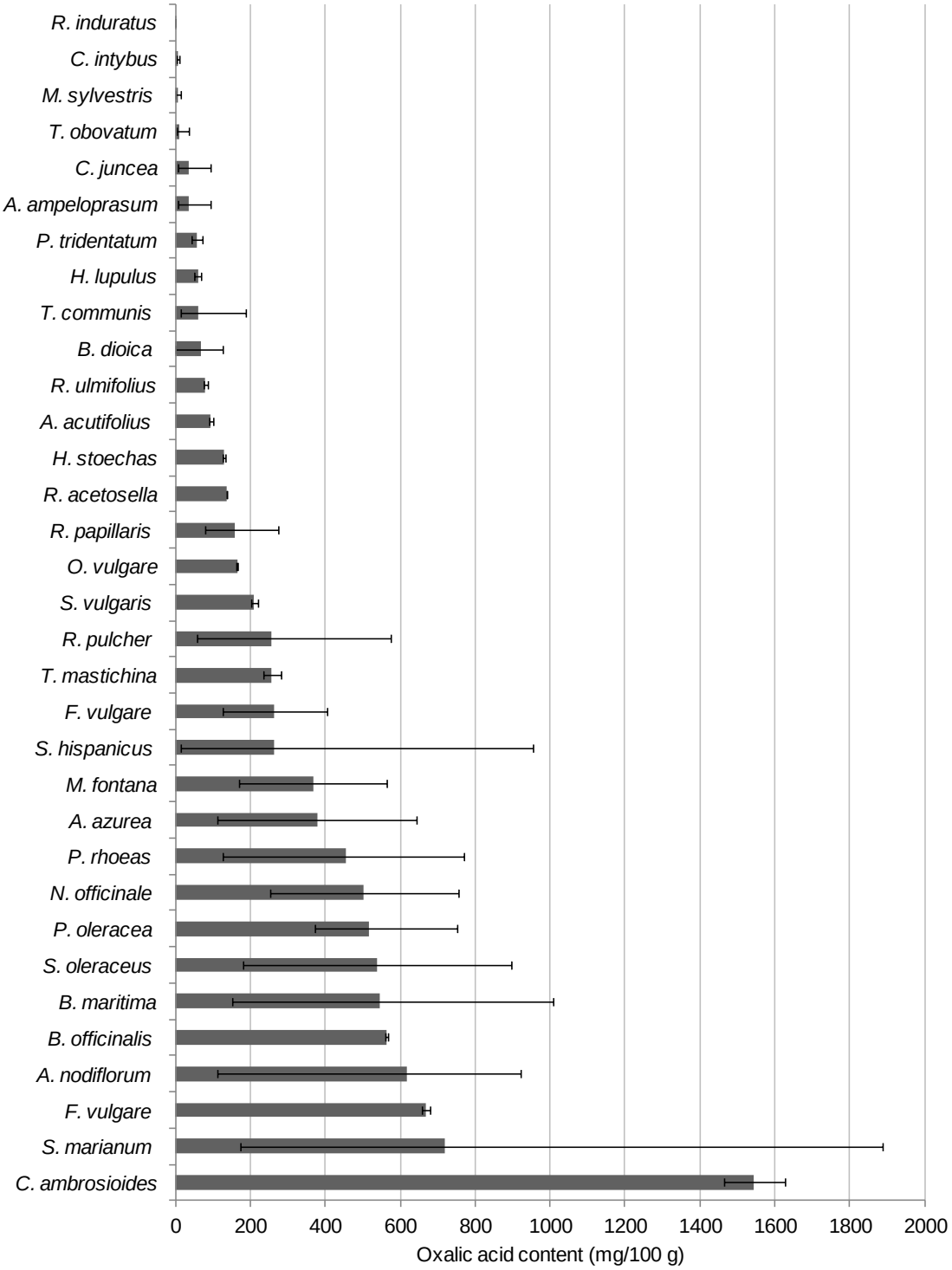


Figure 4 Oxalic acid levels (mg/100 g) reported in the selected WEPs. It is presented the mean value (dark bar) and the minimum and maximum values reported in the literature. The represented values are shown in **Annexe 2**.

Table 8 Potentially toxic compounds that can be found in WEPs.

Plant species	Part of possible concern	Potentially toxic compounds	References
A. azurea	Unspecified parts	Pyrrolizidine alkaloids: lycopsamine, laburnine and acetyllaburnine	Roeder (1999), EFSA (2009)
	Seeds (oil)	-	Roeder (1999), EFSA (2009), Vacillotto *et al.* (2013)
B. officinalis	Seeds (oil)	Pyrrolizidine alkaloid: amabiline	EFSA (2009), Vacillotto *et al.* (2013), EMA (2016a)
B. dioica	Root	Cucurbitacins B, D, E, I, J, K, L and S; cucurbitacin glycosides: bryodulcoside, bryoside and bryonoside; cucurbitacin saponins: brydioside A, B and C; dihydrocucurbitacins B and E; tetrahydrocucurbitacin I	Pohlmann (1975), Hylands and Salama (1976), Hylands and Mansour (1982), Oobayashi *et al.* (1992), Chen *et al.* (2005), Gry *et al.* (2006), EFSA (2009)
	Fruit	Brydiofin	Muñoz *et al.* (1992), Tardío *et al.* (2016)
C. ambrosioides	Aerial part (essential oil)	Peroxygenated monoterpene: ascaridole; phenylpropanoid: safrole	Tucker and Maciarello (1998), Kiuchi *et al.* (2002), EFSA (2009, 2012)
F. vulgare	Aerial part (essential oil)	Phenylpropanoids: *trans*-anethole and estragole (2.3 – 4.9%)	EFSA (2009, 2012)
	Fruit (essential oil)	Phenylpropanoid: estragole (0.8 – >80%)	EFSA (2009, 2012)
	Seeds (essential oil)	Phenylpropanoid: estragole (from 11.9 – 56.1% in unripe seeds to 61.8% in ripe seed)	García-Jiménez *et al.* (2000), EFSA (2012)
G. hederacea	Aerial part	Pyrrolidine alkaloids: hederacines A and B	Kumarasamy *et al.* (2003), EFSA (2012)
	Flowering aerial part (essential oil)	Monoterpene etheroxide: 1,8-cineole (eucalyptol, 1.9 - 4.6%)	EFSA (2012)
H. lupulus	Inflorescence	Prenylflavonoids: 8-prenylnaringenin (hopein), xanthohumol, isoxanthohumol	EFSA (2009)
M. pulegium	Aerial part (essential oil)	Monocyclic monoterpene ketone: pulegone (71.3 – 90%); bicyclic monoterpenes: menthofuran and thujones; monoterpene etheroxide: 1,8-cineole (eucalyptol)	EFSA (2009, 2012), Teixeira *et al.* (2012), Kristanc and Kreft (2016a)
O. vulgare	Aerial part (essential oil)	Bicyclic monoterpene: beta-thujone (0 – 0.6%); monoterpene etheroxide: 1,8-cineole (eucalyptol, 0-6.5%)	EFSA (2012)
P. rhoeas	Whole plant	-	Al-Qura'n (2005), EFSA (2012), Kemal *et al.* (2015), Tardío *et al.* (2016)
Rumex sp.pl.	Whole plant	Oxalates and hydroxyanthracene derivatives: chrysophanol, physcion, emodin, aloe-emodin, rhein, barbaloin (aloin A and B) and sennosides A and B	EFSA (2012)
S. vulgaris	Root	Triterpenoid saponins: silenosides A, B and C	Glensk *et al.* (1998)
S. marianum	Flowering top and seed	-	EFSA (2012)
T. communis	Rhizome	Steroidal saponins: dioscin and gracillin; phenanthrene derivatives	Tardío (2005), Réthy *et al.* (2006), Kovács *et al.* (2007), EFSA (2009)
	Fruit	-	Tardío *et al.* (2016)
Thymus sp.pl.	Aerial part (essential oil)	Monoterpene etheroxide: 1,8-cineole (eucalyptol)	EFSA (2012)

2.1.5.2. Pyrrolizidine and pyrrolidine alkaloids

Pyrrolizidine alkaloids are of special concern to human health. More than 660 compounds have been identified in more than 6000 plant species worldwide, belonging mainly to the families Asteraceae, Boraginaceae, Fabaceae (tribe Crotalarieae) and Orchidaceae. Roughly half of these compounds exhibit chronic toxicity (Smith and Culvenor 1981, Chen *et al.* 2010, Kristanc and Kreft 2016b). Curiously, species containing these alkaloids are probably the most common poisonous plants affecting livestock and wildlife (Chen *et al.* 2010). Structurally, pyrrolizidine alkaloids are ester alkaloids composed of necine (two fused five-membered rings joined by a single nitrogen atom) and necic acid (one or two carboxylic ester arms). The toxic ones are esters of unsaturated necines having a 1,2-double bond in their base moiety. These compounds are well known for their hepatotoxic effects, but many of them are often pneumotoxic, genotoxic and carcinogenic (Roeder 1999, EFSA 2011, Kristanc and Kreft 2016b). Although pyrrolizidine alkaloids themselves show a relatively low acute toxicity, they undergo a toxication process in the liver, which is the first organ to be affected. First, after absorption, a hydroxyl group is introduced in the necine at positions 3 or 8 by cytochrome P450 enzymes in the liver, originating unstable compounds that undergo a rapid dehydration process, resulting in a second double-bond in the necine followed by spontaneous rearrangement to an aromatic pyrrole system (Wiedenfeld 2011). Acute poisoning by pyrrolizidine alkaloids causes haemorrhagic necrosis, hepatomegaly and ascites. The subacute toxicity is characterised by occlusion of the hepatic veins and subsequent necrosis, fibrosis and liver cirrhosis. Hence, death is caused by liver failure. As shown in **Table 8**, pyrrolizidine alkaloids can be found in *A. azurea* (namely lycopsamine, laburnine (**Figure 5**) and acetyllaburnine) (Roeder 1999, EFSA 2009) and *B. officinalis* seed oil (namely the hepatotoxic amabiline) (Dodson and Stermitz 1986, Wretensjö and Karlberg 2003, EFSA 2009, Vacillotto *et al.* 2013, EMA 2016a). Both species are listed in the "*compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements*" of the European Food Safety Authority (EFSA 2009).

Pyrrolidine alkaloids linked with a tropane-like skeleton (namely hederacines A and B, showed in **Figure 5**) were already isolated from *G. hederacea* aerial parts and revealed prominent toxicity in the brine shrimp lethality bioassay (Kumarasamy *et al.* 2003).

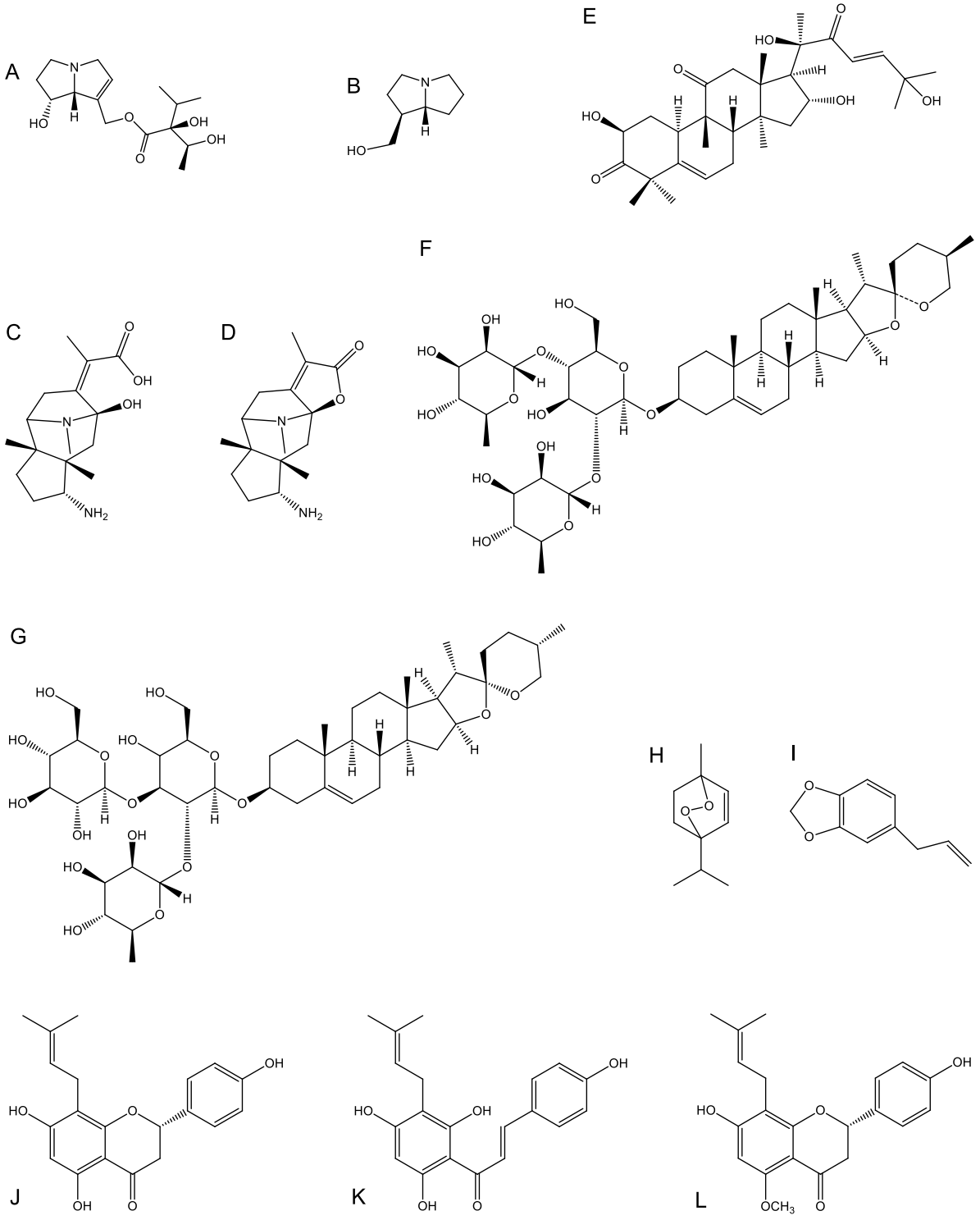


Figure 5 Chemical structure of potentially toxic compounds that can be found in some WEPs presented in **Table 8**. A: lycopsamine; B: laburnine; C: hederacine A; D: hederacine B; E: cucurbitacin D; F: dioscin; G: gracillin; H: ascaridole; I: safrole; J: 8-prenylnaringenin; K: xanthohumol; and L: isoxanthohumol.

2.1.5.3. Brydiofin and cucurbitacins

In Portugal and Spain, the crushed fruits of *B. dioica* are applied topically to mitigate rheumatic symptoms (Aceituno-Mata 2010, Carvalho 2010). However, Muñoz *et al.* (1992) identified a toxic protein called brydiofin in that fruits (**Table 8**) and verified that their extract have a highly toxic effect after intraperitoneal injection of 0.4 mg per Balb/c mouse that was killed in 18 min (Muñoz *et al.* 1992). This lethal effect was not presented by the root and leaf extracts. However, different cucurbitacins can be found in the root of this plant (**Table 8**), part that is not consumed as food. Cucurbitacins are highly toxic and bitter compounds that can be isolated from about 100 species of Cucurbitaceae, being also found in plants belonging to the families Scrophulariaceae, Begoniaceae, Primulaceae, Liliaceae, Tropaeolaceae and Rosaceae (Gry *et al.* 2006, Kaushik *et al.* 2015). Due to the selection of non-bitter plants for food use made by our ancestors and later by plant breeders, the cucurbits currently consumed generally do not synthesize these compounds. Structurally, cucurbitacins are tetracyclic triterpenes with a cucurbitane skeleton and differ from most other tetracyclic triterpenes because they are highly unsaturated and contain many keto, hydroxy and acetoxy groups (Chen *et al.* 2005, Gry *et al.* 2006). A chronic exposure to these compounds is not expected in humans given their extremely bitter and disagreeable taste and the accidental occurrence in plant foods (Gry *et al.* 2006). For cucurbitacins D (**Figure 5**) and I, the oral LD_{50} in mice is ~5 mg/kg body weight; for cucurbitacin E and cucurbitacin E glycoside, which are the most common cucurbitacins identified in food plants, the oral LD_{50} values in mice are 340 and 40 mg/kg body weight, respectively (Gry *et al.* 2006). Animal studies have shown that exposure to cucurbitacin D promotes increased capillary permeability, irritation of the intestinal mucosa, and increased intestinal mobility. The animal's death is caused by congestion of the intestine, pancreas, liver and kidneys. In humans, mucosal irritation occurs within minutes of ingestion of cucurbitacin-containing preparations (Gry *et al.* 2006).

Although highly toxic, cucurbitacins began to attract much interest in the 1960s for their immense pharmacological potential (Chen *et al.* 2012). Using a long array of *in vitro* and *in vivo* cancer cell models, it has been shown that certain natural cucurbitacins and their derivatives have anti-proliferative and pro-apoptotic effects (with no toxicity to non-tumour tissues), being therefore recognized as promising antitumor compounds for several types of cancer (Kaushik *et al.* 2015, Sinha *et al.* 2016, Marostica *et al.* 2017).

2.1.5.4. Saponins

Saponins are a diverse group of secondary metabolites characterized by a structure containing a triterpene or steroid aglycone called sapogenin and one or more sugar chains (Güçlü-Üstündağ and Mazza 2007, Podolak *et al.* 2010), which have already been identified in more than 100 plant families. The consumption of saponin-containing plant foods may be

associated with beneficial and adverse effects. Saponins can act as antinutrients and, in some cases, reduce the consumption of some foods because of their bitter taste (Muzquiz *et al.* 2012). All legumes have triterpene-type saponins (Muzquiz *et al.* 2012). In fact, while triterpenoid saponins are mainly found in Magnoliopsida (former dicotyledons), steroidal saponins are more abundant in Liliopsida (former monocotyledons) (Güçlü-Üstündağ and Mazza 2007, Podolak *et al.* 2010). The structural complexity of these compounds results in a wide range of biological properties (Güçlü-Üstündağ and Mazza 2007) among which *in vitro* haemolytic activity has been one of the most investigated ones. This activity is mainly attributed to their ability to interact with membrane cholesterol that leads to membrane destabilization (Podolak *et al.* 2010). According to Oda *et al.* (2000), the haemolytic effect of saponins can be related to the presence of an acyl residue or oxide-ring moiety. However, both aglycone and sugar chain play an important role for cytotoxic activity (Podolak *et al.* 2010).

As presented in **Table 8**, the steroidal saponins dioscin and gracillin (**Figure 5**) were identified in rhizomes of *T. communis* (EFSA 2009) and the triterpenoid saponins silenosides A, B and C were extracted from roots of *S. vulgaris* (Glensk *et al.* 1998). The pharmacokinetics of dioscin was studied by Li *et al.* (2005). The authors found that its bioavailability after oral administration to rats was very low (0.2%) and sustained prolonged absorption in the intestines, where it remained at high concentrations even after 120 h after administration. Other studies indicated that dioscin has anti-inflammatory properties (Wu *et al.* 2015) and inhibits proliferation and induces apoptosis in cancer cells (Zhang *et al.* 2016, Zhao *et al.* 2016), highlighting this compound as a promising anti-inflammatory and anti-cancer drug.

2.1.5.5. Phenanthrenes and hydroxyanthracenes

Phenanthrenes have been reported in higher plants, mainly in the family Orchidaceae, but also in Dioscoreaceae, Combretaceae and Betulaceae families and Hepaticae class (Kovács *et al.* 2008). Phenanthrene derivatives with a pronounced cytotoxic activity on cervix adenocarcinoma (HeLa) cells were isolated from rhizomes of *T. communis* (Réthy *et al.* 2006, Kovács *et al.* 2007). Despite all the potential of this group of natural bioactive compounds as cytotoxic, anti-inflammatory, antimicrobial, spasmolytic, and antiplatelet aggregation agents (Kovács *et al.* 2008), their pharmacological potential is not yet sufficiently studied.

In addition to oxalates, some *Rumex* species may also contain hydroxyanthracene derivatives (EFSA 2012). Eight anthracene derivatives were described in six Rumex species (**Table 8**), including *R. acetosa*, *R. acetosella*, *R. crispus* and *R. obtusifolius*, and the highest levels were detected in the roots (Smolarz *et al.* 2011).

2.1.5.6. Monoterpenes, phenylpropanoids and prenylflavonoids

As shown in **Table 8**, potentially toxic monoterpenes and phenylpropanoids can be found in the essential oil of some of the wild plants under review. All plants containing these constituents appear on the EFSA compendium of botanicals that have been reported to contain toxic or other substances of concern (EFSA 2009, 2012). The bicyclic monoterpene ascaridole and the phenylpropanoid safrole (**Figure 5**) can be found in *C. ambrosioides* essential oil (Tucker and Maciarello 1998, Kiuchi *et al.* 2002). This is an essential oil with anthelmintic activity that has been used to treat parasitosis and leishmaniasis (Monzote *et al.* 2009). Its toxicity is mainly attributed to the presence of caryophyllene oxide, while the toxicity of ascaridole is potentiated by the availability of redox-active iron (Monzote *et al.* 2009, Gille *et al.* 2010). 1,8-Cineole (eucalyptol) is an essential oil constituent of *G. hederacea*, *M. pulegium* and *O. vulgare* (EFSA 2009, 2012, Kristanc and Kreft 2016a). It is used as food flavouring and as excipient in the pharmaceutical industry. However, this organic compound presents possibly maternal and foetal toxicity (Caldas *et al.* 2016).

Pulegone can be found in Mentha sp.pl., especially in *M. pulegium* essential oil, which also contains the bicyclic monoterpenes menthofuran and thujones (Anderson *et al.* 1996, EFSA 2009, 2012, Da Rocha *et al.* 2012, EMA 2016b, Kristanc and Kreft 2016a). Menthofuran is a hepatotoxin produced biosynthetically from pulegone (a compound with strong insecticidal activity) (Abdelli *et al.* 2016). Menthofuran is metabolically activated to an electrophilic gamma-ketoenal that is capable of covalent binding to cellular proteins to form covalent adducts (Thomassen *et al.* 1992). In turn, thujones are known for their neurotoxic effects (Radulović *et al.* 2017).

Regarding phenylpropanoids, estragole and *trans*-anethole have been reported as constituents of the *F. vulgare* essential oil (García-Jiménez *et al.* 2000, EFSA 2009, 2012). The genotoxic and carcinogenic (mainly hepatocarcinogenic) effects of estragole are well established in several rodent models. Although *trans*-anethole is not genotoxic, it induces hepatocellular carcinomas in rats (Kristanc and Kreft 2016b).

The prenylflavonoids 8-prenylnaringenin (hopein), xanthohumol and isoxanthohumol (**Figure 5**) were identified as the major phytoestrogenic constituents in *H. lupulus* inflorescences (EFSA 2009, Kristanc and Kreft 2016b), compounds which impart an endocrine-disrupting potential to this plant (Kristanc and Kreft 2016b). Costa *et al.* (2017) demonstrated that the ingestion of 8-prenylnaringenin and xanthohumol could ameliorate diabetic-related metabolic dysfunctions in mice by regulating glucose and lipid pathways. Isoxanthohumol exhibits antiproliferative activity against different human cancer cell lines, including those of the breast, ovarian, prostate, and colon. It also inhibits the production of prostate-specific antigen (Żołnierczyk *et al.* 2015).

2.1.6. Safety precautions

Most poisonous wild plants are recognized as dangerous by local populations due to the empirical knowledge acquired over centuries and registered in ethnobotanical studies (Aceituno-Mata 2010, Carvalho 2010). In addition, the consumption of botanicals of potential concern for human health is cautioned by EFSA (EFSA 2009, 2012). Seed oils of *A. azurea* and *B. officinalis*, essential oils of *C. ambrosioides*, *F. vulgare*, *M. pulegium*, *O. vulgare* and *Thymus* sp.pl., roots and rhizomes of *S. vulgaris*, *B. dioica* and *T. communis*, fruits of *B. dioica* and *T. communis*, and some *Rumex* sp.pl., among others presented in **Table 8** and **Figure 4**, are plants of possible concern. Some of these botanicals are very bitter and, in some cases, the traditionally consumed part is not where the potentially toxic compounds accumulate. For example, cucurbitacins and steroidal saponins can be found in the roots of *B. dioica* and *T. communis*, respectively (**Table 8**), but the young shoots with immature leaves are the traditionally consumed parts of these wild asparagus (**Table 2**) (Tardío *et al.* 2006). Traditional knowledge warns against the toxicity of these species and considers the young shoots as the least toxic part (Carvalho 2010). According to some informants, these wild foods should not be eaten in the flowering or fruiting stage because they can cause diarrhoea or other adverse effects (Tardio *et al.* 2002). In fact, ethnobotanical information describes that young shoots should be gathered only in spring before the development of immature floral buds (Carvalho 2010). Therefore, just cooked young shoots should be consumed (Tardío *et al.* 2016).

In the case of plants traditionally prepared as medicinal infusions/decoctions (**Table 2**), empirical knowledge and healers indicate that the long-term use of these preparations in humans should be avoided to prevent possible adverse effects, which may differ according to the dose and physical condition of the consumer (Carvalho 2010).

2.1.7. Valorisation strategies, challenges and trends

WEPs have provided a key source of nutrients to humans since prehistoric times; but their relevance to the human diet decreased first with the agricultural expansion and then with the industrial revolution (Łuczaj 2010, Reyes-García *et al.* 2015). In addition, wild foods are often undervalued because of former associated perception of poverty and scarcity and their relatively low presence in contemporary urban diets. However, several studies have shown that WEPs: i) continue to contribute to food sovereignty in many regions of the world, especially in non-industrialized countries; ii) are key components of extensive farming systems and of subsistence economy; iii) hold potential to reactivate rural economies; and iv) help maintain local identities, with likely effects on biodiversity conservation (Menendez-

Baceta *et al.* 2017). Indeed, the current limited use of wild plants is a lost opportunity for local economy and population well-being of different regions of the world (Bacchetta *et al.* 2016).

Interestingly, some wild species are now re-emerging in gardens and kitchens of urban areas around Europe, being increasingly found in farmers' markets, gourmet food shops, and restaurants (Tardío 2005, Łuczaj *et al.* 2012, Vasquez 2016). Nevertheless, WEPs lack recognition as significant contributors to the human diet in developed countries. Therefore, agro-ecological, nutritional, bioactivity and safety studies are of high importance for their revival and commercialization (Bacchetta *et al.* 2016). The revalorisation strategies herein proposed are based on the following criteria:

- *Nutritional and chemical characterization:* The evaluation of the nutritional value (macro- and micronutrients) and chemical composition (secondary metabolites) will highlight the most promising wild species to be included in contemporary diets or to be use as an alternative source of natural ingredients (Barros, Carvalho, and Ferreira 2010b). The presence of anti-nutritional and toxic compounds should be investigated to ensure the safety of WEPs and based products.
- *Evaluation of bioactivities and health promoting effects:* Several WEPs are considered functional foods (Pinela, Carocho, *et al.* 2016), *i.e.,* foods that exert beneficial physiological effects that go beyond the basic nutrition capacity. Thus, in parallel with the chemical prospecting studies (which highlight the most promising species in terms of bioactive molecules), the *in vitro* and *in vivo* evaluation of antioxidant, anti-inflammatory, antitumor, antimicrobial, antidiabetic, and cardioprotective potential of wild plant-based extracts or isolated compounds should be performed (*e.g.,* the studies of Pereira *et al.* (2016), Guimarães *et al.* (2016) and Nikolić *et al.* (2014) with *Tuberaria lignosa* (Sweet) Samp., *Chamaemelum nobile* (L.) All. and *Thymus* sp.pl., respectively). The impact of a regular consumption of wild plant foods in the prevention of diet-related and chronic diseases should be accessed. The absence of toxicity must be always ensured to safeguard human health and well-being.
- *Application of innovative methods and technologies:* Application of non-conventional and emerging treatments (innovative packaging systems, light treatments, irradiation, high pressures, pulsed electric fields, among others) for quality preservation and shelf-life extension of wild plant foods (Pinela, Barreira, Barros, Antonio, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio, Carvalho, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016, Pinela, Barros, *et al.* 2016), in order to promote their commercialization and the consumer’s acceptance for minimally processed and new food products. Establishment of optimized protocols for recovery of high added-value compounds (polyphenols, terpenoids, polysaccharides,

and others) from wild species and their by-products and production of enriched extracts to be used in industrial sectors (Pinela, Prieto, Antonio, *et al.* 2017, Pinela, Prieto, Barreiro, *et al.* 2017).

- *Development of new products, recipes and characteristic dishes:* Development of wild plant-based foods and beverages, such as *C. intybus*-based coffee (Street *et al.* 2013), as well as functional ingredients to be used as nutraceuticals, preservatives, colorants, sweeteners, or antifungals in new food, pharmaceutical, or cosmeceutical formulations (Barreira *et al.* 2013, Barroso *et al.* 2014, Martins *et al.* 2014, Caleja *et al.* 2015, Caleja, Barros, *et al.* 2016, Caleja, Ribeiro, *et al.* 2016). Some of these natural ingredients (antioxidants, antifungals, colorants and sweeteners) may replace specific artificial food additives used in the industry (Carocho *et al.* 2015, Martins *et al.* 2016). New recipes and characteristic dishes based on traditional uses and modes of consumption of wild edibles might raise some interest in these foods as wild products are becoming fashionable. In the Iberian peninsula and Italy, dishes incorporating *T. communis*, *A. acutifolius*, *A. ampeloprasum*, *N. officinale*, *M. fontana*, *B. officinalis*, *M. sylvestris*, *P. rhoeas*, *S. hispanicus*, *Rumex crispus* L., and *Urtica dioica* L. (**Figure 2**) can be found in some luxury restaurants (Łuczaj *et al.* 2012, Bicho 2015).
- *Implementation of alternatives to wild-harvesting practices:* Wild harvesting may represent a source of income for rural communities. However, an uncontrolled and irrational overexploitation promoted by economic interests may become a risk and lead to species extinction and erosion of some components within different ecosystems. Although the challenges are great, domestication and cultivation of wild species is the most sustainable alternative that offers several advantages over wild harvesting. Additionally, cultivation provides reliable botanical identification, guarantees a steady source of raw material, allows controlled postharvest handling and, therefore, the quality control can be assured. Moreover, cultivated plant materials can be easily certified as organic or biodynamic (Schippmann *et al.* 2002). However, further agronomic and biotechnological research is needed.
- *Agricultural and in vitro production:* The most promising wild plants (native or introduced and naturalized) should be selected for cultivation in order to diversify the on-farm production. Since most of them are autochthonous plants adapted to local environments, they support the seasonal variations and adverse climatic conditions of the region, which ensure more stable productions (Molina *et al.* 2016). For example, *S. hispanicum* is already cultivated and commercialized in some regions of Spain and its shoots are sold as gourmet food product (Łuczaj *et al.* 2012). The agricultural interest for non-food crops for obtaining high-added value products is also attractive,

namely *n*-3 fatty acids from seeds of *B. officinalis* (Guil-Guerrero *et al.* 2013), tocopherols from *G. hederacea* (Barros *et al.* 2010) and *C. ambrosioides* (Barros *et al.* 2013), phenolic compounds from *R. ulmifolius* (Barros *et al.* 2010), inulin from *C. intybus* (Amaducci and Pritoni 1998), cucurbitacins from *B. dioica* roots (Pohlmann 1975, Hylands and Salama 1976, Hylands and Mansour 1982, Oobayashi *et al.* 1992, Chen *et al.* 2005, Gry *et al.* 2006), and prenylflavonoids from *H. lupulus* inflorescences (Kristanc and Kreft 2016b).

- *Breeding and genetic characterization of crop wild relatives:* The genetic improvement of crop wild relatives is a scientific challenge that should be pursued. Advanced biotechnological and molecular tools could facilitate the discovery of new genes and open new opportunities for plant breeding (Amirul *et al.* 2014). In fact, wild relatives maintain a much higher level of genetic diversity than domesticated cultivars (Zhang *et al.* 2017). Using omics-scale technologies and advanced techniques, genes of interest can be transferred from wild species to cultivated crops. The development of novel crop varieties can help overcame critical challenges of food production related to the deleterious effects of climate change and human activities as well as diverse environmental stresses (Zhang *et al.* 2017).
- *In situ and ex situ conservation:* Plant genetic resources strategies and techniques play an important role in wild species conservation and management. Particularly on-farm conservation and protected areas defined (*in situ* conservation measures), as well as *ex situ* conservation measures supporting *in situ* conservation, have great relevance to wild edibles use and prospective research. *In situ* strategies actively involve local people and might be representative samples of unaltered species and ecosystems, limiting, to some extent, the potential for environmental degradation and loss of resources. *Ex situ* approaches preserve genetic resources of wild and cultivated species, using a diverse body of techniques and facilities (*e.g.,* gene banks, *in vitro* plant collections, field collection), providing excellent research opportunities on the components of biological diversity.
- *Application for environmental remediation:* Some wild species have high potential to be used in phytoremediation programs such as *N. officinale* due to their capacity to bioaccumulate environmentally toxic substances in their tissues (Torbati *et al.* 2014).
- *Publicizing and divulgation:* Drawing public attention to and promoting the potential of WEPs in restaurants and gourmet food shops, since, nowadays, there are consumers looking for food with differentiated organoleptic properties from those daily eaten.

The revival of WEPs will promote the conservation of people's local identity and traditions, since wild plants are part of the cultural heritage of the regions where they are used (Carvalho 2010, Carvalho and Morales 2010), increase the range of available foods

and plant-based products, and diversify the agricultural productivity and small-scale and familiar agriculture. In fact, wild plants are alternative potential crops to enhance the agricultural productivity within extensive farming systems and homegardens, at a local and regional level. For instance, the particular case of North-eastern Portugal, where the mountain farming which is largely based on family farming, might use the diversity of agro-ecosystems to promote diversification of crops, integration of forests non timber products and livestock activities, furthermore including sustainable gathering of wild edibles and cultivation of some wild species.

The strategies herein proposed for the valorisation of wild plant species should be carried out by a multidisciplinary team composed by farmers, agronomists, ecologists, ethnobotanists, biochemists, nutritionists, pharmacists, food engineers, chefs (for creating new dishes and recipes) and decision makers, among other stakeholders. This collaborative endeavour between different disciplines and scientists and the industrial sector will generate knowledge, new practices and relevant outputs, as well as an ecologically sustainable development.

2.2. Bioactive compounds of tomatoes as health promoters

2.2.1. Tomato as a versatile functional food

The tomato (*Lycopersicon esculentum* Mill.) plant was imported from the Andean region to Europe in the 16th century. It belongs to the Solanaceae family that includes many other plants of economic importance, including potatoes, eggplants, peppers and tobacco (Bergougnoux 2014). Today, this species is widespread throughout the world, representing the most economically important vegetable crop worldwide. In fact, tomato is the most consumed vegetable after potatoes and probably the most preferred garden crop. In 2013, about 164 million tonnes of tomatoes were produced in the world, having been registered an increase above 2.6 million tonnes over 2012. The three main producing countries are China, India and United States of America, but it is in the Mediterranean and Arabian countries that their consumption is higher (FAOSTAT 2015).

Tomato is a very versatile fruit, being consumed fresh but also processed as paste, soup, juice, sauce, powder, or concentrate. In addition, there are several tomato cultivars and varieties with a wide range of morphological and sensorial characteristics which affect the way how they are prepared and consumed (Guil-Guerrero and Rebolloso-Fuentes 2009, Pinela *et al.* 2012, Bergougnoux 2014). Tomatoes and tomato-based food products are an important source of nourishment for the world's population. Regarding its nutritional value, if one takes into consideration only the proteins, fat, carbohydrates, or sugars content, it appears clearly that it does not have a high nutritional value. However, it represent an important source of other nutrients and non-nutrients endowed with important health promoting properties, namely carotenoids such as β-carotene (provitamin A) and mostly lycopene, which provides the deep red colour, vitamins such as ascorbic acid (vitamin C) and tocopherols (vitamin E), phenolic compounds including hydroxycinnamic acid derivatives and flavonoids, lectins, and minerals (K, Mn, Ca, Cu and Zn) (Guil-Guerrero and Rebolloso-Fuentes 2009, Barros *et al.* 2012, Pinela *et al.* 2012).

Tomatoes are the most important component of the Mediterranean diet, known to be beneficial for human health (Vallverdú-Queralt *et al.* 2013). A relationship between the consumption of tomatoes and tomato-based foods and the prevention of chronic degenerative disease induced by oxidative stress and inflammation has been indicated in several studies (Hadad and Levy 2012, Samaras *et al.* 2014, Li *et al.* 2015, Stajčić *et al.* 2015). However, the bioaccessibility and bioavailability of tomato compounds is affected by the way how tomatoes are consumed (*i.e.,* raw or processed), which affects its subsequent bioactivity. Clinical trials performed in the last years elucidate the positive effects and mechanisms involved in the activity of tomato compounds against cardiovascular disease and various types of cancer (Ghavipour *et al.* 2015, Li *et al.* 2015, Stajčić *et al.* 2015, Vilahur

et al. 2015). Indeed, tomato extracts, as well as lycopene, α-tomatine and some phenolic compounds have been highlighted as having increased potential for the development of new drugs, nutraceuticals and functional foods.

This chapter highlights the tomato fruit as a functional food and as a source of nutraceutical ingredients of industrial value. In this sense, the major health promoting compounds of tomatoes are described chemically and its bioavailability, bioactivity and impact on human health are discussed. Recent *in vitro* and *in vivo* clinical trials are presented, with particular attention paid to the mechanisms behind the protective effects of tomato bioactive compounds against the most common degenerative diseases associated to oxidative stress and inflammation, including cardiovascular and hepatocellular diseases, diabetes and various types of cancer, among other health problems.

2.2.2. Tomato bioactive compounds

Nowadays, consumers are increasingly made aware and better informed about the health benefits provided by food beyond its basic nutritional role. Actually, they are looking for foods with health promoting properties called “functional foods”. The tomato fruit is a good example, whose functionality or health claim properties are conferred by biologically active ingredients responsible for decreasing the risk of susceptibility to certain diseases. The major compounds of this fruit (**Figure 6**) are carotenoids (β-carotene and mainly lycopene), vitamins (ascorbic acid and tocopherols), and phenolic compounds including hydrocinnamic acids (mainly caffeic acid and its ester chlorogenic acid) and flavonoids such as narigenin and rutin (Barros *et al.* 2012, Pinela *et al.* 2012, García-Valverde *et al.* 2013). Other bioactive compounds, such as glycoalkaloids and lectins also present in tomato fruits have shown relevant biological effects *in vitro* and *in vivo* (Friedman 2013, 2015). Nevertheless, the content of bioactive compounds in tomatoes is affected by environmental and genetic (cultivar or variety) factors, geographical location, agricultural practices, processing conditions, among others (Aherne *et al.* 2009, Pinela *et al.* 2012, Koh *et al.* 2013, Masetti *et al.* 2014, Raiola *et al.* 2014, Iglesias *et al.* 2015). The main bioactive compounds of tomato fruit are described below.

2.2.2.1. Carotenoids

Carotenoids are a class of hydrocarbons consisting of eight isoprene units, which are joined in a head-to-tail pattern (except at the centre) to confer symmetry to the molecular structure. This way, the two central methyl groups are in a 1,6-positional relationship and the remaining non-terminal methyl groups are in a 1,5-positional relationship. Most carotenoids derive from a 40-carbon polyene chain structure, which is considered as the backbone of these compounds. This chain can terminate with cyclic end-groups (rings) and may have oxygen-

containing functional groups. The long unsaturated alkyl chains make carotenoids highly lipophilic molecules. In higher plants, carotenoids can be found in chloroplasts of photosynthetic tissues and in chromoplasts of flowers and fruits. Generally, they occur in the free form in leaves and in the esterified form in other tissues. These natural pigments play a central role in photosynthesis; they are involved in photosystem assembly and light harvesting, and protect from excessive light through energy dissipation and free radical elimination, thereby reducing membrane damage. In humans, carotenoids are part of the antioxidant defence system and interact synergistically with other bioactive compounds (Namitha and Negi 2010). Once animals cannot synthesize carotenoids, they need to be incorporated through diet, being tomatoes and tomato based-products one of the most common sources of carotenoids available for the human population.

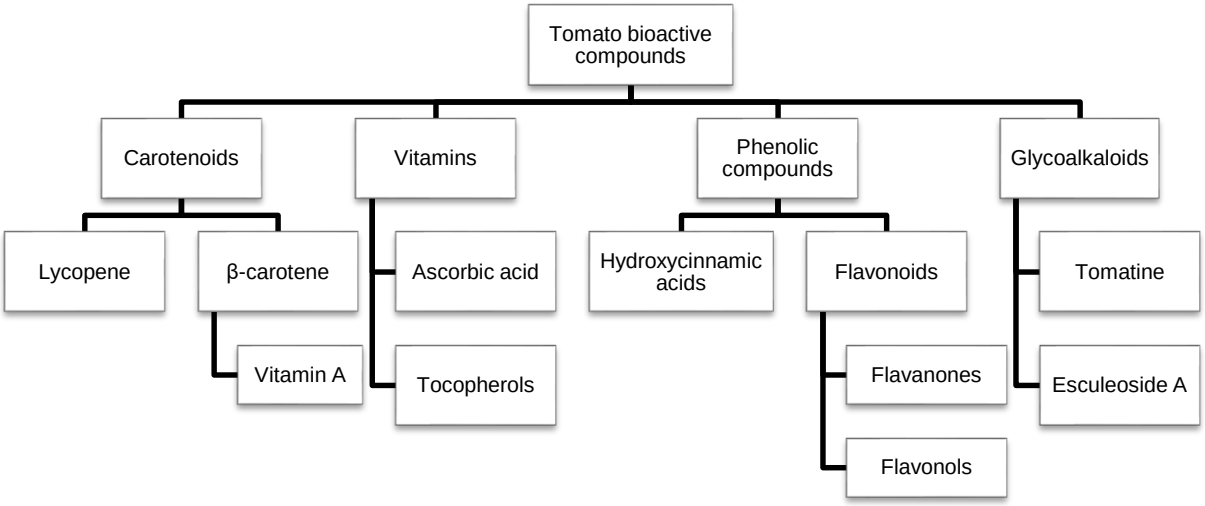


Figure 6 Groups of bioactive compounds of tomatoes.

Tomatoes and tomato-based foods account for over 85% of all the dietary sources of lycopene (Palozza *et al.* 2012). Lycopene is the most abundant carotenoid in ripe tomatoes, representing about 80 to 90% of these pigments (Shi and Maguer 2000). Normally, tomatoes contain up to 10 mg lycopene per 100 g of fresh weight (Guil-Guerrero and Rebolloso-Fuentes 2009), depending on some factors such as those mentioned above. Additionally, the lycopene content increase as the fruit ripens (Tohge *et al.* 2014). Chemically, lycopene (**Figure 7**) is a polyunsaturated (polyene) straight-chain molecule with 11 conjugated and 2 nonconjugated double bonds. Thus, it can be found in both the *cis* and *trans* configurations because of the presence of the double bonds (Hernandez-Marin *et al.* 2013). Additionally, its straight structure facilitates its incorporation into some organs such as the liver, adrenal glands and prostate, where it has a role of preventing oxidative reactions associated with the outbreak of different diseases (Zaripheh *et al.* 2003). The *trans* configuration is the most

common isomer and the largely responsible for the deep red colour of the ripe red tomatoes (Hernandez-Marin *et al.* 2013, Navarro-González *et al.* 2014). Nevertheless, the *trans* form is prone to isomerisation under the influence of some processing conditions, which include the action of light, heat, oxygen and acids, and, after ingestion, it is partly transformed *in vivo* to the more bioactive *cis* form (Boileau *et al.* 2002, Friedman 2013). Lycopene have strong antioxidant activity and other *in vitro* and *in vivo* beneficial effects because of their capacity to act as a free radical scavenger (Zhang *et al.* 2014) that is twice that of β-carotene (**Figure 7**) (Palozza *et al.* 2012). In ripe red tomato fruits, the ratio of lycopene to β-carotene content varies widely between 1.5 and 40 (Darrigues *et al.* 2008, Viskelis *et al.* 2008). Along with α-carotene and β-cryptoxanthin, β-carotene is a provitamin A carotenoid, *i.e.,* they can be converted by the human body into two molecules of vitamin A (**Figure 7**). Actually, what we generally call vitamin A is a group of naturally-occurring molecules, structurally similar to retinol, that are capable of exerting biological activity (Defo *et al.* 2014). In addition to lycopene and β-carotene, phytoene, phytofluene, ζ-carotene, γ-carotene, neurosporene and lutein are other carotenoids reported in tomatoes and tomato-based products (Khachik *et al.* 2002).

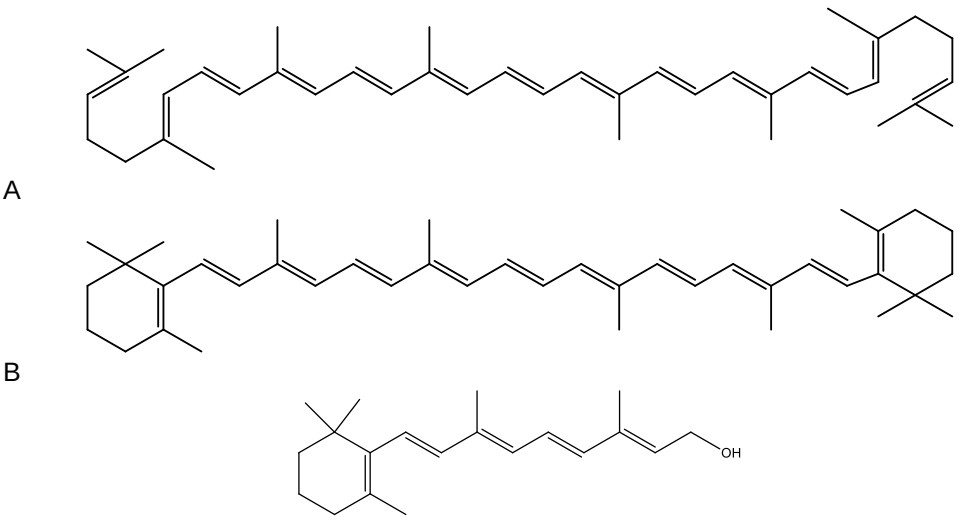


Figure 7 Chemical structures of (A) lycopene, (B) β-carotene and (C) vitamin A.

2.2.2.2. Vitamins

Vitamin C and E are the respective generic names for ascorbic acid and tocopherols. Ascorbic acid, a 6-carbon lactone ring structure with a 2,3-enediol moiety, can be found in all living and actively metabolising plant parts and cell compartments (Baiano and Del Nobile 2015). It comprises two compounds endowed with bioactivity: L-ascorbic acid and L-dehydroascorbic acid (**Figure 8**). Both are easily absorbed by the gastrointestinal tract and can interchange enzymatically *in vivo*. In biological systems, ascorbic acid exists as the

monovalent anion $_L$-ascorbate (Davey *et al.* 2000). However, this vitamin is highly susceptible to oxidation in the presence of metal ions like Cu^{2+} and Fe^{3+}. Its oxidation is also influenced by light, heat, pH, oxygen and water activity (Lee *et al.* 2004). This vitamin has the ability to act as electron donor, being a potent *in vivo* antioxidant; it protects low-density lipoproteins (LDL) from the oxidation caused by different oxidative stress reactions and inhibits the LDL oxidation caused by vascular endothelial cells. The high volume of consumption of tomato all the year round makes this fruit one of the main sources for this vitamin. Different levels of have been reported in tomatoes (8-21 mg/100 g of fresh weight) (Abushita *et al.* 2000, Frusciante *et al.* 2007, Pinela *et al.* 2012), since it is affected by different factors. In turn, vitamin E includes eight chemically distinct molecules (**Figure 9**), four tocopherol isoforms (α-, β-, γ- and δ-tocopherol) and four tocotrienol isoforms (α-, β-, δ- and γ-tocotrienol) (Carocho and Ferreira 2013). Tocopherols differ from the corresponding tocotrienols in the aliphatic tail; tocopherols have a phytyl side chain attached to the chromanol head, whereas the tail of tocotrienols contains three *trans* double bonds at the 3', 7' and 11' positions and forms an isoprenoid chain. These unsaturations in the tail of tocotrienols give only a single stereoisomeric carbon, whereas tocopherols have eight possible stereoisomers per structural formula. The various isoforms differ in the methyl substituents on the chromanol head; the α-forms contain three methyl groups, the β- and γ- have two and the δ-forms have only one methyl group. Together, tocopherols and tocotrienols are called tocochromanols. All these compounds feature a chromanol ring with a hydroxyl group capable of donating a hydrogen atom, and a lipophilic side chain that allows for penetration into cell membranes (Baiano and Del Nobile 2015). The donation of hydrogen atoms to the peroxyl radicals forms unreactive tocopheroxyl radicals ($TO^{\bullet}$) unable to continue the oxidative chain reaction (Burton and Traber 1990). The human body absorbs all forms of vitamin E, but maintains only the α-tocopherol (Packer *et al.* 2001). The amounts of tocopherols also vary in tomatoes, having been reported values from 0.17 to 1.44 mg/100 g of fresh weight (Frusciante *et al.* 2007, Pinela *et al.* 2012). Nevertheless, neither vitamin C nor vitamin E can be synthesized by humans, so their intake must be guarantee through the diet (Giovannoni 2007, Baiano and Del Nobile 2015).

Figure 8 Chemical structures of (A) $_L$-ascorbic acid and (B) $_L$-dehydroascorbic acid.

The tomato fruit also presents folates (12-18 µg/100 g of fresh weight) (Martin *et al.* 2010, Tyagi *et al.* 2015), a complex group of water-soluble compounds known as vitamin B_9. Folic acid (**Figure 10**) consists in an aromatic pteridine ring attached by a methylene bridge to a residue of *p*-aminobenzoic acid which, in turn, is joined by an amide bond to a glutamic acid residue (Morales *et al.* 2015). Folate vitamers are involved in multiple physiological mechanisms in the field of andrology and gynecology (Forges *et al.* 2007) and are essential for fetal growth (Wagner 2001). Folates also are involved in the homocysteine metabolism regulation (Selhub *et al.* 1993) and some authors consider the hyperhomocysteinemia condition as a marker or risk factor for cardiovascular diseases (Hackam and Anand 2003, Splaver *et al.* 2004). As folates are synthesised just by plants and microorganisms, humans are dependent on dietary sources like the tomato fruit for the intake of this vitamin.

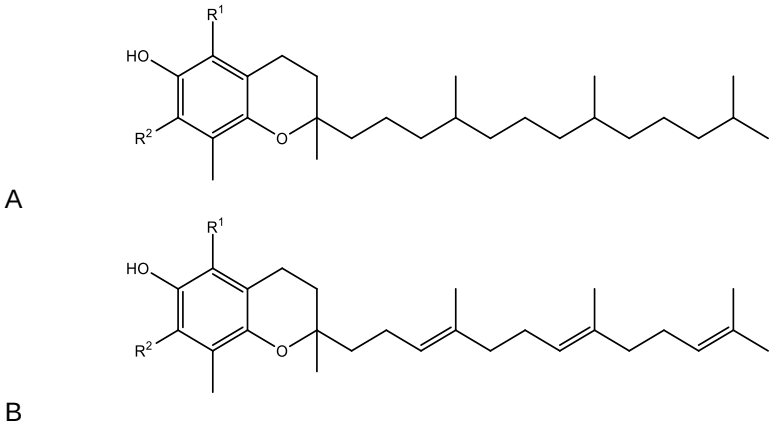


Figure 9 Chemical structures of (A) tocopherols (R^1=R^2=Me: α-tocopherol; R^1= Me, R^2=H: β-tocopherol; R^1=H, R^2=Me: γ-tocopherol; R^1=R^2=H: δ-tocopherol) and (B) tocotrienols (R^1=R^2=Me: α-tocotrienol; R^1= Me, R^2=H: β-tocotrienol; R^1=H, R^2=Me: γ-tocotrienol; R^1=R^2=H: δ-tocotrienol).

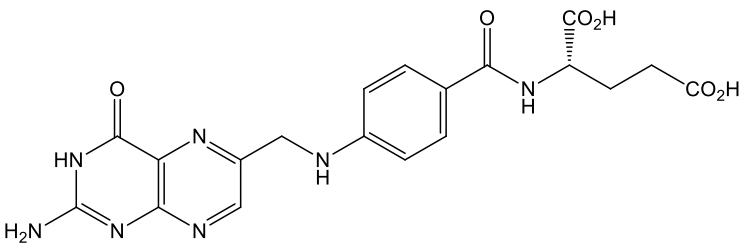


Figure 10 Chemical structure of folic acid.

2.2.2.3. Phenolic compounds

Phenolic compounds are broadly spread throughout the plant kingdom, representing more than 8000 different phenolic structures. They have at least one aromatic ring with one or more hydroxyl groups attached and vary from low molecular weight molecules to large and complex ones. Phenolic compounds generally appear as esters and glycosides rather than

as free compounds due to the conferred stability of these molecules. Phenolic (hydroxycinnamic) acids and flavonoids are the most abundant phenolic compounds in tomatoes (Barros *et al.* 2012), as well as in the diet (Escarpa and Gonzalez 2001). The phenolic acids represent a group of compounds that derive from cinnamic acid through the phenilpropanoid pathway. They display a C_6-C_3 skeleton of *trans*-phenyl-3-propenoic acid with one or more hydroxyl groups bonded to the phenyl moiety, some of which may be methylated. According to literature, caffeic acid and its ester chlorogenic acid are the main phenolic acids in tomato (**Figure 11**) and the most extensively studied (Vallverdú-Queralt *et al.* 2010, Barros *et al.* 2012). Both compounds have *in vitro* antioxidant activity (Sato *et al.* 2011) and might inhibit the formation of mutagenic and carcinogenic *N*-nitroso compounds (Kono *et al.* 1995). Curiously, the antioxidant mechanism of chlorogenic acid is analogous to that of lycopene. The flavonoids are the largest group of molecules within the phenolic compounds. These polyphenolic compounds display an arrangement of three aromatic rings with 15 carbons and a C_6-C_3-C_6 skeleton which can have numerous substituents. The A ring is a benzene, condensed with a six-member ring (ring C), which carries a phenyl benzene in position 2 (ring B) as a substituent. Sugars in the form of glycosides are normally joined with flavonoids and while they increase water solubility along with hydroxyl groups, other substituents like methyl groups and isopentenyl units increase their lipophilic properties (Carocho and Ferreira 2013). In tomatoes, flavonoids are represented by flavanones, including naringenin glycosylated derivatives, and flavonols such as quercetin, rutin and kaempferol glycosylated derivatives (**Figure 12**) (Le Gall *et al.* 2003, Bahorun *et al.* 2004, Slimestad *et al.* 2008, Gómez-Romero *et al.* 2010). They are commonly found in the skin and in small amount in the other parts of the fruit.

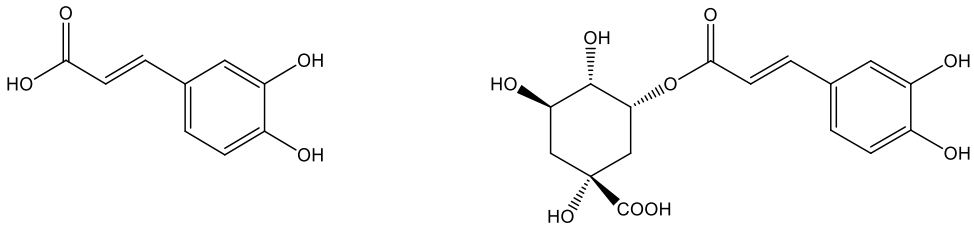


Figure 11 Chemical structures of (A) caffeic acid and (B) chlorogenic acid.

2.2.2.4. Glycoalkaloids

Glycoalkaloids are characteristic secondary metabolites in plants of the Solanaceae family. They are involved in host-plant resistance and have pharmacological and nutritional effects in humans and animals. In tomato plants, the glycoalkaloids tomatine and esculeoside A (**Figure 13**) are synthesized. Tomatine comprises a junction of α-tomatine and

dehydrotomatine (**Figure 13**). Structurally, dehydrotomatine differs from α-tomatine by having a double bond in the steroidal ring B of the aglycone. However, both glycoalkaloids have the same tetrasaccharide (lycotetraose) side chain; α-tomatine has lycotetraose bonded to the aglycone tomatidine, whereas dehydrotomatine has the side chain attached to an aglycone tomatidenol (Friedman 2013, 2015). Unripe tomatoes may contain up to 500 mg of tomatine per kg of dry weight; but the levels are decreased with the ripening of tomatoes and, therefore, ripe red tomatoes present lower levels (~5 mg/kg fw) (Friedman 2004). Besides, the tomatine content of cherry tomatoes is several fold greater than that of larger size standard varieties. On the other hand, the content of esculeoside A, which is stored in ripe fruits of cultivated tomatoes, is comparable to or higher than that of lycopene (Fujiwara *et al.* 2004, Nohara *et al.* 2010). Thus, the levels of esculeoside A increase as the fruit matures, contrary to that observed for tomatine (Iijima *et al.* 2008, Friedman 2013).

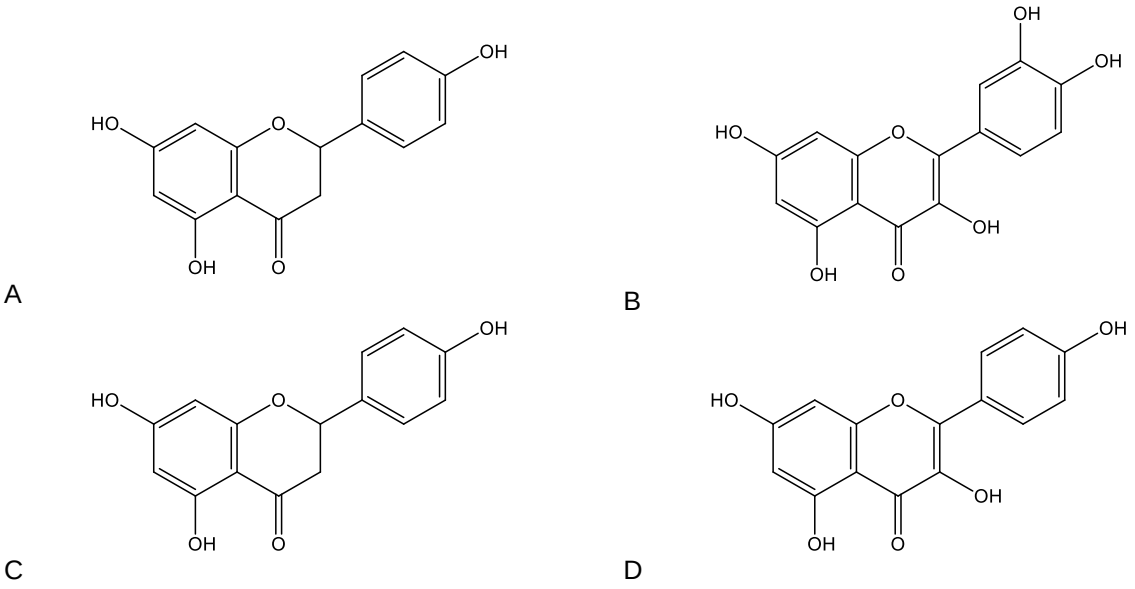
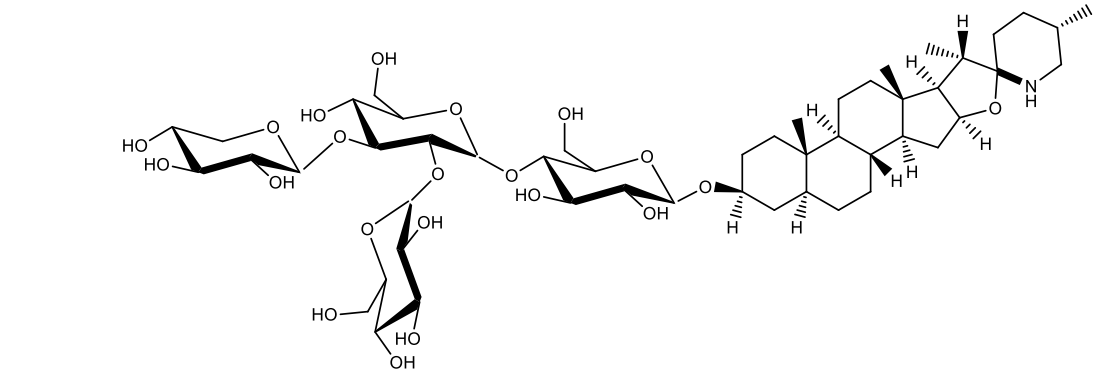


Figure 12 Chemical structures of (A) naringenin, (B) quercetin, (C) rutin and (C) kaempferol.

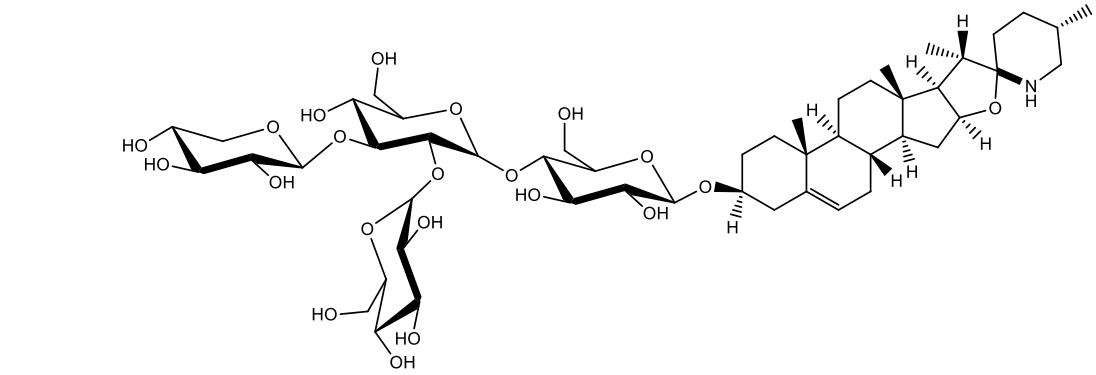
2.2.3. Bioavailability of tomato compounds

The bioavailability of bioactive compounds is crucial to their physiologic effect. Before becoming bioavailable, the tomato bioactive compounds must be released from the plant matrix and modified in the gastrointestinal tract. The digestive transformations that involve the conversions of tomato into substances ready to be absorbed and assimilated are called bioaccessibility. It is commonly assessed using *in vitro* digestion assays, which simulate the gastric and small intestinal digestion processes. Differently, the term bioavailability can be defined as the fraction of a compound or metabolite that reaches the systemic circulation. It is evaluated using *in vivo* assays in animals or humans by measuring the concentration of a

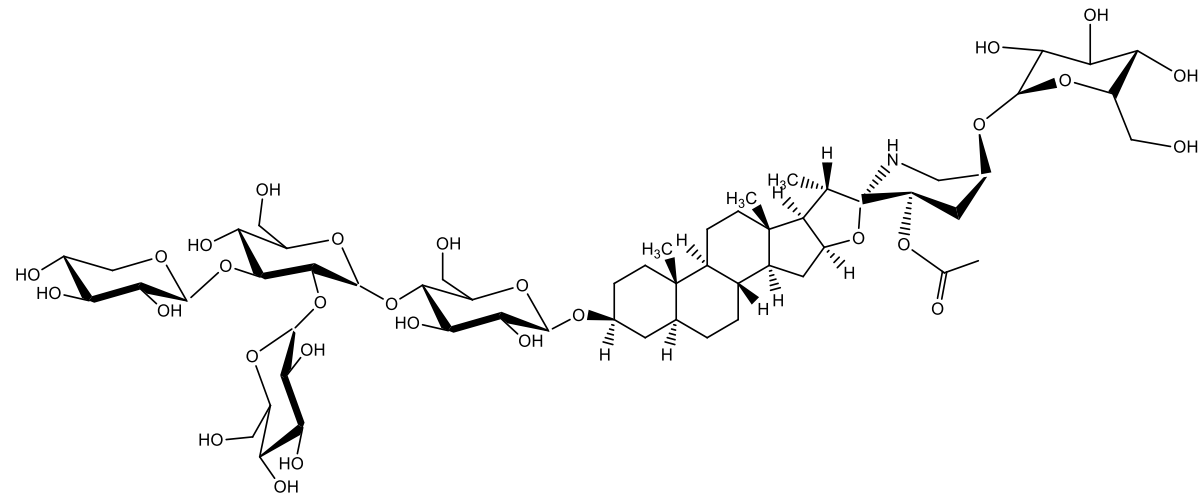
compound in plasma or urine after administration of an acute or chronic dose of the isolated compound or compound-containing food (Carbonell-Capella *et al.* 2014).



A



B



C

Figure 13 Chemical structures of (A) α-tomatine, (B) dehydrotomatine and (C) esculeoside A.

2.2.3.1. Bioavailability of carotenoids

The bioavailability of the tomato carotenoids is widely affected by endogenous (tomato-related) and exogenous (processing-related) factors. Firstly, in order to become bioaccessible, carotenoids need to be released from the tomato matrix in which they are embedded. Thereafter, they need to solubilise into an oil phase either during processing

and/or during the gastric digestion. The release of carotenoids from the tomato matrix and its subsequent incorporation in the oil and micellar phase are decisive steps during digestion in order to become bioavailable. In fact, the mixed micelles formed in the small intestine are the primary vehicle for the absorption of carotenoids via intestinal mucosa (Rich *et al.* 2003). That's why the bioavailability is greatly affected by dietary composition, *i.e.,* the co-ingestion of carotenoids and fat is very important and necessary for absorption (Story *et al.* 2010, Fernández-García *et al.* 2012, Arranz *et al.* 2015). Actually, the Mediterranean way of preparing tomatoes, by cooking them in olive oil, is a smart way to promote the absorption of these bioactive compounds. Regarding exogenous factors, it is known that the lycopene bioaccessibility increases under processing conditions because cell membranes are disrupted, which increases its release from the tomato matrix (Namitha and Negi 2010). Thermal treatments also promote the lycopene isomerisation of *trans* to *cis* form, isomer that is described as being more bioavailable (Boileau *et al.* 2002, Unlu *et al.* 2007). Thus, lycopene from tomato-based processed foods is generally more bioavailable than from fresh tomatoes. Nevertheless, inadequate processing and storage conditions can cause isomerisation during the formation of by-products, which can reduce the absorption of carotenoids and make the food less desirable to the consumer.

2.2.3.2. Bioavailability of vitamins

The ingestion of ascorbic acid causes a dose-dependent increase of this vitamin in the plasma. Its absorption from the gastrointestinal tract occurs by a sodium-dependent active transport mechanism (mainly in the jejunum) but also by a passive absorptive pathway. The active transport predominates when ascorbic acid is at low gastrointestinal concentrations, but when at high concentrations the active transport becomes saturated allowing only passive diffusion (Davey *et al.* 2000). In turn, the absorption of tocopherols is similar to that observed for other fat-soluble vitamins, being necessary it’s packaging into micelles (emulsified in the presence of bile salts and amphipathic lipids available in the intestinal lumen) to facilitate their absorption during digestion, the same as carotenoids. After entry into the intestinal absorptive cells (enterocytes), tocopherols are packaged into chylomicrons and enter the circulation through the lymph-vascular system. Thereafter, chylomicrons triglycerides are hydrolysed by endothelial bound lipoprotein lipases, resulting in the transfer of tocopherols and lipids to peripheral tissues (Lodge 2005). However, the main steps and molecular mediators of the tocopherols transport from the luminal micellar phase into the enterocytes are not yet fully elucidated.

2.2.3.3. Bioavailability of phenolic compounds

The bioavailability within the class of phenolic compounds is widely variable and the most abundant in our diet do not always correspond to those with better bioavailability. The ability of the human body to absorb and metabolize these compounds varies widely depending primarily on their physicochemical properties, such as the basic structure, molecular size, degree of polymerization or glycosylation, solubility and conjugation with other phenolics (Carbonell-Capella *et al.* 2014). Phenolic acids of low-molecular weight such as gallic acid are easily absorbed by the small intestine, as well as flavones and quercetin glycosides (Martin 2009). Conversely, larger polyphenols are poorly absorbed. In addition, a large number of phenolic compounds is found in the glycosylated form or as esters or polymers which must be hydrolysed before the free aglycone can be absorbed. The human metabolism also greatly affects the bioavailability of these bioactive compounds. Once absorbed, polyphenols undergo biotransformations in the small intestine, and later in the liver, into various *O*-sulfated, *O*-glucuronidated and *O*-methylated forms. Thus, the chemical structure of the resultant metabolites could be quite different from that of the parent compounds and, therefore, these metabolites may or not have the initial biological activity (Heleno *et al.* 2015). Moreover, the bioavailability of the phenolic compounds can be influenced by different food processing steps. For example, it has been demonstrated a significant increase in the levels of chlorogenic acid and naringenin in plasma after consumption of cooked tomatoes in comparison with fresh tomatoes (Bugianesi *et al.* 2004). Actually, mechanical and thermal treatments involved in the manufacture of tomato sauce helps to release these bioactive compounds from the tomato matrix, thus increasing the bioavailability more efficiently than through the addition of oil (Martínez-Huélamo *et al.* 2015).

2.2.3.4. Bioavailability of glycoalkaloids

Very limited studies have been conducted to study the bioavailability of glycoalkaloids in humans, but it is known that these compounds are poorly absorbed by the gastrointestinal tract of mammals. An appreciable amount of the ingested glycoalkaloids is hydrolysed in the gut to less toxic aglycones, and the originated metabolites are rapidly excreted via urine and feces (Hoffmann 2003).

2.2.4. Bioactivity of tomato compounds

Bioactivity can be defined as the effect caused upon exposure to an active ingredient. It comprises tissue uptake, as previously referred, and the resulting physiological response, which can be evaluated *in vivo*, *ex vivo* or using *in vitro* assays. The tomato compounds are known for their capacity to act as free radical scavengers of reactive oxygen and nitrogen species (ROS/RNS). These species include free radicals and other non-radical reactive

substances also called oxidants. ROS and RNS are generated as a normal part of human metabolism and its production can be promoted by external factor. The accumulation of these species in the body gives rise to a phenomenon known as oxidative stress, which results from an imbalance between generation and neutralization of reactive species in the cells. The main targets of these species are proteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) molecules, lipids and sugars. The lipid peroxidation is one of the most undesirable effects of ROS because of the consequent formation of free radicals. This phenomenon is initiated by an attack towards a fatty acid side chain by a radical in order to abstract a hydrogen atom. A higher number of double bonds in the fatty acid facilitate the removal of hydrogen atoms and consequently the formation of radicals. After that, the carbon-centred lipid radical can undergo molecular rearrangement and react with oxygen forming a peroxyl radical. These highly reactive species can abstract hydrogen atoms from surrounding molecules and propagate the lipid peroxidation chain reaction. Hydroxyl radicals are major radicals in lipid peroxidation mechanisms (Pham-Huy *et al.* 2008, Carocho and Ferreira 2013). Products generated by these chain reactions are toxic, *e.g.,* malondialdehyde (MDA) may be involved in the onset of mutagenic damage. ROS can also activate the transcription nuclear factor kappa B (NF-κB), which leads to the expression of pro- and anti-inflammatory cytokines genes and their subsequent production (Hayden *et al.* 2006). As a consequence, these processes play a key role in the development of several degenerative and chronic diseases, as well in aging. The main mechanisms and properties inherent to the bioactivity and protective effects of the major tomato compounds are discussed below.

2.2.4.1. Bioactive properties of carotenoids

Carotenoids can inhibit the lipid peroxidation due to their capacity to act as free radical scavengers (Zhang *et al.* 2014). The basic antioxidant properties of these pigments are conferred by its singlet oxygen quenching capacity, by which the carotenoids are excited. The excited carotenoids can dissipate the excess energy through a sequence of rotational and vibracional interactions that allows them to return to the unexcited state, and so quench more radicals. Indeed, these bioactive compounds are known for its capacity to scavenge peroxyl radicals more efficiently as compared to others ROS (Nimse and Pal 2015); however, these radicals are the only ones able to annihilate these pigments (Carocho and Ferreira 2013). Carotenoids may also decay and form non-radical compounds able to stop free radical attacks through its binding to these radical species (Paiva and Russell 1999, Carocho and Ferreira 2013). Nevertheless, its effects in humans are quite complex and it is still unclear whether these biological effects result from their antioxidant capacity or of a non-antioxidant mechanism (Baiano and Del Nobile 2015). According to Navarro-González *et al*. (2014), the mechanism of action of lycopene include: its role as an antioxidant, decreasing

the LDL oxidation and the lipid peroxidation and lowering the LDL cholesterol and the total cholesterol, and as a modulator of inflammatory response through the reduction of cytokines implicated in cardiovascular disease. Lycopene has also influence on cellular proliferation and differentiation as well as in the immune response (Heber and Lu 2002). Curiously, lycopene as the capacity to inhibit the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells both *in vitro* and *in vivo* (Kim *et al.* 2004). It also reduces the oxidative stress and intestinal inflammation in experimental models of colitis in rats (Reifen *et al.* 2004). As mentioned above, the high number of conjugated double bonds in lycopene structure provides the singlet oxygen quenching capacity. Lycopene and β-carotene reduce the production of LDL cholesterol oxidized products that are associated with coronary heart disease; β-carotene also protects the skin against deleterious effects of sunlight (Nimse and Pal 2015). The antioxidant potential of carotenoids is commonly linked to its capacity to prevent free radical triggered diseases, including atherosclerosis, multiple sclerosis, age-related muscular degeneration and cataracts (Lee *et al.* 2004). In fact, the consumption of tomatoes and tomato-based foods has been significantly connected to a low incidence of prostate cancer (Kolberg *et al.* 2015).

2.2.4.2. Bioactive properties of vitamins

The antioxidant activity of tocopherols is conferred by the chromanol head group. The phytyl side chain has no activity; it is embedded within the cell membrane bilayer while the active chromanol ring is closely positioned to the surface. This ingenious arrangement allows tocopherols to act as powerful antioxidants and to be regenerated through reaction with other antioxidants, *e.g.,* ascorbic acid (Lee *et al.* 2004). However, the activity of these antioxidants is affected by its orientation within the membrane. Thus, the tocopherols halts lipid peroxidation in cell membranes and various lipid particles through donation of the phenolic hydrogen of the chromanol ring to lipid peroxyl radicals, thereby forming unreactive tocopheroxyl radicals unable to continue the oxidative chain reaction (Morlière *et al.* 2012). This vitamin protects LDL and cell membrane polyunsaturated fatty acids, and inhibits smooth muscle cell proliferation and protein kinase C activity (Lee *et al.* 2004). Curiously, tocopherols are the major lipid-soluble antioxidants found in plasma, red cells and tissues (Burton and Traber 1990). They have been associated with lower incidence of heart disease, delay of Alzheimer's disease, and prevention of several types of cancer. The α-tocopherol, for example, can reduce the nitrogen dioxide levels more effectively than the other isoforms, a compound implicated in arthritis and carcinogenic processes (Lee *et al.* 2004). However, tocopherols are not efficient scavengers of hydroxyl radicals *in vivo* (Niki 2014).

The bioactivity of ascorbic acid is conferred by the 2,3-enediol. The antioxidant mechanisms are conferred by its ability to donate a hydrogen atom to free radicals, to

eliminate molecular oxygen and quench singlet oxygen. The capacity to scavenge aqueous radicals and regenerate α-tocopherol from tocopheroxyl radicals are other well-known antioxidant mechanisms of this vitamin (Lee *et al.* 2004, Niki 2014). In biological systems, ascorbic acid changes to the ascorbate radical through the donation of an electron to a lipid radical in order to stop the lipid peroxidation chain reaction. Thereafter, the originated ascorbate radicals react rapidly and produce one molecule of ascorbate and other of dehydroascorbate. The last one is devoid of bioactivity, but is converted back into ascorbate (Nimse and Pal 2015). Ascorbic acid is efficient in scavenging the superoxide radical anion, hydroxyl radicals, singlet oxygen, hydrogen peroxide and reactive nitrogen oxide. However, the ascorbic acid may also act as a prooxidant, for example during the reduction of ferric iron (Fe^{3+}) to the more active ferrous iron (Fe^{2+}) (Lee *et al.* 2004).

The vitamin A has high antioxidant activity and can protect lipids against rancidity. In humans, it plays a vital role in protecting LDL from oxidation stimulated by copper (Livrea *et al.* 1995). Retinoids are essential to diverse physiological functions including vision, immune response, bone mineralization, reproduction, cell differentiation, and growth (Defo *et al.* 2014).

2.2.4.3. Bioactive properties of phenolic compounds

The antioxidant capacity of phenolic acids comes from its ability to chelate transition metals and to scavenge free radicals, having a significant impact over hydroxyl and peroxyl radicals, peroxynitrites and superoxide anions (Carocho and Ferreira 2013). The hydroxycinnamic acid derivatives display bioactivity due to the hydroxylation and methylation patterns of the aromatic ring, *e.g.,* the *o*-dihydroxy group in the phenolic ring of caffeic acid improves its antioxidant capacity (Meyer *et al.* 1998). The free radical scavenging mechanism of the hydroxycinnamic acids is analogous to that of flavanoids, which is attributed to its capacity to donate a hydroxyl hydrogen and resonance stabilization of the resulting radicals. The *o*-dihydroxy substituents also have iron-chelating ability (Nimse and Pal 2015).

The bioactivity of flavonoids is conferred by hydroxyl groups attached to the ring structures. They can act as reducing agents, hydrogen donators, superoxide radical scavengers, singlet oxygen quenchers, and metal chelators. Flavonoids have capacity to protect the DNA from damage caused by hydroxyl radicals, reduce tocopheroxyl radicals, activate or inhibit bioactive enzymes, and alleviate the nitrosative stress (Procházková *et al.* 2011, Carocho and Ferreira 2013, Nimse and Pal 2015). Rutin, also known as vitamin P, has antioxidant, anti-inflammatory, and anticarcinogenic properties. It reduces the fragility of blood vessels (Ihme *et al.* 1996). At a low concentration of cupric ions, quercetin is capable of protecting DNA from oxidative damage resulting from the attack of the hydroxyl and superoxide radicals and hydrogen peroxide (Nimse and Pal 2015).

2.2.4.4. Bioactive properties of glycoalkaloids

Glycoalkaloids are perceived as potentially toxic, but the studies conducted over the past decade indicate that they may also have health-promoting effects, depending on dose and conditions of use. They can be used because of its anti-inflammatory, anticancer, antipyretic, anticholesterol, antinociceptive, and antimicrobial effects (Milner *et al.* 2011). Its bioactivity derives mainly from the capacity to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), and from its ability to complex with membrane 3*β*-hydroxy sterols, which causes the rupture of the membrane. Its capacity to inhibit AChE and BuChE makes them important compounds, but the source of enzymes to be tested (plasma vs. serum) and differences in the purity of the glycoalkaloid and aglycones have affected the published results (Fletcher *et al.* 2004, Milner *et al.* 2011). However, the aglycone alone is practically inactive against the cholinesterase enzymes. The sugar unit is required for activity, but it is the structure of the aglycone which determines the extent of inhibition. The existence of heterocyclic nitrogen is also a necessary condition for activity (Alozie *et al.* 1978). Regarding the second mechanism of action, and with respect to the aglycone subunit, an intact E ring and an unshared pair of electrons on the nitrogen of the F ring, as well as solanidane and spirosalane rings are necessary for membrane lytic activity (Roddick *et al.* 2001). In general, the glycoalkaloids bioactivity increases when they are administered as mixtures (depending on the relative proportion used) (Milner *et al.* 2011). However, the synergistic activity of α-tomatine and dehydrotomatine remains unknown. In inflammatory processes, the aglycone tomatidine has the capacity to reduce inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression through blocking NF-κB and JNK signalling in lipopolysaccharide-stimulated macrophages (Chiu and Lin 2008). In turn, α-tomatine has the ability to decrease the cholesterol and triglycerols levels, enhance the immune system, and inhibit the growth of cancer cells (Lee *et al.* 2011, 2013, Kim *et al.* 2015). Actually, this compound has been recognized as a potential anticancer drug (Kúdelová *et al.* 2013). Nevertheless, a deeper understanding of the implications of glycoalkaloids in the human diet is still necessary.

2.2.5. Tomato and human health

The regular consumption of tomatoes and tomato-based foods has been associated with several positive effects on human health. Current studies demonstrate the several benefits of tomato bioactive compounds, either isolated or in combined extracts, namely anticarcinogenic (Kolberg *et al.* 2015, Stajčić *et al.* 2015), cardioprotective (Armoza *et al.* 2013, Samaras *et al.* 2014, Vilahur *et al.* 2015), anticholesterolemic (Choi *et al.* 2013, Vinha *et al.* 2014), antidiabetic (Ghavipour *et al.* 2013), and hepatoprotective (Hermenean *et al.* 2014, Kujawska *et al.* 2014) effects among other health benefits, mainly due to its antioxidant

(Pinela *et al.* 2012, Li *et al.* 2014, Samaras *et al.* 2014) and anti-inflammatory (Hadad and Levy 2012, Li *et al.* 2014) properties. Indeed, the production of ROS and RNS during oxidative stress and inflammatory processes is widely associated with the development and progression of chronic diseases such as cancers, cardiovascular diseases, diabetes, and other disorders associated with aging (Visioli *et al.* 2003, Uttara *et al.* 2009). The tomato bioactive compounds can neutralize the generated reactive species and, therefore, prevent the associated diseases. In fact, low levels of antioxidants have been associated with heart diseases and different types of cancer (Pal *et al.* 2012). Examples of *in vitro* and *in vivo* clinical trials highlighting the crucial role of tomato and tomato-derived compounds on human health are presented below.

2.2.5.1. Tomato consumption improves the oxidative status

The involvement of oxidative stress in the pathogenesis of various degenerative diseases is evidenced by an altered expression of enzymatic antioxidant defences, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Marrazzo *et al.* 2014). A recent study conducted by Li *et al.* (2014) demonstrated that the anti-inflammatory effect of the purple tomato extract might be caused by the decreased levels of MDA and nitric oxide (NO) and increased GPx and SOD activity in oedematous tissue. These results were attributed to the direct antioxidant activities of the bioactive compounds towards the free radicals, and indirect elevation of the enzyme activity. Supplementation of ultra-marathon runners for a period of two months with a whey protein bar and tomato juice also improved the oxidative status, decreasing thiobarbituric acid reactive substances (TBARS) and protein carbonyls (Samaras *et al.* 2014). Other study evaluated the effect of tomato sauces with different amounts of lycopene on oxidative stress biomarkers (Abete *et al.* 2013). Healthy participants consumed 160 g/day of tomato sauce, while maintaining constant their usual diet and physical activity. The regular consumption of the lycopene-enriched tomato sauce caused a considerable decrease of the oxidized-LDL cholesterol levels and increased the total plasma antioxidant capacity. Thus, the putative role of lycopene in combination with other tomato bioactive ingredients in the prevention of oxidative stress related diseases was evidenced.

2.2.5.2. Tomato suppresses the NF-κB activation and reduces inflammation

Inflammation is a normal protective response of the innate immune system to an injury. However, when the oxidative damage is out of control, inflammation may become chronic leading to tissue damage. During inflammation, immune cells are activated and release increased levels of ROS to eliminate invading pathogens. The intracellular ROS production is linked to various cellular processes controlled by NF-κB (which is the central orchestrator of

the inflammatory response), including activation of NAD(P)H oxidase, matrix metalloproteinases (MMP-1, MMP-2 and MMP-9), nitric oxide synthases (NOS), xanthine oxidase, cyclooxygenase-2 (COX-2), and lipooxygenases. Additionally, immune cells release a number of proinflammatory mediators such as cytokines (tumour necrosis factors (TNF-α) and interleukin (IL-6, IL-1β)), chemokines (IL-8), cell adhesion molecules (CAMs), C-type lectin receptors, prostaglandins, leukotrienes, and NO (Oka *et al.* 2010, Shalini *et al.* 2012).

Clinical trials demonstrated that the anti-inflammatory effects of tomato compounds are attributed to their capacity to suppress of the activation of NF-κB. De Stefano *et al*. (2007) investigated the effect of PS (a polysaccharide from unripe tomato peels) on nitrite and ROS production in J774 macrophages stimulated by bacterial lipopolysaccharide (LPS) for one day. Results demonstrated that PS inhibits NF-κB activation and inducible nitric oxide synthase (iNOS) gene expression by preventing the reactive species production; thus, the involvement of this compound in the control of the oxidative stress and/or inflammation was suggested. Joo *et al.* (2009) studied the effects of a tomato lycopene extract on the LPS-induced innate signalling and on the acute and spontaneous chronic intestinal inflammation. Mice were fed a diet containing 0.5 and 2% tomato lycopene extract or an isoflavone free control. The tomato lycopene extract prevented LPS-induced proinflammatory gene expression by blocking of NF-κB signalling, through aggravation of dextran sulfate sodium-induced colitis by enhancing epithelial cell apoptosis. The effectiveness of the combination of carotenoids and phenolics in inhibiting the release of inflammatory mediators from macrophages exposed to LPS and the anti-inflammatory effect in an *in vivo* mouse model of peritonitis was evaluated by Hadad and Levy (2012). Pre-incubation of macrophages with the evaluated compounds, 1h before the addition of LPS for one day, caused a synergistic inhibition of NO, prostaglandin E(2), and superoxide anion production derived from down-regulation of iNOS, COX-2, and NADPH oxidase protein and mRNA expression and synergistic inhibition of TNF-α secretion. The supplementation of mouse resulted in attenuated neutrophil recruitment to the peritoneal cavity and inhibited inflammatory mediator production by peritoneal neutrophils and macrophages.

2.2.5.3. Tomato reduces inflammation linked to obesity, diabetes and cholesterol

Obesity is a chronic inflammatory state in which the augmented level of body fat leads to an increase in circulating inflammatory mediators (Ghavipour *et al.* 2013). Ghavipour *et al.* (2013) demonstrated that tomato juice reduces inflammation in overweight and obese females. In this study, inflammatory biomarkers were analysed in an intervention group that consumed 330 mL/day of tomato juice for 20 days. Serum levels of IL-8 and TNF-α decreased considerably in the intervention group compared with the control one. Curiously, this effect was confined to overweight subjects. Among obese subjects, the levels of serum

IL-6 were reduced in the intervention group, while the levels of IL-8 and TNF-α showed no difference from the control group. Thus, the authors concluded that increased tomato intake may reduce the risk of inflammatory diseases associated with obesity such as cardiovascular disease and diabetes. Another study showed beneficial effects of tomato juice consumption on oxidative stress status of overweight females (Ghavipour *et al.* 2015). Some evidence suggests that oxidative stress, in addition to being a consequence of fat accumulation with subsequent inflammatory response, may be a prerequisite for adipogenesis (Tormos *et al.* 2011). The authors verified that the plasma total antioxidant capacity and erythrocyte antioxidant enzymes increased and serum MDA decreased after the 20 days of intervention period. Thus, it was concluded that the verified reduction of oxidative stress in weight females may prevent from obesity related diseases and promote health.

The supplementation effect of tomato juice on indices associated with metabolic health and adipokine profiles in young healthy women, to which was given 280 mL of tomato juice (containing 32.5 mg of lycopene) daily for 2 months, was studied by Li *et al*. (2015). It was found that the tomato juice supplementation significantly reduced the body weight, body fat, body mass index, waist circumference, and the serum levels of cholesterol and TBARS; while the serum levels of adiponectin, triglyceride, and lycopene were significantly increased. Other authors evaluated the effect of pre-meal tomato intake in the anthropometric indices and blood levels of triglycerides, cholesterol, glucose, and uric acid in an intervention group consisting of young adult women (Vinha *et al.* 2014). The intervention group ingested a raw ripe tomato (~90 g) before lunch for 4 weeks. At the end of that period, it was observed a positive effect in body weight, fat percentage, and blood levels of glucose, triglycerides, cholesterol, and uric acid of the participants.

Regarding studies in animal models, Seo *et al.* (2014) investigated the anti-obesity properties of a tomato vinegar beverage in diet induced obese C57BL/6 mice. The prepared beverage not only reduced fat accumulation, but also insulin resistance; these changes were mediated by the AMP-activated protein kinase and peroxisome proliferator-activated receptor alpha up-regulation. In a similar study conducted by Choi *et al.* (2013) it was concluded that green tomato extracts attenuate high-fat diet-induced obesity in C57BL/6 mice through activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway, and that green tomato extracts may be a potential candidate for anti-obesity drugs. Besides, the results indicated that tomatine may be responsible for the observed reduction of body weight. It has been reported that the glycoalkaloid tomatine forms insoluble complexes with cholesterol *in vitro*. In this sense, to evaluate the capacity of tomatine in reducing dietary cholesterol absorption and the plasma levels of cholesterol and triglycerides, hamsters were fed a high-fat, high-cholesterol diet containing 0.05-0.2% of tomatine (Friedman *et al.* 2000). The tomatine diet decreased the serum LDL levels without changing HDL cholesterol, being

more cholesterol and coprostanol excreted in feces corresponding to the ingested quantity of tomatine. Moreover, these findings suggest that due to the formation and excretion of tomatine-cholesterol complexes, just a very small amount of dietary tomatine is absorbed by the human body.

2.2.5.4. Tomato prevents cardiovascular diseases, atherosclerosis and hypertension

Currently, cardiovascular disease still represents a major cause of morbidity and death in the world (McCullough *et al.* 2012). The endothelium plays a crucial role in cardiovascular health; its dysfunction is associated with the development of atherosclerosis, hypertension and heart failure. Endothelial cells respond to different stimuli through the synthesis and release of several molecules capable of regulating vascular tone, permeability, and inflammation, as well as the blood fluidity and coagulation (Widlansky *et al.* 2003). Indeed, endothelial dysfunction is a key early step in the development of cardiovascular diseases. It is characterized by an impairment of endothelium-dependent relaxation and a predisposition to a proinflammatory and prothrombotic state (Herrmann and Lerman 2008, Vanhoutte 2009). NO contributes to the maintenance of vascular integrity thanks to its antithrombotic, antiatherogenic, and antiproliferative properties. For this reason, decreased levels of NO have been linked with various cardiovascular disorders including hypertension, atherosclerosis and ischaemic disease. The tomato bioactive compounds could contribute to cardiovascular health, and its benefits have been reported in several *in vitro* and *in vivo* studies.

Regarding *in vivo* studies, Kim *et al.* (2011) demonstrated that elevated levels of serum lycopene reduce the oxidative stress correlated to endothelial function. Clinical trials were conducted in healthy men who received 15 mg/day of lycopene for 8 weeks. After treatment, serum lycopene levels increased in a dose-dependent manner. The group who received lycopene showed a greater increase in plasma SOD activity and reduction in lymphocyte DNA comet tail length, as well as an increase in reactive hyperemia peripheral arterial tonometry (RH-PAT) index. Moreover, high-sensitivity CRP (hs-CRP), systolic blood pressure, soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were significantly decreased, and β-carotene and LDL-particle size were increased. A remarkable beneficial effect of lycopene supplementation on the endothelial function (*i.e.,* RH-PAT and sVCAM-1) in subjects with an initially relatively impaired endothelial cell function was also observed. Another study showed that the vascular endothelial function of ultra-marathon runners is improved by its supplementation with tomato juice for a period of 2 months (Samaras *et al.* 2014).

The suitability of tomato paste (a concentrated of bioactive compounds) for modifying postprandial oxidative stress, inflammation, and endothelial function in healthy weight individuals was evaluated by Burton-Freeman *et al*. (2012). Participants consumed high-fat

meals on two separate occasions containing a processed tomato product or a placebo. Both meals increased the plasma levels of glucose, insulin and lipids. Tomato significantly attenuated the high-fat meal-induced LDL oxidation and the increase in interleukin-6 (IL-6), a proinflammatory cytokine and inflammation marker. Thus, it was demonstrated that the inclusion of tomatoes or tomato-based foods at the meal reduces the postprandial lipemia-induced oxidative stress and the associated inflammatory response. Furthermore, these findings highlighted the potential protective role of tomato fruit in reducing the risk of cardiovascular disease. Beneficial effects of daily tomato paste consumption on endothelial function were also demonstrated by Xaplanteris *et al*. (2012) in a group of healthy young men and women. The tomato supplementation led to an overall flow-mediated dilatation increase particularly in individuals with lower baseline values, and a decrease of total oxidative status past 15 days.

Recently, Vilahur *et al*. (2015) reported that the intake of cooked tomato sauce protects against low-density lipoprotein-induced coronary endothelial dysfunction by reducing oxidative damage (diminished DNA damage in the coronary arteries), enhancing endothelial NOS (eNOS) expression and activity, and improving HDL functionality (associated with protein profile changes in apolipoprotein A-I (Apo A-I) and apolipoprotein J (Apo J)). The study was performed in pigs that received a hypercholesterolemic diet and a supplement of cooked tomato sauce (100 g, 21.5 mg lycopene) for 10 days.

Regarding *in vitro* studies, Armoza *et al.* (2013) demonstrated the protective role of lycopene and lutein in improving the basic endothelial function. The study was performed in two cultured endothelial cell models and it was verified an increase in the NO levels and a decrease in the endothelin (ET-1) release. Both carotenoids were efficient in attenuating the inflammatory NF-κB signalling, in particular decreasing the TNF-α-induced leukocytes adhesion, the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and nuclear translocation of NF-κB components and some revert of the inhibitor of kappa B (IκB) ubiquitination. Furthermore, both compounds inhibited the NF-κB activation in transfected endothelial cells. This study demonstrated that the prevention of the overexpression of adhesion molecules through inhibition of NF-κB signalling may be one of the main mechanisms driving carotenoids to attenuate inflammatory leukocyte adhesion to endothelium.

2.2.5.5. Tomato has antitumor and anticarcinogenic properties

Cancer is a very complex disease caused by cells without the usual control over growth. The apparent causes of this disease can diverge case by case; there are two classes of genes capable of controlling its development, namely oncogenes and tumour suppressor genes. Healthy cells follow a normal growth and proliferation pathway with a definite lifespan,

whereas cells with an oncogenic activation undergo much faster division and have an indefinite lifespan. Generally, the cancer formation occurs when an oncogene and a tumour suppressor gene are activated and inactivated in a cell at the same time, respectively. The tumour suppressor genes are involved in the unregulated cell growth inhibition and caretaker genes control the rate of mutation in the genome. Thus, accumulation of mutation in the genome and consequent higher rate of tumour formation can be caused by a defective caretaker gene. Therefore, cancer arises due to functional deformities in multiple genes (Pal *et al.* 2012, Weed 2013). Furthermore, the DNA damage by reactive species can also lead to increased risk of cancer.

In turn, degradation and penetration of the cell extracellular matrix by tumour cells under the action of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA) are key steps in the metastatic cascade of cancer cells. MMPs are the most important proteases involved in tumour cell migration, spreading, tissue invasion and metastasis, and its expression is regulated by extracellular signal regulating kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) (Itoh and Nagase 2002). Among them, MMPs, MMP-2 and MMP-9 are key enzymes and are involved in metastasis processes (Yan *et al.* 2013). Furthermore, the inhibition of the mitogen-activated protein kinase (MAPK) pathway (which is involved in signalling cascades) can prevent angiogenesis, proliferation, invasion and metastasis in many tumours (Chien *et al.* 2010, Chen *et al.* 2011). The metastasis process is also regulated by the phosphatidylinositide-3 kinase (PI3K)/Akt signalling pathway (Shukla *et al.* 2007). The NF-κB can also facilitate cell proliferation, angiogenesis and metastasis. This protein complex consists of a p50/p65 heterodimer that is masked by the inhibitor I kappa B alpha (IκBα), which causes its retention in the cytoplasm under resting conditions. The IκBα kinase can be activated by various stimuli, including those induced by TNF-α and LPS (Lee *et al.* 2013).

Tomato extracts and derived compounds have shown promising effects over different cancer cell lines. Stajčić *et al*. (2015) investigated the cell growth activity of tomato waste extracts obtained from different tomato genotypes. Anti-proliferative effects (determined by sulforhodamine B test) were observed in all cell lines (HeLa, MCF7 and MRC-5) at higher concentrations. The authors also correlated the carotenoids content with the antiproliferative and antioxidant activities.

Tang *et al*. (2009) verified that low concentration of lycopene and eicosapentaenoic acid could inhibit in a synergistic way the proliferation of human colon cancer HT-29 cells. The inhibitory effects were, partly, associated with the down-regulation of the PI-3K/Akt/mTOR signalling pathway, known to play an important role in tumour progression. Therefore, the inhibition of this pathway is a promising approach for discovery of novel chemotherapeutic agents. More recently, Kim *et al.* (2015) investigated the effect of α-

tomatine on CT-26 colon cancer cells *in vitro* and *in vivo* in an intracutaneously transplanted mouse tumour. It was demonstrated that α-tomatine in pure form and in tomatine-rich green tomatoes might prevent colon cancer; α-tomatine induced about 50% lysis of the colon cancer cells at 3.5 μM after 24 h of treatment. It was also found that α-tomatine induced cell death through caspases-independent signalling pathways. Intraperitoneally administered α-tomatine also clearly inhibited tumour growth.

Prostate cancer is the second most common cause of male cancer death in the world (World Cancer Research Fund and American Institute for Cancer Research 2007). A role for NF-κB in the progression of this cancer has been suggested; NF-κB is activated in androgen-insensitive prostate carcinoma cells, and overexpression of NF-κB p65 protein has been detected in the nuclear fraction of prostate cancer clinical specimens (Yemelyanov *et al.* 2006). The chemopreventive potential of α-tomatine on androgen-independent human prostatic adenocarcinoma PC-3 cells was evaluated by Lee *et al.* (2011). The treatment with α-tomatine caused a concentration-dependent inhibition of cell growth. It was less cytotoxic to normal human liver WRL-68 cells and normal human prostate RWPE-1 cells. α-Tomatine exhibited its cytotoxic effects against adenocarcinoma PC-3 cells as early as one hour after treatment, which were assessed by the real-time growth kinetics. The glycoalkaloid α-tomatine induced apoptosis and inhibited NF-κB activation, as well as the activation of caspase-3, -8 and -9, suggesting the involvement of both intrinsic and extrinsic apoptosis pathways. Subsequently (Lee *et al.* 2013) it was shown that α-tomatine suppresses NF-κB activation through inhibition of IκBα kinase activity, which leads to sequential suppression of IκBα phosphorylation, IκBα degradation, NF-κB p65 phosphorylation, and NF-κB p50/p65 nuclear translocation. As indicated, α-tomatine was able to induce apoptosis; it reduced the TNF-α induced activation of the pro-survival mediator Akt, and the NF-κB inhibition caused a reduction in expression of NF-κB-dependent anti-apoptotic proteins. Moreover, intraperitoneal administration of this bioactive glycoalkaloid clearly attenuated the growth of PC-3 cell tumours (grown subcutaneously and orthotopically) in mice. These effects were accompanied by increased apoptosis, lower proliferation of tumour cells, and low nuclear translocation of the p50 and p65 components of NF-κB. Recently, Kolberg *et al.* (2015) investigated whether tomato paste has the ability to modulate NF-κB activity and cancer-related gene expression in human prostate cancer cells (PC-3) and PC-3 xenografts. PC-3 cells were stably transduced with an NF-κB-luciferase construct and then treated with tomato extract or a placebo. Mice bearing PC-3 xenografts received a high-fat diet with or without 10% tomato paste for 6.5 weeks. The tomato extract considerably inhibited the TNF-α stimulated NF-κB activity in the PC-3 cells, and modulated the expression of genes associated with inflammation, apoptosis, and cancer progression. Mice fed tomato paste diet revealed accumulation of lycopene in liver, xenografts and serum. The tomato paste had no

effect on tumour size in mice; but there was a trend toward inhibition of NF-κB activity in the xenografts. Gene expression, most prominent in xenografts, was higher after tomato treatment.

Lung cancer is becoming increasingly common. About 40% of these cancers are adenocarcinoma, a type of non-small cell lung cancers with a low prognosis and highly potential for metastatic (Shivapurkar *et al.* 2003). A study carried out by Yan *et al.* (2013) examined the effect of tomatidine on the migration and invasion of human lung adenocarcinoma A549 cells. *In vitro* treatments with non-toxic doses of tomatidine resulted in markedly suppressed cell invasion, while cell migration was not affected. Tomatidine reduced the mRNA levels of MMP-2 and MMP-9 and increased the expression of reversion-inducing cysteine-rich protein with kazal motifs (RECK, a protein involved in the proteolytic degradation of extracellular matrix in tumour metastasis), as well as the tissue inhibitor of MMP-1. It also inhibited the ERK and Akt signalling pathways and NF-κB activity.

2.2.5.6. Tomato protects liver from hepatotoxicity and hepatocarcinogenesis

Oxidative damage caused by free radicals formed during the metabolism of nitrosamines has been suggested as one of the main cause for the initiation of hepatocarcinogenesis (Gupta *et al.* 2013). *N*-Nitrosamines are a class of chemical compounds that are metabolised to prooxidant and carcinogenic substances. *N*-Nitrosodiethylamine is a representative of this class capable of generating carbocations and ROS during the cytochrome P450-mediated biotransformation. Liver injury induced by *N*-nitrosodiethylamine is a well-known model of hepatotoxicity commonly used for screening of hepatoprotective effects of natural matrices. A lycopene-enriched tomato paste tested by Kujawska *et al.* (2014) was suitable for suppressing the *N*-nitrosodiethylamine-induced oxidative stress in rats. Pre-treatment with tomato paste protected antioxidant enzymes (SOD, CAT and glutathione reductase) and decreased the DNA damage in leucocytes and the plasma concentration of protein carbonyls. The microsomal lipid peroxidation was also decreased in liver of rats pre-treated with a lower dose of tomato paste. Gupta *et al*. (2013) investigated the involvement of tomato lycopene against oxidative stress induced by the deleterious effect of *N*-nitrosodiethylamine on cellular macromolecules of mice, having been demonstrated the intervention of lycopene on the initiation of carcinogenesis. Indeed, lycopene has influence on multiple dysregulated pathways during initiation of carcinogenesis; in particular, it helps in the membrane fluidity normalization, improvement of antioxidant enzymes activity and reduction of GSH which accounted for reduced oxidative damage.

The antioxidant and hepatoprotective properties of naringenin and its β-cyclodextrin formulation at a dose of 50 mg per kg of body weight were evaluated by Hermenean *et al.* (Hermenean *et al.* 2014). Serum-enzymatic and liver antioxidant activity and

histopathological and ultrastructural changes were investigated in mice subjected to acute intoxication with carbon tetrachloride (CCl_4), one of the most potent hepatotoxins. The authors verified that both naringenin and naringenin/β-cyclodextrin complex have antioxidant and hepatoprotective effects against injuries caused by CCl_4. Particularly, 24 h after the CCl_4 administration, the activity of the transaminases aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and the levels of MDA were increased. A considerable decrease in SOD, CAT and GPx activities and in the glutathione (GSH) levels were also detected. Additionally, extended centrilobular necrosis, steatosis, fibrosis, and an altered ultrastructure of hepatocytes were also verified.

The primary liver cancer has become the fifth most common malignancy in the world (Bugianesi 2007). Due to lack of early detection or screening biomarkers, its diagnosis is made at an advanced stage of the disease. Thus, the identification of potential risk factors for early hepatocarcinogenesis and the search for preventive and/or protective measures against them at an early stage are urgently needed. Growing evidence has associated hepatocellular carcinoma and nonalcoholic steatohepatitis (NASH), a chronic and often "silent" liver disease characterized by fat accumulation and infiltration of inflammatory cells in the liver (Mori *et al.* 2004). Wang *et al*. (2010) studied the efficacy of an equivalent dosage of dietary lycopene from either a pure compound or a tomato extract against NASH-induced hepatocarcinogenesis. In this study, rats were injected with diethylnitrosamine and then fed either a Lieber-DeCarli control diet or a high-fat diet with or without lycopene or tomato extract for 6 weeks. Both lycopene and tomato extract supplementations considerably decreased the number of altered hepatic foci, being expressed the placental form of glutathione-S transferase in the liver of rats that received a high-fat diet. Decreased activation of NF-κB was verified. Both supplementations reduced the lipid peroxidation induced by the high-fat diet in the liver; it was observed also a significantly decreased inflammatory foci and mRNA expression of proinflammatory cytokines (TNF-α, IL-1β and IL-12) in the group that received a high-fat diet and the tomato extract. Thus, it was concluded that lycopene and tomato extract inhibit the NASH-induced hepatocarcinogenesis mainly as a result of reduced oxidative stress.

Given the beneficial effect of antioxidant supplementation in metal-induced toxicity, and concerns regarding the benefits of tomatoes on different target tissues, Nwokocha *et al.* (2012) elucidated the effect of tomato extract on reducing the accumulation of heavy metal in the liver of rats. The tomato extract administration was beneficial in reducing heavy metal accumulation in the liver, namely reducing uptake and enhancing the elimination of these metals in a time dependent manner. The hepatoprotective effect against cadmium toxicity was very high. Among the tomato bioactive compounds, vitamin C has been reported to decrease liver damage from cadmium, mercury and lead (Tariq 2007, Donpunha *et al.* 2011).

2.2.6. Safety precautions

Solanum is probably the most economically important genus, containing familiar crop species, as well as many species containing poisonous or medicinally useful secondary compounds. Because of this, the tomato plant was long used only for ornamental purposes (Bergougnoux 2014). However, there are no reports of any toxic effect caused by the consumption of tomato fruit, but some reports correlate potato glycoalkaloids with noxious effects in humans. Potatoes with high levels of glycoalkaloids can severely affect the consumer health or even cause death. Mild poisoning cases can cause headache, vomiting and diarrhea. Some neurological symptoms have also been described, namely drowsiness, visual disturbances, apathy, hallucinations, mental confusion, dizziness, trembling and restlessness (Milner *et al.* 2011). Thus, to ensure safety to consumers, it is necessary to perform further studies of toxicity and bioavailability of the tomato glycoalkaloids considering different maturation stages. Besides glycoalkaloids, other antinutritional and potentially harmful compounds, such as oxalic acid (Pereira *et al.* 2013) and nitrate (Simion *et al.* 2008), have been detected in different concentrations in tomato samples.

2.2.7. Industrial applications

Despite the undeniable importance of tomato in the food processing industry, this fruit as well as its by-products can have other applications on biotechnology, chemical, pharmaceutical, and cosmetic industries. Industrial tomato by-products contain significant amounts of the bioactive compounds endowed with different bioactivities and important health promoting effects (Kalogeropoulos *et al.* 2012, Stajčić *et al.* 2015, Pinela, Prieto, Barreiro, *et al.* 2016, Pinela, Prieto, Carvalho, *et al.* 2016). Therefore, as tomato wastes are bioorganic materials and being in line with the trend for sustainability, these value-added molecules can be isolated to be used as natural bioactive ingredients for different industries. Extracts or isolated compounds from tomato by-products can also be used as anti-inflammatory, cardioprotective, anticholesterolemic, antidiabetic, and antitumour agents to develop new products and drugs. The isolated compounds can also be applied in the food industry to develop new functional foods and nutraceuticals, and used as food additives to extend their shelf-life.

2.3. Nonthermal physical technologies to decontaminate and extend the shelf-life of fruits and vegetables

2.3.1. The emergence of food preservation technologies

As a response to consumers' demand for fresh, healthy and minimally processed foods, conjoint with an altered lifestyle characterized by less time for planning and preparing convenient meals, a wide variety of minimally processed fruits and vegetables has been developed, being one of the major growing sector in food industry (Patrignani *et al.* 2015). In fact, consumers are more concerned about the nutritional and sensory properties of the food they eat, as well its safety (Ramos *et al.* 2013). However, the growing preference for minimally processed fruits and vegetables with fresh-like properties is accompanied by concerns surrounding efficacy of the available sanitizing methods to appropriately deal with food-borne diseases. These products are susceptible to microbial proliferation due to their high water and nutrients content and the loss of their natural resistance, having been repeatedly sources of foodborne illnesses. Additionally, the processing steeps of peeling, cutting or slicing favour the microbial growth due to the release of nutritive substances (Patrignani *et al.* 2015). Bacteria such as *Escherichia coli* O157: H7, *Salmonella* spp. and *Listeria* spp. are the pathogens most frequently linked to fruits and vegetables produce-related outbreaks, being a public health concern (Abadias *et al.* 2011, Drissner and Zuercher 2014).

Fresh fruits and vegetables cannot be conveniently decontaminated using conventional thermal treatments without affecting their physical, nutritional or bioactive properties that may reduce the content or bioavailability of bioactive ingredients (Rawson *et al.* 2011). Besides, washing and chemical sanitizing treatments do not provide an efficient microbial reduction to afford safety consumers (Rowan *et al.* 2015) and are perceived negatively by the consumers, dangerous for human health and harmful to the environment (*e.g.,* sodium hypochlorite generates environmental and health risks associated with the formation of carcinogenic halogenated compounds (Odabasi 2008). The emergence of more resistant microorganisms to conventional food preservation techniques also increases the need for developing new decontamination processes (Abadias *et al.* 2011). Therefore, to meet these demands, the industry is investigating alternative nonthermal physical technologies, as well as possible combinations between them or with other preservation factors (hurdles).

2.3.2. Postharvest decay of fresh produce

Fruits and vegetables are important inputs of nutrients and health-promoting compounds, including vitamins, carotenoids, minerals, fibre, polyphenols, etc. (Pereira *et al.* 2011, Pinela

et al. 2012). After harvest, they remain living tissues and continue to carry out metabolic processes such as respiration and transpiration. Thus, from the moment they are detached from its source of nutrients, they become entirely dependent on their own organic reserves; they will consume carbohydrates, lipids, and organic acids, as well as oxygen (O_2), producing simple molecules of carbon dioxide (CO_2) and water with release of energy (Heldt and Piechulla 2011). As a consequence, unwanted visual symptoms appear on the product, namely weight loss/dehydration, formation of wrinkles, and changes on colour and texture (Hodges and Toivonen 2008).

The quality parameters of minimally processed fruits and vegetables can be affected by both internal and external factors. The internal factors represent metabolic processes and include morphological, physiological, and biochemical defence mechanisms, stress-induced senescence programs, and genotype. The external factors represent environmental situations which inhibit or exacerbate the manifestation of the internal ones; examples include processing treatments (washing, decontamination, peeling, cutting, slicing, wedging, etc.) and storage conditions (packaging system, temperature, humidity, light exposure, etc.) (Hodges and Toivonen 2008, Tiwari and Cummins 2013). Additionally, fresh-cut fruits and vegetables deteriorate faster than intact ones, because wounding plant cells and tissues increases the ethylene (C_2H_4) production and exacerbates water loss. The elevated C_2H_4 production stimulates respiration and consequently accelerates deterioration and senescence, and promotes ripening (Damodaran and Parkin 2017). Moreover, some stressful abiotic conditions may cause the *de novo* synthesis of antioxidant compounds (polyphenols, tocopherols, etc.) to fight against the reactive species that are produced under such unfavourable conditions, being a plant strategy to withstand to those situations (Munné-Bosch 2005, Yusuf *et al.* 2010, Pérez-Gregorio *et al.* 2011). Postharvest changes in fresh produce cannot be stopped, but they can be slowed within certain limits. Therefore, the reduced shelf-life of minimally processed fruits and vegetables requires a search for appropriated (effective and safe for health and environment) techniques to counteract the metabolic processes leading to rapid senescence, so that they can be of high quality for longer times, in order to be profitable for the enterprises dealing with them; as well to promote its decontamination and safety.

2.3.3. Nonthermal physical technologies

Different packaging systems, ionizing radiation, ultraviolet (UV) radiation, pulsed light (PL), high-power ultrasound (US), cold plasma (CP), high hydrostatic pressure (HHP), and dense phase carbon dioxide (DP-CO2), are novel or emerging technologies that have already found application in the food industry or related sectors (**Figure 14**). An overview on the basic principles of these technologies inherent in the decontamination and shelf-life extension of

fresh fruits and vegetables is presented below, as well as the main advantages, limitations and drawbacks concerning their impact on microorganisms, and quality parameters of these perishable foods. Some challenges and future needs are also identified in this paper. **Table 9** and **Table 10** summarize the critical parameters/variables of control, the inactivation mechanism/effect, advantages, limitations and drawbacks, and challenges inherent to the revised preservation technologies.

2.3.3.1. Packaging systems

Food packaging continues to evolve in response to the advancement of material science and technology, as well as the changing consumers' demand. In today's industrialized world, packaging not only is essential to enable effective distribution and preservation of food, but also to facilitate their end-use convenience and communication at the consumer levels (Galić *et al.* 2011, Mihindukulasuriya and Lim 2014). Furthermore, the development of eco-friendly food packaging materials with improved mechanical and barrier properties has been an active area of research, in which the nanotechnology plays a fundamental role (Mihindukulasuriya and Lim 2014, Hannon *et al.* 2015).

Vacuum packaging

Today is possible to find in the market several vacuum-packaged minimally processed fruits and vegetables. Vacuum-packaging involves removing the air from the package prior to sealing. This simple procedure reduces the O_2 level in the package and limits the growth of aerobic microorganisms, as well as the occurrence of oxidation reactions, which spoil the food (Deepa *et al.* 2013).

Modified atmosphere packaging

The use of modified atmosphere packaging (MAP) has greatly expanded during the last years and is now used around the world to extend the shelf-life of a wide range of foods, including minimally processed and ready-to-eat fruits and vegetables. After harvest, fresh produce continue respiration and transpiration processes and, since they are detached from its nutritional source, they become entirely dependent on their own reserves. MAP has the potential to delay these biological processes and the consequent rate of substrates depletion (Sandhya 2010).

The MAP technology consists in changing the air surrounding the food in the package to a desired composition in order to slow down natural deterioration processes, reduce microbial growth and retain all attributes that consumers consider as freshness markers (Niemira and Fan 2014). In contact with food, the O_2 promotes several types of oxidative reactions and grow of spoilage microorganisms. Thus, for fresh fruits and vegetables, desired atmospheres

consist usually in lowered levels of O_2 and heightened levels of CO_2 and/or nitrogen (N_2). Noble or inert gases (including argon (Ar), helium (He), neon (Ne), and xenon (Xe)) can also be used (Char *et al.* 2012, Silveira *et al.* 2014), but the literature on their application and benefits is still limited. CO_2 has significant implications in MAP; it is high soluble in water, which leads to the production of carbonic acid and consequent increased acidity, and may also cause package collapse due to the reduction of headspace volume (Sandhya 2010). N_2 has a low solubility in water and other food constituents and does not support the growth of aerobic microorganisms (Sandhya 2010). Regarding Ar, it is biochemically active, probably due to its enhanced solubility in water, and appears to interfere with enzymatic oxygen receptor sites, thus reduces the metabolic activity of the food product (Char *et al.* 2012). This gas has also been reported to reduce microbial growth and to improve quality of fresh produce (Jamie and Saltveit 2002). These gases can be injected singly or in combination to balance with the metabolic activity of the product.

Once after harvest fresh fruits and vegetables remain living tissues, their interaction with the package needs to be considered to design appropriated packaging conditions, *i.e.,* the gas and moisture permeability of the packaging film needs to be adapted to the product respiration in order to establish an equilibrium MAP for shelf-life extension. The storage temperature also needs to be considered since it has influence on plant metabolic processes; both ripening and C_2H_4 production rates are proportional to the temperature increase (Luengwilai *et al.* 2014). Additionally, sufficient light can promote photosynthesis in green vegetables, leading to the CO_2 consumption and O_2 production, as well as to weight loss and browning (Martínez-Sánchez *et al.* 2011).

Although there are several studies regarding MAP, it is still necessary to evaluate the influence of different packaging atmospheres and storage temperatures on quality parameters, microbial survival and shelf-life of selected fresh commodities, as well as possible combinations with other preservation treatments such as those described below.

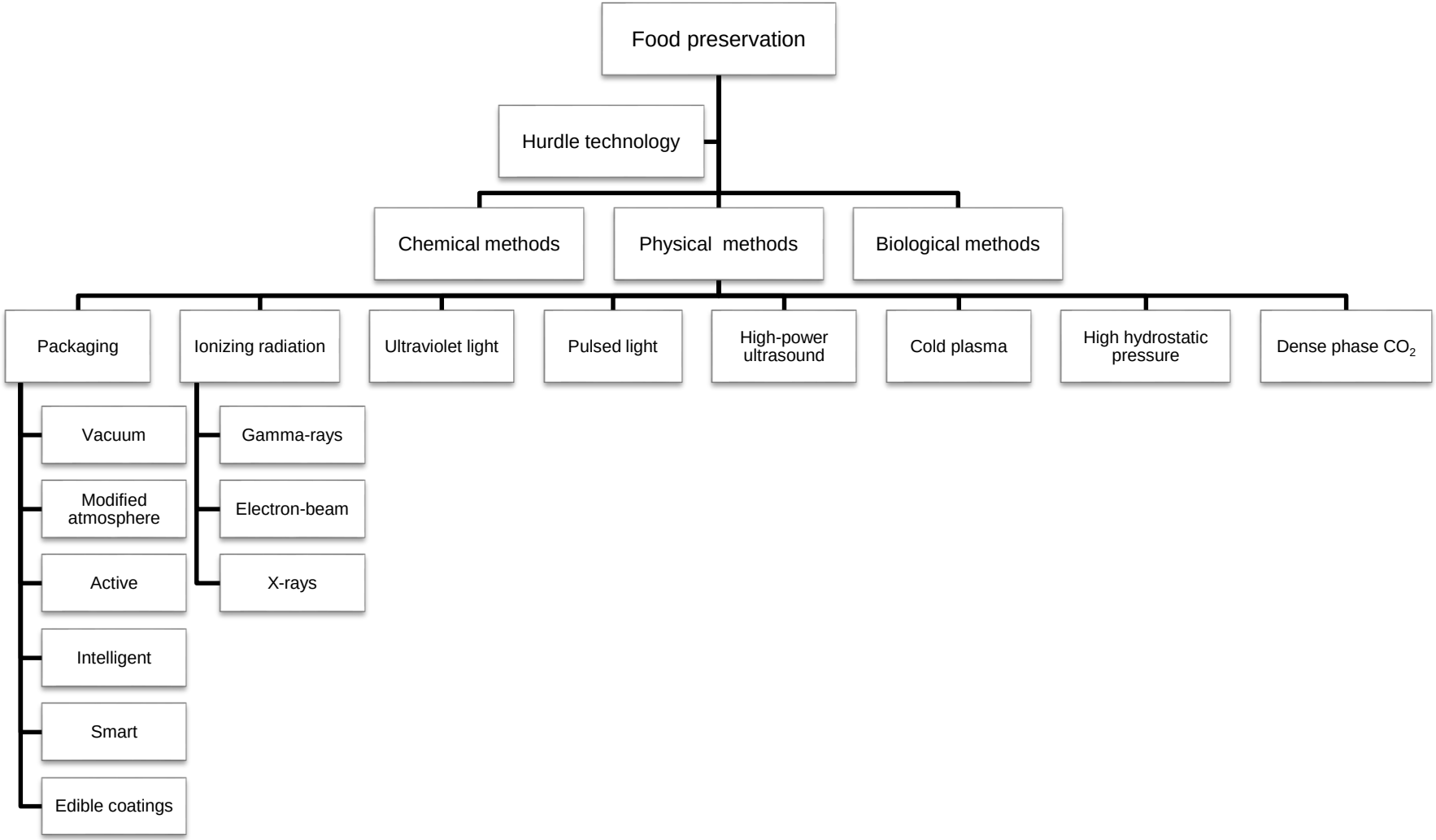


Figure 14 Non-thermal physical technologies to preserve fresh and minimally processed fruits and vegetables.

Table 9 Main advantages, limitations/drawbacks and challenges of the different packaging systems.

Advantages	Limitations/drawbacks	Challenges	References
Vacuum packaging			
Residual O_2 levels; Limits the growth of aerobic microorganisms and the occurrence of oxidation reactions; Economic and easy to apply.	Permeability of the packaging film.	-	Deepa *et al*. (2013)
Modified atmosphere packaging			
Lowered levels of O_2. Controlled respiration rate and deterioration processes. Controls the microbial growth. Provides high-quality foods. Improved shelf-life.	The headspace gas composition depends on the food product. The permeability of the packaging film needs to be adapted to the product respiration.	Determine suitable packaging atmospheres and films for different food commodities.	Jamie & Saltveit (2002); Sandhya (2010); Luengwilai *et al*. (2014); Niemira & Fan (2014)
Active packaging			
Controlled respiration rate and deterioration processes. Controls the microbial growth. Improved food safety and shelf-life.	Possible migration of substances from the package.	Research on the migration behaviours of nanomaterials and their potential impacts on health/safety, as well as the environment. Optimization for specific food commodities. Labelling requirements.	Mehyar & Han (2010); Mihindukulasuriya & Lim (2014)
Intelligent packaging			
Capacity to monitor the integrity and safety of the packaged foods. Improved food safety and shelf-life.	Early stage of development. The increased cost per package.	Development of new devices and technologies is required. Labelling requirements.	Mihindukulasuriya & Lim (2014); Vanderroost *et al*. (2014)
Smart packaging			
Both of active and intelligent packaging. Better control of the food supply chains and improvements on overall performance and security.	Both of active and intelligent packaging.	Both of active and intelligent packaging,	Vanderroost *et al*. (2014)
Edible coatings			
Control of respiration processes. Reduced exposure to O_2 and moisture losses. Replacement and/or fortification of natural layers. Improve mechanical and handling properties and structural integrity. Vehicle for the incorporation of active ingredients.	Difficulty in applying regular thickness layers. Sensory and organoleptic properties of the EC affect its consumer acceptance.	Achieve a controlled release of active ingredients under specific conditions using nanoencapsulation techniques. Creation of multilayered systems (nanolaminates) to coat highly hydrophilic foods.	Olivas & Barbosa-Cánovas (2009); Andrade *et al*. (2012); Carocho *et al*. (2014)

Table 10 Summary of critical parameters/variables, inactivation mechanism/effect, advantages, limitations and drawbacks, and challenges inherent to the revised technologies.

Parameters/variables	Inactivation mechanism/cause/effect	Advantages	Limitations and drawbacks	Challenges	References
Ionizing radiation					
Radiation source Dose (kGy) Dose rate (kGy h^{-1})	Formation of free radicals and other reactive species. DNA damage and loss of reproductive capability and other functions of the cell.	Availability of different systems (gamma-rays, electron beams, x-rays). Excellent penetration into foods. Insect disinfestation and parasites inactivation. Delay ripening and senescence. Sprout inhibition. Post-packaging treatments. Suitable for sterilisation. Reliable and safe. Low energetic inputs. Suitable for large-scale processing. Suitable for packaging materials.	Efficacy depends on food composition and type of microorganism. Possibility of affecting quality parameters. High capital cost. Strict safety standards. Needs training to operate. Consumer acceptance.	Consumer education and regulatory approval required.	Rawson *et al*. (2011); Fernandes *et al*. (2012); Moosekian *et al*. (2012); Lacroix (2014)
Ultraviolet radiation					
UV wavelength and source Treatment time Distance of product from the radiation source	DNA damage, membranes and enzyme activity induced by radiation absorption.	Delay ripening and senescence. Equipment of moderate to low cost and easy to use. Little changes in quality at low doses. Stimulates the synthesis of health-promoting compounds. Suitable for food contact surfaces.	Pre-treatment can be necessary. Low degree of penetration (surface treatment). Occurrence of shadow effects. Process parameters difficult to standardize. The efficacy depends on food composition and microbial concentration.	Evaluate the effects on specific pathogens and the impact on water soluble vitamins.	Gómez *et al*. (2011); Ramos *et al*. (2013); Du *et al*. (2014); Severo *et al*. 2015 ; Syamaladevi *et al*. (2015)
Pulsed light					
Intensity (J cm^{-2}) Number of pulses delivered Treatment time	Photochemical damage of DNA, protein denaturation, agglutination of the cytoplasmatic content and disruption of cell membranes	Significant and rapid microbial inactivation in short-time treatments. Lack of residual compounds. Great flexibility. Little or no changes on foods. Stimulates the synthesis of health-promoting compounds. Suitable for packaging materials. Medium cost and low energetic input. Availability of water-assisted systems.	Low degree of penetration (surface treatment). Occurrence of shadow effects. Efficacy depends on food composition and physical properties, and microbial concentration; Possible occurrence of some appearance defects.	Evaluate the effects on specific pathogens and the impact on water soluble vitamins.	Elmnasser *et al*. (2007); Krishnamurthy *et al*. (2010); Izquier and Gómez-López (2011); Ramos-Villarroel *et al*. (2012); Rodov *et al*. (2012); Xu *et al*. (2013); Huang & Chen (2014 and 2015); Rowan *et al*. (2015)
High power ultrasound					
Power, amplitude and frequency Treatment time Temperature	Acoustic cavitation. Mechanical energy. Production of reactive species. DNA damage.	Enhanced penetration to inaccessible sites. Effective against vegetative cells, spores and enzymes. Reduced processing time. Heat transfer increased.	Efficacy affected by food size and composition. Efficacy depends on type, shape and size of the microorganisms. Possible physical	Optimization of adequate treatment conditions for different foods, which demands	Bermúdez-Aguirre *et al.* (2011); Knorr *et al*. (2011) ; Awad *et al*. (2012); Bilek &

Parameters/variables	Inactivation mechanism/cause/effect	Advantages	Limitations and drawbacks	Challenges	References
Pressure			changes on food. Possible food damage by reactive species. Problems related to scaling-up and lack of suitable industrial scale processing units.	a close collaboration between researchers, equipment suppliers and the food industry.	Turantaş (2013); Gao *et al*. (2014) ; São José *et al*. (2014)
Cold plasma					
Type of plasma generated Energy (which varies with the gas used, density and temperature) Treatment time	Chemical interaction of radicals, reactive species or charged particles with the cell membranes, by damage to membranes and internal cellular components or broken the DNA strands	High efficiency. No shadow effects. In-package treatments.	Efficacy depends on the type of microorganism, inactivation medium, number and physiological state of the cells. Efficacy also affected by physical and chemical properties of foods, as well as operating gas mixture and flow. Technology in an early development stage.	Understand the mode of action of the treatment and the possible interactions with food constituents and properties.	Niemira (2012); Bermúdez-Aguirre *et al*. (2013); Ziuzina *et al*. (2014).
High hydrostatic pressure					
Pressure (MPa) Treatment time Adiabatic heating Compression and decompression rate	Membrane damage. Proteins denaturation. Leakage of cell content. Dissociation of ribosomes.	Independent of food shape or size. Uniformity of treatment throughout food. The primary structure of molecules remains intact. Kills vegetative bacteria. Colour, flavour and nutrients are preserved. Reduced treatment times. Post-packaging treatments; Positive consumer appeal. Easy to use. Commercial systems available. Approved by regulatory agencies.	Foods should have ~40% free water for antimicrobial effect. Efficacy depends on type of microorganism and physiological state of cells, as well as the food composition. Spores not inactivated. Mixed effects on enzymes. Limited packaging options. Batch processing. High cost of equipment.	Evaluation of the behaviour of nutrients, allergens, and microorganisms under specific foods and conditions, as well as the process conditions necessary to inactivate bacterial spores.	Russell (2002); Patterson (2005); Considine *et al*. (2008); Rivalain *et al*. (2010); Castro and Saraiva (2014); Huang *et al*. (2014)
Dense phase carbon dioxide					
Pressure (MPa) Treatment time Temperature Compression and decompression rate	Physical disruption due to a rapid depressurization and expansion of CO_2 within the cells. CO_2 increases the membrane fluidity and permeability.	At supercritical conditions CO_2 has effective penetration capacity into solid matrices. CO_2 can easily be removed from the solutes by mere expansion to ambient pressure. Relatively low operating costs.	High capital costs are associated with this technology.	Elucidation of the mechanisms underlying changes on food properties, especially bioactive ingredients.	Choi *et al*. (2008); Calvo & Torres (2010); Rawson *et al*. (2011); Rawson *et al*. (2014)

Active packaging

Active packaging (AP) plays an additional role in maintaining the quality and safety of fresh foods compared with conventional packaging systems. AP involves an interaction between package and food product in order to provide favourable conditions for quality preservation and shelf-life extension. It is designed to control food deterioration processes by incorporating active ingredients that have been deliberately included in the package film or headspace, such as scavengers for O_2, CO_2 and C_2H_4, moisture absorbers, CO_2 emitters, antimicrobials, antioxidants, preservatives, and so on (Mehyar and Han 2011, Mihindukulasuriya and Lim 2014). Despite being necessary to design and optimize this technology for each specific food, it is expected to be ready for implementation by the food industry soon.

Intelligent packaging

An intelligent packaging (IP) system can interact with internal factors (food and headspace) and/or external environmental factors through innovative devices or sensors. As a result of this interaction, the sensor will generate a response that correlate with the state of the packaged food. The information generated not only is useful for communication with the consumers by informing them about the safety and quality of the products, but can also be utilized by the producers in their decision support systems to determine when and what actions are to be taken during the product distribution chain. Examples include indicators for integrity, freshness and time-temperature, sensors for O_2 and CO_2, radio frequency identification tags and security tags (Mehyar and Han 2011, Mihindukulasuriya and Lim 2014, Vanderroost *et al.* 2014). Despite all the potential of this technology, the use of IP for fresh fruits and vegetables preservation is still at an early development stage.

Smart packaging

The concepts of smart and IP are often used interchangeably in the literature, but they are not the same. Smart packaging is an immature technology that could potentially be applied in the context of IP in the far future, being therefore a major challenge for the coming times. Smart packaging consists in the combination and integration of AP and IP concepts in one packaging system. This innovative combination offers the possibility to monitor changes in the food, package and/or environment, and to respond appropriately on these changes through a feedback mechanism. Sooner or later, smart packaging will undoubtedly result in a better control of food supply chains and in consequent improvements of their overall performance and security, but the development of new devices and technologies is still required (Vanderroost *et al.* 2014).

Edible coatings and films

The application of an edible coating (EC) on the food surface has become popular among the available techniques to preserve fresh or minimally processed fruits and vegetables. Although the concept is not new, recently considerable interest and advanced research activity has been observed in this field, which has been driven by an increasing consumer demand for safer, high quality, and convenient foods (Ciolacu and Nicolau 2013). This technique is close to the AP technology, but EC do not act as a package by itself, they just reduce the barrier requirements of the package (Gómez-Estaca *et al.* 2014). Although many authors do not make distinction between EC and edible films (EF), the latter are used as covers, wraps or separation layers, while the EC are considered part of the final product and designed to protect or enhance its properties (Carocho *et al.* 2014). Actually, their mode of formation and application to foods is different. EC are applied and formed directly on the food, while EF are freestanding structures, formed and later applied as a wrapping on the food product (Olivas and Barbosa-Cánovas 2009).

As with other MAP systems, EC can create a very low O_2 environment. Additionally, on fruits and vegetables, EC could provide replacement and/or fortification of natural layers to prevent moisture losses and to control exchange gases involved in respiration processes such as O_2, CO_2 and C_2H_4. Therefore, EC have the potential to improve food quality and shelf-life (Olivas and Barbosa-Cánovas 2009, Andrade *et al.* 2012). These protective layers may also improve mechanical handling properties and structural integrity, and can be used as a vehicle for incorporating several active ingredients, including anti-browning, antioxidants, antimicrobials, colorants, flavours, fortifying nutrients, plant extracts, etc. (Carocho *et al.* 2014). These ingredients are more efficient into EC or EF than when directly added to foods, since they can be gradually and selectively released from the wrapping matrix to the food surface, keeping effective concentrations where and when they are needed; thus, smaller amounts of these ingredients will be required to achieve the desired effect or aspect (Olivas and Barbosa-Cánovas 2009, Falguera *et al.* 2011). Therefore, EC can also enhance nutritional and sensory attributes.

The structural matrix of these edible layers can be developed from polysaccharides (starch, cellulose, alginate, carrageenan, chitosan and pectin), proteins (gelatine, casein, gluten, zein, keratin, albumin and soy protein), and lipids (waxes, acetylated monoglycerides, fatty alcohols and fatty acids) or blends between hydrocolloids (polysaccharides or proteins) and lipids, called composites (Han 2014). Hydrocolloids can form cohesive molecular networks by strong interactions between molecules, imparting good barrier properties to gases and good mechanical properties. One of the most investigated compounds for EC is chitosan due to its antimicrobial properties. Regarding the application of EC, different methods such as panning, fluidized bed, dipping and spraying can be used (Andrade *et al.* 2012). The EC remains on the food during storage and will be disintegrated or dissolved

during cooking or the mastication process. Thus, once the consumer acceptance of the coated product is influenced by the sensory and organoleptic properties of EC, its acceptability needs to be carefully evaluated (Andrade *et al.* 2012). A weakness of this technology relates to the difficulty in applying a regular thickness layer over the surface of the food, which can lead to irregular dispersion of the added active ingredients (Carocho *et al.* 2014). This can occur mainly in foods with irregular surfaces, which may present not covered areas that expose the food surface to unfavourable preservation conditions. In turn, EF are formed by casting and drying film-forming solutions on a levelled surface, drying a film-forming solution on a drum drier, or using traditional plastic processing techniques such as extrusion (Falguera *et al.* 2011).

Despite the benefits of EC, commercial applications on a broad range for different foods are still very limited, and research have focused mainly on searching for new coating materials. In order to overcome some limitations of this technology, a new generation of EC is under development by using nanotechnological processes. The main objectives are to improve mechanical and barrier properties through nanocomposite coatings, achieve a controlled release of active ingredients under specific conditions using nanoencapsulation techniques, and create multi-layered systems (nanolaminates) to coat highly hydrophilic foods (Andrade *et al.* 2012).

2.3.3.2. Ionizing radiation

Food irradiation may be considered as the second big breakthrough after pasteurization. This novel physical treatment involves food exposure to ionizing radiations, such as gamma, electron-beam or X-ray, to eliminate microbial contamination, inhibit the germination of crops, and delay the ripening rate of fruits and vegetables, allowing to ensure safety and extend the shelf-life (ICGFI 1999, Lacroix 2014, Lung *et al.* 2015). It is considered as a safe and effective postharvest treatment by several international authorities (WHO 1999), and can be applied as an alternative to chemical fumigants. The irradiation technology can not only improve food safety but also reduce crop-related economic losses (Lung *et al.* 2015). In Europe as well as in Asia Pacific region it has been observed a positive trend in order to meet phytosanitary requirements in the international trade (Kuan *et al.* 2013). Nevertheless, the adoption of ionizing radiation for food applications has been a slow process due to some misunderstandings by the consumer who often chooses non-irradiated foods.

In food processing, the permitted sources of gamma-rays are mainly cobalt-60, a radioactive isotope produced from cobalt-59, and caesium-137, a spent fuel from nuclear reactors. Beta-rays, a stream of electrons, are another source; but, due to their low energy levels, they need to be accelerated to make them acquire the required energy to be used in food preservation (Lacroix 2014). According to Clemmons *et al*. (2015), electron accelerators

appear to be more successful compared to gamma-rays because the source can suspend the irradiation at any time; non-nuclear energy can accelerate the generation of radiation when required; little risk for occupational injuries; and applicability in high-flow and high-dose irradiation.

Gamma-rays and electron-beams produce the so-called ionizing radiation constituted by electronically charged atoms or molecules. During the food exposure to the irradiation field, the quantity of energy absorbed by the food must be measured (in Gray or kiloGray (kGy)) to establish correct procedures for food preservation and quality control (Antonio *et al.* 2012). For this reason, dosimeters are placed within the food product to be irradiated to measure the distribution of the absorbed energy and to determine the maximum and the minimum dose absorbed by the food. The desired dose is achieved by the time of exposure and by the location of the food product relative to the source. The amount of energy absorbed by the food product also depends on its mass, bulk density and thickness (Lacroix 2014). Additionally, gamma irradiation treatments can be divided into three levels based on dosage: low-dose treatment up to 1 kGy for insect disinfestation and parasite inactivation, delay in fruit maturity and prevention of germination; intermediate-dose treatment from 1 to 10 kGy to reduce non-spore forming pathogens and spoilage microorganisms and extend shelf-life of fresh commodities; and high doses above 10 kGy to reduce microorganisms to the point of sterility (ICGFI 1999, Fan *et al.* 2012). Likewise, the gamma irradiation treatment provides an alternative way to eliminate pesticide residues from plant products (Wen *et al.* 2010).

The principal target of ionizing radiation is water that produces free radicals and other reactive species, able of breaking chemical bonds and modifies various molecules, leading to destruction or deactivation of bacterial components (Ramos *et al.* 2013). The inactivation of microorganisms by irradiation is primarily due to DNA damage, which destroys the reproductive capabilities and other functions of the cell (Rawson *et al.* 2011). Besides, changes in food quality parameters might vary depending on the basic raw food material, irradiation dose delivered, and type of radiation source employed (Lacroix 2014). Depending upon the radiation dose, foods may be pasteurized to reduce or eliminate food-borne pathogens.

During irradiation, the dose, the food composition and the type of microorganism affect differently the efficacy of treatment (Moosekian *et al.* 2012). In general, the irradiation dose is positively proportional to the degree of killing microorganisms. When the food properties are adverse to microbial growth, their resistance to the radiation is reduced and can be killed by low irradiation doses. Regarding microorganisms, they show different tolerance levels towards specific doses of irradiation; normally, Gram-positive bacteria

display stronger resistance than Gram-negative bacteria, while prokaryotic microorganisms are more resistant than eukaryotic microorganisms (Moosekian *et al.* 2012).

For fresh and minimally processed fruit and vegetables, low-doses (up to 1 kGy) are approved by the Food and Drug Administration (FDA) for shelf-life extension purposes. The treatment is applied for sprout inhibition, delay ripening, and to reduce bacterial, parasitic and protozoan pathogens (ICGFI 1999, Ramos *et al.* 2013). If the dose is less than appropriate, the intended preservation effect may not be achieved, and if the dose is excessive, the food may be damaged and unacceptable for consumption. Additionally, for post-packaging irradiation, the package materials should be chemically stable to prevent polymer degradation and low molecular weight hydrocarbons and halogenated polymers formation which can migrate into foods (Galić *et al.* 2011). Moreover, all irradiated products need to be labelled with the RADURA-logo and a statement “treated by irradiation” or “treated with irradiation” either on the package or at the point of sale.

Currently more research is needed to evaluate the dose-response effects on different quality parameters of specific fresh produce, as well as to educate retailers and consumers about irradiation processing to advance commercial applications of this technology. The use of this technology as a hurled for packaged fresh produces also needs more research.

2.3.3.3. Ultraviolet radiation

Ultraviolet (UV) radiation can be used as a nonthermal treatment to decontaminate and reduce decay of fresh or minimally processed fruits and vegetables. UV radiation has been tested with UV-A (long waves, 400-315 nm), UV-B (medium waves, 315-280 nm) and most commonly with UV-C (short-waves, 280-100 nm) since it has more energy (US-FDA 2017). The treatment can induce resistance mechanisms against pathogens or damage directly the bacterial DNA (Ramos *et al.* 2013). UV-C radiation also produces significant damage in the cytoplasmic membrane integrity and cellular enzyme activity (Gómez *et al.* 2011). However, the microbial inactivation is limited solely to the food surface, as UV-C has extremely low penetration into solids. Recent studies reported positive effects induced by this postharvest treatment on physiological, microbiological and quality parameters of fresh fruits and vegetables. Martínez-Lüscher *et al*. (2014) demonstrated that the UV-B radiation has an overall positive impact on grape berry composition. Du *et al*. (2014) verified that the treatment can be used as an additional processing step on selected specialty crops to enhance their soluble phenolic content, and that the changes are species-dependent. Regarding medium waves, Syamaladevi *et al*. (2015) showed the UV-C radiation efficacy to reduce *Penicillium expansum* inoculated onto the surface of organic apples, cherries, strawberries and raspberries, and that the efficacy depends on the fruit surface morphology. The treatment also inhibits C_2H_4 production and delays the softening of tomato fruit during

ripening (Bu *et al.* 2013, Severo *et al.* 2015). Additionally, an increase on the beneficial effect of tomatoes for human health was reported by Bravo *et al*. (2012) after UV-C irradiation, namely increased total phenolic levels and antioxidant activity. This novel technology also has high potential for surface decontamination of apricots (Yun *et al.* 2013) and fresh-cut melon (Manzocco *et al.* 2011).

2.3.3.4. Pulsed light

Pulsed light (PL), or high intensity light pulses, is a fast and environmentally friendly emerging technology to decontaminate food surfaces by inactivating microorganisms using pulses of an intense broad spectrum rich in UV-C light (Gómez-López *et al.* 2007). PL works with xenon lamps that can produce intense and short time pulses of broad spectrum "white light", from ultraviolet wavelengths of 200 nm to infrared wavelengths of 1000 nm, with peak emissions between 400-500 nm. The power is magnified many times by storing energy in a high power capacitor over relatively long times (fractions of a second) and releasing it over a short period of time (millionths or thousandths of a second) producing several high energy pulses per second. This phenomenon increases the instantaneous energy intensity that contributes to the inactivation of both spoilage and pathogenic microbial cells. In addition, it can limit the negative effects on food quality in terms of nutritional and organoleptic properties since there is no substantial increase in temperature during the treatment. This novel technique is proposed for the decontamination of solid and liquid foods, packages, medical devices, packaging and processing equipments for the food, medical and pharmaceutical industries (Gómez-López *et al.* 2005a, Rajkovic *et al.* 2010, Palgan *et al.* 2011, Luksiene *et al.* 2012, Ramos-Villarroel *et al.* 2012, Charles *et al.* 2013).

The inactivation efficacy of PL depends on the intensity (measured in J cm^{-2}) and numbers of pulses delivered. The UV-C part of the light spectrum (200-280 nm) emitted by the flash lamp is the most lethal and consequently the most important for microbial inactivation. As a result, cells are inactivated by photochemical damage of DNA, protein denaturation, agglutination of the cytoplasmatic content leading to a disruption of cell membranes, and other photothermal and photophysical effects (**Figure 15**) (Krishnamurthy *et al.* 2010, Ramos-Villarroel *et al.* 2012). Thus, significant and rapid microbial inactivation in short-time treatments, lack of residual compounds and great flexibility are some of the advantages of this technology. Additionally, recent evidence demonstrates that PL kills yeast through a multi-hit or mechanistic process that affects the cell membrane permeability along with DNA and macromolecule stability and functionality depending on the dose applied (Rowan *et al.* 2015). Nevertheless, the food composition affects the effectiveness of this technology. The presence of proteins and oil decreases the decontamination effect, because they absorb the effective wavelengths. On the other hand, foods rich in carbohydrates such

as fruit and vegetables seem to be more suitable for PL treatments (Gómez-López *et al.* 2005b, Elmnasser *et al.* 2007). However, few studies have focused on the effects of PL on physiological and quality parameters of fruit and vegetables.

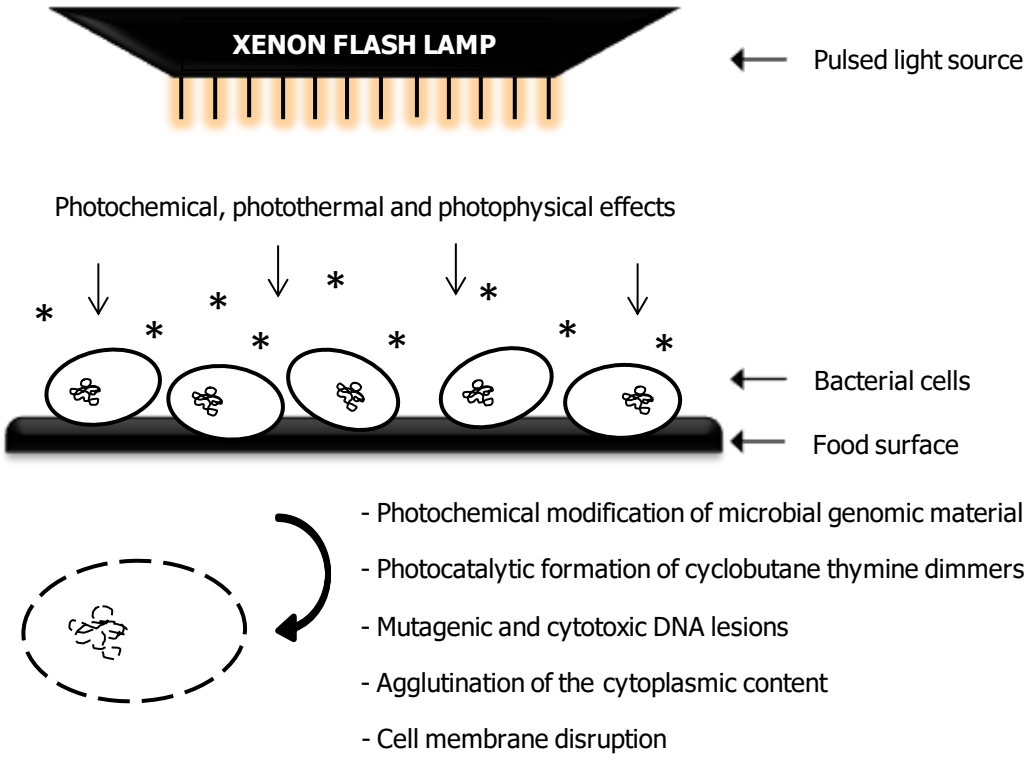


Figure 15 Mechanism of microbial inactivation by pulsed light.

The inactivation of microorganisms naturally present or inoculated on fruits and vegetables surfaces by PL has been achieved and demonstrated in the last years. Izquier and Gómez-López (2011) investigated the dose-effect relationship between PL fluence (J cm^{-2}) and inactivation of naturally occurring microorganisms present on fresh-cut iceberg lettuce, white cabbage and carrot surfaces. They verified that a single low energy pulse (0.72 J cm^{-2}) was enough to achieve one log reduction, with an ultrafast treatment time of 0.5 ms, and a satisfactory inactivation level for shelf-life extension. Other study carried out by Charles *et al*. (2013) demonstrates that PL is an effective strategy to preserve the firmness, the colour and the carotenoid content of fresh-cut mangoes, compared to the control where quality decreased. They also reported an increased polyphenol oxidase (PPO) activity after 3 days, maintained phenylalanine ammonia lyase activity and unaffected phenolic and total ascorbic acid contents. On the other hand, Aguiló-Aguayo *et al*. (2013) achieved a surface reduction of natural and inoculated (*Saccharomyces cerevisiae*) microorganisms in fresh tomatoes without losses of nutritional quality, as vitamin C was unaffected and the carotenoids concentration was actually slightly increased. However, PL severely reduced the

acceptability product quality by causing an important weight loss and visible wrinkles after three days. Likewise, a negative effect on surface colour and texture in fresh-cut watermelon, was reported by Ramos-Villarroel *et al*. (2012) after exposure to 30 light pulses (12 J cm^{-2}). The authors also found that inoculated *E. coli* (Gram-negative) is more sensitive to PL than *Listeria innocua* (Gram-positive) probably due to the cell wall composition of these bacteria. The cell walls of Gram-positive bacteria are more rigid and thicker than those of Gram-negative bacteria, which may give an extra protection against the PL treatment. Nevertheless, the inactivation of microorganisms present on food surfaces does not necessarily result in shelf-life extension. Gómez-López *et al*. (2005b) failed to prolong the shelf-life of minimally processed white cabbage and iceberg lettuce in spite of the reduction in the initial microbial load.

The effectiveness of the PL treatment is limited by its low degree of penetration (~2 µm) or because of a shadow effect. Indeed, microorganisms may penetrate via crevices or irregularities present on the product surface or through the epidermis of the fruits or vegetables (Gómez-López *et al.* 2005a, 2005b, Lagunas-Solar *et al.* 2006, Huang and Chen 2014). For this reason, Luksiene *et al*. (2012) evaluated the possibility to decontaminate fruit and vegetables with different irregular surfaces by PL, including plums, tomatoes, cauliflowers, sweet peppers and strawberries, from food pathogens and mesophiles. They verified that the surface irregularities just slightly, but not significantly, reduce the treatment efficiency, once significant decontamination effect was observed compared to control. Additionally, no effects on nutritional quality (vitamin C, total phenolics and antioxidant capacity), colour and texture have been observed in the processed samples. Therefore, an ideal food product surface would be smooth, clear and without roughness, pores and grooves which can “shadow” the microbial cells from the light, causing less complete light diffusion and thus reducing process effectiveness. For the same reason, food products to be treated should be clean and free of contaminating particulates (Lagunas-Solar *et al.* 2006, Cacace and Palmieri 2014). Likewise, the PL decontamination efficacy decreases at high contamination levels. Thus, once at high population densities microorganisms overlap each other’s, only the upper layers will become inactivated, which shadow the remaining from the light (Gómez-López *et al.* 2005a, 2007).

In order to overcome these limitations is possible to add devices to the PL system to create multidirectional light pulses, or generate the random movement/rotation of the food product to provide uniform PL exposure of the entire surface and any protected microorganism can be attained. To achieve this, a novel setup using a water-assisted PL system was developed, in which the food samples are immersed in agitated water and can randomly move and rotate, allowing more uniform PL exposure of all the food samples surfaces. This wet PL treatment was already applied successfully to decontaminate green

onions (Xu *et al.* 2013), blueberries (Huang and Chen 2014), strawberries and raspberries (Huang and Chen 2015) showing better results than the dry PL system, and being a promising chemical-free alternative to other sanitization treatments. Additionally, whenever possible or if appropriate, the food product could be cut into thin slices or pieces so that light can penetrate almost through the entire product.

Another study suggests that a brief postharvest PL treatment stimulates coloration and anthocyanin accumulation in figs, and seems to be a feasible means of compensating for insufficient sunlight stimulation of colour development in figs and possibly other fruits as well (Rodov *et al.* 2012). However, current literature is scarce, therefore more research is needed to evaluate the effectiveness and suitability of this technology, namely to understand the effect of spectral range on specific pathogens and the impact on water soluble vitamins that are known to be sensitive to UV light, and then optimize its use.

2.3.3.5. High-power ultrasound

Ultrasound (US) is a green processing technology very promising in the food industry because of its potential to inactivate microorganisms present in fruit and vegetable surfaces (Chemat *et al.* 2011). The US treatment is simple, relatively cheap and energy saving (Awad *et al.* 2012); it also offers advantages in terms of cost, productivity and selectivity, with better processing time, improved quality, and reduced physical risks (Chemat *et al.* 2011). The US treatment is based on energy generated by sound waves with frequency beyond the limit of human hearing (Awad *et al.* 2012). Regarding the US system, it consists of an electrical power generator (source of energy), a transducer (to convert electrical energy at US frequencies) and a coupler or emitter (to emit the US waves from the transducer into the medium) (Chemat *et al.* 2011). The US band can be divided into low-power (high frequencies) and high-power (low frequencies) US (Kentish and Ashokkumar 2011). The first one includes frequencies higher than 1 MHz and has non-destructive effects in the food properties through which they pass. On the other hand, the low frequencies, between 18 kHz and 100 kHz, induce mechanical, physical and chemical changes, such as physical disruption, which supports the inactivation mechanism on food surfaces (Awad *et al.* 2012).

In food preservation, high-power US is used to inactivate microorganisms. The antimicrobial effects are attributed to intracellular acoustic cavitation (**Figure 16**), phenomenon characterized by the formation, growth and implosion of small bubbles generated by the US waves. These bubbles pass through the biological structure and promote a series of compression and rarefaction (expansion) cycles leading to the production of localized energy (hot spots). Two cavitation phenomena may occur, the stable cavitation and the transient cavitation. The stable cavitation requires thousands of cycles of oscillating US waves to originate tiny and non-linear bubbles forming large bubble clouds

whose collapse does not occur. In the transient cavitation (**Figure 16**), the bubbles are increased in size within a few oscillatory cycles and collapse quickly, releasing energy that contributes for the generation of reactive species endowed with bactericidal effects (Bilek and Turantas 2013, São José *et al.* 2014). These hot spots originated by the bubbles implosion events, characterized by presenting high temperature (up to 5500 °C) and pressure (up to 1000 MPa), occur in very short time periods (μs) and lead to the release of reactive species (*e.g.,* hydroxyl radicals) as a result of the dissociation of vapour trapped in the bubbles. Thus, apart from the physical effect responsible for the microbial cell death, the originated reactive species also contributes to the microbial inactivation (Gao *et al.* 2014, São José *et al.* 2014). Furthermore, the released energy allows reaching food surfaces that are difficult to access using other sanitizing methods (Gao *et al.* 2014).

In microbial cells, the cavitation phenomenon can lead to the pore formation, cell membrane disruption and selectivity loss, and consequent cell breakage (**Figure 16**). Besides, the originated reactive species can demerge the DNA (Bermúdez-Aguirre *et al.* 2011, São José *et al.* 2014). Nevertheless, the type, shape and size of the microorganisms may influence the treatment efficacy. In general, Gram-positive bacteria are more resistant than the Gram-negative ones, because of its thicker cell walls and probably due to a more tightly adherent peptidoglycan layer in Gram-positive cells. Additionally, cocci are more resistant than rod-shaped bacteria, and larger cells are more sensitive than smaller ones. Moreover, spores appear to be more resistant than vegetative forms while enzymes are reported to be inactivated by US due to a depolymerisation effect (Chemat *et al.* 2011). The pH also affects the decontamination efficiency, being the inactivation rate increased at lower pH (São José *et al.* 2014). Additionally, since the frequency affects the cavitation phenomenon and larger bubbles are produced at lower frequencies, these are more effective in high viscous foods (Bermúdez-Aguirre *et al.* 2011, São José *et al.* 2014).

Recent studies demonstrated that the amplitude of ultrasonic waves, exposure time, treatment temperature, and volume and composition of the food can affect differently the fresh fruits and vegetables properties. Aday *et al*. (2013) evaluated the effects of different US powers (30, 60 and 90 W) and treatment times (5 and 10 min) on quality parameters of strawberry. Significant differences between treatment times were not found. Power levels between 30 and 60 W resulted in improved strawberry quality (colour, texture, pH and total soluble solids (TSS)), being useful for shelf-life extension. On the other hand, 90 W resulted in detrimental effects on the evaluated parameters. Nevertheless, decay incidence analysis confirmed that all treatment conditions were effective to reduce mould growth. Cao, Hu, Pang, *et al*. (2010) also investigated the effects of the US treatment on strawberry decay and physiological quality. Fresh fruits were treated with 0, 25, 28, 40 or 59 kHz frequency (operating at a power of 350 W), at 20 ºC for 10 min, and then stored at 5 ºC for 8 days. It

was found that 40 kHz significantly reduced decay incidence and microbial population, and inhibited the decrease of firmness and maintained significant higher levels of TSS, total titratable acidity and vitamin C. It was also found that treatments with 25 and 28 kHz had no significant effect on decay and quality deterioration of this fruit. Other study demonstrated that 250 W and 9.8 min were the optimal conditions to treat strawberries, highlighting its suitability for quality maintenance and shelf-life extension (Cao, Hu, and Pang 2010).

The decontamination effectiveness is not always severe enough when using the US treatment alone. Thus, combining US with other preservation factors may be advantageous due to the hurdle effect (Chemat *et al.* 2011). If combined with pressure, heat, both pressure and heat, or UV irradiation, the treatment is called manosonication, thermosonication, manothermosonication, or photosonication, respectively. However, a very few data regarding these combinations to preserve fresh or minimally processed fruits or vegetables are available. Cruz *et al*. (2006) determined the degradation kinetics of vitamin C in watercress (*Nasturtium officinale*) by thermosonication. The treatment was found to be a better blanching process, since it inactivates watercress peroxidase at less severe blanching conditions and consequently retained vitamin C content at higher levels. Additionally, the heat blanching required 70 s to produce the same degree of peroxidase inactivation (90% reduction), that was about 14-fold the processing time of the thermosonication treatment (5 s). This huge difference leads to a higher retention of vitamin C in the thermosonication treatment (about 94%) as compared to heat blanching, which reduced the content to 29%.
Since the ultrasonic equipment has to be custom designed for each application, so far only a few treatments have reached industrial level (Knorr *et al.* 2011). Thus, future challenges are based on the optimization of adequate treatment conditions for different foods, which demands many research capacities and a close collaboration between researchers, equipment suppliers, and the food industry.

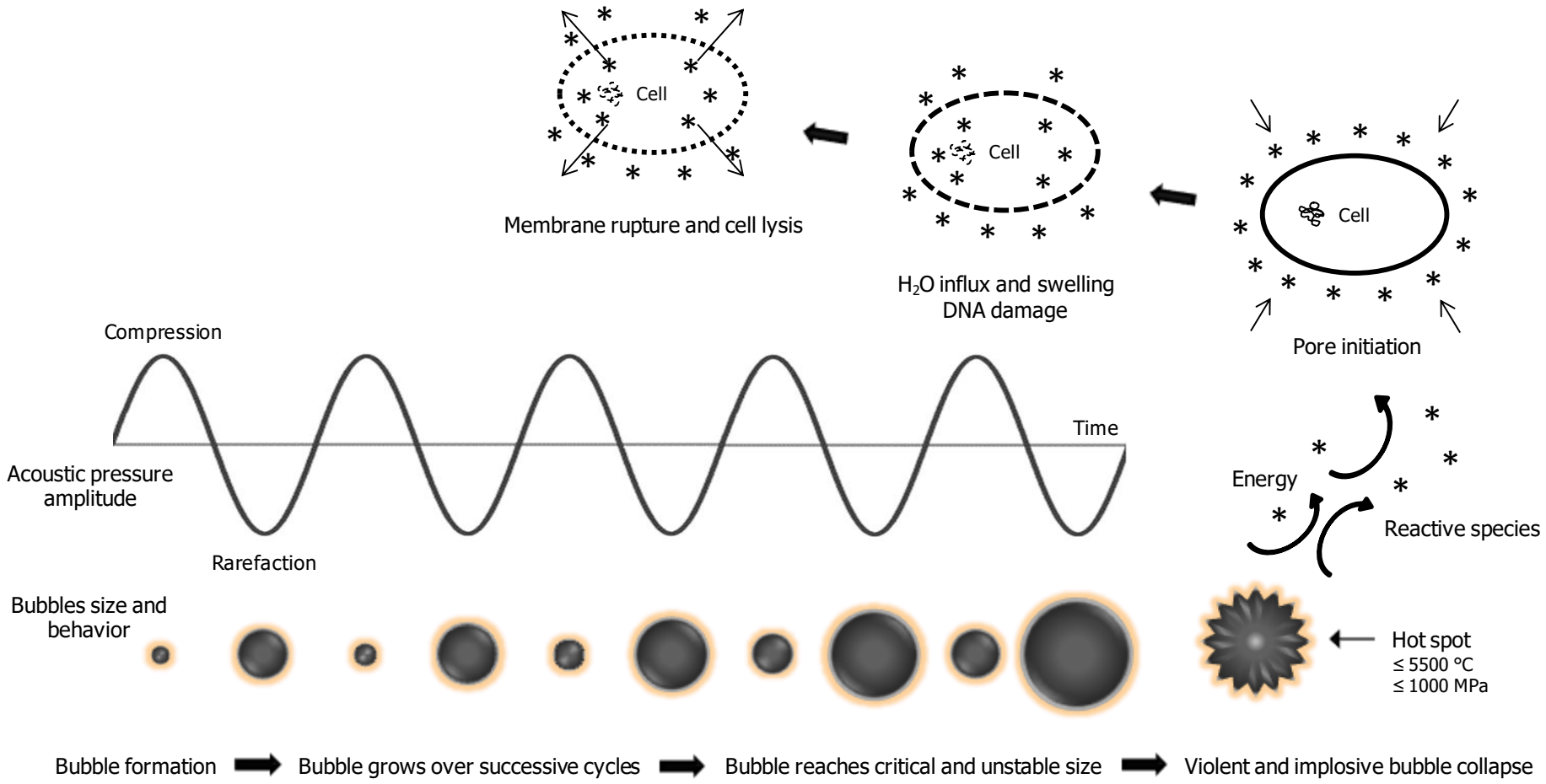


Figure 16 Mechanism of transient acoustic cavitation and microbial cells inactivation. Adapted from Leonelli and Mason (2010) and Chemat *et al*. (2011).

2.3.3.6. Cold plasma

Cold plasma (CP) is a new and green food preservation (decontamination) technology with the potential for application to a wide variety of foods. It is so recent that the terminology is still evolving and has only been applied at very small scales. Actually, different CP technologies have been developed for food processing, as well as technologies used to generate it (Niemira 2012). The nonthermal plasma is formed by quasi-neutral ionized gases which are dissociated by an energy input. These ionized gases comprise particles such as photons, free electrons, positive and negative ions, atoms in their fundamental or excited states, and free radicals that, in combination, have the capacity to inactivate microorganisms on food surfaces (Niemira 2012, Pankaj *et al.* 2014, Baier *et al.* 2015). The gas being ionized may be air, O_2 or N_2, or a mixture containing some proportion of noble gases (Ar, He or Ne). In turn, heat, electricity, laser light and radiation, among others, are used as energy sources (Niemira 2012). Plasmas can be generated at atmospheric pressure and close to ambient temperature, thus, allowing nonthermal treatment conditions (Moreau *et al.* 2008, Ziuzina *et al.* 2014, Lacombe *et al.* 2015).

As a cloud of active particles, the recombination process of these species generates energy that is retained in the plasma for a period of time, and then is released as visible and UV light. The active particles in the plasma can react with the food substrate, releasing the stored energy into the target bacteria or viruses. Therefore, the microbial inactivation may occur by chemical interaction of radicals, reactive species, or charged particles with the cell membranes, by damage to membranes and internal cellular components by the UV radiation, or broken the DNA strands also by the UV light. However, the antimicrobial modes of action depend on the source and type of plasma generated, whose total energy varies with the gas used, density and temperature. Additionally, one mode of action may be more significant than another on a given commodity, and therefore the sanitizing efficiency can be improved by using plasmas with multiple antimicrobial mechanisms, taking advantage of synergistic effects (Niemira 2012).

According to Niemira (2012), the CP systems can be classified in three categories based on where the food to be treated is placed with respect to the CP source and the nature of the CP chemistry, which delineates the half-life and reactivity of the charged active species within the plasma. The first category is the remote treatment system, where the food product is placed at some distance from the generation source, which is treated with plasma rich in secondary chemical species; the second is known as direct treatment system where the product is placed relatively close to the generation source and treated with active plasma; and in the third category, the electrode contact systems, the product is within the CP generation field.

Effective treatment time ranges from less than 3 s to 300 s, depending on physical (food shape) and chemical properties of foods and processing conditions. The decontamination efficacy also depends on the type of microorganism, inactivation medium, number and physiological state of cells, and operating gas mixture and flow (Bermúdez-Aguirre *et al.* 2013, Ziuzina *et al.* 2014). The early development stage of this technology also represents a limitation, as well as the diversity and complexity of the necessary equipment and the scarce information about physicochemical and functional changes that might occur in treated food product (Knorr *et al.* 2011, Bermúdez-Aguirre *et al.* 2013, Ramos *et al.* 2013).

Most studies on the application of CP in the food field are focused mainly on inactivation of microorganisms, often neglecting the effects on quality parameters. The antimicrobial activity of CP against Gram-negative and Gram-positive bacteria, yeast and fungi, as well as spores and biofilms that are generally very difficult to inactivate was shown in various studies (Montie *et al.* 2000, Laroussi and Lu 2005, Vleugels *et al.* 2005, Brandenburg *et al.* 2007). Indeed, it is effective in reducing some human pathogens. Ziuzina *et al*. (2014) demonstrated that the atmospheric CP treatment for 10, 60 and 120 s resulted in reduction of *Salmonella enterica*, *Escherichia coli* and *Listeria monocytogenes* populations on packaged tomatoes to undetectable levels from initial populations of 3.1, 6.3, and 6.7 $\log_{10}$ CFU/sample, respectively. However, an extended treatment time (up to 300 s) was necessary to reduce bacterial populations attached on the more complex surface of strawberries. Baier *et al*. (2014) showed that keeping a distance of 17 mm to an Ar plasma-jet, corn salad leaves could be treated for up to 60 s, without exceeding the leaves surface temperature of 35.2 ºC. The authors also performed antibacterial tests on corn salad, cucumber, apple and tomato, achieving an inactivation of inoculated *E. coli* of at least 3 log cycles, after 60 s of treatment. However, the inherent inactivation mechanisms are not yet fully understood.

Once the emitted reactive species from plasma react with bacteria, they may also affect food properties. Nevertheless, the application of CP to improve shelf-life and safety of fresh or minimally processed fruits and vegetables is new and little is known about the effect on physicochemical or functional properties. Baier *et al*. (2015) studied the impact of CP on the external quality of apples, cucumbers, tomatoes and carrots, and its antimicrobial efficacy on indigenous and inoculated microorganisms. Significant changes were found on colour of tomatoes and carrots and on chlorophyll fluorescence parameters of cucumbers, whereas elasticity remained almost unaffected in all matrices. The treatment was suitable for apples, but for more susceptible produces such as carrots, some unwanted surface effects were induced. In turn, Lacombe *et al*. (2015) observed that CP can inactivate microorganisms on blueberries. However, after 60, 90 and 120 s, the treatment affected the firmness, reduced the anthocyanins content, and changed the surface colour, respectively. Contrariwise, other

study demonstrated that the effects on strawberry colour and firmness were insignificant (Misra, Patil, *et al.* 2014). The suitability of CP has also been demonstrated for lettuce (Jahid *et al.* 2015) and cherry tomatoes (Misra, Keener, *et al.* 2014).

Today, this technology shows promises and is subject of active research to enhance efficacy. Nevertheless, optimization and scale up to commercial treatment levels require a more comprehensive understanding of the inherent chemical processes, namely in order to understand the mode of action of the treatment and the possible interactions with food constituents and properties. As the technology moves from the lab scale to industrial scale, the capital costs will be high but may be offset by advances in energy efficiency and overall engineering scale efficiencies (Niemira 2012). Furthermore, with the technology evolution, it is also expected that the terminology and experimental methodologies will become more standardized.

2.3.3.7. High hydrostatic pressure

High hydrostatic pressure (HHP) processing is a relatively new and very promising nonthermal food processing technology that subjects liquid or solid foods, with or without packaging, to pressures between 50 and 1000 MPa. It is a good alternative to heat treatments to destroy food-borne pathogens and inactivate enzymes because, as a cold pasteurisation process, it does not lead to substantial modification of the nutritional, functional and organoleptic properties of foods, and enhance safety and shelf-life of perishable foods like fresh or minimally processed fruits and vegetables (Mújica-Paz *et al.* 2011, Castro and Saraiva 2014, Huang *et al.* 2014).

In a HHP process, the food product to be treated is placed in a pressure vessel capable of sustaining the required pressure and submerged in a liquid (typically water), which acts as the pressure-transmitting medium (Knorr *et al.* 2011, Castro and Saraiva 2014). The pressure is transmitted in a uniform (isostatic) and quasi-instantaneous manner throughout the food sample. Therefore, the time necessary for pressure processing is independent of food shape or size, in contrast to thermal treatments (Huang *et al.* 2014, Medina-Meza *et al.* 2014). Moreover, there is little variation in temperature with increasing pressure (the temperature increases approximately 3 ºC per 100 MPa, depending on the food composition). These particularities prevent the food from being deformed or heated, which would modify its organoleptic or nutritional properties (Considine *et al.* 2008).

In general, low-molecular-weight compounds are only slightly affected by HHP because the break of covalent bonds does not occur at pressure below 2 GPa, due to its very low compressibility. Therefore, the primary structure of volatile compounds, pigments, vitamins, and other compounds connected with the sensory, nutritional, and health-promoting aspects of foods are less affected or rarely affected (Oey *et al.* 2008, Huang *et al.* 2014).

This effect is important for fruits and vegetables once they are rich sources of bioactive ingredients (including vitamins) and pigments (Pereira *et al.* 2011, Pinela *et al.* 2012). However, the ionic bonds and hydrophobic interactions, responsible for maintaining the secondary and tertiary structure of proteins, are weakened/disrupted (in general above 200 MPa) and this event is associated with decreases in volume. The additional rupture of hydrogen bonds and electrostatic interactions can change the structure of large molecules and complex organized structures (Considine *et al.* 2008, De Roeck *et al.* 2010, Rendueles *et al.* 2011). The global consequence for the food product is diverse, since the nutrient digestibility and bioavailability can be modified (Briones-Labarca *et al.* 2011, Linsberger-Martin *et al.* 2013), the activity of certain enzymes inhibited (Liu *et al.* 2013), or functional properties altered (Kim *et al.* 2014). For example, the structure of starch undergoes changes, such as gelling, similar to thermal-induced structure changes. Although, the physical structure of most high-moisture foods remains unchanged after exposure to HPP, since no shear forces are generated by pressure (Considine *et al.* 2008, Knorr *et al.* 2011, Rendueles *et al.* 2011).

The HHP effect on the microbial inactivation has been reported and reviewed (Patterson 2005, Considine *et al.* 2008, Rivalain *et al.* 2010, Mota *et al.* 2013, Huang *et al.* 2014). In general, yeasts and fungi are more sensitive to pressure than vegetative bacteria, which can be inactivated at relatively low pressures (normally 200-400 MPa), at room temperature. Ascospores of heat-resistant moulds appear to be more pressure resistant, and many of them are not inactivated at pressures below 800 MPa, and conidia are much more pressure sensitive, with a sensitivity similar to yeasts. Nevertheless, bacterial spores are the most pressure-resistant life forms known; therefore, it is needed a combination of higher pressure and temperature once pressure treatment alone does not achieve a substantial inactivation of spores (Raso and Barbosa-Cánovas 2003, Rivalain *et al.* 2010, Mor-Mur *et al.* 2014). Commonly, a pressure of 50 MPa can inhibit protein synthesis in microorganisms and reduce the number of ribosomes, 100 MPa can induce partial protein denaturation, and 200 MPa cause damage to the cell membrane and internal cell structure. Increasing the pressure above 300 MPa induces irreversible denaturation of enzymes and proteins, which causes rupturing of the cell membrane and the excretion of internal substances, resulting in bacterial death (Huang *et al.* 2014). However, the pressure resistance of the different bacterial species and strains is quite different. Generally, Gram-positive bacteria are more resistant to heat and pressure than Gram-negatives, probably due to the different chemical composition and structural properties of the cell envelope, and cocci are more resistant than rod-shaped bacteria (Russell 2002, Patterson 2005, Rivalain *et al.* 2010). Cells in the stationary phase of growth are also generally more resistant than those in the exponential phase. This is because microorganisms in the exponential phase undergo continuous cell division and

synthesis, and the stress tolerance of cells in an adverse environment is lower (Considine *et al.* 2008, Huang *et al.* 2014). Additionally, the chemical composition of the food product can have a significant effect on the response of microorganisms to pressure, due to the ability to provide essential vitamins and amino acids to stressed cells. For example, proteins, carbohydrates and lipids can confer a protective effect against pressure. The low water activity of the system also protects microorganisms against the pressure effects, except those that are injured. It is also known that pH and pressure can act synergistically leading to an increased microbial inactivation (Patterson 2005, Considine *et al.* 2008, Mor-Mur *et al.* 2014).

Regarding viruses, there is relatively little information in comparison to other microorganisms, but it is known that its pressure resistance varies greatly among their structural diversity and within related taxonomic groups or even strains. Enveloped viruses are usually more sensitive to pressure treatments than naked viruses. HPP can cause damage to the virus envelope preventing the virus particles binding to cells or even complete dissociation of virus particles, which may be either fully reversible or irreversible, depending on the pressure. Prions, associated with neurological disorders in animals and humans, are generally even more difficult to destroy than bacterial spores. The treatment requires very drastic conditions, with the application of pressure up to 1200 MPa for 10 min at a temperature up to 135 °C (Considine *et al.* 2008, Rivalain *et al.* 2010, Rendueles *et al.* 2011).

The effect of HPP on enzyme activity seems to be variable. In whole fruit and vegetables, enzymes are usually confined to compartments; but in fresh-cut fruits or vegetables this compartmentalization is destroyed and the enzymes released in the food product causing unwanted changes. Thus, it is expected that the pressure treatment changes the structural conformation of the enzyme/protein, which sometimes leads to a partial or complete inactivation or, in other cases, to its activation. In general, a relatively low pressure (~100-200 MPa) may activate some enzymes, while a high pressure (400-1000 MPa) may induce their inactivation. Indeed, in most vegetables, the pressure alone is insufficient to inactivate enzymes and hence needs to be combined with heat (Raso and Barbosa-Cánovas 2003, Mújica-Paz *et al.* 2011, Mor-Mur *et al.* 2014).

Although this novel technology is being increasingly investigated, the main targets of research regarding plant-based foods are purees (González-Cebrino *et al.* 2013) and juices (Queirós *et al.* 2014), while few studies have been focused on whole or minimally processed fruits and vegetables. Apart from its use for preservation purposes, HHP might also influence the biosynthesis pathways and could lead to the formation of product variants with novel functional properties. In fact, HPP is used to develop tailor-made foods. Nevertheless, there is a lack of data about the behaviour of nutrients, allergens, and food-spoiling

microorganisms under defined food matrices and treatment conditions, as well as the process conditions necessary to inactivate pressure-resistant bacterial spores.

2.3.3.8. Dense phase carbon dioxide

Recently, some attention has been paid to dense phase carbon dioxide (DP-CO_2), a collective term for liquid, high pressurized, or supercritical CO_2, as an alternative nonthermal treatment to preserve fresh fruits and vegetables among other foods (Calvo and Torres 2010, Rawson *et al.* 2012, Zhou *et al.* 2015). It utilizes pressures in combination with CO_2 to kill microorganisms as a mean of food preservation. DP-CO_2 denotes phases of matter that remain fluid, yet are dense with respect to gaseous CO_2. CO_2 is inert, nontoxic, non-flammable, inexpensive, readily available in high purity, and leaves no residues, being generally recognized as safe. This gas is also a natural constituent of many foods. When operating at supercritical conditions (7.4 MPa and 31.1 ºC), it acquires very good mass-transfer properties (low viscosity and zero surface tension), providing effective penetration into solid matrices (Rawson *et al.* 2011). At the end of treatment, it can easily be removed from the solutes by mere expansion to ambient pressure (Choi *et al.* 2008, Calvo and Torres 2010).

The microbial inactivation may occur by physical disruption due to a rapid depressurization and expansion of CO_2 within the cells. The CO_2 increases the membrane fluidity, due to an order loss of the lipid chains, resulting in increased permeability. The dissolution of CO_2 in the aqueous component of foods originates carbonic acid which lowers extracellular pH and consequentially inhibits many metabolic systems of the cell. However, microbial inactivation can also be achieved even when cells remain intact or exhibit only limited signs of deformation. In general, and as mentioned for some of the other reviewed technologies, Gram-positive bacteria are more difficult to inactivate than Gram-negatives, mainly due to the thickness and composition of the cell wall (Garcia-Gonzalez *et al.* 2007, Rawson *et al.* 2012).

One of the first food applications was the treatment of whole fruits to inhibit mould growth (Haas *et al.* 1989). Recent published data demonstrated that this technology can effectively inactivate microorganisms and enzymes in minimally processed fruits (Valverde *et al.* 2010) and vegetables (Calvo and Torres 2010, Bi *et al.* 2011), being considered as a promising and sustainable technology for preservation of a wide range of foods. Nevertheless, studies on the effects of this technology on food quality parameters are still limited. Thus, more research is necessary, especially on the elucidation of mechanisms underlying changes on food properties, especially on bioactive ingredients. Given the strong benefits of this technology, it is likely to be increasingly adopted in the food industry.

2.3.4. The hurdle concept

The hurdle concept provides a framework for combining (simultaneously or sequentially) more than one milder preservation factor in order to achieve an enhanced level of product safety and stability. These factors are hurdles, which microorganisms and pathogens need to overcome to survive in the food environment. Thus, they should be “high enough” so that the microorganisms cannot overcome them, and its selection must take into account the initial numbers and types of microorganisms. The correct combination of hurdles can ensure microbial safety, stability and quality of foods. Additionally, the hurdles can be physical, chemical or biological factors, and its intensity can be adjusted individually, depending on the objective, to meet consumer preferences in an economically sensible way, without compromising product safety. This concept fits with the actual consumer trend for minimally processed fruits and vegetables and has gained popularity regarding practical applications and research (Mukhopadhyay and Gorris 2014). The combination of emerging technologies with conventional or novel preservation techniques has been explored in the last years; but a deeper understanding of the hurdle effect is crucial to obtain high quality and safety foods, and to support hurdles selection and their levels (Gómez *et al.* 2011).

2.4. Innovative extraction methods for bioactive compounds

2.4.1. Recovery of valuable compounds from plant materials

The strict legislation for human health and environmental safety implemented today, and the emergence of novel methodologies for the extraction, fractionation, and recovery of biomolecules have caused great interest in plant-derived waste valorisation. Different kinds and amounts of agri-food wastes are produced within the food-supply chain, representing a disposal problem for the industry (FAO 2013), but promising sources of nutrients and phytochemicals (Kalogeropoulos *et al*. 2012, Stajčić *et al.* 2015, Pinela, Prieto, Barreiro, *et al.* 2016). Thus, the sustainable use of plant-derived wastes and by-products for recovery of high added-value compounds with potential application in the food, feed, biotechnological, and pharmaceutical industries may help to tackle the societal challenges of the 21st century.

The recovery of valuable molecules from plant materials and its recycling inside the food chain as food ingredients can be carried out following the so-called “5-stages universal recovery process” (Galanakis 2012, 2013). This holistic approach includes: (1) macroscopic pretreatment; (2) separation of macro- and micromolecules; (3) extraction; (4) purification/isolation; and (5) encapsulation or product formation. Recent trends on extraction, one of the most important steps of the recovery process, have focused on finding more efficient and green technologies that minimize the extraction time and solvent consumption. Among them, microwave-assisted extraction, ultrasound-assisted extraction, and extraction with electrotechnologies (such as pulsed electric fields, high-voltage electrical discharges and pulsed ohmic heating) and high pressures (pressurized liquids) generally meet these requirements (**Figure 17**). The extraction mechanisms of these technologies and examples of their application are discussed below.

2.4.2. Conventional methods

Conventional extraction (**Figure 17**) of bioactive compounds from foodstuffs is time and solvent consuming, representing a serious energetic and environmental issue. Solid-liquid extraction can be defined as a mass transport phenomenon where compounds contained in foodstuff structure migrate into the solvent up to equilibrium. Examples include heating reflux, Soxhlet and solvent extraction, maceration, percolation, and hydrodistillation (Pinela *et al.* 2012, Golmakani *et al.* 2014, Parniakov *et al.* 2014, Altemimi *et al.* 2015, Uribe *et al.* 2015). In plants, bioactive compounds exist enclosed in insoluble structures such as cell vacuoles, which are not accessible to solvents. The use of heat to enhance mass transfer and reduce the extraction time can have deleterious effects, since high temperature can degrade thermolabile compounds (Ramli *et al.* 2014, Fernández-ponce *et al.* 2015). Besides, the use of solvent mixtures containing acetone or methanol, pure or combined with water or an acid,

is not in accordance with the green chemistry concept (Barba, Boussetta, *et al.* 2015). Because of this, alternative green solvents and novel extraction technologies based on physical mechanisms are being adopted.

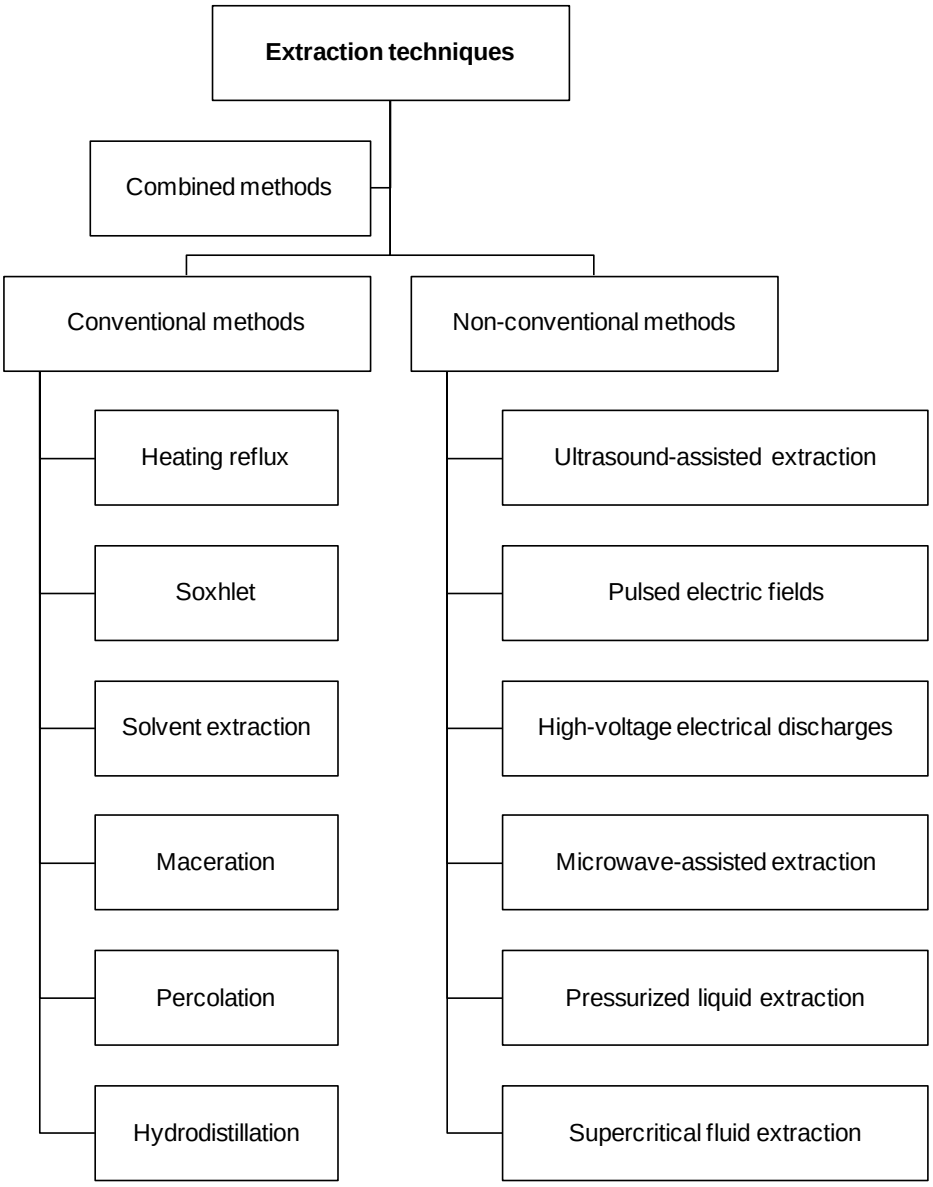


Figure 17 Conventional and innovative methods used in the extraction of bioactive compound from plant materials.

2.4.3. Non-conventional methods

The increasing need to extract bioactive molecules from natural sources has led to deeper interest in new processes that can reduce the extraction time, processing temperature and solvent consumption and contribute to higher extraction efficiency (*i.e.,* yield and quality) and lower energy consumption as compared to conventional methods.

2.4.3.1. Microwave-assisted extraction

Microwave-assisted extraction (MAE) has been rapidly developed as one of the hot-spot techniques for extracting high added-value compounds from different matrices. The extraction mechanism is based on the impact of microwaves on molecules by ionic conduction and dipole rotation inside target materials. The microwaves interact directly with the free water molecules presents inside the materials, resulting in a rapid build-up of pressure within cells, and a pressure-driven enhanced mass transfer of target compounds out of the source material, which causes rupture of the plant tissue and release of the active compounds into the extracting solvent (Haddadi-Guemghar *et al.* 2014, Setyaningsih *et al.* 2015). During extraction, the solvent volume must be enough to ensure that the solid matrix is entirely immersed. The solvent choice is dictated by the solubility of the extracts of interest, by the interaction between solvent and plant matrix, and by the microwave absorbing properties of the solvent determined by its dielectric constant (Dahmoune *et al.* 2015). However, MAE can also be performed without the addition of any solvent (Li *et al.* 2013, Oroian and Escriche 2015). Solvent-free microwave extraction (SFME) is based on a combination of microwave heating and dry distillation. The inherent benefits of SFME are reduction of pollution and handling costs, as the result of the simplified manipulation procedure, easy clean-up and labour saving (Li *et al.* 2013). MAE has been applied to obtain phenolic compounds from myrtle (*Myrtus communis* L.) leaves (Dahmoune *et al.* 2015), horsetail (*Equisetum arvense* L.) (Milutinović *et al.* 2014), orange peels (Nayak *et al.* 2015), false daisy (*Eclipta prostrata* Linn) (Fang *et al.* 2015), plum (Haddadi-Guemghar *et al.* 2014), tomatoes (Li *et al.* 2012) and rice (*Oryza sativa* L.) grains (Setyaningsih *et al.* 2015), lycopene from tomato peels (Ho *et al.* 2015), and phenolic compounds, vitamin C and carotenoids from stevia (Barba, Grimi, *et al.* 2015).

2.4.3.2. Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is one of the most used techniques in industry to enhance the mass transfer phenomena. The mass transfer rate is increased by cavitation forces, in this case the bubbles in the liquid-solid extraction can explosively collapse and generate localized hot spots (characterized by high temperature and pressure) causing plant tissue rupture and improving the release of intracellular substances into the solvent. UAE has been considered an economic method (Chen *et al.* 2015) and has already been applied to extract phenolic compounds from spinach (*Spinacea oleracea* L.) (Altemimi *et al.* 2015), grape pomace (González-Centeno *et al.* 2014), orange peels (Nayak *et al.* 2015), sugar beet molasses (Chen *et al.* 2015) and phenolics and betacyanins from red pitaya (*Hylocereus polyrhizus* (Weber) Britton & Rose) (Ramli *et al.* 2014).

2.4.3.3. Pulsed electric fields

The pulsed electric fields (PEF) treatment consists on the application of pulses of high voltage (kV) and short duration (µs-ms) to a biological material placed between two electrodes. This voltage results in an electric field of which intensity depends on the voltage delivered and the gap between the electrodes. The originated phenomenon, called electroporation, consists on the increment of the cell membrane permeability to ions and macromolecules (Segovia *et al.* 2014). Therefore, PEF increases the extraction of high-added value compounds and can replace the enzymatic maceration of the process. PEF have been applied to extract phenolics from papaya (*Carica papaya* L.) peels (Parniakov *et al.* 2014), polyphenols from grapes (Delsart *et al.* 2014) and borage (*B. officinalis*) leaves (Segovia *et al.* 2014), phenolic compounds and ascorbic acid from plum (*Prunus domestica* var. Casselman) and grape peels (Medina-Meza and Barbosa-Cánovas 2015), as well as phenolic compounds, vitamin C and carotenoids from stevia (*Stevia rebaudiana* Bertoni) leaves (Barba, Grimi, *et al.* 2015).

2.4.3.4. High-voltage electrical discharges

High-voltage electrical discharges (HVED) have physical and mechanical effects on the product caused by shock waves. This technique introduces energy directly into an aqueous solution through a plasma channel formed by a high-voltage electrical discharge between two submerged electrodes (Oroian and Escriche 2015). The HVED mechanism is based on the electrical breakdown in water (Boussetta and Vorobiev 2014). Air bubbles that are initially present in water or formed due to local heating are involved into this phenomenon and accelerate the process. If the electrical field is too intense, the avalanche of electrons becomes a starting point of streamer propagation from the high voltage needle electrode to the grounded one. The electrical breakdown is accompanied by secondary phenomena (*e.g.,* high-amplitude pressure shock waves, cavitation, and liquid turbulence) that cause particle fragmentation and cell structure damage, thus facilitating the release of intracellular compounds (Boussetta and Vorobiev 2014, Barba, Boussetta, *et al.* 2015). When compared to other physical treatments, HVED results in a higher extraction rate than that obtained with PEF and ultrasounds. However, this process can originate very small particles that can lead to a more difficult solid-to-liquid separation step (Boussetta and Vorobiev 2014). This technology has been used to extract phenolics from rapeseed (*Brassica napus*) (Barba, Boussetta, *et al.* 2015) and papaya peels (Parniakov *et al.* 2014), anthocyanins from blackberries (*Rubus fruticosus* L.) (Barba, Galanakis, *et al.* 2015), and phenolic compounds, vitamin C and carotenoids from stevia (Barba, Grimi, *et al.* 2015).

2.4.3.5. Pressurized liquid extraction

Pressurized liquid extraction (PLE), also known as accelerated or enhanced fluid extraction, or subcritical water extraction, is increasingly used in the extraction of antioxidants from different foodstuffs. This green technology involves the use of pressurized liquid solvents, including water or ethanol, below their critical point. The combination of high temperature and pressure enhances the extraction performance as compared with processes carried out at room temperature and atmospheric pressure. The temperature promotes higher analyte solubility by increasing both solubility and mass transfer rate and also decrease the viscosity and surface tension of solvents, thus improving extraction rate (Azmir *et al.* 2013). The set up of the technique also maintains samples in an O_2 and light-free environment, which makes it preferable for use in the nutraceutical industry (Saha *et al.* 2015). Furthermore, the addition of high concentrations of CO_2 to pressurized liquid solvents enhances the kinetic desorption of analytes, inactivates unwanted enzymes that may destroy bioactive compounds, minimizes the use of liquid solvents, and reduces undesired concentration steps. Unlike MAE, PLE is not limited to solvents that can absorb microwaves (Fernández-ponce *et al.* 2015). Additionally, for extraction of polar compounds, PLE is considered as a potential alternative to supercritical fluid extraction (SFE). Recent studies evaluate the suitability of this technology for the extraction of phenolic compounds from mango (*Mangifera indica* L.) leaves (Fernández-ponce *et al.* 2015) and roots of *Scutellaria pinnatifida* A. Hamilt. subsp *alpina* (Bornm) Rech. f. (Golmakani *et al.* 2014), as well as carotenoids from kaki (*Diospyros kaki* L.), peach, apricot (Zaghdoudi *et al.* 2015) and carrots (Saha *et al.* 2015).

2.4.3.6. Supercritical fluid extraction

Supercritical fluid extraction (SFE) has been used to extract target compounds from a variety of matrices at laboratory and commercial scale. Carbon dioxide is the main solvent used in this technique, especially when the target molecule is apolar. At supercritical conditions, above its critical temperature (31.1 °C) and pressure (7.4 MPa), CO_2 presents unique physicochemical properties because of their duality between liquid and pure gas (Cardenas-Toro *et al.* 2014). Thus, it acquires very good mass-transfer properties because of a high, more gas-like diffusion coefficients and low viscosity values, providing effective penetration into solid matrices, and preserves the integrity and stability of more delicate compounds due to its relatively low surface tension (Rawson *et al.* 2012, Yang *et al.* 2013). In fact, this technique is adequate for thermolabile and oxidation-susceptible compounds (Cardenas-Toro *et al.* 2014). Solvents other than CO_2 are not necessarily required and the expense of removing organic solvents can be eliminated. Another advantage is its high selectivity and easy separation process from the extracted compounds without leaving toxic residues in

extracts. Although high capital costs are associated with SFE, operating costs are relatively low and is very simple to be scaled-up to industrial scale.

SFE has been used for the isolation of antioxidant compounds, including polyphenols (Sanjaya *et al.* 2014), carotenoids, tocopherols and sitosterols (Vági *et al.* 2007). These studies demonstrate that SFE can be used to extract both hydrophilic and lipophilic compounds from plant matrices. However, compounds of appreciable polarity (*e.g.,* phenols and flavonoids) are sparingly soluble in pure CO_2; therefore, a polar modifier (co-solvent such as ethanol, methanol or water) must be added to obtain the necessary polarity to improve the extraction efficiency of polar compounds and to overcome interactions between the analyte and the matrix, facilitating the release of target compounds into the extraction medium. Accordingly, an efficient extraction of phenolics from *Golden delicious* apple peels and resveratrol from grape pomace using ethanol as co-solvent was achieved by Massias *et al.* (2015) and Casas *et al.* (2010), respectively. The extraction of protocatechuic acid from *Scutellaria barbata* D. Don (Yang *et al.* 2013) and phenolics from jatoba (*Hymenaea courbaril* L.) bark (Veggi *et al.* 2014) was successfully achieved using water as co-solvent. Likewise, the extraction of proanthocyanidins from grape marc using CO_2 as solvent and 10% ethanol-water mixture (57%, v/v) as co-solvent proved to be an efficient extraction method (Da Porto *et al.* 2014). As alternative to the addition of polar modifiers, is possible to increase the pressure (and consequently the temperature) to decrease the interaction of polar compounds with the matrix and improve solubility (Zaghdoudi *et al.* 2015).

2.4.3.7. Extraction solvents

The preference of phenolic antioxidants phenolics for ethanol and methanol may be caused by their non-polar part and the aliphatic fragment of alcohols. Bigger molecules prefer ethanol, as it could better “cover” the gaps between the hydrogen bonds (Oroian and Escriche 2015). Ethanol possesses GRAS (Generally Recognized as Safe) status and the extracts can be used in the food industry. Aqueous ethanol offer many advantages as hydrophilic and lipophilic compounds are recovered together in different proportions and synergistic interactions occur inside the medium. Methanol is cheaper than ethanol but due to its toxicity it is not favoured in the food industry (Oroian and Escriche 2015). On the other hand, carotenoids such as lycopene are more lipophilic and thereby polar aprotic (*e.g.,* acetone) or non-polar solvents (*e.g.,* ethyl acetate) are preferred (Strati and Oreopoulou 2011). But, in this case, the solvent should be removed completely from the extract prior to its reutilization in food products. In fact, short-chain alcohols, especially ethanol and isopropanol, have been proposed as alternative extraction solvents for carotenoids and vitamin E (Baiano and Del Nobile 2015).

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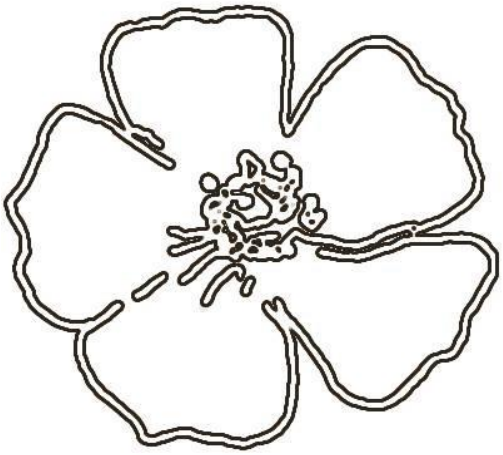
3. Medicinal Plants



This **3rd chapter** covers the work performed with two medicinal plants traditionally prepared in herbal beverages, dwarf mallow (*Malva neglecta* Wallr.) and perennial spotted rockrose (*Tuberaria lignosa* (Sweet) Samp.). The chemical composition and antioxidant activity of these species is described, as well as the effects induced by the ionizing radiation treatment on these parameters. The impact of this technology on the extraction kinetics of phenolic compounds from *T. lignosa* is also reported.

Dwarf mallow (*Malva* neglecta Wallr.)

3.1. Quality control of gamma irradiated dwarf mallow based on colour, organic acids and antioxidant parameters

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Article

Quality Control of Gamma Irradiated Dwarf Mallow (*Malva neglecta* Wallr.) Based on Color, Organic Acids, Total Phenolics and Antioxidant Parameters

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Abstract

This study addresses the effects of gamma irradiation (1, 5 and 8 kGy) on colour, organic acids, total phenolics, total flavonoids, and antioxidant activity of dwarf mallow (*Malva neglecta* Wallr.). Organic acids were analysed by ultra fast liquid chromatography (UFLC) coupled to a photodiode array (PDA) detector. Total phenolics and flavonoids were measured by the Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively. The antioxidant activity was evaluated based on the DPPH• scavenging activity, reducing power, β-carotene bleaching inhibition and thiobarbituric acid reactive substances (TBARS) formation inhibition. Analyses were performed in the non-irradiated and irradiated plant material, as well as in decoctions obtained from the same samples. The total amounts of organic acids and phenolics recorded in decocted extracts were always higher than those found in the plant material or hydromethanolic extracts, respectively. The DPPH• scavenging activity and reducing power were also higher in decocted extracts. The assayed irradiation doses affected differently the organic acids profile. The levels of total phenolics and flavonoids were lower in the hydromethanolic extracts prepared from samples irradiated at 1

kGy (dose that induced colour changes) and in decocted extracts prepared from those irradiated at 8 kGy. The last samples also showed a lower antioxidant activity. In turn, irradiation at 5 kGy favoured the amounts of total phenolics and flavonoids. Overall, this study contributes to the understanding of the effects of irradiation in indicators of dwarf mallow quality, and highlighted the decoctions for its antioxidant properties.

Keywords: *Malva neglecta*; irradiation; colour parameters; organic acids; antioxidant activity; decoction; quality control.

3.1.1. Introduction

Organic acids are primary metabolites widely spread throughout the plant kingdom. Chemically, they are low weight molecules and are considered to be any organic carboxylic acid with a general structure R-COOH. Although they are weak acids, these water-soluble compounds may confer acidic properties to foods and influence its organoleptic properties (flavour, colour and aroma) and consequent acceptability by the consumers (Andrés *et al.* 2015). From a practical point of view, the organic acids profile (levels and relative ratios) is important for food quality control. It allows determining the plant percentage added to final products, thus detecting adulterations or possible microbial deteriorations that occurred during storage, giving a practical advantage for its use as an authenticity index in plant-based foods and beverages (Silva *et al.* 2002, Ehling and Cole 2011, Andrés *et al.* 2015). The relative amounts and the presence of each of these compounds are also useful as means to evaluate food processing (Silva *et al.* 2002, Nawirska-Olszańska *et al.* 2014). Thus, qualitative and quantitative analyses of these ingredients are of great importance.

During the production process (from harvesting and drying to packaging and storage), raw plants are prone to various contaminations and infestations, which can lead to spoilage, quality deterioration and consequent economic losses (Darfour *et al.* 2014). Besides constituting health hazards to consumers, contaminated products can also adversely affect the efficacy and stability of their bioactive ingredients and lead to spoilage of final products (Aouidi *et al.* 2011). The presence of organic acids and phenolic compounds is advantageous, as they contribute to the natural preservation process (through its antimicrobial and antioxidant activities), but is not enough. The search for new preservation treatments with a minimum impact on physical, chemical and functional parameters highlighted the gamma irradiation as a safe, effective and sustainable option to sanitize plant products (WHO 1999, Fernandes, Antonio, Oliveira, *et al.* 2012).

Nowadays there is a growing scientific interest in irradiation-induced modifications on the antioxidant properties and compounds responsible for such effects. The antioxidant activity is strongly linked the presence of phenolic compounds (Dalar *et al.* 2012, Guimarães

et al. 2013, Martins *et al.* 2015, Pinela *et al.* 2015), secondary metabolites frequently found attached to sugars (glycosides), which increases its water solubility. These compounds have the ability to donate a hydrogen atom from the aromatic hydroxyl group to a free radical and/or the capacity to support an unpaired electron in their aromatic structures. Therefore, the methods used to evaluate the antioxidant activity can be classified according to the mechanism of action, *i.e.,* single-electron transfer or hydrogen atom transfer (Carocho and Ferreira 2013). However, phenolic compounds, organic acids and other bioactive constituents may be affected by the irradiation treatment if applied inappropriately. It is known that the ionizing radiation interacts with water molecules generating free radicals, in a reaction commonly known as radiolysis (Harder *et al.* 2016). Then, these free radicals can interact with different biomolecules, leading to breakdown of chemical bonds and changes in its structure and, consequently, in its bioactivity and extractability from the plant material. The referred compounds may also be affected by the direct impact of the gamma-rays (Alothman *et al.* 2009, Harder *et al.* 2016). As a consequence, the colour of the processed samples may change. However, this quality attribute is directly related to consumers' appreciation of a product, as they tend to associate its colour with its taste, hygienic security, shelf-life and personal satisfaction (Śledź *et al.* 2013, Sturm *et al.* 2014). A very stringent plant selection based on colour parameters also occurs in the food, pharmaceutical and cosmeceutical industries (Jo, Son, Lee, *et al.* 2003). Therefore, the colour evaluation is important in quality control of irradiated products.

Dwarf mallow (*Malva neglecta* Wallr.) is an annual herbaceous plant of the family Malvaceae traditionally eaten raw as leafy vegetable or prepared in herbal beverages (mainly decoctions) due to its disinfectant and anti-inflammatory claims (Carvalho and Morales 2010). It is also used to treat multiple medical conditions such as asthma, colds, digestive and urinary problems, and abdominal pains (Türker and Dalar 2013)]. Scientific works have reported antioxidant (Dalar *et al.* 2012, Türker and Dalar 2013), antibacterial (Seyyednejad *et al.* 2010) and anti-ulcerogenic (Gürbüz *et al.* 2005) properties on this plant. Nevertheless, as far as we know, the organic acids profile of this plant remains unknown, as well as the effects induced by the gamma irradiation treatment on its physical or chemical properties. Thus, the main purpose of this study was to investigate the dose-response effects of gamma irradiation on colour, organic acids, total phenolics and flavonoids, and antioxidant properties (quality control indicators) of *M. neglecta* samples. The influence of the preparation method (decoction) was also investigated.

3.1.2. Materials and methods

3.1.2.1. Dosimeters, standards and reagents

Amber Perspex routine dosimeters, Batch V, were purchased from Harwell Company (Oxfordshire, UK). Organic acids (oxalic, quinic, malic, citric, succinic and fumaric acids) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

3.1.2.2. Samples gathering and irradiation

Plant material (leafy flowering stems) from dwarf mallow (*Malva neglecta* Wallr. Fam. Malvaceae) was sustainable wild harvested in June, Miranda do Douro, North-eastern Portugal, considering local medicinal uses as well as healers' and selected consumers' criteria, which are related to particular gathering sites and requirements for safe herbal dosages forms, namely decoctions (Carvalho 2010). Taxonomic identification of the plant material was confirmed by the specialist Dr. Ana Maria Carvalho from the Polytechnic Institute of Bragança, Portugal. A voucher specimen was deposited in the Herbarium of the School of Agriculture of Bragança. Samples were then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain a homogenate sample.

The obtained powdered sample was divided into four portions and submitted to 1, 5 and 8 kGy of gamma-rays (predicted doses). A non-irradiated control (0 kGy) followed all the experiment. The irradiation was performed in a cobalt-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) located at C2TN (Centre for Nuclear Sciences and Technologies, University of Lisbon, Portugal), with four sources and a total activity of 177 TBq (4.78 kCi; January 2014). During the irradiation process, Amber Perspex routine dosimeters were used to measure the distribution of the absorbed energy and to determine the maximum (D_{max}) and the minimum (D_{min}) dose absorbed by the samples, following the procedure previously described by Fernandes *et al.* (2013). The measured average doses were 1.10 ± 0.16 kGy, 4.82 ± 0.10 kGy and 8.07 ± 0.46 kGy for the samples irradiated at the predicted doses of 1, 5 and 8 kGy, respectively. The estimated dose rate for the irradiation position, obtained with a Fricke dosimeter (Fernandes *et al.* 2013), was 1.9 kGy/h and the dose uniformity ratio (D_{max}/D_{min}) was 1.2. For simplicity, the predicted doses were considered in the text.

3.1.2.3. Colour measurement

The powdered samples were placed on an adapter for granular materials (model CR-A50) to reduce external interferences and data were collected in three different points on each set of samples with a colorimeter (model CR-400; Konica Minolta Sensing, Inc., Sakai, Osaka, Japan) previously calibrated using the standard white plate. Using illuminant C and the diaphragm opening of 8 mm, the CIE *L*a*b** colour space values were registered through the computerized system using the colour data software "SpectraMagic Nx" (version CM-S100W). Average values were considered to determine the colour coordinates, where *L** represents lightness, *a** represents chromaticity on a green (–) to red (+) axis, and *b** represents chromaticity on a blue (–) to yellow (+) axis. The total colour difference (Δ*E**) was calculated according to the CIEDE2000 equation (ISO/CIE 2014).

3.1.2.4. Preparation of decoctions and hydromethanolic extracts

Decoctions were prepared according to folk recipes/formulations (Carvalho 2010). Briefly, each powdered sample (1 g) was added to 200 mL of distilled water and boiled for 5 min. The mixture was left to stand at room temperature for 5 min more and then was filtered through Whatman No. 4 paper. The obtained decoctions were frozen and lyophilized.

Hydromethanolic extractions were performed by stirring each powdered sample (1 g) with 30 mL of methanol/water (80:20, *v*/*v*) at 25 °C and 150 rpm for 1 h. After filtering the supernatant through Whatman No. 4 paper, the residue was extracted with an additional portion (30 mL) of the hydromethanolic mixture. The combined extracts were concentrated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then frozen and lyophilized.

The lyophilized decocted and hydromethanolic extracts were re-dissolved in water and methanol/water (80:20, *v*/*v*), respectively, to obtain stock solutions of 4 mg/mL which were successively diluted to different concentrations for evaluation of the antioxidant activity and total phenolics and flavonoids.

3.1.2.5. Analysis of organic acids

Organic acids were analysed by ultra-fast liquid chromatography (UFLC) coupled to a photodiode array detector (PDA) according to the procedures previously described by Pereira *et al.* (2013). Briefly, the powdered samples (~1 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 °C at 150 rpm) for 45 min and then subsequently filtered through Whatman No. 4 paper. For decoctions, the lyophilized extracts (~10 mg) were dissolved in 1 mL of meta-phosphoric acid. All samples were filtered through 0.2 μm nylon filters before analysis. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards

of each compound. The results were expressed in mg per g of dry weight or lyophilized decoction (dw).

3.1.2.6. Evaluation of the total phenolic and flavonoid content

The total phenolic content was determined by the Folin-Ciocalteu method (Singleton and Rossi 1965) with slight modifications (Pereira *et al.* 2011) in the hydromethanolic and decocted extracts concentrated at 1.25 mg/mL. This assay is based on the formation of a blue-coloured complex between the molybdenum and tungsten present in the Folin-Ciocalteu reagent upon reaction with reducing agents, which is monitored at 765 nm. The standard curve was calculated using gallic acid and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract.

The total flavonoid content was determined using the aluminium chloride colorimetric method (Chang *et al.* 2002) with slight modifications as described by the authors (Barros *et al.* 2010) in the hydromethanolic and decocted extracts concentrated at 2.5 mg/mL. This assay is based on the formation of a flavonoid-aluminium complex, which is monitored at 510 nm. The standard curve was calculated using catechin and the results were expressed as mg of catechin equivalents (CE) per g of extract.

3.1.2.7. Evaluation of the antioxidant activity

The hydromethanolic and decocted extracts at different concentrations were submitted to four distinct *in vitro* assays to evaluate its antioxidant capacity (Pinela *et al.* 2015). Briefly, the $DPPH^{\bullet}$ scavenging activity and the reducing power assays were performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA). The reduction of $DPPH^{\bullet}$ was determined after incubation of the different extracts with a methanolic solution containing $DPPH^{\bullet}$ (6×10^{-5} M) for 60 min in the dark by measuring the absorbance at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of $DPPH^{\bullet}$ discoloration using the Equation (1):

$$RSA\ (\%) = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100, \quad (1)$$

where A_{DPPH} is the absorbance of the $DPPH^{\bullet}$ solution and A_S is the absorbance of the solution containing the sample extract. The reducing power was evaluated by the ferricyanide/Prussian blue assay, whereby the capacity of the extracts to convert potassium ferricyanide ($K_3[Fe(CN)_6]$) into potassium ferrocyanide ($K_4[Fe(CN)_6]$), which then reacts with ferric chloride ($FeCl_3$) to form a ferric-ferrous complex that is measuring spectrophotometrically, was monitored at 690 nm. The β-carotene bleaching inhibition (CBI) was evaluated by measuring the capacity of the extracts to neutralize linoleate free radicals.

The different extracts were mixed with an emulsion containing β-carotene, linoleic acid and Tween 80 emulsifier and the absorbance was immediately measured at 470 nm in a Model 200 spectrophotometer (AnalytikJena, Jena, Germany). After 2 h of incubation at 50 °C, the absorbance was read again. The CBI was calculated using the Equation (2):

$$CBI\ (\%) = (A_{120}/A_0) \times 100 \quad (2)$$

where A_{120} is the absorbance of the emulsion after 2 h of incubation and A_0 is the initial absorbance. The thiobarbituric acid reactive substances (TBARS) formation inhibition was evaluated in porcine brain homogenates. The colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex formed during heating of the reaction mixture at 80 °C was measured at 532 nm. The inhibition ratio was calculated using the Equation (3):

$$\text{Inhibition ratio}\ (\%) = [(A - B)/A] \times 100 \quad (3)$$

where A and B correspond to the absorbance of the control and the sample solution, respectively. The results were expressed in EC_{50} values (mg/mL), *i.e.,* sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

3.1.2.8. Statistical analysis

In all cases, analyses were carried out using three samples separately processed, and all the assays were carried out in triplicate. The results were expressed as mean±standard deviation and analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α = 0.05. This treatment was carried out using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA).

3.1.3. Results and discussion

3.1.3.1. Effects on colour parameters

The CIE *L*a*b** colour values for non-irradiated and irradiated samples of *M. neglecta* are presented in **Figure 18**. The 5 and 8 kGy doses had no influence on colour parameters. However, significant differences were found for samples irradiated with the 1 kGy dose. These samples revealed lower lightness (*L** = 43.43 ± 0.89) and yellowness (*b** = 22.29 ± 0.96) and higher redness (*a** = −12.69 ± 0.45), which induced the more pronounced total colour difference (Δ*E** = 1.81), compared with the non-irradiated control samples (*L** = 45.07 ± 0.97, *b** = 23.98 ± 0.55 and *a** = -13.84 ± 0.29). Furthermore, while the Δ*E** up to 0.5 and from 0.5 to 1.5 indicate trace colour differences and slight differences, respectively, the Δ*E**

corresponding to the 1 kGy dose indicates a noticeable difference detectable by the human eye (Chen and Mujumdar 2008).

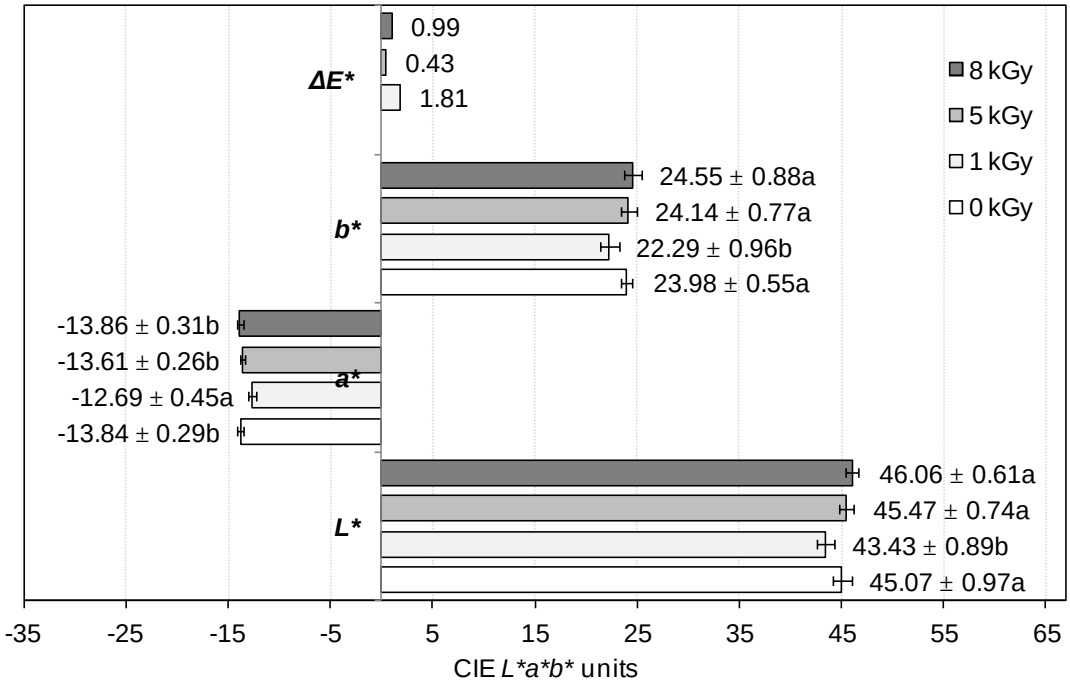

Figure 18 Impact of the gamma irradiation treatment on colour parameters of *M. neglecta* powdered samples. Δ*E**: total colour difference; *b**: blueness (–) ↔ yellowness (+); *a**: greenness (–) ↔ redness (+); and *L**: darkness (0) ↔ lightness (100). For each colour parameter, different letters (a,b) indicate statistically significant differences ($p < 0.05$).

Similar results were previously reported by Kirkin *et al*. (2014). The authors found that the 7 kGy dose had a greater effect on colour parameters of *Rosmarinus officinalis* L. than the higher doses of 12 and 17 kGy. In these samples, the *L** value was decreased and the *a** value was increased, in accordance to our results. The discoloration of *Piper nigrum* L. by decreasing the *a** and *b** values and increasing the *L** value was also reported, as well as the suitability of the applied doses to maintain the colour parameters of *Thymus vulgaris* L. and *Cuminum cyminum* L. A tendency for lightness decrease was also reported by Pereira, Barros, Antonio, *et al.* (2015) in *Ginkgo biloba* L. samples irradiated at 10 kGy. Nevertheless, browning is unwanted in dried products (Rahimmalek and Goli 2013). On the other hand, Pinela *et al*. (2015) reported that the gamma irradiation treatment up to 10 kGy had no effect on colour parameters of shade- and freeze-dried samples of *Tuberaria lignosa* (Sweet) Samp. Therefore, we can conclude that the irradiation-induced modifications on colour parameters not only depend on the applied dose, but also on the plant material under investigation.

3.1.3.2. Effects on organic acids

Table 11 shows the organic acids content found in *M. neglecta* dry material submitted to gamma irradiation, as well as in the decocted extracts (the form traditionally used in folk medicine) prepared from that material. Oxalic, quinic, malic, citric, succinic and fumaric acids were quantified, being oxalic acid the most abundant organic acid (**Figure 19**). The total amounts recorded in the decocted extracts were always higher than those found in the plant dry material, as well as the individual levels of each organic acid (except for citric acid) (**Table 11**). This difference may be related to a better extraction efficiency achieved at elevated temperature, but that can lead to citric acid degradation (Reda 2011). These results are in agreement with those previously reported by Guimarães *et al*. (2013) for *Matricaria recutita* L. dry material and decocted extracts.

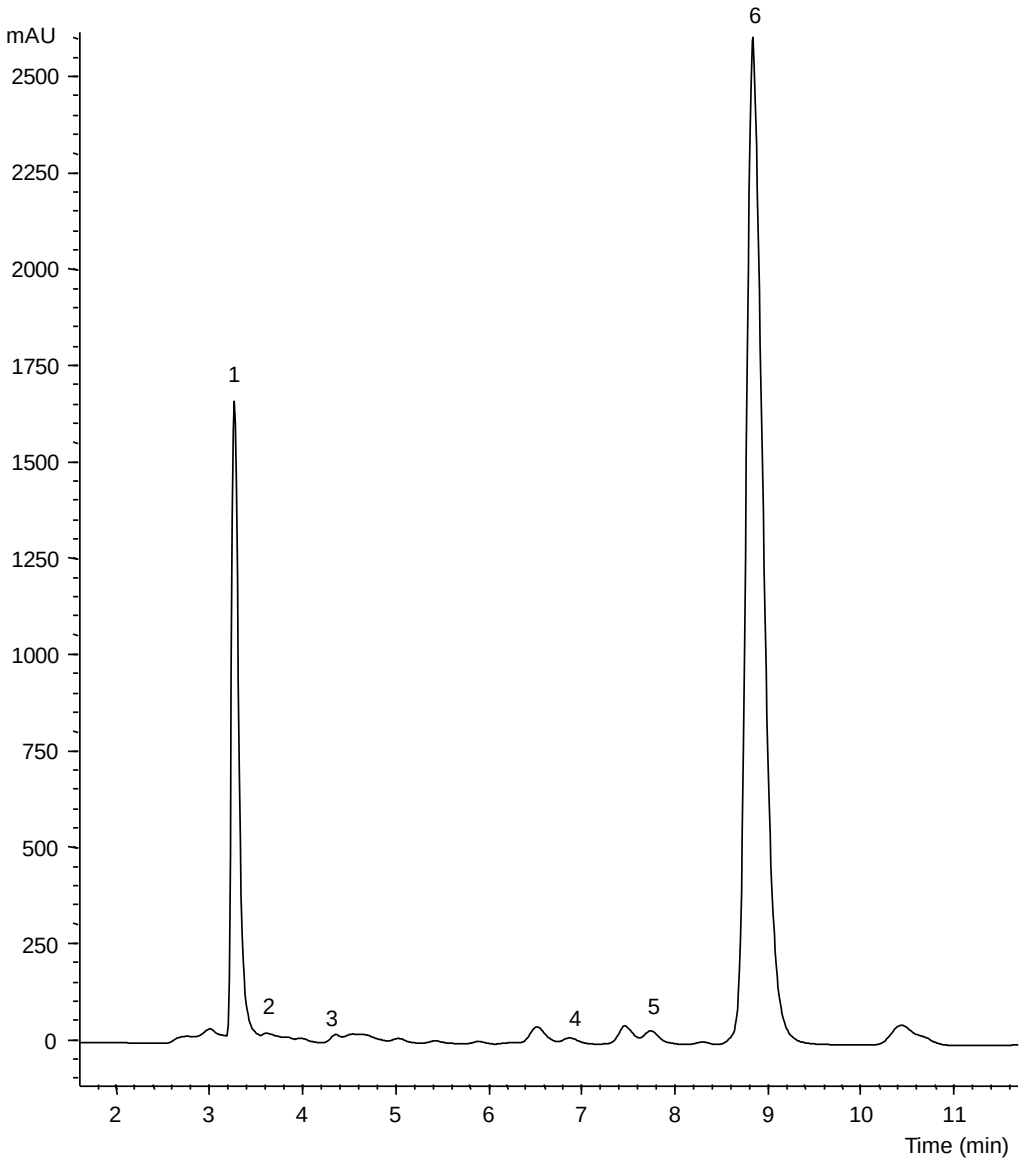

Figure 19 Chromatographic profile of organic acids in the decoction prepared from *M. neglecta* samples irradiated at 5 kGy, obtained using UFLC-PDA. 1: oxalic acid, 2: quinic acid, 3: malic acid, 4: citric acid, 5: succinic acid, and 6: fumaric acid.

Table 11 Organic acids content in the dry material and decocted extracts of *M. neglecta* samples submitted to gamma irradiation.

	Oxalic acid	Quinic acid	Malic acid	Citric acid	Succinic acid	Fumaric acid	Total
Dry material (mg/g dw)							
0 kGy	67.50 ± 0.16 b	6.63 ± 0.21 b	3.16 ± 0.28 b	21.56 ± 0.41 b	7.50 ± 0.13 a	15.07 ± 0.03 c	121.43 ± 1.17 b
1 kGy	67.29 ± 0.05 b	7.50 ± 0.42 a	3.09 ± 0.16 b	19.84 ± 0.14 c	7.30 ± 0.41 a	18.04 ± 0.05 a	123.07 ± 0.02 a
5 kGy	65.30 ± 0.17 c	6.34 ± 0.28 b	3.42 ± 0.33 ab	22.28 ± 0.31 a	7.29 ± 0.12 a	13.59 ± 0.07 d	118.22 ± 1.15 c
8 kGy	71.75 ± 0.10 a	6.45 ± 0.08 b	3.64 ± 0.05 a	19.02 ± 0.08 d	6.23 ± 0.13 b	15.85 ± 0.05 b	122.94 ± 0.30 ab
Decocted extracts (mg/g dw)							
0 kGy	106.71 ± 0.50 a	3.59 ± 0.25 d	9.78 ± 0.67 b	7.92 ± 0.09 d	47.43 ± 0.67 b	20.61 ± 0.14 c	196.03 ± 0.80 b
1 kGy	79.80 ± 0.05 c	6.96 ± 0.20 c	10.52 ± 0.08 a	9.80 ± 0.15 b	47.36 ± 0.38 b	21.54 ± 0.19 b	175.99 ± 0.89 c
5 kGy	88.72 ± 0.03 b	8.90 ± 0.09 a	9.72 ± 0.19 b	12.76 ± 0.07 a	54.17 ± 0.06 a	20.81 ± 0.08 c	195.09 ± 0.01 b
8 kGy	106.33 ± 0.10 a	8.37 ± 0.39 b	7.89 ± 0.29 c	8.58 ± 0.35 c	41.42 ± 0.50 c	25.17 ± 0.09 a	197.76 ± 0.95 a

The results are presented as mean±standard deviation. In each column and for each sample (dry material and decoction), different letters (a–d) indicate statistically significant differences ($p < 0.05$).

In the plant material, the relative amounts of oxalic, citric and fumaric acids were the most affected by the irradiation treatment (**Table 11**); however, this variation was more marked in the decocted extracts, mainly in the oxalic and succinic acids, probably due to the combined effects induced by gamma irradiation and preparation method. Therefore, the levels of organic acids and relative ratios in the decocted extracts were somewhat different from those found in the dry material, *e.g.,* citric acid was the second most abundant organic acids in the plant material and presented a reduction of ~12% in the decocted extracts, while the relative ratios of succinic acid increased ~19% in those preparations. Nevertheless, despite the oxalic, malic, citric and fumaric acids have been already described in flowers and flowering shoots of *Malva sylvestris* L. (Pereira *et al.* 2013), the quinic and succinic acids were not detected or present in trace amounts in this species. So, a qualitative analysis of the organic acids profile can be used to detect possible adulterations in the samples.

In the dry material, the 1 kGy dose did not induce any adverse effect on organic acids profile and increased the total levels. Lower amounts of total organic acids were however detected in samples irradiated at 5 kGy, due to a decrease of oxalic and fumaric acids. The highest levels of citric and succinic acids and the reduction of fumaric acid were also associated with the 5 kGy dose, detected in both samples (dry material and decocted extract). Additionally, the samples irradiated at 8 kGy gave the highest levels of total organic acids. It can be concluded that, in general, the effects induced by the different doses were positive, once the decrease in total organic acids was mainly associated with the oxalic acid content. Despite the importance of this acid in pharmaceutical (Lian *et al.* 1999), it has been associated with some health problems, *e.g.,* oxalic acid can combine with calcium in the kidneys to form kidney stones in susceptible people (Robitaille *et al.* 2009).

The identified organic acids have been used as food additives or pharmaceutical and cosmetic excipients (Sauer *et al.* 2008, Carocho *et al.* 2014). Citric acid is a widely used food additive in many kinds of beverages due to its mild and refreshing sourness (Liu *et al.* 2014, Andrés *et al.* 2015), succinic acid is also used in the food industry as well as in pharmaceuticals and antibiotics (Sauer *et al.* 2008), fumaric acid is used due to its effectiveness against psoriasis and inflammation and due to its neuro- and chemoprotective effects (Baati *et al.* 2011), and malic and citric acids are reported to have bactericidal effects (Raybaudi-Massilia *et al.* 2009). Additionally, the consumption of these compounds in moderate amounts can promote appetite, help digestion and be beneficial to human health (Nawirska-Olszańska *et al.* 2014). However, excessive doses of certain organic acids should be avoided (Carocho *et al.* 2014).

3.1.3.3. Effects on total phenolics, total flavonoids and antioxidant activity

The effects of gamma irradiation on the total phenolic and flavonoid contents and antioxidant activity of the *M. neglecta* hydromethanolic and decocted extracts can be accessed from the analysis of **Table 12**. The results of the antioxidant activity were expressed in EC_{50} values; thus, the lower the EC_{50} value, the higher the antioxidant activity. In general, the decocted extracts revealed higher amounts of total phenolics and flavonoids than the hydromethanolic extracts, as well as an increased DPPH$^{\bullet}$ scavenging activity and reducing power. The samples irradiated at 1 kGy and extracted with the hydromethanolic mixture revealed lower levels of both total phenolic and flavonoids and a decreased in the DPPH$^{\bullet}$ scavenging activity, reducing power, and TBARS formation inhibition capacity. This result is in agreement with the higher ΔE^* found in these samples. Contrariwise, the hydromethanolic extracts prepared from the samples irradiated at 5 kGy revealed the higher levels of total phenolics and flavonoids and an increased DPPH$^{\bullet}$ scavenging activity and lipid peroxidation inhibition capacity (accessed by the β-carotene bleaching inhibition and TBARS formation inhibition in brain cell homogenates) in comparison with those prepared from the non-irradiated control samples. In fact, the lipid peroxidation inhibition capacity of these extracts was favoured by the irradiation treatment (except for the TBARS formation inhibition of the extracts prepared from the samples irradiated at 1 kGy). This result can be explained by the affinity of these two *in vitro* assays for lipophilic antioxidants and by the fact that the irradiation treatment affects differently the different antioxidant molecules (Alothman *et al.* 2009, Harder *et al.* 2016).

Regarding the decocted extracts, the 8 kGy dose decreased the antioxidant properties and levels of total phenolics and flavonoids. However, the other assayed doses reinforced the reducing power, the TBARS formation inhibition capacity, and the levels of total phenolics and flavonoids. Similar trends have been observed in other studies. A higher DPPH$^{\bullet}$ scavenging activity and reducing power of decocted and infused extracts of *T. vulgaris*, comparatively to the hydroalcoholic ones, was found by Martins *et al.* (2015). The decoction was also the preferable preparation method for obtaining increased levels of phenolic acids and flavonoids. The suitability of decoctions and infusions for extracting phenolic compounds from commercial samples of *Achillea millefolium* L. was demonstrated by Dias *et al.* (2013), aqueous extracts that also had interesting antioxidant properties. Furthermore, despite plant extracts riches in phenolic compounds are commonly referred as having increased antioxidant properties (Dalar *et al.* 2012, Guimarães *et al.* 2013, Pinela *et al.* 2015), the presence of organic acids, as phenolics, may also contribute to these effects (Silva *et al.* 2004).

Table 12 Antioxidant activity (EC_{50} values, mg/mL) and total phenolic (mg GAE/g Extract) and flavonoid (mg CE/g Extract) content of the hydromethanolic and decocted extracts prepared from the *M. neglecta* dry material submitted to irradiation.

	DPPH• scavenging activity	Reducing power	β-Carotene bleaching inhibition	TBARS formation inhibition	Total phenolics	Total flavonoids
Hydromethanolic extracts						
0 kGy	1.15 ± 0.02 ^{c}	0.52 ± 0.01 ^{c}	0.46 ± 0.01 ^{a}	0.56 ± 0.05 ^{b}	69.54 ± 0.21 ^{b}	22.85 ± 0.52 ^{b}
1 kGy	1.57 ± 0.02 ^{b}	0.69 ± 0.01 ^{a}	0.41 ± 0.03 ^{b}	0.58 ± 0.03 ^{a}	55.04 ± 0.36 ^{d}	19.56 ± 0.08 ^{c}
5 kGy	1.06 ± 0.07 ^{d}	0.57 ± 0.02 ^{b}	0.41 ± 0.01 ^{b}	0.11 ± 0.03 ^{d}	78.55 ± 0.67 ^{a}	27.30 ± 0.20 ^{a}
8 kGy	1.75 ± 0.04 ^{a}	0.56 ± 0.01 ^{b}	0.40 ± 0.01 ^{b}	0.22 ± 0.01 ^{c}	64.78 ± 1.51 ^{c}	22.70 ± 0.51 ^{b}
Decocted extracts						
0 kGy	0.37 ± 0.01 ^{c}	0.268 ± 0.002 ^{b}	0.16 ± 0.01 ^{c}	0.403 ± 0.004 ^{b}	91.05 ± 1.14 ^{b}	25.14 ± 0.53 ^{b}
1 kGy	0.40 ± 0.01 ^{b}	0.264 ± 0.003 ^{c}	0.17 ± 0.02 ^{c}	0.35 ± 0.01 ^{c}	96.92 ± 3.73 ^{a}	28.03 ± 0.07 ^{a}
5 kGy	0.36 ± 0.01 ^{c}	0.253 ± 0.003 ^{d}	0.41 ± 0.01 ^{b}	0.32 ± 0.01 ^{d}	96.76 ± 0.60 ^{a}	27.63 ± 0.35 ^{a}
8 kGy	0.46 ± 0.02 ^{a}	0.326 ± 0.001 ^{a}	0.46 ± 0.01 ^{a}	0.69 ± 0.02 ^{a}	78.99 ± 0.30 ^{c}	21.98 ± 0.47 ^{c}

The results are presented as mean±standard deviation. In each column and for each sample (hydromethanolic and decocted extracts), different letters (a–d) indicate statistically significant differences ($p < 0.05$). EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox EC_{50} values: 42 µg/mL (DPPH• scavenging activity), 41 µg/mL (reducing power), 18 µg/mL (β-carotene bleaching inhibition) and 23 µg/mL (TBARS inhibition). GAE: gallic acid equivalents; CE: catechin equivalents.

The total phenolic and flavonoid contents of different parts of *M. neglecta* were already reported by Dalar *et al*. (2012) in samples from the Eastern Anatolia Region of Turkey. Lower total phenolic contents of 17.4 ± 0.3 mg GAE/g extract and 6.6 ± 0.3 mg GAE/g extract were reported in leaf and whole plant extracts, respectively, obtained using acidified methanol (80% methanol and 1% HCl (*v*/*v*) in water) as extraction solvent. Regarding total flavonoids, 7.21 ± 0.28 mg RE (rutin equivalents)/g extract and 2.95 ± 0.16 mg RE/g extract were found in the leaf and whole plant extracts, respectively. The same authors also evaluated the antioxidant activity through the FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity) assays. The highest bioactivity was assigned to the leaf extracts and then to the flower extracts. These differences in the phenolic content may be justified by variations on edafoclimatic conditions of the locations where the samples were collected, which may affect the plant composition during the growing season (Nikolopoulou *et al.* 2007). Additionally, as verified in our study, the extraction method also causes significant variations in the evaluated responses. The used of different variables in obtaining hydromethanolic and decocted extracts, namely different solvents, extraction times, temperatures, and sample to solvent ratios, may have favoured the extraction of different molecules.

Although nothing has been reported on the impact of gamma irradiation on the antioxidant activity or phenolic composition of *M. neglecta*, different effects have been reported in other plant materials. A study conducted by Pinela *et al.* (2015) concluded that irradiation doses up to 10 kGy did not significantly affect the antioxidant activity and phenolic composition of decoctions and infusions prepared from *T. lignosa* samples. On the other hand, increased antioxidant properties in infusions and methanolic extracts of borututu (*Cochlospermum angolensis* Welw.) and *G. biloba* samples irradiated at 10 kGy were reported by Pereira *et al*. (2014) and Pereira, Barros, Antonio, *et al.* (2015), respectively. The authors also verified that the 10 kGy dose improves the extractability of phenolic compounds from the *G. biloba* samples (Pereira, Barros, Dueñas, *et al.* 2015). An increased total phenolic content and enhanced antioxidant activity of gamma irradiated almond skins extracted with 40% ethanol was reported by Harrison and Were (2007). The authors attributed these results to the release of phenolic compounds from glycosidic components and degradation of larger phenolic compounds into smaller ones by the gamma irradiation treatment. In fact, the direct impact of gamma-rays and the indirect action of radiolytic products may change the structure of different antioxidant molecules and/or break some chemical bonds, thus leading to its decomposition or altered extractability from the plant material (Harrison and Were 2007, Alothman *et al.* 2009, Pereira, Barros, Antonio, *et al.* 2015). That is why the bioactivity of irradiated samples can decrease or be improved.

3.1.4. Conclusions

This study demonstrated the adequacy of the irradiation at 5 and 8 kGy to preserve the colour parameters of the *M. neglecta* dry material. Moreover, it was confirmed that the irradiation-induced modifications on colour parameters not only depend on the applied dose, but also on the plant material under study. Oxalic, quinic, malic, citric, succinic and fumaric acids were identified for the first time. The total levels recorded in decocted extracts were always higher than those found in the plant dry material, as well as the individual levels of each organic acid (except for citric acid). Irradiation at 5 kGy increased the amounts of citric and succinic acids and decreased the fumaric acid levels in both matrices. In general, decoctions were preferred for their higher levels of total phenolics and flavonoids, $DPPH^{\bullet}$ scavenging activity and reducing power. In these preparations, the antioxidant properties and levels of total phenolics and flavonoids were decreased with the 8 kGy dose. In turn, the hydromethanolic extracts obtained from samples irradiated at 1 kGy showed decreased levels of total phenolic, total flavonoids, and lower antioxidant properties. Thus, decoctions were highlighted by interesting antioxidant properties and levels of total phenolics and organic acids. Nevertheless, further studies are of interest to evaluate the decontamination effectiveness of this technology and to investigate the effect in other quality parameters.

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Perennial spotted rockrose (*Tuberaria lignosa* (Sweet) Samp.)

3.2. Antioxidant activity, ascorbic acid, phenolic compounds and sugars of wild and commercial *Tuberaria lignosa* samples: Effects of drying and oral preparation methods

Antioxidant activity, ascorbic acid, phenolic compounds and sugars of wild and commercial *Tuberaria lignosa* samples: Effects of drying and oral preparation methods

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Abstract

The antioxidant activity and phytochemical composition (ascorbic acid, free sugars and phenolic compounds) of decoctions and infusions of wild and commercial samples of *Tuberaria lignosa* (Sweet) Samp. (Fam. Cistaceae) aerial parts were evaluated and compared. Among wild samples, the effects of the drying method (freeze or shade-drying) on those parameters were studied. Infusion of the freeze-dried wild sample gave the highest levels of sugars, while infusion of shade-dried wild sample and decoction of the freeze-dried sample presented higher ascorbic acid and phenolic compounds content (including ellagitannins and flavonoids) than the other samples. The last two samples also revealed higher antioxidant activity and, in some cases, even higher than trolox. Decoctions gave lower amounts of disaccharides than infusions, which seemed to be hydrolysed increasing the content in monosaccharides in the first samples. Commercial samples showed the lowest content in phenolic compounds, mainly in ellagitannins and flavonoids, as also the lowest antioxidant activity. This work gives scientific evidences to the traditional medicinal uses of

wild *Tuberaria lignosa*, highlighting the interest of its decoctions and infusions as bioactive compounds source and functional beverages.

Keywords: *Tuberaria lignosa*; Iberian ethnobotany; drying method; oral preparations; antioxidants.

3.2.1. Introduction

The adverse effects of oxidative stress on human health have become a serious issue. Under stress, the concentration of reactive oxygen species (ROS) (*e.g.,* superoxide anion radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide) in our bodies is higher than the concentration of enzymatic antioxidants (*e.g.,* superoxide dismutase, glutathione peroxidase and catalase) and non-enzymatic antioxidants (*e.g.,* ascorbic acid, vitamin E and glutathione) (Ferreira and Abreu 2007, Krishnaiah *et al.* 2011). This imbalance leads to damage in lipids, proteins, carbohydrates and DNA and consequently induces degeneration, destruction and toxicity of various biomolecules (Halliwell 1996, Valko *et al.* 2007). The development of many diseases including atherosclerosis, cardiovascular disease, cataracts, rheumatoid arthritis, inflammatory disorders, anaemia, asthma, cancer, ageing and Parkinson's and Alzheimer's diseases is connected with oxidative stress (Halliwell 1996, Valko *et al.* 2007, Dasgupta *et al.* 2014). One solution to this problem is to supplement the diet with antioxidant compounds that are contained in natural plant sources. These natural plant antioxidants can therefore serve as a type of preventive medicine (Krishnaiah *et al.* 2011).

The plant kingdom offers a wide range of natural antioxidants. However, little is known about the practical usefulness of most of them. Many herbal beverages, frequently used in folk medicine, have antioxidant and pharmacological properties linked with the presence of phenolic compounds, especially flavonoids (Cabrera *et al.* 2006, Pinto 2010). The recognition of herbal beverages as functional drinks might be related with the plant species from which they are prepared and with formulation or preparation methods (Henning *et al.* 2004, Milašienė *et al.* 2007).

In Portugal as well as in Spain, some of the most popular medicinal plants have been traditionally gathered for preparing herbal infusions or decoctions (Pardo de Santayana *et al.* 2005). Such is the case of *Tuberaria lignosa* (Sweet) Samp. (Fam. Cistaceae), a species native to western and southern Europe and mainly present in the western regions of the Iberian Peninsula (Castroviejo 2005). It is one of the most quoted medicinal plant in the North-eastern Portuguese region where it is popularly known as "alcária" or "erva-loba" (Carvalho 2010). In traditional folk medicine, different parts (roots and aerial parts) of this plant are used for treating various diseases and ailments, such as gastrointestinal disorders,

heart-burn, wounds, influenza, skin infections and warts due to its vulnerary, anti-inflammatory and anti-infectious properties (Martín-Aragón *et al.* 1994, Novais *et al.* 2004, Bedoya *et al.* 2010, Carvalho 2010). Flowers, leaves or whole plant are prepared fresh or shade-dried in medicinal infusions and decoctions (Novais *et al.* 2004, Carvalho 2010). Commonly, infusions of leaves or inflorescences are used for hepato-depurative disorders (internal use) and decoctions of the whole plant for skin inflammations (external and topical use). However, in Portugal local healers and key informants avoid using in humans the *T. lignosa* plant extracts for long periods so as to prevent to a certain degree toxicity and adverse effects, which can vary considerably according the method of preparation, doses and physical condition intrinsic to the individual (Carvalho 2010).

In this study, the antioxidant activity and phytochemical composition (ascorbic acid, free sugars and phenolic compounds) of decoctions and infusions of wild and commercial samples of *T. lignosa* flowering aerial parts (*e.g.,* basal leaves, stems and inflorescences) were evaluated and compared. Furthermore, among wild samples, the effects of the drying method (freeze or shade-drying) on those parameters were studied.

3.2.2. Materials and methods

3.2.2.1. Samples

Tuberaria lignosa Sweet Samp. (synonym of *Xolantha tuberaria* (L.) Gallego, Muñoz Garm & C. Navarro) is a perennial species in the rockrose family Cistaceae, native to western and southern Europe, occurring mainly in dry, stony sites of the western Mediterranean area. The leaves are in a rosette at the base of the plant and the yellow flowers are organized in relaxed determinate inflorescences (Castroviejo 2005).

Wild samples of *T. lignosa* were collected in the flowering season in Miranda do Douro (Trás-os-Montes, North-eastern Portugal), considering the local medicinal uses as well as healers and selected consumers' criteria, which are related to particular gathering sites, and requirements for safe herbal dosages forms, such as infusion and decoction. In the area of sample collection annual rainfall is about 600-800 mm, the average temperature 12.5≥ T> 10 ºC and altitude 600-700m above sea level (Agroconsultores and Coba 1991). Samples were submitted to two different drying processes: 1) Freeze-drying (7750031 FreeZone 4.5, Labconco, Kansas, USA) immediately after being collected; 2) shade-drying, being stored in a dark and dry place in cellophane or paper bags kept at room temperature (more or less 21ºC and 50% of relative humidity) for 30 days, simulating informants' general conditions of use. Voucher specimens are deposited in the Herbarium of the Escola Superior Agrária de Bragança (BRESA, ETBO55/2011).

Commercial samples were obtained in a local herbal shop, in Bragança (North-eastern Portugal) and were available as dried rosettes of leaves and inflorescences. Usually

herbal shops order vegetal materials for processing and pack from local inhabitants of the villages, then wild and commercial samples may have the same origin.

3.2.2.2. Preparation of decoctions and infusions

Decoctions: Each sample (15 g, according to Portuguese informants' practices; Carvalho 2010) was added to 500 mL of distilled water, heated in an Are heating plate (VELP scientific, Usmate, Italy) and boiled for 5 min. The mixture was left to stand at 25 ºC for 5 min more, and then filtered under reduced pressure. The obtained decoctions were frozen and lyophilized.

Infusions: Each sample (7.5 g, respecting local practices; Carvalho 2010) was added to 500 mL of boiling distilled water and left to stand at 25 ºC for 5 min, and then filtered under reduced pressure. The obtained infusions were frozen and lyophilized.

3.2.2.3. Standards and reagents

Acetonitrile 99.9 % was of HPLC grade from Lab-Scan (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). L-ascorbic acid, sugar standards (D(-)-fructose, D(+)-glucose anhydrous, D(+)-raffinose pentahydrate, D(+) sucrose and D(+)trehalose) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). The phenolic compound standards (apigenin-6-*C*-glucoside, *p*-coumaric acid, ellagic acid, gallic acid, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, luteolin-6-*C*-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) were purchased from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, CA, USA).

3.2.2.4. In vitro antioxidant properties

General: Lyophilized preparations of the decoctions and infusions were redissolved in water at a concentration of 2 mg/mL and further diluted to different concentrations to be submitted to distinct *in vitro* assays (Pinela *et al.* 2011) to evaluate their antioxidant properties. The extract concentrations providing 50 % of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

DPPH free-radical scavenging activity: This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Bedfordshire, UK). The reaction mixture in each one of the 96-wells consisted of one of the different concentration solutions (30 µL) and methanolic solution

(270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation:

$$RSA\ (\%) = [(A_{DPPH}-A_S)/A_{DPPH}] \times 100 \quad (1)$$

where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution.

Reducing power: This methodology was performed using the Microplate Reader described above. The different concentration solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1 % w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10 % w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionised water (0.8 mL) and ferric chloride (0.1 % w/v, 0.16 mL), and the absorbance was measured at 690 nm.

Inhibition of β-carotene bleaching: A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm in a spectrophotometer 200 (AnalytikJena, Jena, Germany). β-Carotene bleaching inhibition was calculated using the following equation:

$$(\text{Abs after 2h of assay/initial Abs}) \times 100 \quad (2)$$

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS): Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000*g* (Centurion K24OR refrigerated centrifuge, West Sussex, UK) for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28 % w/v, 0.5 mL), followed by thiobarbituric

acid (TBA, 2 %, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = [(A - B)/A] \times 100\% \quad (3)$$

where A and B were the absorbance of the control and the compound solution, respectively.

3.2.2.5. Phytochemical characterization

Vitamin C: Each lyophilized decoction/infusion (100 mg) was extracted with metaphosphoric acid (1 %, 10 mL) for 45 min at room temperature and filtered through Whatman Nº 4 filter paper. The filtrate (1 mL) was mixed with 2,6-dichloroindophenol (9 mL) and the absorbance was measured after 15 min at 515 nm (Pinela *et al.* 2011). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (6.0×10^{-3}-1.0×10^{-1} mg/mL), and the results were expressed as mg of ascorbic acid per g of lyophilized decoction/infusion.

Free Sugars: Each decoction/infusion (1 mL) was filtered through 0.2 µm nylon filters from Whatman, and analysed by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described by the authors (Pinela *et al.* 2011). The equipment consisted of an integrated system with a Smartline system 1000 pump (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD) and a Smartline 2300 RI detector. Data were analysed using Clarity DataApex 2.4 Software. The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6×250 mm, 5 mm, Knauer, Berlin, Germany) operating at 35 ºC in a 7971 R Grace oven. The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (0.375-24 mg/mL) of different standard compounds: D(-)-fructose (y=1.04x, R^2=0.9999), D(+)-glucose anhydrous (y=0.935x, R^2=0.9991), D(+)-sucrose (y=1.087x, R^2=0.9999), trehalose (y=0.991x, R^2=0.9999) and raffinose (y=0.891x, R^2=0.9999). The results were expressed as mg per g of lyophilized decoction/infusion.

Phenolic compounds: Each lyophilized decoction/infusion (1 mg) was dissolved in water:methanol (80:20 v/v), filtered through 0.2 µm nylon filters from Whatman, and analysed by HPLC (Hewlett-Packard 1100 chromatograph, Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem

Station (rev. A.05.04) data-processing station. A Spherisorb S3 ODS-2 C_{18} (Waters, Dinslaken, Germany), 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1 % formic acid in water, (B) acetonitrile. The elution gradient established was 10 % B to 15 % B over 5 min, 15-25 % B over 5 min, 25-35 % B over 10 min, isocratic 50 % B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive scan modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to obtain full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the decoctions/infusions were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations (2.5-100 µg/mL) of different standard compounds: apigenin-6-*C*-glucoside (y=246.05x-309.66; R^2=0.9994); *p*-coumaric acid (y=321.99x+98.308; R^2=0.9984); ellagic acid (y=35.695x-265.7; R^2=0.9991) gallic acid (y=556.94x+738.37; R^2=0.9968); kaempferol-3-*O*-glucoside (y=190.75x-36.158; R^2=1); kaempferol-3-*O*-rutinoside (y=175.02x-43.877; R^2=0.9999); luteolin-6-*C*-glucoside (y=365.93x+17.836; R^2=0.9997); quercetin-3-*O*-glucoside (y=316.48x+2.9142; R^2=1), and quercetin-3-*O*-rutinoside (y=222.79x+243.11; R^2=0.9998). The results were expressed in mg per g of lyophilized decoction/infusion.

3.2.2.6. Statistical analysis

The results are expressed as mean value and mean standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test

with α = 0.05. This treatment was carried out using SPSS Version 16.0 (SPSS, New York, USA).

3.2.3. Results

As far as we know, this is the first evaluation of the effects of drying (freeze or shade-drying) and preparation (oral dosage forms in water) methods in the antioxidant activity and phytochemical composition of wild and commercial samples of *Tuberaria lignosa* materials. For wild samples, shade-drying was performed simulating informants' general conditions of use and freeze-drying was carried out immediately after being collected; for both wild and commercial samples, decoctions and infusions were prepared according to folk recipes/formulations (Carvalho 2010).

The antioxidant activity of the extracts obtained from the decoction/infusion preparations of wild (freeze or shade-dried) and commercial *T. lignosa* samples are shown in **Table 13**. The wild samples showed higher antioxidant activity than the commercial samples. However, the influence of the preparation procedure (decoction or infusion) on antioxidant activity was not clear. The infusion of the shade-dried wild sample showed the highest DPPH radical scavenging activity (EC_{50}=52 μg/mL) and reducing power (EC_{50}=21 μg/mL), while the decoction of the freeze-dried wild sample gave the highest lipid peroxidation inhibition in TBARS assay (EC_{50}=4 μg/mL). Both types of samples gave similar β-carotene bleaching inhibition (EC_{50}=12 μg/mL). It has to be highlighted that the studied samples revealed interesting antioxidant properties, particularly the infusion of shade-dried wild sample that gave higher reducing power than the standard trolox. The antioxidant effects herein reported for *Tuberaria lignosa* support recommendations for its traditional use.

Table 13 Antioxidant activity (EC_{50} values, μg/mL) of decoctions/infusions of different samples of *Tuberaria lignosa*.

	Wild sample				Commercial sample		Standard
	Freeze-dried		Shade-dried				
	Decoction	Infusion	Decoction	Infusion	Decoction	Infusion	Trolox
Yield (%)	21.16±0.89	11.83±0.44	19.72±1.02	15.99±0.27	20.81±1.44	17.07±0.11	-
DPPH scavenging activity	57.28±2.17^{c}	56.42±0.89^{c}	58.72±2.98^{cb}	52.06±0.44^{d}	61.08±0.95^{b}	65.85±3.24^{a}	43.03±1.71^{e}
Reducing power	27.06±0.78^{d}	30.38±0.23^{c}	32.79±0.55^{b}	21.31±0.16^{e}	48.54±0.57^{a}	32.13±1.00^{b}	29.26±3.15^{c}
β-carotene bleaching inhibition	11.56±2.55^{d}	15.06±3.05^{c}	20.65±1.60^{b}	11.72±1.47^{d}	20.37±1.55^{b}	25.77±3.49^{a}	2.63±0.14^{e}
TBARS inhibition	3.99±0.31^{d}	12.63±0.49^{b}	10.14±0.60^{c}	10.03±0.49^{c}	19.18±1.96^{a}	18.22±0.11^{a}	3.73±1.90^{d}

In each row different letters mean significant differences (p<0.05).

Ascorbic acid and free sugars were analysed and quantified (**Table 14**). Infusions gave higher amounts of ascorbic acid than decoctions, except in the freeze-dried wild sample. In fact, the decoction of this sample and the infusion of the shade-dried sample

revealed higher ascorbic acid concentration (~5 mg/g) than the other samples. Regarding free sugars, it should be noted that the levels of the disaccharides sucrose and trehalose decreased in decoctions, being hydrolysed into fructose and glucose that increased in those samples in comparison with the corresponding infusions (**Table 14)**. This phenomenon can also be observed in **Figure 20A** for the shade-dried wild sample decoction that gave the highest fructose content (68 mg/g), while the infusion contained the highest level of sucrose (36 mg/g). The freeze-dried sample infusion showed the highest total sugars content (144 mg/g) as also the highest glucose concentration (49 mg/g). Fructose was the most abundant sugar in all the samples whatever their origin or preparation. The commercial sample (decoction or infusion) presented the lowest total sugars content (94-95 mg/g), despite the relatively high levels of trehalose found in its infusion (**Table 14**). Curiously, raffinose was not detected in the commercial sample (**Figure 20B**).

Table 14 Ascorbic acid and free sugars contents (mg/g) in the decoctions/infusions of different samples of *Tuberaria lignosa*.

	Wild sample				**Commercial sample**	
	Freeze-dried		**Shade-dried**			
	Decoction	**Infusion**	**Decoction**	**Infusion**	**Decoction**	**Infusion**
Ascorbic acid	4.75 ± 0.02^{a}	3.62± 0.01^{d}	4.47 ± 0.01^{b}	4.75 ± 0.01^{a}	3.27 ± 0.11^{e}	3.94 ± 0.03^{c}
Fructose	53.94 ± 0.76^{b}	53.44 ± 2.91^{b}	67.60 ± 1.93^{a}	53.13 ± 0.60^{b}	52.05 ± 1.70^{b}	45.46 ± 1.06^{c}
Glucose	43.70 ± 0.43^{c}	48.81 ± 1.30^{a}	45.83 ± 0.12^{b}	25.04 ± 0.36^{e}	30.60 ± 0.62^{d}	21.44 ± 0.12^{f}
Sucrose	7.72 ± 0.33^{d}	32.14 ± 1.55^{b}	11.17 ± 0.32^{c}	36.01 ± 0.91^{a}	3.58 ± 0.14^{e}	9.11 ± 0.43d^{c}
Trehalose	11.35 ± 0.09^{c}	12.07 ± 0.51^{c}	9.04 ± 0.74^{d}	13.66 ± 0.76^{b}	7.94 ± 0.00^{d}	19.19 ± 0.17^{a}
Raffinose	1.40 ± 0.10^{a}	2.37 ± 0.90^{a}	1.67 ± 0.05^{a}	2.51 ± 0.16^{a}	nd	nd
Total sugars	123.22 ± 1.92^{c}	143.72 ± 5.28^{a}	135.31 ± 3.05^{b}	130.35 ± 2.78^{cb}	94.17 ± 1.22^{d}	95.20 ± 1.44^{d}

nd- not detected. In each row different letters mean significant differences ($p<0.05$).

Figure 21 shows the chromatographic profile of the phenolic compounds detected in *Tuberaria lignosa* (exemplified for the infusion of the shade-dried sample), which includes mainly ellagitannins, followed by flavonoids (flavonols and flavones) and phenolic acid derivatives. **Table 15** presents the data obtained from HPLC-DAD-MS analysis (retention time, λ_{max} in the visible region, mass spectral data) used for the compound identification.

Ellagitannins were the majority compounds found in the samples (peaks 1, 3, 4, 5 and 6). *Tuberaria lignosa* was already reported as a source of those compounds, which were suggested to be responsible for its antiviral activity against HIV (Bedoya *et al.* 2010). Peak 1 ($[M-H]^-$ at *m/z* 781) was assigned to punicalin (Seeram *et al.* 2005), whereas the rest of ellagitannins were associated to punicalagin derivatives. Peaks 3 and 5 ($[M-H]^-$ at *m/z* 1083) were identified as punicalagin isomers. Both compounds showed similar the MS^2 fragmentation yielding ions at *m/z* 781, 601 and 301, corresponding to their cleavage to

punicalin, gallagic acid and ellagic acid, respectively, as also reported by Seeram *et al.* (2005) and Fernández-Arroyo *et al.* (2010). Peaks 4 and 6 ([M-H]$^-$ at *m/z* 1251) were tentatively identified as derivatives of punicalagin attached to gallic acid (punicalagin gallate isomers), as previously reported by Saracini *et al.* (2005). In these compounds, the gallic acid would not be bound to punicalagin by the carboxyl group through ester linkage, as denoted by the fragment at *m/z* 1083 corresponding to the loss of gallic acid itself.

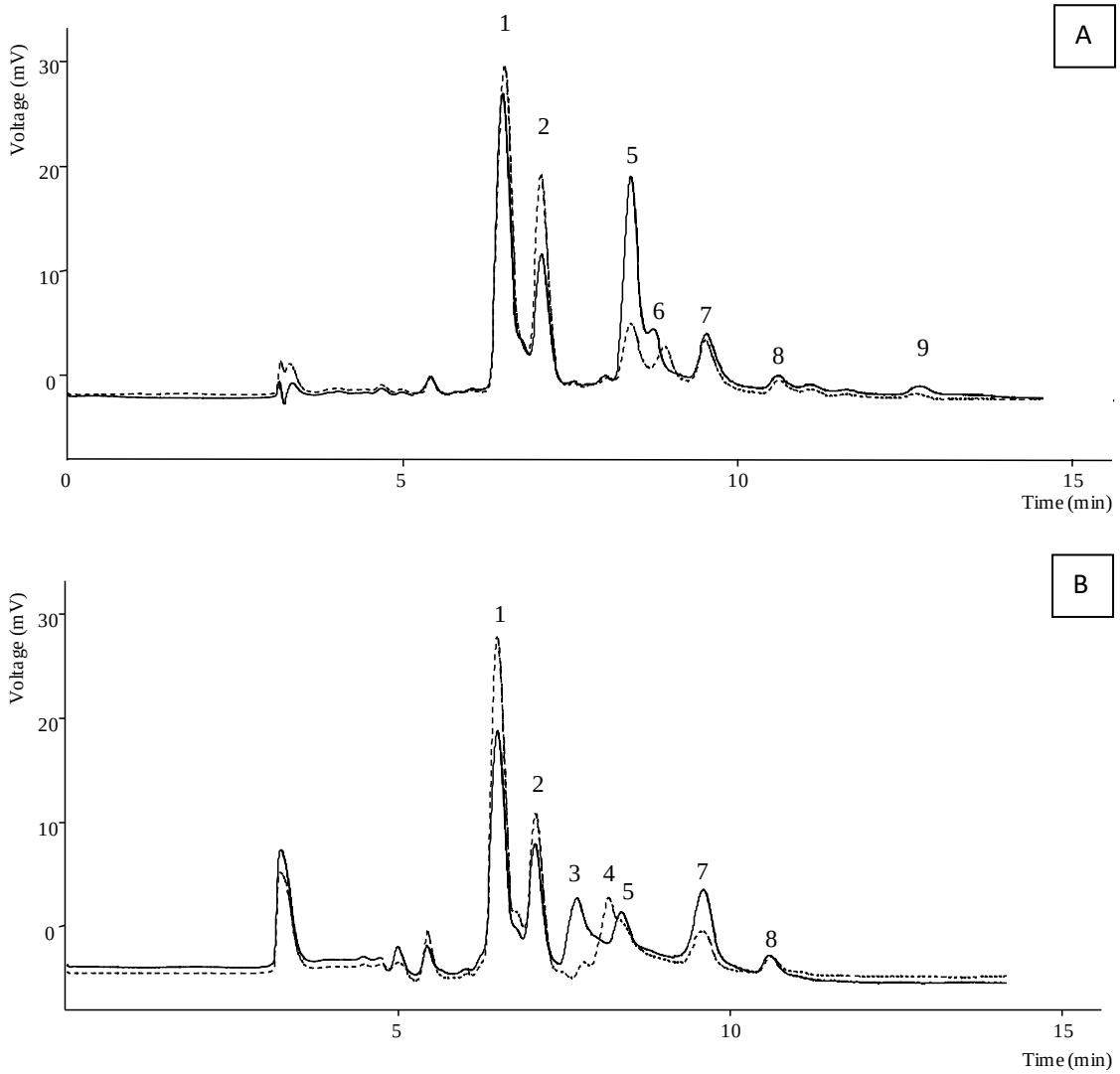

Figure 20 (A) Free sugars in the infusion (—) and decoction (----) of *Tuberaria lignosa* shade-dried sample: 1-fructose; 2-glucose; 5-sucrose; 7-trehalose and 9-raffinose. (B) Free sugars in the infusion (----) and decoction (—) of commercial sample: 1-fructose; 2- glucose; 5-sucrose; 7-trehalose. Peaks 3, 4, 6 and 8 were not identified.

Flavones and flavonols were also found in the studied samples. Peak 7 showed a pseudomolecular ion [M-H]$^-$ at *m/z* 609 that released three MS^2 fragments ions at *m/z* 489 and 399, corresponding to losses of 120 and 90 mu, characteristic of *C*-hexosyl flavones, and at *m/z* 369 that might correspond to the luteolin aglycone bearing some sugar residues [luteolin + 83 mu] that remained attached to it (Ferreres *et al.* 2003, 2004). The fact that no

relevant fragment derived from the loss of a complete hexosyl residue (-162 mu) was detected suggested that both sugars were *C*-attached, which allowed tentatively identifying the compound as luteolin-6-*C*-glucose-8-*C*-glucose. Peak 8 had a similar fragmentation pattern with a pseudomolecular ion $[M-H]^-$ at *m/z* 593, being tentatively identified as apigenin-6-*C*-glucose-8-*C*-glucose. Peaks 10 and 15 showed the same pseudomolecular ion $[M-H]^-$ at *m/z* 447 giving place to three MS^2 fragment ions, a major one at *m/z* 357 $[M-90]^-$, and other two at *m/z* 327 $[M-120]^-$ and at *m/z* 429 $[M-18]^-$. The fragmentation pattern was characteristic of *C*-glycosylated flavones at C-6/C-8, and the relative abundance of fragments pointed out to sugar substitution at C-8 (peak 10) at C-6 (peak 15) according to the fragmentation patterns described by Ferreres *et al*. (2003, 2004). These peaks were respectively identified as luteolin-8-*C*-glucoside and luteolin-6-*C*-glucoside; the identity of this latter was further confirmed by comparison with an authentic standard. Similarly, peaks 11 and 13, with the same pseudomolecular ion ($[M-H]^-$ at *m/z* 431) and similar MS^2 behaviour, were identified as apigenin-8-*C*-glucoside and apigenin-6-*C*-glucoside, this latter also confirmed by comparison with a standard.

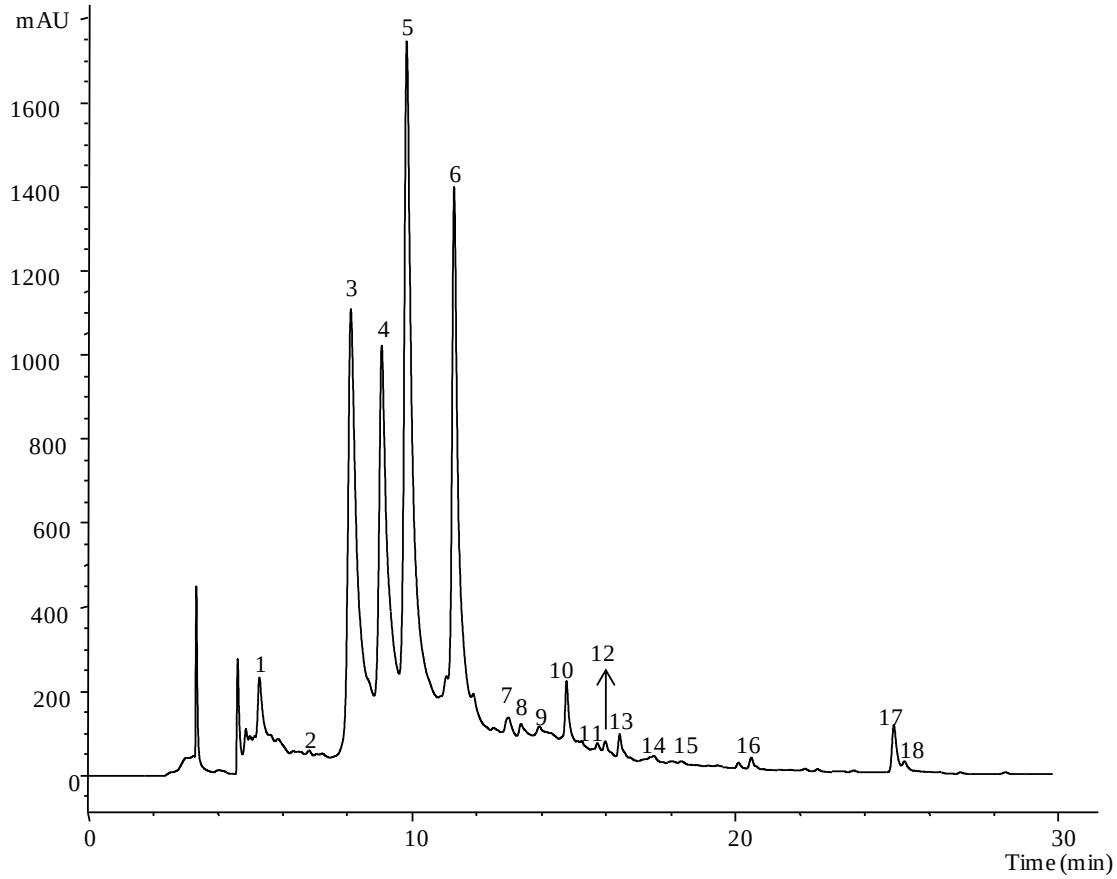

Figure 21 HPLC profile of phenolic compounds in the infusion of *Tuberaria lignosa* shade-dried sample, recorded at 280 nm.

Peaks 12 and 14 were identified respectively as quercetin 3-*O*-rutinoside (rutin) and kaempferol-3-*O*-rutinoside according to their retention time, UV spectra and mass characteristics compared with authentic standards. Peak 16 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 739, 146 mu greater than a kaempferol-rutinoside, which may correspond to either a rhamnosyl or a *p*-coumaroyl moiety. No good UV spectrum could be obtained for this peak so as to confirm or discard the presence of a *p*-coumaroyl moiety (additional peak or shoulder around 310 nm). However, since the presence of such a hydroxycinnamoyl residue should be reflected in a greater loss of polarity and subsequent delay in its elution, the peak was tentatively identified as a kaempferol-*O*-rhamnoside-*O*-rutinoside.

Table 15 Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds in the decoctions/infusions of *Tuberaria lignosa*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.2	268/378	781	781(100), 721(7), 601(20), 299(40)	Punicalin
2	6.94	270	325	325(36), 169(100), 125(27)	Monogalloylglucose
3	8.2	259/378	1083	1083(100), 781(6), 601(14), 301(4)	Punicalagin isomer 1
4	9.2	258/378	1251	1251(72), 1083(23), 781(3), 601(10), 301(22)	Punicalagin gallate isomer 1
5	9.98	258/378	1083	1083(100), 781*, 601(12), 301(10)	Punicalagin isomer 2
6	11.42	264/378	1251	1251(60), 1083(11), 781(6), 601(40), 301(51)	Punicalagin gallate isomer 2
7	12.03	350	609	609(100), 489(33), 399(9), 369(16)	Luteolin-6-*C*-glucose-8-*C*-glucose
8	13.03	334	593	593(100), 473(13), 383(6), 353(21)	Apigenin-6-*C*-glucose-8-*C*-glucose
9	14.07	310	337	191(100), 173(16), 163(6)	5-*O*-*p*-Coumaroylquinic acid
10	14.92	350	447	429(7), 357(60), 327(100), 285(8)	Luteolin-8-*C*-glucoside
11	15.40	356	431	413(4), 341(37), 311(100)	Apigenin-8-*C*-glucoside
12	16.11	354	609	301(100)	Quercetin-3-*O*-rutinoside
13	16.58	334	431	413(8), 341(60), 311(100)	Apigenin-6-*C*-glucoside
14	17.58	348	593	285(100)	Kaempferol-3-*O*-rutinoside
15	18.57	350	447	357(4), 327(17), 285(24)	Luteolin-6-*C*-glucoside
16	20.93	-	739	593(100), 285(11)	Kaempferol-*O*-rhamnoside-*O*-rutinoside
17	25.20	314,354 sh	707	593(100), 285(14)	Kaempferol-*p*-coumaroylglucoside-glutarate
18	25.52	314,354 sh	593	447(10), 285(69)	Kaempferol-*p*-coumaroylglucoside

* relative abundances <1.

Peak 17 presented a pseudomolecular ion at *m/z* 707 releasing two MS^2 fragments at *m/z* 593 ($[M-H-114]^-$, corresponding to a possible glutarate moiety) and at *m/z* 285 ($[M-H-114-308]^-$, loss of a possible rutinoside or *p*-coumaroylglucoside moiety). The UV spectrum of the peak was not characteristic of a kaempferol-rutinoside as it presents maximum wavelength at 314 nm could confirm the presence of the *p*-coumaroyl residue, so that the

peak could be tentatively identified as kaempferol-*p*-coumaroylglucoside-glutarate. Peak 18 showed similar characteristics as peak 14, but it released an additional minor MS^2 fragment at *m/z* 447 (loss of 146 mu that may correspond to either a rhamnosyl or a *p*-coumaroyl moiety). Its delayed retention time and UV spectrum suggested that it may be kaempferol-*p*-coumaroylglucoside.

Finally, peaks 2 and 9 were associated to phenolic acid derivatives. The characteristics of peak 2, with a pseudomolecular ion $[M-H]^-$ at *m/z* 331 and a majority MS^2 fragment at *m/z* 169 ($[M-162]^-$, loss of a hexosyl moiety) consistent with gallic acid, allowed assigning it as a monogalloylglucose. Peak 9 ($[M-H]^-$ at *m/z* 337) was identified as 5-*O*-*p*-coumaroylquinic acid according to its MS^2 fragmentation pattern as reported by Clifford *et al.* (2003) and Clifford *et al.* (2006) for caffeoylquinic acid isomers.

As for the other analysed compounds (ascorbic acid and sugars), the type of sample preparation did not show a clear influence on the phenolic composition of the extracts (**Table 16**). Little differences were found among the concentrations and distribution of phenolics in the different extracts (decoction or infusion) obtained from the two types of wild *Tuberaria* samples (shade- or freeze-dried). However, much lower amounts of phenolic compounds, namely ellagitannins, were found in the preparations of the commercial sample. Punicalagin derivatives were the most abundant compounds in all the samples.

3.2.4. Discussion

In general, the antioxidants (phenolic compounds, vitamins, etc.) content of fresh plant materials is higher than that of dried plant materials due to their degradation during drying. However, some recent studies have shown that dried plant materials (including air-drying) contain higher antioxidants, such as polyphenolics, and antioxidant activity as compared to fresh plant materials (Suvarnakuta *et al.* 2011). Medicinal plants are often dried and stored for a long time before use in manufacturing various types of products (Lin *et al.* 2011). Dehydration is an important preservation method for plant material, as it inhibits enzymatic degradation and limits microbial growth of plants (Müller and Heindl 2006, Harbourne *et al.* 2009). Drying can be performed using different methods. Traditionally, in folk medicine, medicinal plants are dried in the shade (Sellami *et al.* 2011). Freeze-drying is generally better to preserve the quality of medicinal plants during processing (Abascal *et al.* 2005, Pinela *et al.* 2011). The length of storage period also has a significant influence in the quality of medicinal plants, reducing the antioxidant activity and antioxidants content (Guimarães *et al.* 2011).

In the present work, the influence of the drying process applied to wild samples was not so evident as previously observed by us and by others in other plant species, where freeze-drying proved to preserve more the composition, including phenolics, ascorbic acid,

sugars, tocopherols, chlorophylls, and lycopene contents, as well as antioxidant activity (Chan *et al.* 2009, Pinela *et al.* 2011). Nevertheless, Hossain *et al.* (2010) reported that air-dried samples had significantly higher antioxidants content and antioxidant capacity than had freeze-dried and vacuum oven-dried samples. Fresh samples had the lowest values for the parameters tested. Oven-drying resulted in higher parameters than did freeze-drying. As stated, the effects of drying methods on antioxidants and antioxidant activity are not conclusive. In fact, in the performed study, the effects of drying process were not evident and, as it can be observed in **Table 14** and **Table 16**, the magnitude of concentrations of the different compounds in wild samples is similar. This absence of marked effects of drying methods was also reported by Hsu *et al*. (2003).

Table 16 Quantification (mg/g) of the phenolic compounds in the decoctions/infusions of the different samples of *Tuberaria lignosa*.

	Wild sample				**Commercial sample**	
	Freeze-dried		**Shade-dried**			
Peak	**Decoction**	**Infusion**	**Decoction**	**Infusion**	**Decoction**	**Infusion**
1	tr	tr	1.24 ± 0.03	tr	11.72 ± 0.94	11.22 ± 0.13
2	tr	tr	tr	tr	tr	tr
3	44.73 ± 2.26	43.28 ± 0.94	46.22 ± 1.29	51.18 ± 0.27	39.56 ± 0.61	38.03 ± 0.57
4	51.76 ± 2.82	49.94 ± 0.03	46.26 ± 0.04	49.98 ± 0.87	7.27 ± 0.44	5.20 ± 0.15
5	78.20 ± 0.91	79.42 ± 1.50	78.47 ± 1.29	89.64 ± 1.10	67.84 ± 0.12	64.10 ± 1.23
6	46.50 ± 1.53	44.85 ± 2.10	40.53 ± 1.98	41.88 ± 0.50	7.79 ± 0.12	6.81 ± 0.63
7	0.68 ± 0.03	1.03 ± 0.06	0.71 ± 0.01	0.69 ± 0.01	0.84 ± 0.11	0.79 ± 0.09
8	1.63 ± 0.03	1.94 ± 0.49	1.81 ± 0.02	1.73 ± 0.11	2.01 ± 0.25	1.87 ± 0.11
9	0.38 ± 0.08	0.53 ± 0.06	0.34 ± 0.01	0.36 ± 0.06	0.84 ± 0.12	0.93 ± 0.02
10	5.26 ± 0.26	3.86 ± 0.36	4.92 ± 0.12	4.20 ± 0.03	2.18 ± 0.18	2.09 ± 0.19
11	1.47 ± 0.01	1.56 ± 0.04	1.55 ± 0.00	1.55 ± 0.02	1.65 ± 0.02	1.57 ± 0.04
12	0.79 ± 0.02	0.60 ± 0.02	0.68 ± 0.02	0.70 ± 0.02	0.53 ± 0.04	0.54 ± 0.05
13	2.47 ± 0.07	2.39 ± 0.01	2.40 ± 0.01	2.15 ± 0.00	1.81 ± 0.09	1.80 ± 0.07
14	0.77 ± 0.02	0.40 ± 0.02	0.51 ± 0.02	0.46 ± 0.00	0.72 ± 0.06	0.80 ± 0.03
15	0.05 ± 0.00	0.03 ± 0.00	0.06 ± 0.00	0.04 ± 0.01	0.21 ± 0.01	0.20 ± 0.01
16	0.33 ± 0.02	0.32 ± 0.00	0.28 ± 0.00	0.27 ± 0.02	0.24 ± 0.01	0.31 ± 0.02
17	4.54 ± 0.08	3.78 ± 0.23	4.12 ± 0.05	3.79 ± 0.04	4.80 ± 0.09	4.18 ± 0.43
18	1.49 ± 0.03	1.35 ± 0.08	1.26 ± 0.05	1.37 ± 0.02	1.16 ± 0.07	1.42 ± 0.15
TPA	0.38 ± 0.08^{c}	0.53 ± 0.06^{b}	0.34 ± 0.01^{c}	0.36 ± 0.06^{c}	0.84 ± 0.12^{a}	0.93 ± 0.02^{a}
TEAD	221.19 ± 7.51^{ba}	217.48 ± 4.65^{b}	212.73 ± 4.63^{b}	232.67 ± 0.47^{a}	134.18 ± 0.13^{c}	125.37 ± 0.90^{c}
TF	19.48 ± 0.50^{a}	17.27 ± 0.19^{cb}	18.31 ± 0.01^{b}	16.96 ± 0.13^{c}	16.16 ± 0.81^{cd}	15.56 ± 1.11^{d}
TP	241.18 ± 7.97^{ba}	235.34 ± 4.65^{b}	231.53 ± 4.64^{b}	250.14 ± 0.57^{a}	151.08 ± 0.74^{c}	141.72 ± 2.13^{c}

tr- traces; TPA- total phenolic acid derivatives; TEAD- total ellagic acid derivatives; TF- total flavonoids; TP- total phenolic compounds. In each row different letters mean significant differences ($p<0.05$).

Regarding the influence of preparation method (decoction/infusion), the expected higher degradation of compounds in decoctions was not always observed. This could be due to the different amounts of sample used to prepare decoctions and infusions. To perform the present study, it was decided to use the amounts exactly indicated by local informants in order to mimic the traditional use of *T. lignosa*. Therefore, a higher amount was used in decoctions preparation, leading to a higher extraction yield (**Table 13**) that probably compensated for the loss of compounds. It should be highlighted that consumers using higher amounts of plant material for decoctions than for infusions, empirically know what was proved experimentally herein.

The most relevant effects were observed between wild and commercial samples that gave completely different amounts of antioxidants and antioxidant activity. The loss of phytochemical properties observed in commercial samples could be linked to processing (drying, preserving and packing) and storage time, since the vegetal material of wild and commercial sample could come from the same localities, although gathered separately in different periods.

3.2.5. Conclusions

Freeze-dried wild sample (infusion) gave the highest levels of sugars, while shade-dried wild sample (infusion) and freeze-dried sample (decoction) presented higher ascorbic acid and phenolic compounds (including ellagic acid derivatives and flavonoids) content than the other samples. The last two mentioned samples also revealed higher antioxidant activity than the other samples and, in some cases, even higher than Trolox. Decoctions gave lower amounts of disaccharides than infusions, which seemed to be hydrolysed, increasing the content in monosaccharides. Commercial samples showed the lowest contents in all the analysed compounds, and especially in ellagitannins and flavonoids, which was also consistent with their lower antioxidant activity in all the assays compared with the wild samples.

As far as we know this is the first detailed characterisation of the antioxidant properties and phytochemical composition of wild and commercial samples of *T. lignosa*. The obtained results give scientific and technical support to the traditional uses of the wild plant in folk medicine, highlighting its decoctions and infusions as a source of bioactive compounds (*e.g.,* phenolic compounds and ascorbic acid) to be used as functional beverages. Further studies should be performed in order to understand the mechanism of action of the mentioned compounds.

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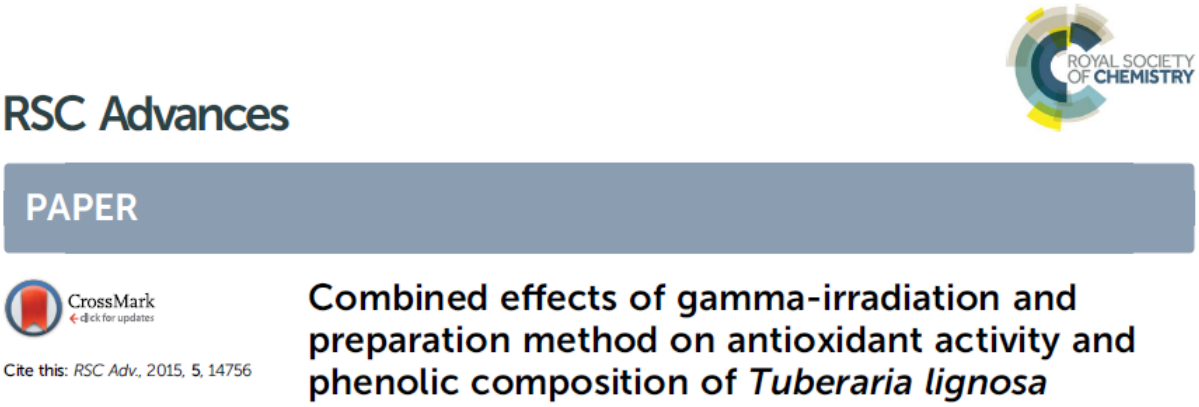
3.3. Combined effects of gamma irradiation and preparation method on antioxidant activity and phenolic composition of *Tuberaria lignosa*



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Abstract

In this study, the effect of different doses of gamma-irradiation (0, 1, 5 and 10 kGy) on colour, antioxidant activity and phenolic compounds of shade-and freeze-dried samples of *Tuberaria lignosa* were evaluated and compared. The last two parameters were performed on decoctions and infusions in order to investigate the influence of the preparation method as well. In general, gamma-irradiation has no influence on colour parameter; changes caused by this technology were only identifiable on the lipid peroxidation inhibition capacity of the shade-dried samples and also on a few phenolic compounds. Differences among preparation method were significant for all assayed parameters, being decoctions preferable over infusions, as indicated by the higher antioxidant activity and levels of phenolic compounds. Overall, the gamma-irradiation treatment (up to 10 kGy) did not significantly affect the

analysed parameters. Nevertheless, other studies are of interest to evaluate the preservation effectiveness of this technology.

Keywords: *Tuberaria lignosa*; infusion/decoction; drying/irradiation; colour parameters; antioxidant activity; phenolic compounds.

3.3.1. Introduction

During the last decades, medicinal and aromatic plants have been extensively studied and found to be excellent sources of bioactive and health-promoting compounds (Guimarães *et al.* 2013, Roriz *et al.* 2014). Actually, many plant extracts rich in phenolic compounds are used as food complements or can be integrated into cosmetic or pharmaceutical formulations (Barroso *et al.* 2014, Martins, Barros, Carvalho, *et al.* 2014). For this reason, the industry is strongly interested in bioactive molecules from natural sources.

Based on ethnobotanical surveys conducted in western regions of the Iberian Peninsula, *Tuberaria lignosa* (Sweet) Samp. (Fam. Cistaceae) arises as one of the most quoted medicinal plants (Castroviejo 2005, Carvalho and Morales 2010). After being dried, this plant is used in herbal preparations (infusion and decoction) for treating various diseases and ailments, such as gastrointestinal and hepato-depurative disorders and skin inflammations (Carvalho and Morales 2010). These local practices are supported by documented biological effects, namely anti-inflammatory and antiulcerogenic (cytoprotective) properties (Martín-Aragón *et al.* 1994), as well as *in vitro* antioxidant (Pinela *et al.* 2012) and antiviral activities (Bedoya *et al.* 2010). Additionally, the phenolic fraction of this plant, mainly composed of ellagitannins and flavonoids, may be linked to the above mentioned effects (Bedoya *et al.* 2010, Pinela *et al.* 2012).

During the entire production process (from harvesting and drying to packaging and storage), raw medicinal plants are prone to chemical and microbial contaminations and insect infestation, which can lead to spoilage, quality deterioration and consequent economic loss (Darfour *et al.* 2014, Pereira *et al.* 2014). Besides being a health hazard to consumers, contaminated medicinal plants can also adversely affect the efficacy and stability of their bioactive compounds, especially during storage (Aouidi *et al.* 2011), and lead to spoilage of pharmaceuticals and food items to which they are added (Machhour *et al.* 2011). Therefore, an effective and sustainable decontamination process must be followed to ensure the hygienic quality of these products, making them suitable for human consumption and commercialization.

Chemical fumigants have been used to decontaminate plant products, being now prohibited or increasingly restricted in several countries due to health, environmental or occupational safety issues (UNEP 2010). Furthermore, once conventional thermal treatments

can damage many plant properties, either chemical or physical (Rawson *et al.* 2011), new and emerging non-thermal technologies are being investigated and applied. Among them, irradiation processing with gamma-rays is in an exceptional position. This physical method, considered safe and effective by several international authorities (namely FAO, IAEA and WHO) (WHO 1999), has been used for insect disinfestations and parasite inactivation (with low doses up to 1 kGy), reduction of non-spore forming pathogens and spoilage microorganisms (with medium doses from 1 to 10 kGy), and reduction of microorganisms to the point of sterility (achieved at high doses above 10 kGy) (ICGFI 1999, Machhour *et al.* 2011). Likewise, the gamma-irradiation treatment provides an alternative way to eliminate pesticide residues from plant products (Wen *et al.* 2010). In the European Union, the maximum dose of gamma-irradiation approved to sanitize dried herbs is 10 kGy (Directive 1999/3/EC of the European Parliament and of the Council of 22 February 1999 on the establishment of a Community list of foods and food ingredients treated with ionising radiation 1999), whereas in USA the maximum is 30 kGy (FDA 2012).

Meanwhile, there is a growing scientific interest in irradiation-induced modifications on antioxidant activity and the compounds responsible for such activity. It is known that during the irradiation process, free radicals and other reactive species are generated due to the interaction with water molecules, capable of breaking chemical bonds and modify various molecules (Aouidi *et al.* 2011). A previous study conducted by our team on *T. lignosa* showed that it has strong antioxidant activity (Pinela *et al.* 2012); however, the effects of gamma-irradiation on the chemical and physical properties of this plant have never been studied. Therefore, the present study was undertaken to explore the effect of different doses of gamma-irradiation (0, 1, 5 and 10 kGy) on the antioxidant activity, phenolic compounds and colour parameters of shade- and freeze-dried *T. lignosa* samples. The first two parameters were performed on decoctions and infusions, forms traditionally used for therapeutic applications, in order to investigate the influence of the preparation method as well.

3.3.2. Materials and methods

3.3.2.1. Standards and reagents

For irradiation: The dose rate of irradiation was estimated by Fricke dosimetry, using a chemical solution sensitive to ionizing radiation prepared in the lab following the standards (ASTM 1992). The irradiation dose was estimated during the process using Amber Perspex routine dosimeters (batch V, from Harwell Company, UK). To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona,

Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

For antioxidant activity evaluation: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system.

For phenolic compounds analysis: Acetonitrile (99.9%, HPLC grade) was purchased from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (apigenin-6-*C*-glucoside, *p*-coumaric acid, ellagic acid, gallic acid, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, luteolin-6-C-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) were purchased from Extrasynthese (Genay, France). Water was treated in a Milli-Q water purification system.

3.3.2.2. Plant samples

Tuberaria lignosa (Sweet) Samp. (synonym of *Xolantha tuberaria* (L.) Gallego, Munoz Garm & C. Navarro) was collected in the flowering season in Miranda do Douro (Trás-os-Montes, north-eastern Portugal), considering the local medicinal uses as well as healers' and selected consumers' criteria, which are related to particular gathering sites and requirements for safe herbal dosages forms, such as infusion and decoction.

The option for wild samples, instead of ones from commercial origin, was supported by a previous work of our research team that highlighted wild *T. lignosa* samples as having higher phenolics content and antioxidant activity than those obtained in a local herbal shop available as dried rosettes of leaves and inflorescences (Pinela *et al.* 2012). While the plant material collected in the field is fresh, the commercial one from herbal shops may have been stored for a long period of time or dried differently, which leads to quality loss.

Voucher specimens were deposited in the Herbarium of the Escola Superior Agrária de Bragança, Portugal.

3.3.2.3. Samples drying

Tuberaria lignosa flowering aerial parts (*e.g.,* basal leaves, stems and inflorescences) were submitted to two different drying methods: freeze-drying (7750031 Free Zone 4.5, Labconco, Kansas City, MO, USA) immediately after being gathered; and shade-drying, being stored in a dark and dry place in cellophane or paper bags kept at room temperature (~21 ºC and 50% relative humidity) for 30 days, simulating informants' general conditions of traditional plant-use.

3.3.2.4. Samples irradiation

Freeze- and shade-dried samples were divided into four groups (conveniently packaged in sterilized polyethylene bags): control (non-irradiated, 0 kGy), sample irradiated at 1 kGy, sample irradiated at 5 kGy, and sample irradiated at 10 kGy, where 1, 5 and 10 kGy were the predicted doses. The samples irradiation was performed in a ^{60}Co experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with four sources, total activity 177 TBq (4.78 kCi), in January 2014. The estimated dose rate for the irradiation position was obtained with a Fricke dosimeter. During irradiation process, the dose was estimated using Amber Perspex routine dosimeters, following the procedure previously described by Fernandes *et al.* (2013). The estimated doses after irradiation were: 0.92 ± 0.01 kGy, 4.63 ± 0.28 kGy and 8.97 ± 0.35 kGy for the freeze-dried samples irradiated at 1, 5 and 10 kGy, respectively; and 1.00 ± 0.04 kGy, 5.07 ± 0.27 kGy and 9.66 ± 0.90 kGy for the shade-dried samples irradiated at 1, 5 and 10 kGy, respectively. The dose rate was 1.9 kGy.h^{-1} and the dose uniformity ratio (D_{max}/D_{min}) was 1.1 for the freeze- and shade-dried sample irradiated at 1 kGy, and 1.2 for the freeze- and shade-dried sample irradiated at 5 and 10 kGy. For simplicity, in the text, tables and figures, the values 0, 1, 5 and 10 kGy were considered for the doses.

3.3.2.5. Colour measurement

A colorimeter (model CR-400; Konica Minolta Sensing, Inc., Japan) with an adapter for granular materials (model CR-A50) was used to measure the colour of the samples. Using illuminant C and the diaphragm opening of 8 mm, the CIE *L**, *a** and *b** colour space values were registered through the computerized system using a colour data software "Spectra Magic Nx" (version CM-S100W 2.03.0006). The instrument was calibrated using the standard white plate before analysis.

The colour of the shade-and freeze-dried irradiated and non-irradiated samples was measured in three different points on each set of samples, being considered the average value to determine the colour coordinates *L** (lightness ↔ darkness), *a** (redness ↔ greenness), and *b** (yellowness ↔ blueness).

3.3.2.6. Preparation of decoctions and infusions

To prepare decoctions, each sample (1 g) was added to 200 mL of distilled water, heated on a heating plate (VELP Scientific, Usmate, Italy) and boiled for 5 min. The mixture was left to stand at room temperature for 5 min more, and then filtered through Whatman No. 4 paper. To prepare infusions, each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered through Whatman No. 4 paper. A portion of the obtained decoctions and infusions was frozen and lyophilized (Free Zone

4.5, Labconco, Kansas City, MO, USA) for subsequent analysis of phenolic compounds. The antioxidant properties were evaluated directly on the decoctions/infusions.

3.3.2.7. In vitro antioxidant properties

General: The decoctions and infusions were redissolved in water (final concentration 1 mg/mL) and further diluted to different concentrations to be submitted to distinct *in vitro* assays (Pinela *et al.* 2012) to evaluate their antioxidant properties. The extract concentration providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching inhibition and TBARS formation inhibition assays) or absorbance at 690 nm (reducing power assay) against extract concentrations. Trolox was used as standard.

DPPH radical-scavenging activity: This methodology was performed using an ELX800 Microplate Reader (Bio-Tek, Potton, UK). The reaction mixture in each one of the 96-wells consisted of one of the different solution concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} M). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $RSA\ (\%) = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the D_{PPH} solution.

Reducing power: This methodology was performed using the microplate reader described above. The different solution concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mM, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in 48-well plates, with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm.

Inhibition of β-carotene bleaching: A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the samples (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm in a Model 200 spectrophotometer

(AnalytikJena, Jena, Germany). β-carotene bleaching inhibition (CBI) was calculated using the following equation: CBI (%) = (Abs after 2 h of assay/ initial Abs) × 100

Inhibition of thiobarbituric acid reactive substances (TBARS) formation: Porcine *(*Sus scrofa) brains were obtained from official slaughtered animals, dissected, and homogenised with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000 g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100, where A and B were the absorbance of the control and the compound solution, respectively.

3.3.2.8. Phenolic compounds

Each lyophilised decoction/infusion (1 mg) was dissolved in water:methanol (80:20 v/v), filtered through 0.2 µm nylon filters from Whatman, and analysed by HPLC (Hewlett–Packard 1100 chromatograph, Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP ChemStation (Rev. A.05.04) data-processing station. A Spherisorb S3 ODS-2 C18 (Waters, Dinslaken, Germany), 3 µm (4.6 mm × 150 mm) column thermostatted at 35 ºC was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15-25% B over 5 min, 25-35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser that was controlled by Analyst 5.1 software. Zero grade air served as the nebuliser gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive scan modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to obtain full scan spectra, to give an overview of all the ions in

sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10 V. Spectra were recorded in negative ion mode between *m/z* 100 and 1500. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25 V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the decoctions/infusions were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations (2.5-100 µg/mL) of different standard compounds: apigenin-6-*C*-glucoside (y = 246.05 x - 309.66; R^2 = 0.9994); *p*-coumaric acid (y = 321.99x + 98.308; R^2 = 0.9984); ellagic acid (y = 35.695x - 265.7; R^2 = 0.9991); gallic acid (y = 556.94x + 738.37; R^2 = 0.9968); kaempferol-3-*O*-glucoside (y = 190.75x - 36.158; R^2 = 1); kaempferol-3-*O*-rutinoside (y = 17 5.02x - 43.877; R^2 = 0.9999); luteolin-6-*C*-glucoside (y = 365.93x + 17.836; R^2 = 0.9997); quercetin-3-*O*-glucoside (y = 316.48x + 2.9142; R^2 = 1), and quercetin-3-*O*-rutinoside (y = 222.79x + 243.11; R^2 = 0.9998). The results were expressed in mg per g of lyophilised decoction/infusion.

3.3.2.9. Statistical analyses

In all cases, analyses were carried out using three samples separately processed, each of which was further measured three times. Data were expressed as mean±standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analysed using 2-way ANOVA, with the factors “irradiation dose” (ID) and “preparation method” (PM). When a statistically significant interaction (ID×PM) was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction was verified, means were compared using Tukey’s honestly significant difference (HSD), or other multiple comparison test (*t*-test).

Principal components analysis (PCA) was applied as pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach’s alpha parameter (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

3.3.3. Results and discussion

3.3.3.1. Colour assessment

The results for CIE colour *L** (lightness), *a** (redness) and *b** (yellowness) are presented in **Table 1**. The colour coordinate *L** measures the lightness of the sample surface and ranges from black at 0 to white at 100. The chromaticity coordinate *a** measures red when positive and green when negative, and chromaticity coordinate *b** measures yellow when positive and blue when negative (Argyropoulos and Müller 2014). The reported values are given as the mean value of each irradiation dose (ID), including results from shade-or freeze-dried samples, as well as the mean value of each drying method (DM), considering all irradiation doses in each case. The significance of the effect of DM was evaluated using a *t*-test for equality of means (after checking the equality of variances through a Levene's test), since there were fewer than three groups. The interaction among factors (ID×DM) was never significant ($p > 0.05$), allowing to compare the effects of each factor *per se*. As it can be concluded from **Table 17**, the effect of ID was not significant in any case, indicating that these physical parameters are not strongly affected by gamma-irradiation. On the other hand, the effect of the DM was always significant, showing that samples dried under shade are prone to present lower lightness and redness and higher yellowness. According to the literature, higher *L** values and lower *a**/*b** values are desirable in dried products (Rahimmalek and Goli 2013). Therefore, freeze-drying may be indicated as the most suitable DM for *T. lignosa* samples. Additionally, the lack of significant changes observed in irradiated samples might be an advantageous feature, since the colour parameters are of great importance in food and cosmetics industry (Jo, Son, Shin, *et al.* 2003). In fact, the colour of dried medicinal and aromatic plants is considered as a primary quality criterion (Argyropoulos and Müller 2014) and is directly related to consumers' appreciation of a product as they tend to associate product colour with its taste, hygienic security, shelf-life and personal satisfaction (Sturm *et al.* 2014).

3.3.3.2. Antioxidant activity

The EC_{50} values obtained for each antioxidant assay are presented in **Table 18**, both for shade-dried and freeze-dried samples. The reported values are given as the mean value of each irradiation dose (ID), including results from samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), containing the results for all assayed doses in each case. The significance of the effect of PM was evaluated using a *t*-test for equality of means (after checking the equality of variances through a Levene's test), since there were fewer than three groups. The interaction among factors (ID×PM) was significant ($p < 0.05$) in all cases, acting as a source of variability. Thereby, no multiple comparison tests could be performed. However, some conclusions could be drawn after

analysing the estimated marginal mean (EMM) plots. For instance, shade-dried (**Figure 22A)** and freeze-dried (**Figure 22B)** samples, further extracted by decoction, gave greater antioxidant activity than infusion ones. Concerning the effect of ID, the only identifiable tendency was the apparently negative effect of the 5 kGy dose on the lipid peroxidation inhibition capacity in shade-dried samples.

Table 17 CIE colour *L** (lightness), *a** (redness) and *b** (yellowness) of non-irradiated and irradiated *T. lignosa* shade- or freeze-dried samples. The results are presented as mean±SD[a].

		L*	***a****	***b****
Irradiation dose (ID)	0 kGy	47 ± 5	0 ± 3	17 ± 3
	1 kGy	46 ± 6	0 ± 3	18 ± 3
	5 kGy	45 ± 5	-2 ± 3	18 ± 2
	10 kGy	43 ± 7	-1 ± 3	18 ± 3
p-value (n = 18)	Tukey's test	0.154	0.252	0.770
Drying method (DM)	Shade-dried	41 ± 5	-2 ± 2	19 ± 2
	Freeze-dried	49 ± 4	1 ± 2	17 ± 3
p-value (n = 36)	*t*-student's test	<0.001	<0.001	<0.001
p-value (n = 72)	ID×DM	0.253	0.262	0.077

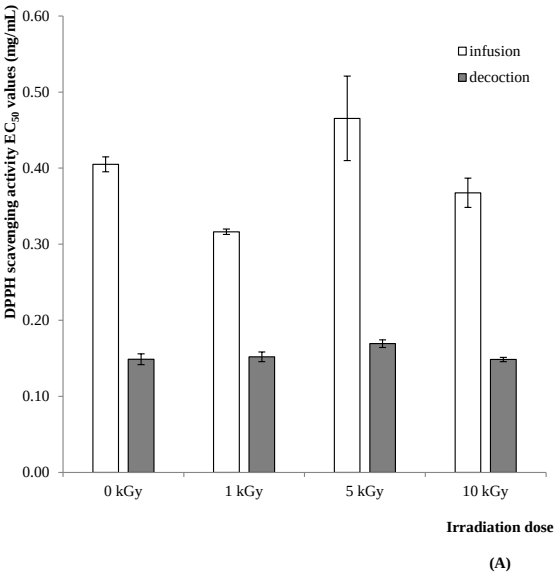
[a]Results are reported as mean values of each irradiation dose (ID), including results from shade- or freeze-dried samples, as well as the mean value of each drying method (DM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or DM), and can be higher than mean values.

The interest of decoctions and infusions from shade- and freeze-dried samples of *T. lignosa* was already highlighted as a source of bioactive compounds and having appreciable antioxidant properties (Pinela *et al.* 2012). The same work also highlighted wild *T. lignosa* samples as having higher phenolics content and bioactivity than those obtained in a local herbal shop available as dried rosettes of leaves and inflorescences. That is why wild samples were chosen for this study instead of ones from commercial origin.

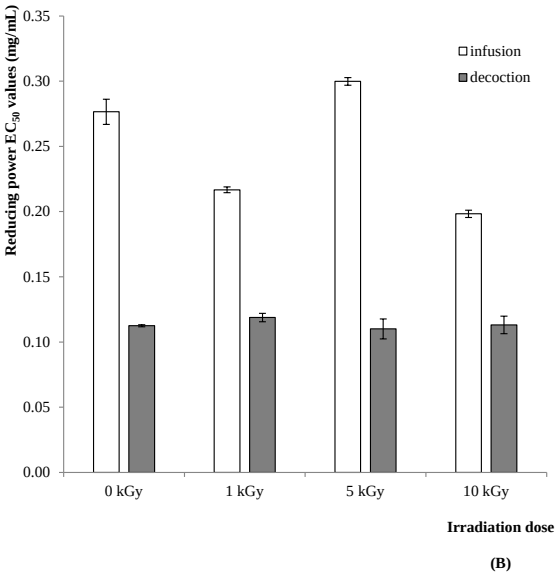
Table 18 Antioxidant properties (EC_{50} values, mg/mL) of non-irradiated and irradiated *T. lignosa* shade- or freeze-dried samples, according to the irradiation dose (ID) and preparation method (PM). The results are presented as mean±SD[a].

		DPPH scavenging activity	Reducing power	TBARS formation inhibition	β-carotene bleaching inhibition
Shade-dried					
Irradiation dose (ID)	0 kGy	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.04 ± 0.02
	1 kGy	0.2 ± 0.1	0.17 ± 0.05	0.2 ± 0.1	0.02 ± 0.01
	5 kGy	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.07 ± 0.04
	10 kGy	0.3 ± 0.1	0.16 ± 0.04	0.3 ± 0.1	0.03 ± 0.01
p-value (n = 18)	Tukey's test	0.242	0.160	<0.001	<0.001
Preparation method (PM)	Infusion	0.39 ± 0.05	0.25 ± 0.04	0.4 ± 0.1	0.05 ± 0.04
	Decoction	0.15 ± 0.01	0.11 ± 0.01	0.2 ± 0.1	0.025 ± 0.002
p-value (n = 45)	*t*-student's test	<0.001	<0.001	<0.001	<0.001
p-value (n = 90)	ID×PM	<0.001	<0.001	<0.001	0.046
Freeze-dried					
Irradiation dose (ID)	0 kGy	0.3 ± 0.1	0.16 ± 0.05	0.02 ± 0.02	0.3 ± 0.1
	1 kGy	0.3 ± 0.2	0.2 ± 0.1	0.03 ± 0.02	0.2 ± 0.1
	5 kGy	0.3 ± 0.1	0.2 ± 0.1	0.02 ± 0.01	0.2 ± 0.1
	10 kGy	0.3 ± 0.2	0.2 ± 0.1	0.03 ± 0.01	0.2 ± 0.1
p-value (n = 18)	*p*-value (n=18)	0.861	0.386	0.430	0.528
Preparation method (PM)	Infusion	0.41 ± 0.05	0.26 ± 0.04	0.04 ± 0.01	0.35 ± 0.05
	Decoction	0.15 ± 0.02	0.10 ± 0.01	0.01 ± 0.01	0.11 ± 0.05
p-value (n = 45)	*t*-student's test	<0.001	<0.001	<0.001	<0.001
p-value (n = 90)	ID×PM	<0.001	<0.001	<0.001	<0.001

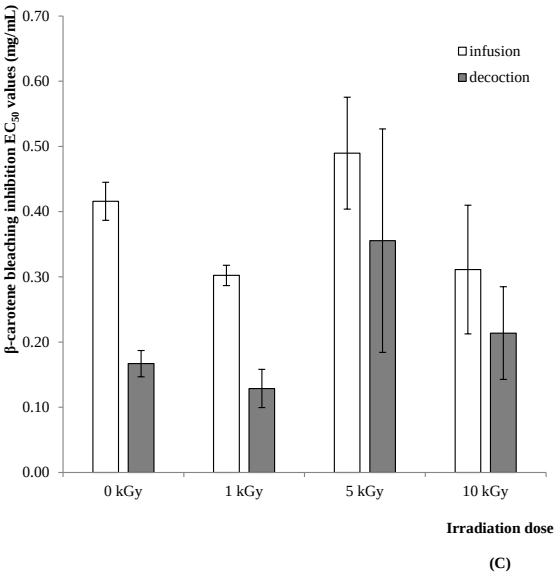
[a]Results are reported as mean values of each irradiation dose (ID), including samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or PM), and can be higher than mean values.


DPPH scavenging activity EC50 values (mg/mL)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
infusion
decoction
0 kGy
1 kGy
5 kGy
10 kGy
Irradiation dose

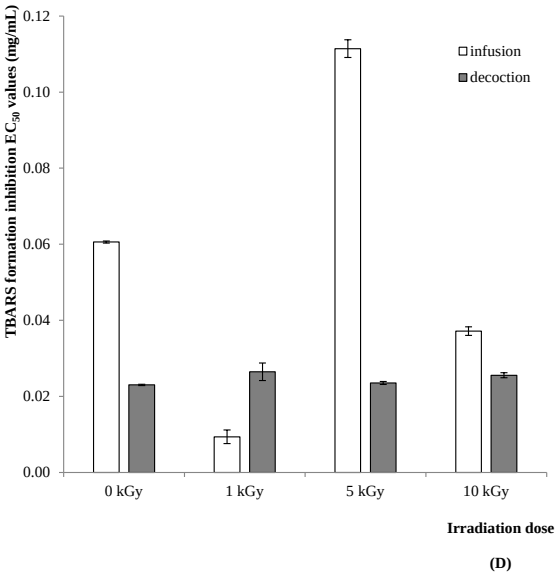
(A)


Reducing power EC50 values (mg/mL)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
infusion
decoction
0 kGy
1 kGy
5 kGy
10 kGy
Irradiation dose

(B)

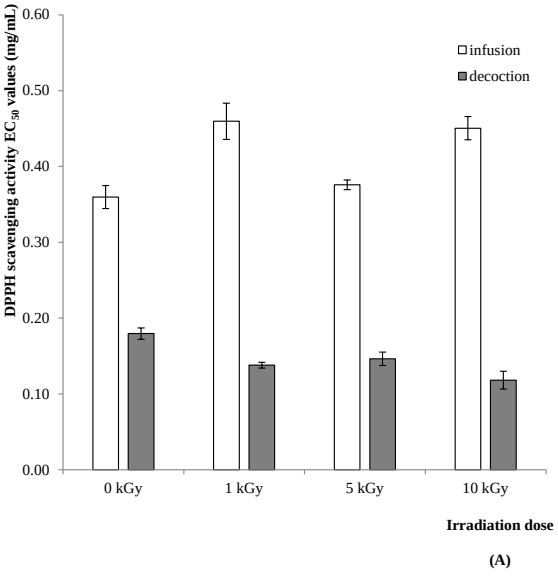

β-carotene bleaching inhibition EC50 values (mg/mL)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
infusion
decoction
0 kGy
1 kGy
5 kGy
10 kGy
Irradiation dose

(C)

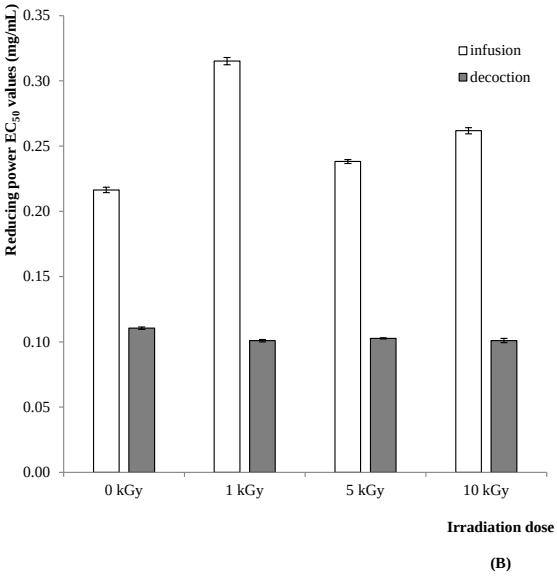

TBARS formation inhibition EC50 values (mg/mL)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
infusion
decoction
0 kGy
1 kGy
5 kGy
10 kGy
Irradiation dose

(D)

A


DPPH scavenging activity EC50 values (mg/mL)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
infusion
decoction
0 kGy
1 kGy
5 kGy
10 kGy
Irradiation dose

(A)


Reducing power EC50 values (mg/mL)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
infusion
decoction
0 kGy
1 kGy
5 kGy
10 kGy
Irradiation dose

(B)

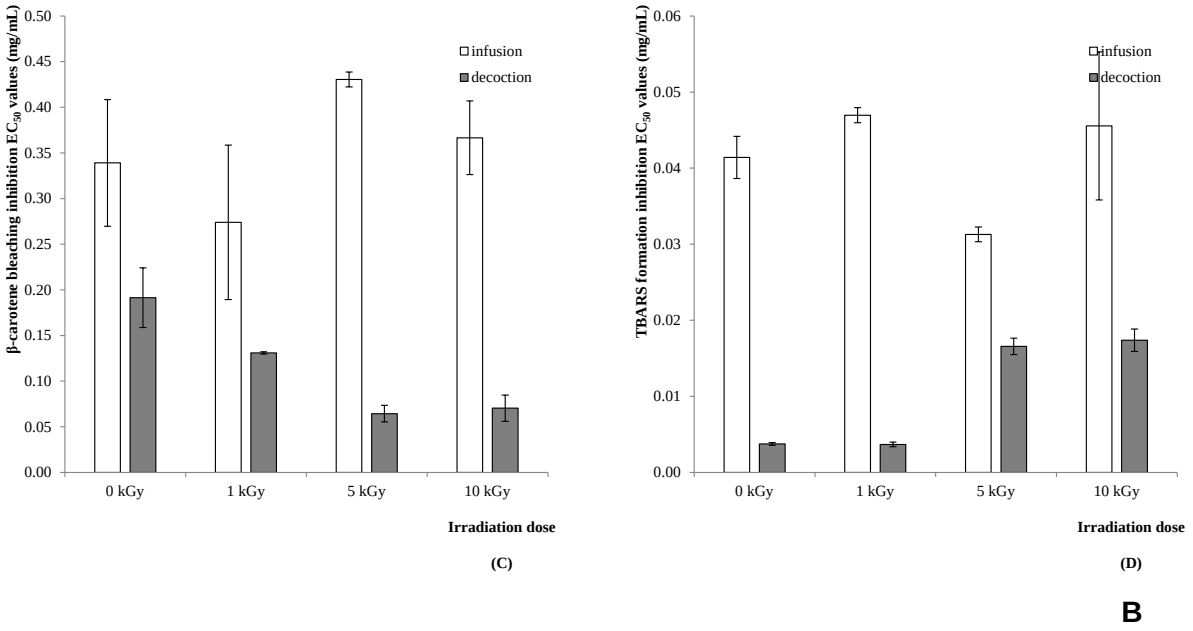


Figure 22 Estimated marginal mean plots representing the effect of the preparation method on the antioxidant properties of shade-dried (A) and freeze-dried (B) samples of *T. lignosa*. A- DPPH scavenging activity; B- reducing power; C- β-carotene bleaching inhibition; D- TBARS formation inhibition.

Although the antioxidant activities of different medicinal and aromatic plants have already been studied (Guimarães *et al.* 2013, Barroso *et al.* 2014, Roriz *et al.* 2014), nothing has been reported on the effect of gamma-irradiation on the antioxidant activity of *T. lignosa*. However, some research studies report different effects of the gamma-irradiation treatment on the antioxidant properties of other plant materials. A study conducted by Pereira *et al.* (Pereira *et al.* 2014) indicated that, in general, the antioxidant properties were increased in borututu (a folk medicine obtained from the African tree *Cochlospermum angolense*) infusions and methanolic extracts with the irradiation dose of 10 kGy. Carocho *et al.*[27] found that the antioxidant potential of Portuguese chestnuts was increased at 3 kGy. As well, Hussain *et al.* (2013) reported a significant decrease in EC_{50} values (corresponding to a higher antioxidant activity) of sun-dried irradiated (3 kGy) apricots. According to Pérez *et al.* (2011) a 30 kGy dose applied to dry sage and oregano for sanitization did not significantly affect the capacity to inhibit the DPPH radical or the reducing power, nor did it affect the total phenolic content of the methanolic and aqueous extract. Similarly, Mustapha *et al.* (2014) observed no significant changes in the free radical scavenging activity of irradiate millet flour up to 5 kGy. In contrast, Kim and Yook (2009) observed that irradiation of kiwifruit up to 3 kGy had negative effects on vitamin C content and antioxidant activity.

Regarding the use of gamma-irradiation for preservation purposes, its suitability for sanitation of *T. lignosa* is unknown; nevertheless some studies support its effectiveness in similar doses for comparable natural matrices, including other dried medicinal and aromatic plants, without affecting their bioactive properties. Chiang *et al.* (2011) demonstrated that 2

kGy is sufficient for the inactivation of enterobacteria and 6 kGy for elimination of yeasts and fungi in *Polygonum multiflorum* Thunb. (an herb used in traditional Chinese medicine), without adversely compromising the total phenols content or the antioxidant potential. Likewise, Kumar *et al.* (2010) concluded that an irradiation dose up to 10 kGy is adequate to ensure the microbiological decontamination of Indian herbs retaining their antioxidant properties. Furthermore, in the European Union, the maximum dose of gamma-irradiation approved to sanitize dried herbs is 10 kGy, assuring its decontamination (Directive 1999/3/EC of the European Parliament and of the Council of 22 February 1999 on the establishment of a Community list of foods and food ingredients treated with ionising radiation 1999).

3.3.3.3. Phenolic compounds

Table 19 and **Table 20** shows the quantified amounts of phenolic compounds in non-irradiated and irradiated samples of *T. lignosa* previously freeze- or shade-dried, respectively. The results are expressed as mean value of each ID for different PM, as well as the mean value of each PM, comprising results for all the assayed ID. In general, despite slight quantitative differences, the phenolic profiles described herein were coherent to those previously characterized in *T. lignosa* (Pinela *et al.* 2012). The most abundant compounds were punicalagin isomers (compounds 1 and 3) and punicalagin gallate isomers (compounds 2 and 4) (**Figure 23**), which accounted for more than 90% of the quantified phenolic compounds. In fact, *T. lignosa* was previously reported as an important source of this type of compounds (Bedoya *et al.* 2010). The interaction among factors (ID×PM) was again significant ($p < 0.05$) in all cases; thus, no multiple comparison tests could be performed. Nevertheless, some observations can be made. In general, shade-dried samples contained lower levels of phenolic compounds than freeze-dried ones. Also a tendency to a decrease in the concentrations of phenolic compounds, especially ellagitannins, was observed in the irradiated samples in relation to non-irradiated ones, which was more accused in the shade-dried samples, although the changes were not statistically significant. Significant differences existed, however, in the levels of compounds depending on the preparation procedure (**Figure 23**). With no exception, higher contents of ellagitannins, flavones and flavonols were found in samples extracted by decoction than by infusion, both in shade- and freeze-dried products.

Table 19 Phenolic compounds (mg/g) of non-irradiated and irradiated *T. lignosa* freeze-dried samples. The results are presented as mean±SD[a].

Compound	Irradiation dose (ID)				Tukey's test *p*-value (n = 18)	Preparation method (PM)		*t*-student's test *p*-value (n = 36)	ID×PM *p*-value (n = 72)
	0 kGy	**1 kGy**	**5 kGy**	**10 kGy**		**Infusion**	**Decoction**		
1) Punicalagin (isomer)	23 ± 2	20 ± 10	21 ± 9	22 ± 7	0.776	15 ± 4	28 ± 3	<0.001	<0.001
2) Punicalagin gallate (isomer)	28 ± 11	25 ± 14	22 ± 12	24 ± 8	0.561	14 ± 3	36 ± 3	<0.001	<0.001
3) Punicalagin (isomer)	47 ± 5	37 ± 13	43 ± 14	43 ± 11	0.058	32 ± 7	53 ± 3	<0.001	<0.001
4) Punicalagin gallate (isomer)	33 ± 13	27 ± 15	27 ± 12	28 ± 9	0.520	17 ± 3	41 ± 3	<0.001	<0.001
5) Luteolin-6-*C*-glucose-8-*C*-glucose	0.27 ± 0.05	0.25 ± 0.05	0.28 ± 0.05	0.29 ± 0.05	0.198	0.22 ± 0.02	0.33 ± 0.02	<0.001	<0.001
6) 5-*O*-*p*-Coumaroylquinic acid	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.4	<0.001	0.8 ± 0.3	0.6 ± 0.1	0.001	<0.001
7) Luteolin-8-*C*-glucoside	1.3 ± 0.4	1.5 ± 0.5	1.5 ± 0.5	1.4 ± 0.4	0.634	0.9 ± 0.1	1.9 ± 0.2	<0.001	<0.001
8) Apigenin-8-*C*-glucoside	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	0.111	1.2 ± 0.1	1.4 ± 0.1	<0.001	0.025
9) Quercetin-3-*O*-rutinoside	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.349	0.16 ± 0.01	0.30 ± 0.04	<0.001	<0.001
10) Apigenin-6-*C*-glucoside	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	0.003	1.2 ± 0.1	1.4 ± 0.1	<0.001	<0.001
11) Kaempferol-3-*O*-rutinoside	0.37 ± 0.04	0.43 ± 0.05	0.44 ± 0.05	0.41 ± 0.05	0.014	0.35 ± 0.03	0.47 ± 0.05	<0.001	<0.001
12) Luteolin-6-*C*-glucoside	0.01 ± 0.01	0.03 ± 0.03	0.01 ± 0.01	0.01 ± 0.01	<0.001	nd	0.02 ± 0.02	-	-
13) Kaempferol-*O*-rhamnoside-*O*-rutinoside	nd	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.002	nd	0.2 ± 0.1	-	-
14) Kaempferol-*p*-coumaroylglucoside-glutarate	nd	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.001	nd	0.2 ± 0.1	-	-
15) Kaempferol-*p*-coumaroylglucoside	nd	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.001	nd	0.2 ± 0.1	-	-
Phenolic acids	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.0 ± 0.4	<0.001	0.8 ± 0.3	0.6 ± 0.1	0.001	<0.001
Flavonols	0.6 ± 0.1	1.1 ± 0.5	1.1 ± 0.5	1.0 ± 0.5	0.005	0.51 ± 0.02	1.4 ± 0.4	<0.001	<0.001
Flavones	4 ± 1	4 ± 1	4 ± 1	4 ± 1	0.680	3.5 ± 0.2	5.0 ± 0.3	<0.001	<0.001
Ellagitannins	130 ± 30	109 ± 52	114 ± 47	118 ± 35	0.469	78 ± 17	158 ± 4	<0.001	<0.001

[a]Results are reported as mean values of each irradiation dose (ID), including results from samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or PM), and can be higher than mean values. nd: not detected

Table 20 Phenolic compounds (mg/g) of non-irradiated and irradiated *T. lignosa* shade-dried samples. The results are presented as mean±SD[a].

Compound	Irradiation dose (ID)				Tukey's test *p*-value (n = 18)	Preparation method (PM)		*t*-student's test *p*-value (n = 36)	ID×PM *p*-value (n = 72)
	0 kGy	1 kGy	5 kGy	10 kGy		Infusion	Decoction		
1) Punicalagin (isomer)	26 ± 14	17 ± 3	17 ± 8	13 ± 13	0.003	9 ± 5	27 ± 7	<0.001	<0.001
2) Punicalagin gallate (isomer)	21 ± 13	14 ± 4	19 ± 10	13 ± 13	0.086	7 ± 4	27 ± 6	<0.001	<0.001
3) Punicalagin (isomer)	50 ± 20	32 ± 6	33 ± 14	24 ± 24	<0.001	19 ± 12	50 ± 12	<0.001	<0.001
4) Punicalagin gallate (isomer)	23 ± 15	15 ± 5	21 ± 12	15 ± 15	0.097	7 ± 4	30 ± 7	<0.001	<0.001
5) Luteolin-6-*C*-glucose-8-*C*-glucose	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.09 ± 0.02	<0.001	0.14 ± 0.04	0.3 ± 0.1	<0.001	<0.001
6) 5-*O*-*p*-Coumaroylquinic acid	1.1 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	<0.001	0.6 ± 0.2	1.0 ± 0.2	0.001	<0.001
7) Luteolin-8-*C*-glucoside	1 ± 1	1 ± 1	1 ± 1	1 ± 1	0.503	0.4 ± 0.2	2.0 ± 0.3	<0.001	<0.001
8) Apigenin-8-*C*-glucoside	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	0.056	1.1 ± 0.1	1.3 ± 0.1	<0.001	<0.001
9) Quercetin-3-*O*-rutinoside	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.479	0.08 ± 0.03	0.32 ± 0.05	<0.001	<0.001
10) Apigenin-6-*C*-glucoside	1.3 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	1.3 ± 0.3	0.218	1.1 ± 0.1	1.5 ± 0.1	<0.001	<0.001
11) Kaempferol-3-*O*-rutinoside	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.188	0.3 ± 0.04	0.53 ± 0.05	<0.001	<0.001
12) Luteolin-6-*C*-glucoside	0.02 ± 0.02	0.002 ± 0.002	0.002 ± 0.002	0.02 ± 0.02	<0.001	nd	0.02 ± 0.02	-	-
13) Kaempferol-*O*-rhamnoside-*O*-rutinoside	0.2 ± 0.2	nd	nd	nd	-	nd	0.1 ± 0.1	-	-
14) Kaempferol-*p*-coumaroylglucoside-glutarate	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	nd	0.001	nd	0.2 ± 0.1	-	-
15) Kaempferol-*p*-coumaroylglucoside	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	nd	0.002	nd	0.2 ± 0.1	-	-
Phenolic acids	1.1 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	<0.001	0.6 ± 0.2	1.0 ± 0.2	0.001	<0.001
Flavonols	1.1 ± 0.5	0.8 ± 0.4	0.9 ± 0.4	0.6 ± 0.3	0.018	0.4 ± 0.1	1.3 ± 0.3	<0.001	<0.001
Flavones	4 ± 1	4 ± 1	4 ± 1	4 ± 2	0.360	2.7 ± 0.4	5.2 ± 0.5	<0.001	<0.001
Ellagitannins	121 ± 62	78 ± 18	90 ± 45	65 ± 65	0.012	42 ± 25	135 ± 31	<0.001	<0.001

[a]Results are reported as mean values of each irradiation dose (ID), including results from samples submitted to infusion or decoction, as well as the mean value of each preparation method (PM), considering all irradiation doses in each case. Therefore, SD reflects values in those samples (with different ID or PM), and can be higher than mean values. nd- not detected.

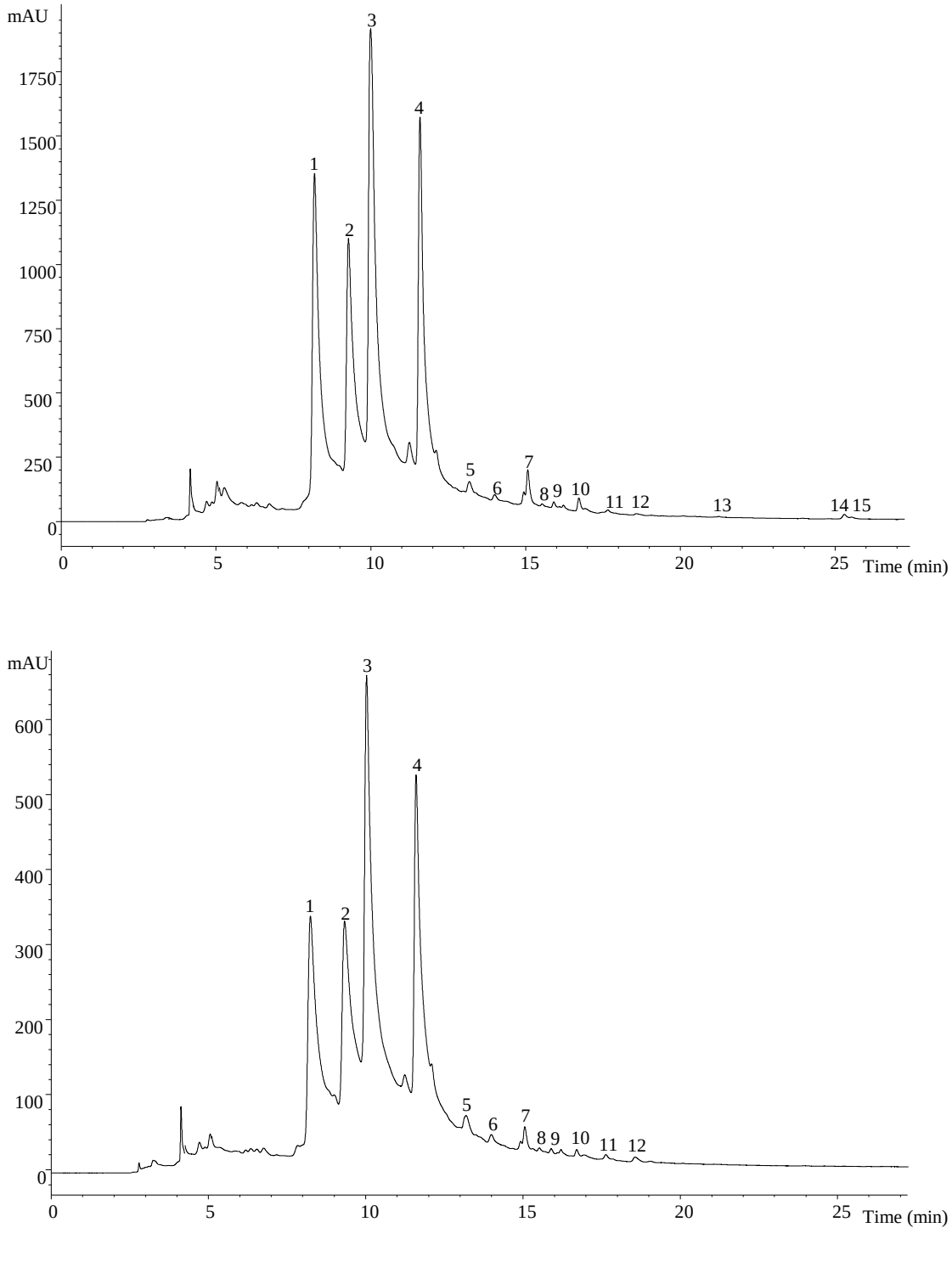


Figure 23 HPLC profile of phenolic compounds in decoction (A) and infusion (B) of *T. lignosa* freeze-dried samples irradiated with 1 kGy, recorded at 280 nm.

According to Khattak *et al.* (2008) the effects of gamma-irradiation on the phenolic content and antioxidant activity would be influenced by plant type and composition, state of the sample (fresh or dry), extraction solvent and procedures, and dose of gamma-irradiation. Furthermore, the irradiation treatment of plant products previously dehydrated under a

selected drying method may be a strategy to maintain or improve some chemical or physical parameters.

In general, from the obtained results, it might be concluded that the decoction methodology is preferable to infusion, as indicated by the higher antioxidant activity and levels of phenolic compounds. This finding may be linked to the higher extraction yield achieved with the longer extraction time of decoction compared to infusion. However, local medicinal uses as well as healers' or selected consumers' criteria should be taken into account during the preparation and use of these herbal beverages. Indeed, infusions are commonly used for internal use while decoctions are used for external and topical application. Furthermore, *T. lignosa* preparations should be avoided during long-term treatments in order to prevent possible side effects or toxicity, which can vary considerably according to the preparation method, doses and physical condition of the individual (Carvalho and Morales 2010). That is why the folk medicine recommends specific dosages and controlled periods of intake with ritual healing practices (Carvalho and Morales 2010).

The obtained results are in agreement with those of Martins, Barros, Santos-Buelga, *et al.* (2014) and Martins *et al.* (2015) who concluded that decoction preparations are preferable over infusions or even hydroalcoholic extracts to achieve higher concentration of flavonoids and total phenolic compounds, as well as greater antioxidant activity, from oregano and thyme plants. Vergara-Salinas *et al*. (2012) reported that for extracting phenolics from thyme with water, 100 °C and 5 min are appropriate operating conditions, whereas antioxidant-active non-phenolic compounds were favoured at higher temperatures and exposure times. Another recent study, conducted by Martínez-Las Heras *et al.* (2014), concluded that the drying method (including shade- and freeze-drying) and preparation procedures have a great influence on the stability and extractability of bioactive compounds from persimmon leaves. The authors showed that increasing the extraction time (up to 60 min) and temperature (from 70 ºC to 90 ºC) during water extraction of the herbal beverage increases the concentration of flavonoids and phenolic compounds. Similarly, He *et al.* (2012) studied the subcritical water extraction of phenolic compounds from pomegranate seed residues and showed that increasing the same variables (extraction time up to 30 min and temperature up to 220 ºC) increases the content of these compounds.

3.3.3.4. Principal component analysis (PCA)

In the previous sections, the effects resulting from ID or PM were compared considering antioxidant properties and phenolic composition separately. Despite, some statistically significant changes were observed in both cases, the true effects of the evaluated factors were not completely clear. Accordingly, the results for those parameters were evaluated simultaneously through principal components analysis (PCA). Chromatic parameters were

not considered in this analysis since they were measured before the preparation of extracts; furthermore, colour parameters after decoction or infusion of herbs are less relevant.

It was intended to verify if differences observed in each evaluated parameter were high enough to correlate with the defined principal components in a way that the geometric distribution of their loadings would lead to the individual clustering of each ID or PM. Regarding shade-dried samples, the first two dimensions (first: Cronbach's α, 17.060; eigenvalue, 0.984; second: Cronbach's α, 2.671; eigenvalue, 0.654) accounted for most of the variance of all quantified variables (74.2% and 11.6%, respectively). Groups corresponding to each gamma-irradiation dose (0 kGy, 1 kGy, 5 kGy and 10 kGy) were not individualized, as it could be hypothesized from **Table 18**, **Table 19** and **Table 20**. In fact, only the group corresponding to those samples irradiated with 10 kGy and prepared by decoction (please confront **Figure 24A** and **B**) were clearly separated from the remaining cases. The other defined groups include objects corresponding to non-irradiated and irradiated samples distributed in a random manner. This mixed grouping did not allow to define which of the assayed parameters better describe each one of applied ID, which might be considered as an indication of the lack of significant effects of gamma-irradiation at the assayed doses (except samples extracted by decoction and further submitted to a 10 kGy ID) on the antioxidant and phenolic profiles of *T. lignosa.* On the other hand, object points corresponding to each PM were clearly separated, proving that the previously highlighted significant differences were high enough to profile each of these methodologies (**Figure 24B**). In an overall analysis, it is clear that samples obtained by decoction have higher amounts of phenolic compounds as also stronger antioxidant activity, as indicated by the diametrically opposed position of their component loadings and the antioxidant activity assays object points.

Concerning freeze-dried samples, the first two dimensions (first: Cronbach's α, 17.383; eigenvalue, 0.985; second: Cronbach's α, 1.739; eigenvalue, 0.444) also accounted for most of the variance of all quantified variables (75.6% and 7.6%, respectively). The obtained outcomes were quite similar, with no separation of object scores according to each of the applied ID (**Figure 25A**). Curiously, a small group of objects was individually clustered, as it was verified in shade-dried samples. Nevertheless, in this case, this group corresponded to non-irradiated samples prepared by decoction. This dissimilarity among samples dried using different methodologies is in agreement with the observed significant interaction among factors (ID×PM). As it can be easily deduced from **Figure 25B**, object points corresponding to each PM were clearly separated. Once again, infusions showed lower levels in phenolic compounds, as also weaker antioxidant activity.

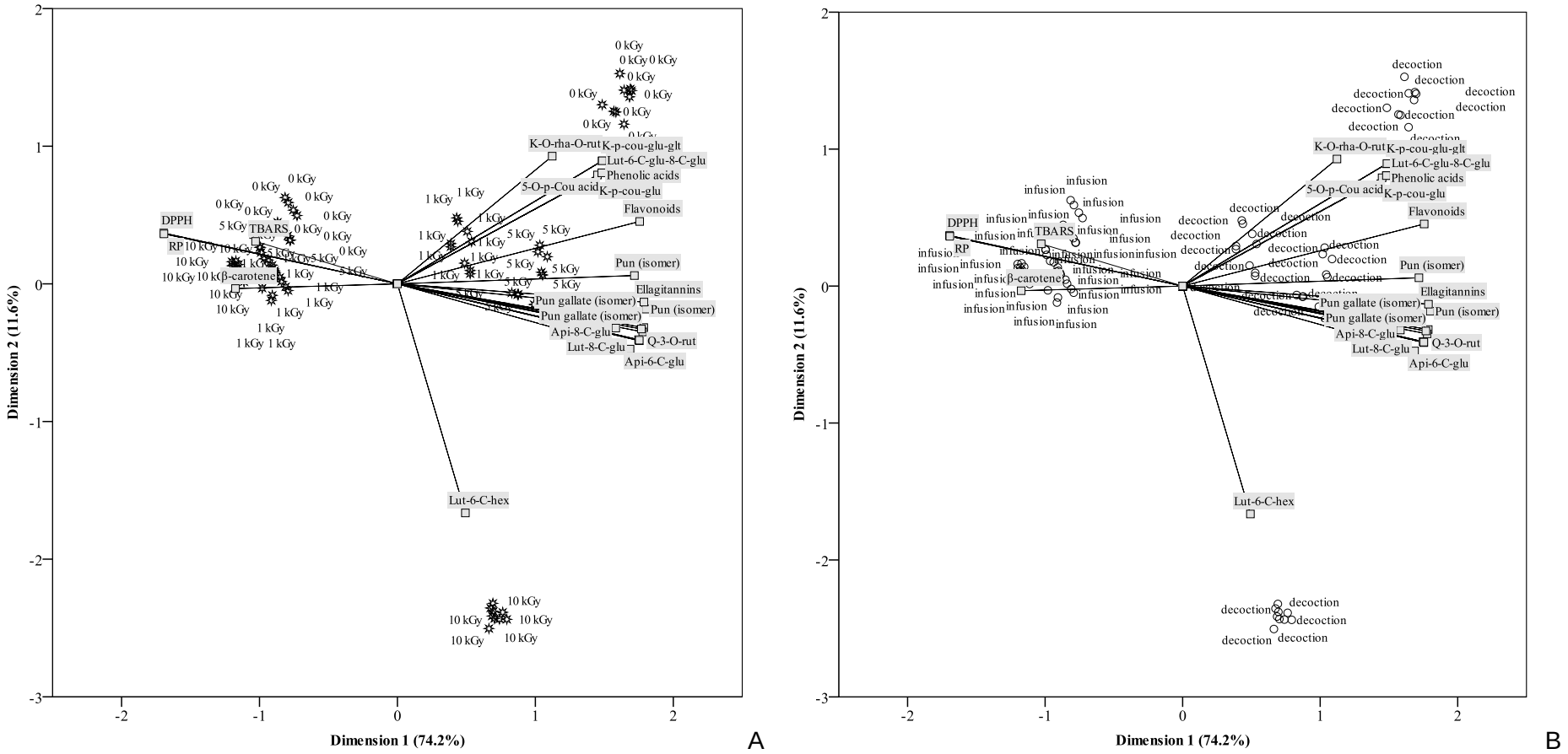


Figure 24 Biplot of object scores (gamma-irradiation doses) and component loadings (evaluated parameters) using gamma-irradiation **(A)** and preparation method (**B**) as labelling variables for shade-dried samples.

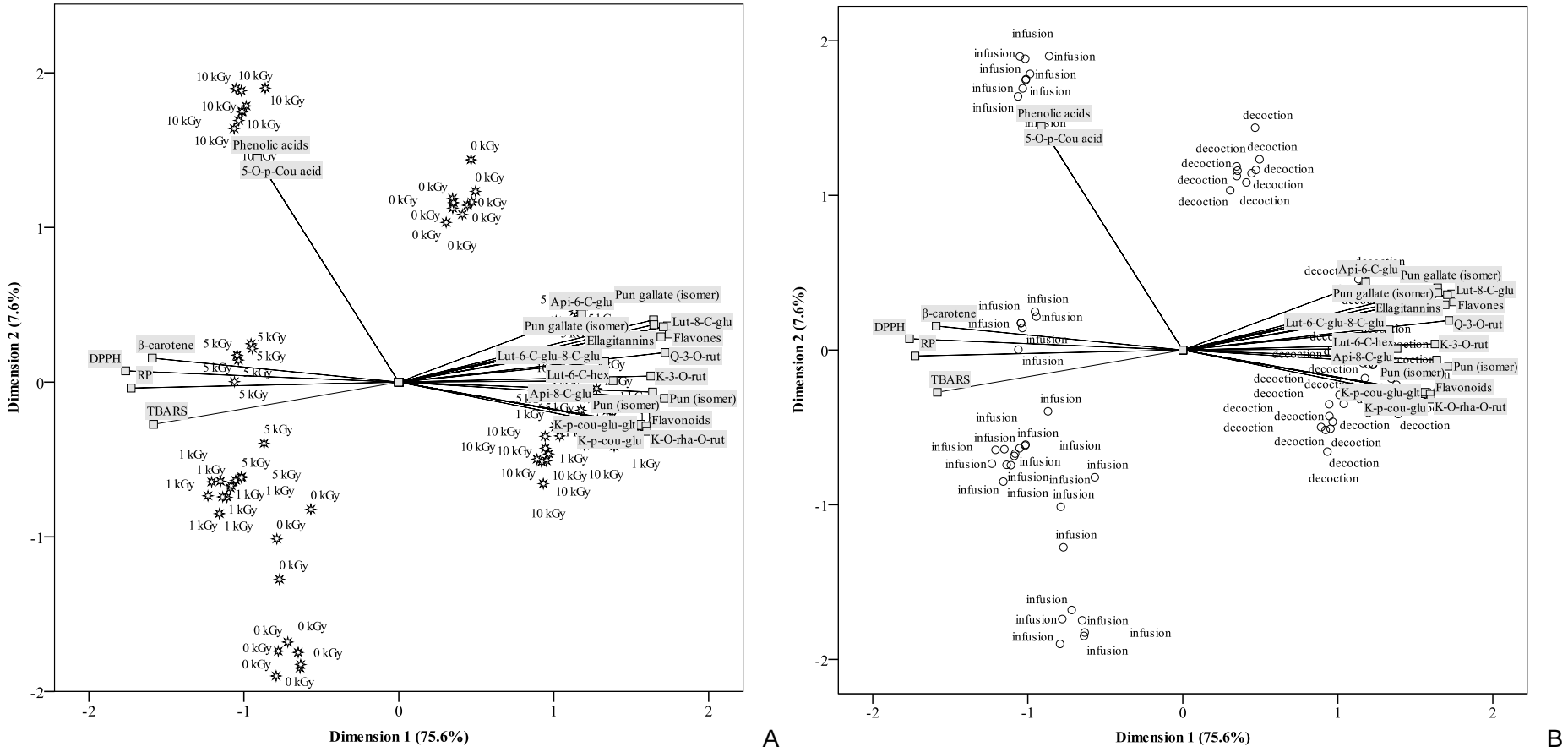


Figure 25 Biplot of object scores (gamma-irradiation doses) and component loadings (evaluated parameters) using gamma-irradiation **(A)** and preparation method (**B**) as labelling variables for freeze-dried samples.

3.3.4. Conclusion

In general, the preparation method (infusion or decoction) had higher influence in the phenolic profile and antioxidant activity than the irradiation treatment at the applied doses. In addition, CIE colour parameters were also more sensitive to the drying method than irradiation. Differences among infusions and decoctions were significant for all assayed parameters, while changes caused by gamma-irradiation were only significant in TBARS formation inhibition, β-carotene bleaching inhibition and a few phenolic compounds. Besides their effects in individual cases, when all parameters were evaluated together, modifications caused by the preparation method were clearly higher than those observed for gamma-irradiation. As it might be depicted from the PCA plots, object points corresponding to different irradiation doses were grouped arbitrarily, while those corresponding to infusions and decoctions were completely separated. The obtained results indicate that the decoction should be the preferable choice to prepare beverages from this plant, in order to obtain the higher antioxidant activity and phenolic content. Furthermore, the gamma-irradiation treatment (up to 10 kGy), if applied as a preservation technology, will not significantly affect the antioxidant properties of dried *T. lignosa* samples. Nevertheless, other studies are of interest to evaluate the preservation effectiveness of this technology.

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3.4. Ellagitannin-rich bioactive extracts of *Tuberaria lignosa*: Insights into the radiation-induced effects in the recovery of high added-value compounds

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Ellagitannin-rich bioactive extracts of *Tuberaria lignosa*: insights into the radiation-induced effects in the recovery of high added-value compounds†

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Abstract

Ellagitannins are polyphenols responsible for a number of bioactivities and health-promoting effects. These industrially important molecules can be affected by postharvest treatments and recovery processes, but little is known about the irradiation-induced effects on its integrity, bioactivity and extractability. Herein, the impact of gamma radiation on the production of ellagitannin-rich extracts as investigated using *Tuberaria lignosa* as a case study. These effects were compared with those induced in flavonoids and organic acids. The extracts were particularly rich in hydrophilic antioxidants (measured by *in vitro* assays). The recovery of the different phytochemicals was favoured by longer extraction times. Ellagitannins (mainly punicalagin derivatives) were better extracted from samples irradiated at 5 kGy and were not significantly affected by the 10 kGy dose. However, totals of flavonoids and organic acids were decreased by the consequent increase in irradiation dose.

Therefore, this study supports the use of gamma radiation for processing *T. lignosa*, aiming obtaining ellagitannin-rich bioactive extracts.

Keywords: gamma radiation; hot water extraction; ellagitannin derivatives; punicalagin derivatives; antioxidant activity; response surface methodology.

3.4.1. Introduction

Medicinal plants play a critical role in the healthcare provision of much of the world's population. In addition to their uses in folk medicine, medicinal plants are used for isolation of bioactive molecules, production of functional ingredients/extracts, or as a starting material for the production of semi-synthetic pharmacologically active substances (Gurib-Fakim 2006, Gull *et al.* 2015). To date, a large number of plant species have been studied for its composition in polyphenols, which have attracted considerable attention due to their health-promoting effects and growing commercial value in food, pharmaceutical and cosmeceutical industries. Among polyphenols, ellagitannins are hydrolysable tannins defined as the esters of hexahydroxydiphenic acid and a polyol, usually glucose or quinic acid, and form the largest group of known tannins (Landete 2011). Punicalagins are the most abundant ellagitannins in perennial spotted rockrose (*Tuberaria lignosa* (Sweet) Samp., Fam. Cistaceae) (Pinela *et al.* 2012). These water-soluble polyphenols contain ellagic and gallagic acids linked to a sugar moiety and are naturally found in the forms of two reversible α- and β-anomers. It has been shown that punicalagins inhibit the oxidative stress, inflammatory responses and apoptosis, along with other beneficial effects (Yaidikar and Thakur 2015, Zhong *et al.* 2015, Rao *et al.* 2016). Therefore, the exploitation of ellagitannin (punicalagin)-rich natural sources is of high industrial interest.

Industrial preservation and decontamination processes are applied to prevent the growth of microorganisms on plant materials and to facilitate their storage and transportation (Pinela *et al.* 2011, 2012, Ihsanullah and Rashid 2017). Conventional decontamination methods are not suitable for dried plant materials. Fumigation with chemical agents was used in the past for disinfestations of dried commodities during storage; but the use of these chemicals is now prohibited or increasingly restricted in several countries due to serious adverse effects on human health and environment (UNEP 2010). Heat treatments are not suitable, while ultraviolet radiation is not effective in decontaminating large volumes due to its low penetration capacity (Pinela and Ferreira 2017). Among the non-thermal and non-chemical methods, gamma radiation is by far the most effective technique to preserve the chemical and hygienic quality of plant commodities and reduce losses associated with microbial contamination and insect infestation. Despite a general bad feeling of consumers about food irradiation, the treatment is widely accepted by the food industry and is a safer

alternative compared to chemical fumigants (Kausar *et al.* 2013). The joint FAO/IAEA/WHO Experts Committee ensures that any food irradiated up to a maximum dose of 10 kGy is safe and wholesome for human consumption (WHO 1999).

The gamma irradiation treatment is well established as a non-thermal processing method. However, an inadequate exposure to gamma rays might result in minimal availability of health-promoting compounds. The integrity of phytochemicals/antioxidants may be affected by the direct action of gamma rays or by interaction with radiolytic products (free radicals and radiation-induced degradation products) (Alothman *et al.* 2009, Harder *et al.* 2016). The chemical bonds of polyphenols can be broken, thereby releasing soluble low-molecular-weight phenols, leading to a variation in the amount of bioactive molecules (Adamo *et al.* 2004, Alothman *et al.* 2009). The levels of physical and chemical modifications might vary depending on the plant material under study, the sensitivity of the phytochemicals/antioxidants towards irradiation, irradiation dose delivered, and type of radiation source employed (Alothman *et al.* 2009, Pereira, Barros, Dueñas, *et al.* 2015, Pereira *et al.* 2016, Pinela *et al.* 2016). Consequently, irradiation may affect the extraction yield and, in some cases, improve the content (extractability) of polyphenols and the antioxidant properties of the obtained extracts (Pereira, Barros, Dueñas, *et al.* 2015, Ito *et al.* 2016, Pinela *et al.* 2016). To evaluate the effects of ionizing radiation on the extraction/degradation of bioactive compounds from plant matrices, it is important to evaluate the extraction kinetics by monitoring the concentration changes over time using chromatographic and spectrometric techniques.

The content of phenolic compounds in infused and decocted extracts prepared from irradiated wild samples of *T. lignosa* was previously evaluated by Pinela *et al.* (2012) However, the impact of the irradiation treatment in the extraction/degradation kinetics of the different phytochemicals was not evaluated. This study was carried out to investigate the effects of gamma radiation on the production of ellagitannin-rich bioactive extracts from *T. lignosa* using response surface methodology (RSM) for analysis. These effects were compared with those induced in other compounds, namely in flavonoids and organic acids. In addition, the presence of hydrophilic and lipophilic antioxidants on the extracts was evaluated by distinct *in vitro* assays.

3.4.2. Material and methods

3.4.2.1. Plant material, standards and reagents

Samples of *T. lignosa*, available as dried rosettes of leaves and inflorescences, were obtained in a local herbal shop in Bragança (North-eastern Portugal). Botanical identification of all plant material used was previously confirmed. Amber Perspex routine dosimeters, Batch V, were purchased from Harwell Company (Oxfordshire, UK). Organic acids (oxalic,

quinic, shikimic and succinic acids) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (99.9%, HPLC grade) was purchased from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (apigenin-6-*C*-glucoside, *p*-coumaric acid, ellagic acid, gallic acid, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, luteolin-6-C-glucoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside) were purchased from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Merck Millipore, model A10, Billerica, MA, USA).

3.4.2.2. Irradiation of plant material

Plant samples were packaged in sterilized polyethylene bags and submitted to the predicted ionizing radiation doses of 5 and 10 kGy. A non-irradiated control (0 kGy) followed all the experiment. The irradiation process was performed in a cobalt-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with four sources and a total activity of 177 TBq (4.78 kCi), located in the Centre for Nuclear Sciences and Technologies (C2TN), in Portugal. During the irradiation process, amber Perspex routine dosimeters were used to measure the distribution of the absorbed energy and to determine the maximum (D_{max}) and the minimum (D_{min}) dose absorbed by the samples, following the procedure previously described by Fernandes, Antonio, Barreira, *et al*. (2012). The measured average doses were 4.85±0.47 and 9.92±0.52 kGy for the samples irradiated at the predicted doses 5 and 10 kGy, respectively. The estimated dose rate for the irradiation position, obtained with a Fricke dosimeter, was 1.9 kGy/h and the dose uniformity ratio (D_{max}/D_{min}) was 1.1.

3.4.2.3. Extraction method

The non-irradiated and irradiated plant material were submitted to a solid-liquid extraction as defined in the 3^2 full factorial design presented in **Table 21**. Boiling water (0.2 L) was used as extraction solvent since it is low-priced, easily available and non-toxic. After extraction, the obtained solutions were filtered through Whatman No. 4 paper and a portion was lyophilized (Free Zone 4.5, Labconco, Kansas City, MO, USA) for the subsequent chromatographic analysis of organic acids and phenolic compounds. The screening of antioxidants was made on the obtained solutions. The extraction yield was expressed as percentage (%, w/w).

Table 21 Results of the three-level full factorial design combining the effects of extraction time (X_1) and irradiation dose (X_2) on the recovery of phytochemicals from *T. lignosa*. The experimental domain of the variables X_1 and X_2 is presented in natural and coded values (used to compute the RSM factorial design)

Experimental design																												
	Runs	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
Natural values	(X_1) : Time (t, min)	0	5	5	0	5	10	0	10	10	0	5	5	0	5	10	0	10	10	0	5	5	0	5	10	0	10	10
	(X_2) : Dose (D, kGy)	0	0	5	5	10	0	10	5	10	0	0	5	5	10	0	10	5	10	0	0	5	5	10	0	10	5	10
Coded values	(X_1) : Time (t)	-1	0	0	-1	0	1	-1	1	1	-1	0	0	-1	0	1	-1	1	1	-1	0	0	-1	0	1	-1	1	1
	(X_2) : Dose (D)	-1	-1	0	0	1	-1	1	0	1	-1	-1	0	0	1	-1	1	0	1	-1	-1	0	0	1	-1	1	0	1
Response variables for RSM application																												
Antioxidant activity (IC_{50}, mg/g P)																												
DPPH$^{\bullet}$ scavenging activity		0.0	270	341	0.0	408	199	0.0	192	215	0.0	269	339	0.0	4074	199	0.0	1916	216	0.0	268	338	0.0	408	199	0.0	190	216
Reducing power		0.0	218	260	0.0	327	162	0.0	135	141	0.0	211	265	0.0	327	161	0.0	136	142	0.0	211	265	0.0	327	161	0.0	136	142
β-Carotene bleaching inhibition		0.0	181	322	0.0	324	294	0.0	175	119	0.0	116	266	0.0	328	355	0.0	156	129	0.0	160	934	0.0	227	280	0.0	150	177
TBARS formation inhibition		0.0	12.4	119	0.0	71.4	71.8	0.0	14.1	31.0	0.0	13.4	115	0.0	78.0	52.3	0.0	18.8	33.3	0.0	12.3	111	0.0	64.3	66.3	0.0	20.6	30.8
Organic acids (mg/g P)																												
Oxalic acid		0.0	1.29	1.28	0.0	3.72	4.14	0.0	3.57	1.47	0.0	1.26	1.28	0.0	3.61	3.94	0.0	3.65	1.38	0.0	1.27	1.28	0.0	3.67	4.04	0.0	3.61	1.43
Quinic acid		0.0	1.82	1.50	0.0	1.75	1.07	0.0	2.69	2.62	0.0	1.77	1.93	0.0	1.74	0.76	0.0	1.85	3.93	0.0	1.79	1.72	0.0	1.75	0.92	0.0	2.27	3.28
Shikimic acid		0.0	5.17	4.19	0.0	4.60	4.93	0.0	6.47	4.85	0.0	5.06	4.18	0.0	4.59	4.98	0.0	6.43	4.89	0.0	5.11	4.18	0.0	4.59	4.96	0.0	6.45	4.87
Succinic acid		0.0	0.0	0.0	0.0	0.0	10.9	0.0	5.61	3.56	0.0	0.0	0.0	0.0	0.0	9.92	0.0	5.64	3.55	0.0	0.0	0.0	0.0	0.0	10.4	0.0	5.62	3.55
TOTAL		0.0	8.28	6.96	0.0	10.1	21.1	0.0	18.3	12.4	0.0	8.09	7.39	0.0	9.95	19.6	0.0	17.5	13.7	0.0	8.18	7.18	0.0	10.1	20.3	0.0	17.9	13.13
Ellagitannin derivatives (mg/g P)																												
Punicalin[1]		0.0	0.64	0.47	0.0	0.49	1.08	0.0	1.24	0.88	0.0	0.64	0.47	0.0	0.48	1.10	0.0	1.28	0.88	0.0	0.64	0.47	0.0	0.48	1.09	0.0	1.26	0.88
Punicalagin (isomer 1)[1]		0.0	1.51	1.32	0.0	1.27	2.26	0.0	3.35	2.39	0.0	1.67	1.32	0.0	1.27	2.31	0.0	3.35	2.40	0.0	1.59	1.32	0.0	1.27	2.28	0.0	3.35	2.40
Punicalagin gallate (isomer 1)[1]		0.0	0.20	0.14	0.0	0.08	0.16	0.0	0.35	0.30	0.0	0.20	0.13	0.0	0.07	0.14	0.0	0.34	0.28	0.0	0.20	0.13	0.0	0.08	0.15	0.0	0.35	0.29
Punicalagin (isomer 2)[1]		0.0	3.24	2.73	0.0	2.70	5.38	0.0	6.77	5.31	0.0	3.25	2.65	0.0	2.69	5.38	0.0	6.78	5.33	0.0	3.25	2.69	0.0	2.69	5.38	0.0	6.77	5.32
Punicalagin gallate (isomer 2)[1]		0.0	0.08	0.07	0.0	0.06	0.10	0.0	0.71	0.81	0.0	0.09	0.06	0.0	0.06	0.09	0.0	0.75	0.84	0.0	0.08	0.07	0.0	0.06	0.09	0.0	0.73	0.82
TOTAL		0.0	5.66	4.74	0.0	4.60	8.96	0.0	12.4	9.69	0.0	5.85	4.63	0.0	4.58	9.01	0.0	12.5	9.73	0.0	5.76	4.69	0.0	4.59	8.99	0.0	12.4	9.71

Experimental design

Runs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Other phenolic compounds (mg/100 g P)																											
Luteolin-6-*C*-glucose-8-*C*-glucose[2]	0.0	5.8	4.7	0.0	4.6	9.5	0.0	8.5	9.2	0.0	6.0	4.6	0.0	4.5	10.2	0.0	8.5	9.2	0.0	5.9	4.7	0.0	4.6	9.8	0.0	8.5	9.2
5-*O*-*p*-Coumaroylquinic acid[3]	0.0	5.3	5.1	0.0	3.9	15.0	0.0	9.4	7.0	0.0	5.3	4.5	0.0	3.3	14.2	0.0	9.3	6.1	0.0	5.3	4.8	0.0	3.6	14.6	0.0	9.3	6.5
Luteolin-8-*C*-glucoside[2]	0.0	8.3	7.4	0.0	7.1	16.4	0.0	27.3	25.6	0.0	8.2	7.4	0.0	7.1	16.4	0.0	28.6	25.4	0.0	8.3	7.4	0.0	7.1	16.4	0.0	28.0	25.5
Apigenin-8-*C*-glucoside[4]	0.0	16.6	12.3	0.0	12.1	24.5	0.0	25.7	23.4	0.0	16.8	12.2	0.0	12.0	25.2	0.0	25.7	23.8	0.0	16.7	12.2	0.0	12.1	24.8	0.0	25.7	23.6
Quercetin-3-*O*-rutinoside[5]	0.0	2.6	1.9	0.0	2.1	4.8	0.0	6.4	7.4	0.0	3.0	2.2	0.0	2.2	5.9	0.0	6.8	7.5	0.0	2.8	2.0	0.0	2.2	5.4	0.0	6.6	7.4
Apigenin-6-*C*-glucoside[4]	0.0	15.1	10.8	0.0	11.2	23.4	0.0	25.2	22.9	0.0	15.1	10.6	0.0	11.1	23.6	0.0	25.2	22.7	0.0	15.1	10.7	0.0	11.2	23.5	0.0	25.2	22.8
Kaempferol-3-*O*-rutinoside[6]	0.0	9.0	6.3	0.0	4.6	19.6	0.0	17.1	15.1	0.0	9.1	6.2	0.0	4.7	19.8	0.0	17.0	14.8	0.0	9.0	6.3	0.0	4.7	19.7	0.0	17.1	15.0
Luteolin-6-*C*-hexoside[2]	0.0	0.3	0.1	0.0	0.1	0.7	0.0	0.8	0.7	0.0	0.3	0.1	0.0	0.0	0.6	0.0	0.7	0.5	0.0	0.3	0.1	0.0	0.0	0.7	0.0	0.8	0.6
TOTAL	0.0	62.9	48.6	0.0	45.7	114	0.0	120	111	0.0	63.9	47.7	0.0	45.0	115	0.0	121	110	0.0	63.4	48.1	0.0	45.4	114	0.0	121	110
Extraction yield (%)	0.0	12.4	9.12	0.0	8.98	17.6	0.0	17.6	15.6	0.0	12.4	9.12	0.0	8.98	17.6	0.0	17.6	15.6	0.0	12.4	9.12	0.0	8.98	17.6	0.0	17.6	15.6

Phenolic compounds used for quantification: 1- ellagic acid (y = 35.695x - 265.7; R^2 = 0.9991); luteolin-6-*C*-glucoside (y = 365.93x + 17.836; R^2 = 0.9997); 3- *p*-coumaric acid (y = 321.99x + 98.308; R^2 = 0.9984); 4- apigenin-6-*C*-glucoside (y = 246.05 x - 309.66; R^2 = 0.9994); 5- quercetin-3-*O*-glucoside (y = 316.48x + 2.9142; R^2 = 1); 6- kaempferol-3-*O*-rutinoside (y = 17 5.02x - 43.877; R^2 = 0.9999).

3.4.2.4. Screening of antioxidants

The obtained solutions were redissolved in water (final concentration 1 mg/mL) and further diluted to different concentrations in order to evaluate the antioxidant capacity in a dose-response format using *in vitro* assays based on hydrophilic and lipophilic reaction mechanisms (Pinela *et al.* 2015).

Hydrophilic antioxidants

DPPH free-radical scavenging activity: The solutions at different concentrations (30 µL) were mixed with a methanolic solution (270 µL) containing DPPH free-radicals (6×10^{-5} M) in a 96-well plate. The reaction mixture was left to stand for 60 min in the dark. After that, the reduction of DPPH free-radicals was determined by measuring the absorbance at 515 nm in an ELX800 Microplate Reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). For each sample, the measured absorbance was transformed in terms of the remaining nM of DPPH radicals.

Reducing power: The reducing power assay evaluates the capacity of the extracts to convert potassium ferricyanide (Fe^{3+}) into potassium ferrocyanide (Fe^{2+}), which reacts with ferric chloride to form a ferric-ferrous complex that can be monitored spectrophotometrically. The solutions at different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mM, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and then trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured into a 48-well plate containing deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm using the microplate reader described above. For each sample, the measured absorbance was transformed in terms of nM of reduced Fe^{2+}.

Lipophilic antioxidants

Thiobarbituric acid reactive substances (TBARS) formation inhibition capacity: The solutions at different concentrations (0.2 mL) were incubated with a brain tissue homogenate (0.1 mL; prepared according to Pinela *et al.* (2012) in the presence of $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL). The mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm. The measured absorbance was transformed in terms of inhibition ratio (IR, %), which was calculated using the following expression:

$$IR(\%) = \left[(C - S)/C \right] \times 100 \quad [1]$$

where *C* and *S* are the absorbance of the control and extract solution, respectively.

β-Carotene beaching inhibition capacity: A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask and the chloroform was removed at 40 ºC under vacuum. Then, linoleic acid (40 mg), Tween 80 emulsifier (400 mg) and distilled water (100 mL) were added and vigorously shaken. The solutions at different concentrations (0.2 mL) were shaken with the prepared emulsion (4.8 mL) and the zero time absorbance was measured at 470 nm. Past 2 h of incubation at 50 ºC, the absorbance of the mixture was measured again. The β-carotene bleaching inhibition as function of the remaining β-carotene (Rβ, %) after 120 min of interaction was calculated using the following expression:

$$R\beta(\%) = (\beta_{120}/\beta_0) \times 100 \quad [2]$$

where β_0 and β_{120} are the remaining equivalent quantities of β-carotene at the start (t = 0) and after 120 min of the oxidation process, respectively.

Quantification of the antioxidant activity through dose-response analysis

Each sample obtained under the experimental design presented in **Table 21** was studied in a dose-response format. Among all the different standardized formats for presenting the results of the hydrophilic and lipophilic antioxidant determinations, the Weibull cumulative distribution function (Weibull and Sweden 1951) was selected to computed the dose-response of each sample as discussed in other studies (Prieto *et al.* 2014). Thus, the variation of each antioxidant response (*A*) as function of increasing concentration of an antioxidant extract (*E*) can be satisfactorily described using the Weibull model rearranged for our own purposes as follows:

$$R(E) = K\left\{1 - \exp\left[-\ln(2)\left(E/IC_{50}\right)^a \right]\right\} \quad [3]$$

The parameter *K* is the maximum asymptotic value of the response, the parameter *a* is a shape parameter related to the slope that can produce potential profiles (*a*<1), first order kinetic ones (*a*=1) and a variety of sigmoidal profiles (*a*>1), and the IC_{50} is the concentration needed to reach 50% of maximum response. The results were expressed in IC_{50} values (mg/g P).

3.4.2.5. Analysis of organic acids

Organic acids were determined following procedures previously described by Pereira *et al.* (2013) and optimized by Barros *et al.* (2013). The lyophilized extracts (~10 mg) were dissolved in 1 mL of meta-phosphoric acid and filtered through 0.2 μm nylon filters. The

analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 µm, 250 × 4.6 mm i.d.) thermostated at 35 ºC. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a photodiode array detector (PDA) at 215 nm. The organic acids found were quantified by comparison of the area of their peaks with calibration curves obtained from commercial standards. The results were expressed in mg per g of plant material (mg/g P).

3.4.2.6. Analysis of phenolic compounds

The lyophilized extracts (~10 mg) were dissolved in water and filtered through 0.22 µm disposable LC filter disks. Chromatographic analysis was performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector coupled to a electrospray ionization mass detector (ThermoFinnigan, San Jose, CA, USA). The chromatographic system and analytical procedures were previously described by Bessada *et al.* (2016) The phenolic compounds were identified using 280 nm and 370 nm as preferred wavelengths and by comparing their retention times and UV-vis and mass spectra with those obtained from authentic standards, when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds. The results were expressed in mg per g of plant material (mg/g P) for the major compounds (ellagitannin derivatives) and in mg per 100 g of plant material (mg/100 g P) for the minor ones (other phenolic compounds).

3.4.2.7. Response surface methodology

The RSM was applied to analyse the impact of the ionizing radiation treatment in the extraction or degradation kinetics of different phytochemicals from *T. lignosa* aerial parts. This statistical tool allows modelling processes in which a response of interest is affected by different independent variables (in this case *t* and *D*) and accessing possible interactions using a reduced number of experimental trials.

Experimental design

The combined effects of the variables extraction time (*t*) and irradiation dose (*D*) were tested under a full factorial design involving the combination of three values for each factor (minimum, mean and maximum) and three replicates per condition. The number of experiments *n* for *k* factors is given as $n=3^k$. Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. The variables were coded according to the following equation:

$$X = \left(x_a - x_0 \right) / \Delta x \quad [4]$$

where X is the coded value for the variables t and D, x_a is the corresponding actual value, x_0 is the actual value in the centre of the domain, and Δx is the increment of x_a corresponding to a variation of 1 unit of X. The natural and coded values are presented in **Table 21**.

Mathematical model

The response surface model was fitted by means of least-squares calculation using the following Box-Behnken design equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad [5]$$

where Y is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} is the coefficients of quadratic effect, and n is the number of variables.

3.4.2.8. Numerical methods and statistical analysis

All fitting procedures, coefficient estimates and statistical calculations were performed using a Microsoft Excel spreadsheet. The model fitting and statistical analysis of the experimental results according to the proposed equations were carried out in four phases:

- *Coefficients determination:* Parametric estimates were obtained by minimization of the sum of quadratic differences between observed and model-predicted values using the nonlinear least-square (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* 2003 (Kemmer and Keller 2010), which allows a quick testing of a hypotheses and its consequences (Murado and Prieto 2013a).

- *Coefficients significance:* The determination of the parametric confidence intervals was done using the "*SolverAid*" (Prikler 2009). The model was simplified by dropping the terms that were not statistically significant for a *p*-value (*p)* > 0.05.

- *Model consistency:* The Fisher *F*-test (α=0.05) was used to determine whether the constructed models were adequate to describe the observed data (Shi and Tsai 2002).

- *Other statistical assessment criteria:* To recheck the uniformity of the model, the following criteria were applied: a) The "*SolverStat*" macro was used for the assessment of parameter and model prediction uncertainties (Comuzzi *et al.* 2003); b) The R^2 was interpreted as the proportion of variability of the dependent variable explained by the model; c) The adjusted coefficient of determination (R^2_{adj}) was a correction to R^2 taking into account the number of variables used in the model; d) Bias and accuracy factors of all equations were calculated to evaluate the quality of the fitting to the experimental data, such as the mean squared error

(MSE), the root mean square error (RMSE) and the mean absolute percentage error (MAPE); e) The Durbin-Watson (DW) coefficient was used to check if the residuals of the model were not autocorrelated; and f) The analysis of variance (ANOVA) table was used to evaluate the explanatory power of each variable.

3.4.3. Results and discussion

3.4.3.1. Response criteria for RSM analysis

Extraction yield

Extraction yields ranging from 8.98 to 17.6% were obtained when the plant material irradiated at 10 and 5 kGy were extracted for 5 and 10 min, respectively (**Table 21**). Slightly higher yields (19.7–21.2) were obtained by Pinela *et al.* (2012) when *T. lignosa* samples were prepared by decoction (method in which the dried plant is boiled for 5 min and the mixture is then left to stand at 25 ºC for 5 min more). Based on the amount of extracted residue, the results were analysed in terms of plant material (**Table 22**) and extracted residue (**Annexe 3**). The last form of expressing the results was selected for the detailed discussion. However, the fitting coefficients obtained after applying the Box-Behnken model and the statistical information of the fitting analysis are similar for both cases.

Hydrophilic and lipophilic antioxidants

Antioxidants may act in various ways, by scavenging free radicals, by chelating metal ions, and by decomposing peroxides (Carocho and Ferreira 2013). Therefore, the presence of hydrophilic and lipophilic antioxidant compounds in the different extracts was evaluated using distinct *in vitro* assays with known reaction mechanisms. The results of the screening of antioxidants are presented in **Table 21**. The hydrophilic responses were measured via DPPH free-radical scavenging activity and reducing power, while the β-carotene bleaching inhibition and TBARS formation inhibition assays were used to evaluate the lipophilic ones. The results were expressed in IC_{50} values (mg/g P), corresponding to the concentration of plant material (or extract) needed to reach 50% of the maximum response. Thus, the lower the IC_{50} value the higher the antioxidant activity. As shown in **Table 22**, no statistically significant results were found for the lipophilic antioxidant responses ($R^2 \leq 0.54$). This demonstrates that hydrophilic antioxidants were predominant in the extracts, what was somewhat expected once the extraction solvent was water. To accurately assess the effects of the different gamma irradiation doses on the lipophilic fraction of antioxidants, the use of apolar extraction solvents would be required. Additionally, the measured antioxidant responses may be attributed to the phenolic fraction constituted by ellagitannins, polyphenols with several hydroxyl functional groups in *ortho* positions in their structures, which could provide hydrogen atoms and support the unpaired electron (Landete 2011).

Table 22 Parametric results of the three-level Box-Behnken full factorial design presented in Eq. [5] combining the effects of extraction time (X_1) and irradiation dose (X_2) on the recovery of phytochemicals from *T. lignosa* plant material. The analysis of significance of the parameters (α=0.05) and the statistical information of the fitting procedure to the model are presented

	Fitting coefficients obtained after applying the Box-Behnken design equation						**Statistical information of the fitting analysis**						
	Intercept	**Linear effect**		**Quadratic effect**		**Interactive effect**	**Obs**	**R^2**	**R^2adj**	**MSE**	**RMSE**	**MAPE**	**DW**
	b_0	b_1 (t)	b_2 (D)	b_{11} (t^2)	b_{22} (D^2)	b_{12} ($t \times D$)							
Extraction yield	10.17±0.56	8.49±0.39	-0.89 ±0.39	-1.69±0.68	*ns*	-0.48±0.27	27	0.9902	0.988	64.1	8.2	5.6	2.3
DPPH• scavenging activity	339.1±19.9	101.1±14.1	25.9 ±14.1	-238.0±24.4	*ns*	*ns*	27	0.9678	0.961	26370	162	8.9	1.3
Reducing power	268.5±19.1	73.4±13.5	15.7 ±13.5	-195.2±23.3	*ns*	*ns*	27	0.9528	0.944	16486	128	10.7	1.3
β-Carotene bleaching inhibition	317.9±105.7	102.3±74.8	*ns*	-215.6±129.5	*ns*	*ns*	27	0.4678	0.408	47560	218	42.4	2.5
TBARS formation inhibition	66.5±20.3	18.8±14.4	*ns*	-47.6±24.9	*ns*	*ns*	27	0.5053	0.462	1885	43	102	2.7
Oxalic acid	1.70±0.36	1.51±0.44	*ns*	*ns*	*ns*	-0.65±0.54	27	0.6988	0.664	3.2	1.8	43.3	2.0
Quinic acid	1.75±0.25	1.08±0.18	0.39 ±0.18	-0.67±0.31	*ns*	0.59±0.22	27	0.9121	0.891	1.6	1.3	19.1	2.0
Shikimic acid	4.63±0.34	2.71±0.24	*ns*	-1.92±0.42	*ns*	*ns*	27	0.9625	0.959	7.6	2.8	9.6	3.3
Succinic acid	1.76±0.17	3.26±0.42	-1.14 ±0.42	1.51±0.71	*ns*	-1.71±0.51	27	0.8986	0.845	15.7	4.0	8.9	1.5
TOTAL	8.53±0.54	8.57±0.66	-0.89 ±0.66	*ns*	*ns*	-1.80±0.81	27	0.9703	0.964	67.4	8.2	12.3	2.0
Punicalin	0.58±0.06	0.54±0.04	-0.06 ±0.04	*ns*	-0.06±0.03	-0.05±0.05	27	0.9734	0.937	0.53	0.73	20.90	2.1
Punicalagin (isomer 1)	1.56±0.25	1.34±0.14	*ns*	*ns*	-0.30±0.14	*ns*	27	0.9500	0.930	1.63	1.28	12.89	3.0
Punicalagin gallate (isomer 1)	0.16±0.04	0.13±0.03	*ns*	*ns*	-0.04±0.04	0.04±0.03	27	0.8449	0.795	0.02	0.14	43.09	1.6
Punicalagin (isomer 2)	3.15±0.27	2.91±0.19	*ns*	*ns*	-0.38±0.34	*ns*	27	0.9757	0.950	7.71	2.78	12.33	2.5
Punicalagin gallate (isomer 2)	0.07±0.01	0.27±0.06	0.12 ±0.06	0.20±0.10	*ns*	0.18±0.07	27	0.8844	0.760	0.02	0.14	75	1.7
TOTAL	5.71±0.81	5.19±0.44	*ns*	*ns*	-0.87±0.04	*ns*	27	0.9641	0.947	26.2	5.1	13.6	2.2
Luteolin-6-*C*-glucose-8-*C*-glucose	4.71±0.31	4.59±0.17	-0.33 ±0.17	-0.47±0.29	0.52±0.29	*ns*	27	0.9931	0.992	18.41	4.29	4.0	2.7
5-*O*-*p*-Coumaroylquinic acid	4.91±0.28	5.08±0.35	-1.63 ±0.35	*ns*	*ns*	-2.02±0.42	27	0.9799	0.980	27.30	5.23	9.8	1.7
Luteolin-8-*C*-glucoside	9.07±1.97	11.65±1.08	1.31 ±1.08	4.07±1.87	-2.24±1.87	2.27±1.32	27	0.9631	0.954	131.8	11.5	16.8	2.5
Apigenin-8-*C*-glucoside	13.66±0.80	12.36±0.57	-0.98 ±0.57	-1.30±0.99	*ns*	*ns*	27	0.9889	0.987	133.7	11.6	6.4	2.2
Quercetin-3-*O*-rutinoside	2.33±0.30	3.23±0.21	0.24 ±0.21	0.90±0.36	*ns*	0.52±0.26	27	0.9798	0.975	9.58	3.09	11.9	1.5

	Fitting coefficients obtained after applying the Box-Behnken design equation						Statistical information of the fitting analysis						
	Intercept	**Linear effect**		**Quadratic effect**		**Interactive effect**	**Obs**	**R^2**	**R^2adj**	**MSE**	**RMSE**	**MAPE**	**DW**
	b_0	b_1 (t)	b_2 (D)	b_{11} (t^2)	b_{22} (D^2)	b_{12} ($t \times D$)							
Apigenin-6-*C*-glucoside	12.05±0.48	11.92±0.59	-0.78 ±0.59	*ns*	*ns*	*ns*	27	0.9865	0.984	123.9	11.1	7.2	2.5
Kaempferol-3-*O*-rutinoside	6.64±0.33	8.62±0.23	-1.51 ±0.23	1.98±0.40	*ns*	-1.18±0.28	27	0.9966	0.996	67.8	8.23	4.7	1.6
Luteolin-6-*C*-hexoside	0.15±0.05	0.34±0.04	-0.05 ±0.04	0.19±0.06	*ns*	*ns*	27	0.9459	0.935	0.12	0.34	47.9	2.0
TOTAL	52.30±3.19	57.79±2.25	-3.72 ±2.25	5.49±3.90	*ns*	*ns*	27	0.9920	0.990	2906	53.9	6.2	2.1

ns: non significant coefficient; *R^2*: Correlation coefficient; *R^2adj*: Adjusted coefficient of determination; *MSE*: Mean squared error; *RMSE*: Root mean square errors; *MAPE*: Mean absolute percentage error; and *DW*: Durbin-Watson statistic.

Organic acids and phenolic compounds

The results of the organic acids and phenolic compounds analysis are presented in **Table 21**. Shikimic acid was the most abundant organic acid in *T. lignosa*. Oxalic, quinic and succinic acids were also quantified. Ellagitannin derivatives, namely punicalin, punicalagin isomers and punicalagin gallate isomers, were found to be the predominant phenolic compounds, representing approximately 90% of the total phenolic fraction. Among them, punicalagin (isomer 2) was the most abundant. A phenolic acid (5-*O*-*p*-coumaroylquinic acid), flavones (luteolin-6-*C*-glucose-8-*C*-glucose, luteolin-8-*C*-glucoside, and luteolin-6-*C*-hexoside) and flavonols (apigenin-8-*C*-glucoside, apigenin-6-*C*-glucoside, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-rutinoside) were also identified in the studied plant material. The presented phenolic profile (**Figure 26**) is coherent to that previously characterized by our research team in commercial samples of this specie (Pinela *et al.* 2012). All quantified phytochemicals were grouped in organic acids, ellagitannin derivatives and others phenolics, and the quantification values were used as dependent variables in the RSM analysis.

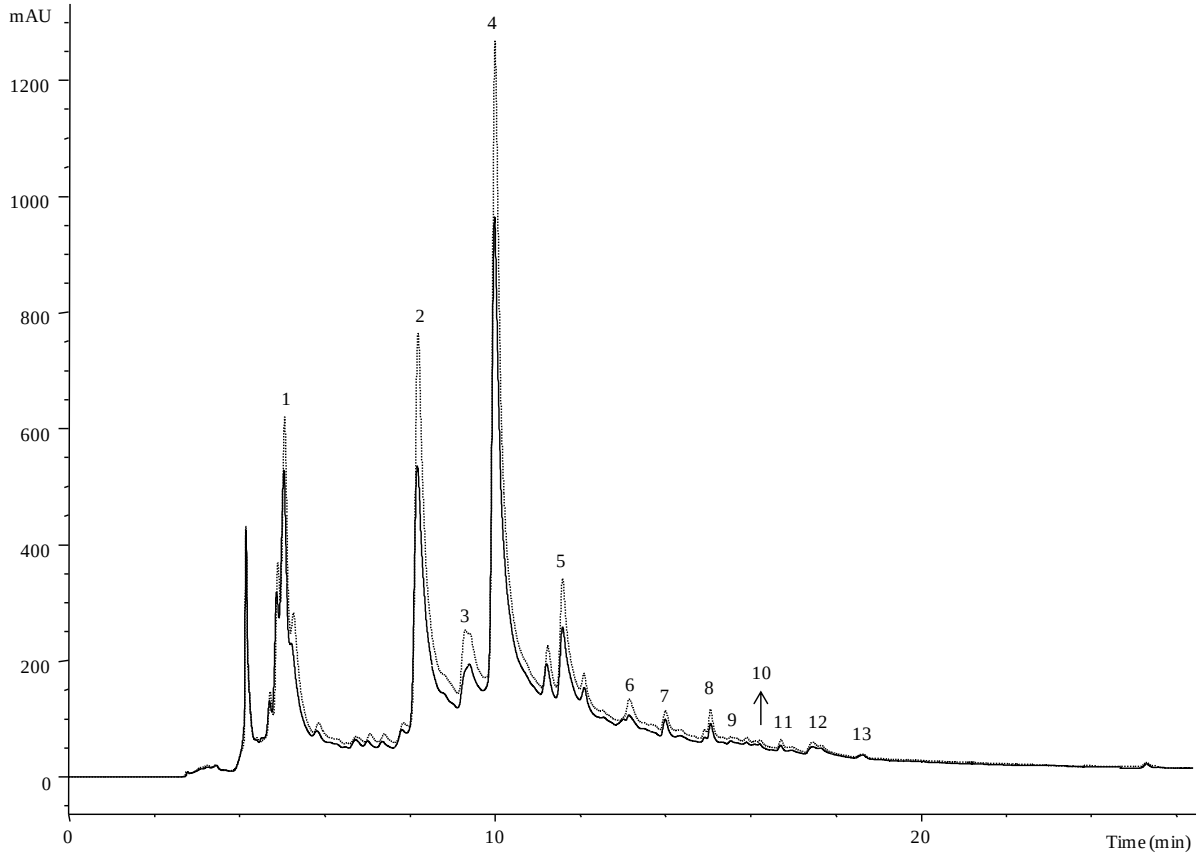

Figure 26 HPLC profile of phenolic compounds in *T. lignosa* extracts obtained from non-irradiated (0 kGy; ____) and irradiated (5 kGy; -----) samples processed for 10 min, recorded at 280 nm. 1: Punicalin; 2: Punicalagin (isomer 1); 3: Punicalagin gallate (isomer 1); 4: Punicalagin (isomer 2); 5: Punicalagin gallate (isomer 2); 6: Luteolin-6-*C*-glucose-8-*C*-glucose; 7: 5-*O*-*p*-Coumaroylquinic acid; 8: Luteolin-8-*C*-glucoside; 9: Apigenin-8-*C*-glucoside; 10: Quercetin-3-*O*-rutinoside; 11: Apigenin-6-*C*-glucoside; 12: Kaempferol-3-*O*-rutinoside; 13: Luteolin-6-*C*-hexoside.

3.4.3.2. Modelling and fitting the model

The RSM experiment was designed based on the responses discussed above. The multivariable characterization performed by RSM technique allows a simultaneous analysis of variables, reduces the number of coefficients used to describe the responses, provides better estimations of parameters, reduces the interval of confidence of the coefficients, and minimizes the effects of experimental errors. This simultaneous description of all curves is very efficient when the experimental results obtained do not span the full range and some of them fail to provide information about one or more of the parameters of the equation. In addition, standardizing the response, optimal data analysis is obtained independently of the experimental values of the variables, which is one of the common issues when analysing several factors (De Lean *et al.* 1978, Murado and Prieto 2013b).

The variables *t* and *D* notably altered the efficiency of the extraction process. A full factorial RSM design of three levels was applied and the second-order polynomial model of Eq. [5] with interactive terms was used to predict each response. The experimental results obtained after running 27 trials (9 genuine combination conditions and 3 replicates per condition) for each response are presented in **Table 21**.

Theoretical response surface models

Table 22 shows, for each response, the estimated coefficient values of Eq. [5] obtained by non-linear least-squares analysis, the coefficients parametric intervals and several numerical statistical criteria used to test the validity of the fitting procedure. The coefficients that showed effects with *p*-values higher than 0.05 were consider not significant (*ns*) at the 95% confidence level and were discarded for the model development. Therefore, mathematical models were built according to the second-order polynomial model of Eq. [5] obtaining the expressions described below.

When the response of extracted residue (%) was considered:

Residue: $$Y = 10.17 + 8.49t - 0.89D - 1.69t^2 - 0.48tD \quad [6]$$

When the antioxidant responses (IC_{50} values, mg/g P) were considered:

DPPH• scavenging activity: $$Y = 339.1 + 101.1t + 25.9D - 238.1t^2 \quad [7]$$

Reducing power: $$Y = 268.5 + 73.4t + 15.7D - 195.2t^2 \quad [8]$$

β-Carotene bleaching inhibition: $$Y = 317.9 + 102.3t - 215.6t^2 \quad [9]$$

TBARS formation inhibition: $$Y = 66.5 + 18.8t - 47.6t^2 \quad [10]$$

When the response of organic acids (mg/g P) was considered:

Oxalic acid: $$Y = 1.70 + 1.51t - 0.65tD \quad [11]$$

Quinic acid: $$Y = 1.75 + 1.08t + 0.39D - 0.67t^2 + 0.59tD$$ [12]

Shikimic acid: $$Y = 4.63 + 2.71t - 1.92t^2$$ [13]

Succinic acid: $$Y = 1.76 + 3.26t - 1.44D + 1.51t^2 - 1.71tD$$ [14]

TOTAL: $$Y = 8.53 + 8.57t - 0.89D - 1.80tD$$ [15]

When the response of ellagitannin derivatives (mg/g P) was considered:

Punicalin: $$Y = 0.58 + 0.54t - 0.06D - 0.06D^2 - 0.05tD$$ [16]

Punicalagin (isomer 1): $$Y = 1.56 + 1.34t - 0.30D^2$$ [17]

Punicalagin gallate (isomer 1): $$Y = 0.16 + 0.13t - 0.04D^2 + 0.04tD$$ [18]

Punicalagin (isomer 2): $$Y = 3.15 + 2.91t - 0.38D^2$$ [19]

Punicalagin gallate (isomer 2): $$Y = 0.07 + 0.27t + 0.12D + 0.20t^2 + 0.18tD$$ [20]

TOTAL: $$Y = 5.71 + 5.19t - 0.87D^2$$ [21]

When the response of other phenolic compounds (mg/100 g P) was considered:

Luteolin-6-*C*-glucose-8-*C*-glucose: $$Y = 4.71 + 4.59t - 0.33D - 0.47t^2 + 0.52D^2$$ [22]

5-*O*-*p*-Coumaroylquinic acid: $$Y = 4.91 + 5.08t - 1.63D - 2.02tD$$ [23]

Luteolin-8-*C*-glucoside: $$Y = 9.07 + 11.65t + 1.31D + 4.07t^2 - 2.24D^2 + 2.27tD$$ [24]

Apigenin-8-*C*-glucoside: $$Y = 13.66 + 12.36t - 0.98D - 1.30t^2$$ [25]

Quercetin-3-*O*-rutinoside: $$Y = 2.33 + 3.23t + 0.24D + 0.90t^2 0.52tD$$ [26]

Apigenin-6-*C*-glucoside $$Y = 12.05 + 11.92t - 0.78D$$ [27]

Kaempferol-3-*O*-rutinoside: $$Y = 6.64 + 8.62t - 1.51D + 1.98t^2 - 1.18tD$$ [28]

Luteolin-6-*C*-hexoside: $$Y = 0.15 + 0.34t - 0.05D + 0.19t^2$$ [29]

TOTAL: $$Y = 52.30 + 57.79t - 3.72D + 5.49t^2$$ [30]

In all cases, *t* corresponds to extraction time, *D* corresponds to irradiation dose, and *Y* is the response. Only the significant parameters of the second-order polynomial model of Eq. [5] were used since some terms were non-significant (**Table 22**). Although the obtained model coefficients are empirical and cannot be associated with physical or chemical significance, their numerical values can be used for direct comparisons because they are presented based on the coded values of the variables in the experimental plan. In addition, the model is useful for many operational purposes, such as predicting results of untested operating conditions. The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level, and when a

factor has a negative effect, the response is lower at high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable.

Statistical and experimental verification of the predictive models

Three basic groups of analysis were used to verify the model significance: 1) Coefficients significance (α=0.05); 2) Model consistency (Fisher *F*-test); and 3) Other statistical criteria (R^2, R^2_{adj}, MSE, RMSE, MAPE, DW coefficient, and ANOVA). Only in three cases (β-carotene bleaching inhibition, TBARS formation inhibition, and oxalic acid), the statistic verification fails in at least one of the groups or subgroups described. In all other cases, the models are workable and can be applied in the subsequent prediction and optimisation stages showing a good agreement between the experimental and predicted values, which implies that the variation is explained by the independent variables.

All significant models presented high values of R^2 and R^2adj (**Table 22**), which indicates the percentage of variability of each response explained by the models. The statistic lack of fit (MSE, RMSE, MAPE, and DW coefficient), used to test the adequacy of the developed models, demonstrated that a non-considerable improvement was achieved by the inclusion of the statistically non-significant (*ns*) parameters (**Table 22**). In addition, the ANOVA results for the regression equations are presented in **Annexe 4, Annexe 5, Annexe 6** and **Annexe 7**. The linear and quadratic terms were highly significant ($p < 0.01$). The lack of fit (used to verify the adequacy of the model) was not significant ($p > 0.05$), which indicates that the model fits adequately to the experimental data.

In general, the behaviour of the extraction kinetics can be understood by the second-order polynomial models described in Eqs. [6] to [30]. To make more explicit the RSM combinations and visually present the tendencies, the effects of irradiation in the kinetics of the extraction process are discussed below in detail.

3.4.3.3. Analysis of the response surfaces

The combined effects of the ionizing radiation dose and extraction time on the extraction yield and on the extractability of organic acids, ellagitannin derivatives and other phenolic compounds (flavonoids and a phenolic acid) from *T. lignosa* are presented in **Figure 27**. In the left-hand side are presented the 3D response surface plots as function of the studied independent variables. The grid surface was build using the theoretical values predicted with the second order polynomial of Eq. [5]. The points (•) represent the experimental results presented in **Table 21**. The statistical information is illustrated in the right-hand side using two basic graphical criteria, depicting the capacity to predict the obtained results (based on R^2 coefficients) and the residual distribution as function of each variable. The distribution of residuals is shown always randomly scattered around zero, and grouped data and

autocorrelations are not observed. This means that these models are workable and can be applied in subsequent prediction stages. It is also indicated a good agreement between the experimental and predicted values (R^2 > 0.95), which implies that the variation can be explained by the independent processing variables. The estimated parametric values are shown in **Table 22**.

The extraction yield was improved by longer extraction times through a nonlinear interactive manner (**Figure 27** and **Table 22**), whereas the irradiation treatment led to a linear decrease in the amount of extracted residue (the extraction process efficacy was decreased in 13.28% by the maximum dose tested; **Table 23**).

The higher levels of total organic acids were obtained from non-irradiated material extracted for 10 min (**Figure 27**). The extraction yield of the total of these compounds decreased 22.16% by the action of the irradiation treatment (**Table 23**). A decrease of the total organic acids content was also verified by Fernandes *et al*. (2014) in dried samples of the wild mushrooms *Boletus edulis* Bull. and *Russula delica* Fr. electron-beam irradiated at 10 kGy. This phenomenon can be explained by the direct decomposition effect of gamma (or electron-beam) radiation on these organic compounds. Regarding the impact on specific organic acids (**Figure 28**), it was observed that the extractability of quinic acid increases with the consequent increase in extraction time and ionizing radiation dose. Due to these non-linear and linear interactive effects, respectively, when extracting the plant material irradiated at 10 kGy for 10 min, the maximum value of 3.93 mg of quinic acid per g of plant material was obtained. Furthermore, it was also found a negative quadratic effect for the extraction time, *i.e.,* when extracting the non-irradiated plant material (0 kGy) for 10 min, the extraction yield of this compound is decreased. On the other hand, the extraction time had a positive quadratic effect on the extractability of succinic acid and interacted negatively with the ionizing radiation dose, which means that increasing the dose decreases the yield of this compound (in 39.32%) in accordance with the trend verified for the total organic acids content. Shikimic acid, the most abundant organic acid in *T. lignosa*, was not affected by the irradiation dose (**Table 23**). The preservation of this compound was also verified by Pereira, Barros, Antonio, *et al.* (2015) in *Ginkgo biloba* L. samples gamma irradiated at doses up to 10 kGy. The results for oxalic acid were not statistically significant ($R^2 \leq 0.69$).

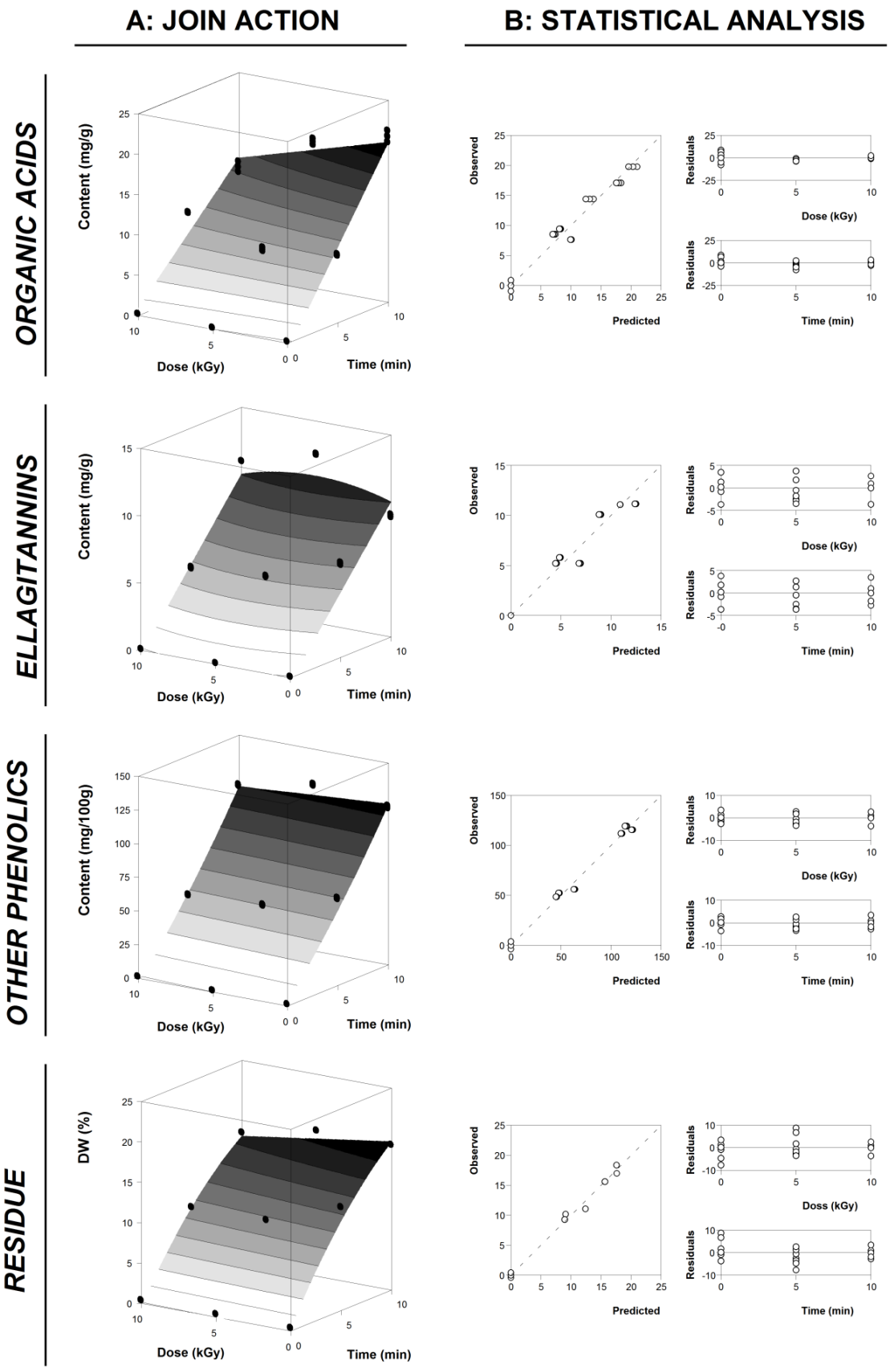

Figure 27 Graphical results of the effects of irradiation dose on the extraction kinetics of different groups of phytochemicals from *T. lignosa* aerial parts. In the left-hand side is presented the joint graphical 3D analysis in unction of each variable involved (*t* and *D*) for total organic acids, total ellagitannins and total other phenolic compounds and for the extracted residue. The grid

surfaces were built using the theoretical values predicted with the second order polynomial of Eq. [3]. Points (•) represent the obtained experimental results presented in **Table 21**. The estimated parametric values are shown in **Table 22**. The goodness of fit is illustrated in the right-hand side based on two basic graphical criteria, depicting the capacity to predict the obtained results (based on R^2 coefficients) and the residual distribution in function of each variable.

Table 23 Global variable effect (%) computed from the developed Box-Behnken model (Eq. [5]) presented in **Table 22** for each evaluated response. The effect of the variable was linear (L) or non-linear (NL) and, in both cases, there was or not an interactive effect (I). The arrows represent the effect of increasing (↑) or decreasing (↓) the extraction. The percentage of this effect is presented between brackets.

Response variables		**Variable effects**	
		Time *(%)*	Dose *(%)*
Extraction yield	Residue	NL-I ↑ (100)	L-I ↓ (13.28)
Antioxidant activity	DPPH• scavenging activity	NL ↑ (79.60)	L ↑ (20.40)
	Reducing power	NL ↑ (82.41)	L ↑ (17.59)
	β-Carotene bleaching inhibition	NL ↑ (100)	*ns*
	TBARS formation inhibition	NL ↑ (100)	*ns*
Organic acids	Oxalic acid	L-I ↑ (100)	L-I ↓ (30.15)
	Quinic acid	NL-I ↑ (63.59)	L-I ↑ (36.41)
	Shikimic acid	NL ↑ (100)	*ns*
	Succinic acid	NL-I ↑ (100)	L-I ↓ (39.32)
	TOTAL	L-I ↑ (100)	L-I ↓ (22.16)
Ellagitannin derivatives	Punicalin	L-I ↑ (100)	NL-I ↓ (16.78)
	Punicalagin (isomer 1)	L ↑ (100)	*ns*
	Punicalagin gallate (isomer 1)	L-I ↑ (78.25)	NL-I ↓ (21.75)
	Punicalagin (isomer 2)	L ↑ (100)	*ns*
	Punicalagin gallate (isomer 2)	NL-I ↑ (56.57)	L-I ↑ (43.43)
	TOTAL	L ↑ (100)	*ns*
Other phenolic compounds	Luteolin-6-*C*-glucose-8-*C*-glucose	NL ↑ (100)	NL ↓ (6.64)
	5-*O*-*p*-Coumaroylquinic acid	L-I ↑ (100)	L-I ↓ (35.22)
	Luteolin-8-*C*-glucoside	NL-I ↑ (77.48)	NL-I ↑ (22.52)
	Apigenin-8-*C*-glucoside	NL ↑ (100)	L ↓ (7.33)
	Quercetin-3-*O*-rutinoside	NL-I ↑ (82.15)	L-I ↑ (17.85)
	Apigenin-6-*C*-glucoside	L ↑ (100)	L ↓ (6.12)
	Kaempferol-3-*O*-rutinoside	NL-I ↑ (100)	L-I ↓ (21.56)
	Luteolin-6-*C*-hexoside	NL ↑ (100)	L ↓ (13.53)
	TOTAL	NL ↑ (100)	L ↓ (6.05)

ns: no significant effect was found

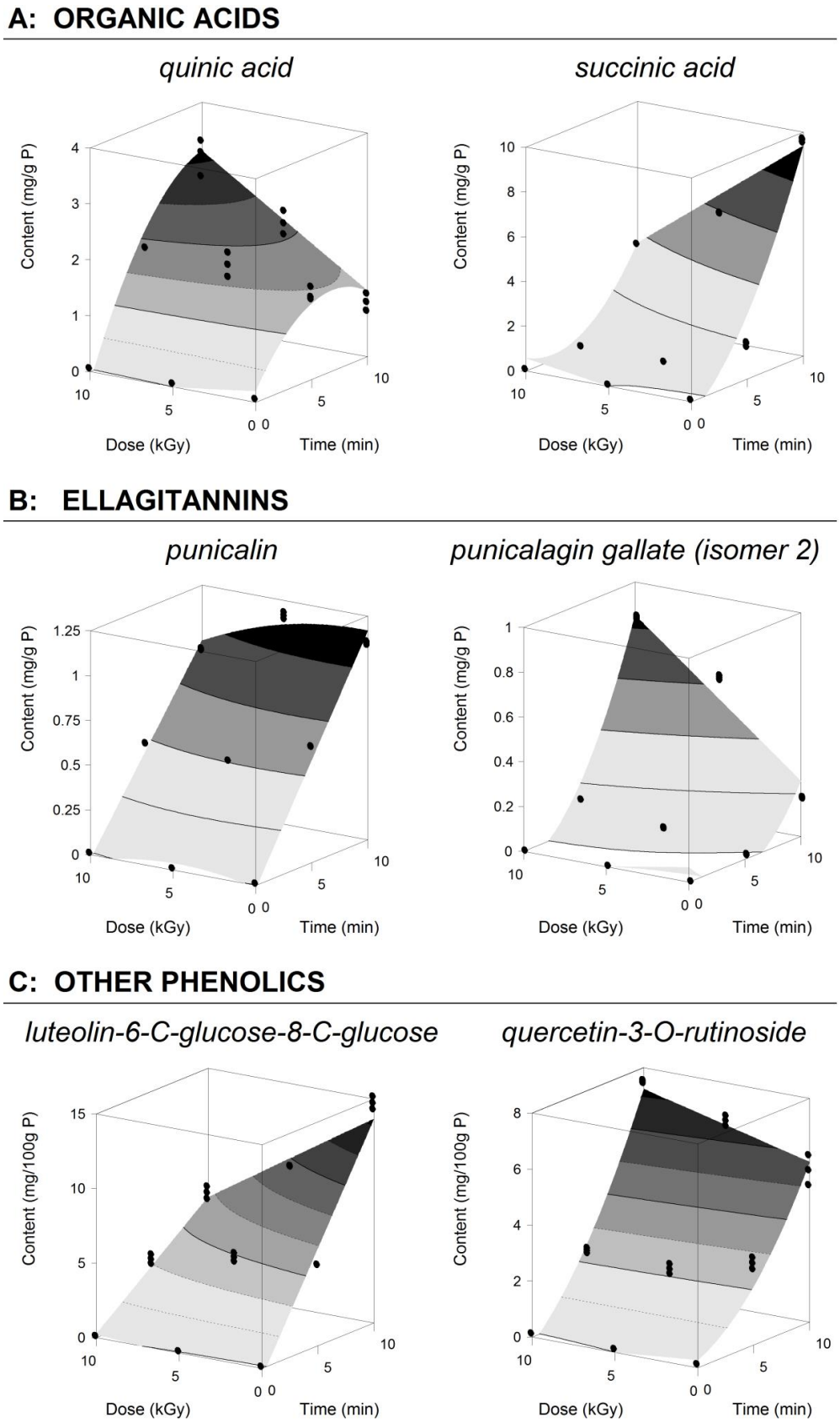

Figure 28 Graphical results of the effects of irradiation dose on the extraction kinetics of selected phytochemicals from *T. lignosa* aerial parts. The grid surfaces were built using the theoretical values predicted with the second order polynomial of Eq. [3]. Points (•) represent the obtained experimental results presented in **Table 21**. The estimated parametric values are shown in **Table 22**.

Concerning the group of ellagitannins (manly punicalagin derivatives), it was verified a linear effect of the extraction time leading to a higher yield of these compounds at 10 min of extraction. Intermediate ionizing radiation doses promoted the extractability of these added-value compounds. Thus, the higher yields (~12.4 mg/g P) were achieved with the observations 8, 17 and 26 corresponding to the 5 kGy dose and 10 min of extraction (**Table 21**). This effect can also be seen in **Figure 26**, which compares the HPLC phenolic profile of the extracts obtained from samples non-irradiated (0 kGy) and irradiated at 5 kGy, processed for 10 min. The 10 kGy dose did not induced significant effects on the total content of ellagitannins (**Table 23**). In the case of punicalin (**Figure 28**), this ellagitannin was degraded with the consequent increase in irradiation dose, being also observed a slight negative interaction between both independent variables (**Table 22**). Contrariwise, punicalagin gallate (isomer 2) was better extracted from plant material irradiated at higher doses. The 10 kGy dose improved the extraction efficacy of this compound in 43.43% (**Table 23**), leading to amounts of 0.82 mg/g P. A positive interaction between both variables was also found for both isomers of punicalagin gallate. As far as we know, studies regarding the impact of processing by ionizing radiation on ellagitannin derivatives are very scarce. In addition, it is important to highlight the antioxidant properties of punicalagin and punicalin. According to previous studies, these compounds have a strong free-radical (DPPH, ABTS and hydroxyl radicals) scavenging capacity (Kulkarni *et al.* 2004, Mena *et al.* 2013, Wang *et al.* 2013, Vora *et al.* 2015) that has been attributed to their high degree of hydroxylation (Landete 2011, Moilanen *et al.* 2016).

The extraction kinetics of the other phenolic compounds present in the phenolic fraction (one phenolic acid and seven flavonoids) was also affected by the applied treatment. As verified for organic acids and ellagitannins, longer extraction times favoured the extraction of the total content of these compounds (**Table 22**), but a linear decrease of 6.05% was caused by the irradiation dose (**Table 23**). Irradiation also decreased the hydrophilic antioxidant responses (which had higher IC_{50} values). Comparable extraction trends were reported by Martins, Barros, Santos-Buelga, *et al.* (2014); longer preparations procedures led to a higher recovery of flavonoids and total phenolic compounds from thyme and oregano (*Origanum vulgare* L.). In the particular case of luteolin-6-*C*-glucose-8-*C*-glucose (**Figure 28**), its extractability decreases 6.64% with the increase of the irradiation dose up to 10 kGy. In turn, the extractability of luteolin-8-*C*-glucoside and quercetin-3-*O*-rutinoside was improved by the dose in 22.52 and 17.85% trough nonlinear interactive and linear interactive effects, respective. The 5-*O*-*p*-coumaroylquinic acid (the only quantified phenolic acid) was greatly affected by the irradiation treatment.

3.4.3.4. Industrial relevance

Ionizing radiation is the most effective technique for preserving the phytochemical composition and bioactive properties of plant commodities, to assure its hygienic quality, and reduce losses associated with insect infestation and microbial contamination (Alothman *et al.* 2009, Pereira, Barros, Antonio, *et al.* 2015, Pinela *et al.* 2015, Pereira *et al.* 2016). For the different industrial sectors interested in plant materials (in particular medicinal plants and herbs), the use of suitable preservation/decontamination treatments is critical to obtain high quality raw material. Ionizing radiation has been increasingly used and, for this reason, it is important to know how the treatment affects the integrity and extractability of different biomolecules. This study provides information, little explored so far, on the ionizing radiation effects on the extraction/degradation kinetics of high added-value phytochemicals from *T. lignosa* aerial parts. This medicinal plant was selected due to its interesting composition in ellagitannins, namely punicalin and punicalagin derivatives (which levels can reach 221 mg/g extract; Pinela *et al.* 2012), bioactive polyphenols with several medical and pharmaceutical applications (Yaidikar and Thakur 2015, Zhong *et al.* 2015, Rao *et al.* 2016).

3.4.4. Conclusions

Chromatographic and spectrometric methods were successfully combined with RSM for evaluating the impact of gamma radiation on obtaining ellagitannin-rich extracts from *T. lignosa*. The extracts were particularly rich in hydrophilic antioxidants (measured by *in vitro* assays) and the content of different phytochemicals was improved by longer processing times. In general, the ellagitannin derivatives (~90% of the phenolic fraction) were not significantly affected by the highest irradiation dose. However, the group consisting of flavonoids and a phenolic acid and the group of organic acids were negatively affected by irradiation. Therefore, depending on the dose, the irradiation treatment (applied for decontamination/quarantine purposes) will preserve the ellagitannins content or improve their extraction. The obtained functional extracts could be used in the development of functional foods and nutraceuticals. This study also highlights *T. lignosa* as a source of high added-value molecules, which may be responsible for the therapeutic properties attributed to this plant highly regarded in the North-eastern region of Portugal.

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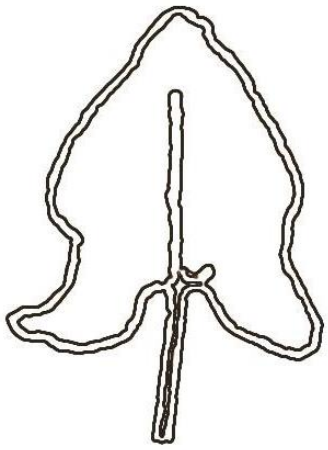
4. Leafy Vegetables



This **4th chapter** covers the work performed with two leafy vegetables, buckler sorrel (*Rumex induratus* Boiss. & Reut.) and watercress (*Nasturtium officinale* R. Br.). The nutritional and chemical composition and the antioxidant activity of these species are described, as well as the effects of post-packaging irradiation and modified atmosphere packaging on these quality parameters and its suitability for shelf-life extension during refrigerated storage. The optimization of the cold extraction of phenolic compounds from watercress by high hydrostatic pressures using response surface methodology is also discussed.

Buckler sorrel (*Rumex induratus* Boiss. & Reut.)

4.1. Modified atmosphere packaging and post-packaging irradiation of *Rumex induratus* leaves: A comparative study of postharvest quality changes

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ORIGINAL ARTICLE

Modified atmosphere packaging and post-packaging irradiation of *Rumex induratus* leaves: a comparative study of postharvest quality changes

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Abstract

The effects of conventional and inert-gas enriched atmospheres, as well as those caused by different γ-irradiation doses, on buckler sorrel (*Rumex induratus*) leaves quality were evaluated and compared after 12 days of storage at 4 ºC. The green colour of the abaxial surface of the leaves was relatively stable, as well as the carbohydrates content and the calorific value. The storage time decreased the amounts of fructose, glucose, trehalose, α-tocopherol, and β-tocopherol and increased the levels of total organic acids and δ-tocopherol. The total tocopherols content was higher in air-packaged non-irradiated leaves, antioxidant compounds that may have contributed to the preservation of polyunsaturated fatty acids (PUFA). Some antioxidant properties were also favoured during storage. It was found that the overall postharvest quality of buckler sorrel leaves is better maintained with the argon-enriched atmospheres, while the 6 kGy dose was a suitable option to preserve PUFA during cold storage. This study highlights the suitability of the applied postharvest treatments and the interest to include buckler sorrel leaves in contemporary diets.

Keywords: *Rumex* induratus; buckler sorrel; modified atmosphere packaging; gamma irradiation; refrigerated storage; postharvest quality.

4.1.1. Introduction

The human longevity associated with the Mediterranean Diet could be partly attributed to the intake of wild plant-based foods (Yannakoulia *et al.* 2015). The consumption of these plants plays an important role in complementing staple agricultural foods in many regions of the world (García-Herrera *et al.* 2014). A good example is buckler sorrel (*Rumex induratus* Boiss. & Reut., Fam. Polygonaceae), a native Iberian plant that occurs mainly in dry and stony sites of the thermo-Mediterranean region. Their tender leaves are appreciated and consumed, especially in salads (Carvalho 2010), constituting a promising dietary source of nutrients (Pereira *et al.* 2011) and biologically active compounds (Ferreres *et al.* 2006, Guerra *et al.* 2008). However, this leafy vegetable is underutilized, due to the altered lifestyle of the modern society and introduction of non-native vegetables, not being found in food composition databases.

The revalorization of traditionally consumed wild species is currently considered as a focus of renewed attention (García-Herrera *et al.* 2014, Bacchetta *et al.* 2016). Besides being an important input of health-promoting compounds and considered as added-value foods for commercialization, its recovery is an important strategy to improve the diversity of available foods. Additionally, consumers are looking for safe, healthy, more sustainable and convenient foods with different organoleptic properties of those daily consumed (Kühne *et al.* 2010). Thus, the resurgence of buckler sorrel consumption can meet this demand, and may be achieved if some challenges are accomplished, like quality assurance and innovation.

To promote buckler sorrel revival and commercialization it is important to improve its shelf-life for an extended period for distribution and storage. This can be achieved using modified atmosphere packaging (MAP), a method which consists of changing the headspace gas composition surrounding the food in the package to prolong the initial fresh state and quality. The consequent reduction in metabolic activity and chemical oxidation prevents compositional changes associated with maturation and senescence, thus retaining the attributes that consumers consider as freshness markers (Pinela and Ferreira 2017). Recently, inert gases such as argon and nitrogen have been tested (Char *et al.* 2012, Pinela, Barreira, Barros, Antonio, *et al.* 2016), but the literature describing their application and benefits is still limited. Besides, modified atmospheres can passively evolve within the package as a consequence of product respiration and diffusion of gases through the film (Choi *et al.* 2015). Postharvest treatments based on ionizing radiation can also be used for preserving fresh fruits and vegetables during shelf-life (Pinela and Ferreira 2017). Low doses of γ-irradiation are ideally applied after packaging to prevent post-contamination and to delay

physiological processes (ICGFI 1999). Nevertheless, the commercial application to fresh products is still limited, despite being considered as a safe and effective technology by several international authorities (WHO 1999).

At the present, there are no reports that any preservation technology has previously been applied to buckler sorrel leaves. In this study, the effects induced by different packaging atmospheres (conventional and inert-gas enriched MAP) and γ-irradiation doses (up to 6 kGy) on postharvest quality parameters of buckler sorrel leaves were evaluated after 12 days of storage at 4 ºC.

4.1.2. Materials and methods

4.1.2.1. Standards and reagents

Amber Perspex routine dosimeters, Batch X, were purchased from Harwell Company (Oxfordshire, UK). Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), other individual fatty acid isomers, tocopherols (α-, β-, γ-, and δ-isoforms), sugars ($_D$(–)-fructose, $_D$(+)-glucose anhydrous, $_D$(+)-melezitose hydrate, $_D$(+)-sucrose, and , $_D$(+)-trehalose), organic acids (quinic, malic, oxalic, and ascorbic acids), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and catechin standards were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH˙) as obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

4.1.2.2. Sampling and samples preparation

Wild specimens of buckler sorrel or French sorrel (*Rumex induratus* Boiss. & Reut.; syn: *Rumex scutatus* subsp. *induratus* (Boiss. & Reut.) Nyman) were gathered in April 2014 in the Bragança region (North-eastern Portugal), considering local consumers' sites, criteria and preferences (Carvalho 2010). Subsequently, healthy and undamaged leaves were selected, rinsed in tap water and drained to eliminate excess water. A portion was immediately analysed (non-stored control), and the remaining fresh material was subjected to the postharvest treatments described below and analysed in the end of the storage period. A voucher specimen was deposited in the Herbarium of the School of Agriculture of Bragança.

4.1.2.3. Samples packaging and irradiation

A low-density polyethylene film (black LDPE resin) with a thickness of 63 μm and permeability to O_2 and CO_2 at 25 ºC of 69 $cm^3/m^2/24$ h/atm and 251 $cm^3/m^2/24$ h/atm,

respectively, was used in the manufacture of packages (VWR, Lisbon, Portugal). Approximately 20 g of buckler sorrel leaves were placed in 11.5 cm × 20 cm sterilized packages (headspace volume of 0.7 L) and applied four different atmospheres: air-packaging (stored control in passive MAP), vacuum-packaging (no atmosphere), and N_2- and Ar-enriched atmospheres. Briefly, air-packaging consisted of sealing without eliminating the air in the package (20.8% O_2 and <0.1% CO_2) and vacuum-packaging was performed by eliminating the air with a vacuum-packaging machine. For non-conventional MAP, the headspace air in the packages was first eliminated and then the target gas (100% N_2 or Ar) was injected.

For the irradiation treatment, air-packaged samples were divided into four groups: a non-irradiated (0 kGy) control group and three groups irradiated at 1, 2 and 6 kGy of γ-rays (predicted doses). The irradiation was performed one day after packaging in an ^{60}Co experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) located at C2TN, with four sources and a total activity of 177 TBq (4.78 kCi; February 2014). Amber Perspex routine dosimeters were used to measure the distribution of the absorbed energy and to determine the maximum (D_{max}) and the minimum (D_{min}) dose absorbed by the samples, following the procedure previously described by Fernandes *et al.* (2012). Although γ-rays penetrates dense materials and can be used to treat boxed commodities and even those stacked on pallets (Hallman 2017), the samples were rotated upside down half of the time to increase the dose uniformity. The measured average doses were 1.02±0.07 kGy, 2.14±0.08 kGy and 5.99±0.20 kGy for the samples irradiated at the predicted doses of 1, 2 and 6 kGy, respectively. The dose uniformity ratio (D_{max}/D_{min}) was 1.18. The desired doses were achieved by the time of exposure and by the location of the samples relative to the source. The estimated average dose rate for the irradiation position was obtained with a Fricke dosimeter and was 1.22 kGy/h.

A total of 70 packages were prepared (10 for each treatment) and stored at 4 ºC for 12 days.

4.1.2.4. Headspace gas composition analysis

The O_2, CO_2 and N_2 concentrations inside the packages were monitored using a portable gas analyser (model Oxybaby 6.0, WITT, Denmark) previously calibrated by sampling atmospheric air. The Ar concentration was calculated according to the equation (1).

$$[Ar] = 100 - ([O_2] + [CO_2] + [N_2]) \quad (1)$$

Measurements were performed after packaging and at the end of the storage period and the values were expressed as a percentage.

4.1.2.5. Quality analysis

Colour parameters

The CIE *L*a*b** colour values were measured on both sides (adaxial and abaxial surfaces) of nine randomly selected leaves with a colorimeter (model CR-400; Konica Minolta Sensing Inc., Japan) previously calibrated using a standard white plate (Pinela *et al.* 2015). Average values were considered to determine the colour coordinates, where *L** represents lightness, *a** represents chromaticity on a green (-) to red (+) axis and *b** represents chromaticity on a blue (-) to yellow (+) axis.

Proximate composition

Samples were analysed using the AOAC procedures (AOAC 2005). Briefly, the crude protein content (N×6.25) was estimated by the macro-Kjeldahl method, using an automatic distillation and titration unit (model UDK152; VELP Scientifica, Italy); the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC; and total carbohydrates were calculated by the difference according to the equation (2). The results were expressed as g per 100 g of fresh weight (fw).

$$m_{carbohydrates} = 100 - (m_{moisture} + m_{proteins} + m_{fats} + m_{ashes}) \quad (2)$$

The calorific value was calculated according to the equation (3) (Regulation (EU) No 1169/2011) and expressed as kcal per 100 g of fresh weight (fw).

$$Energy = 4 \times (m_{proteins} + m_{carbohydrates}) + 9 \times (m_{fats}) \quad (3)$$

Hydrophilic compounds

Free sugars were determined by high performance liquid chromatography (HPLC) coupled to a refraction index detector as described by Pereira *et al.* (2011). The identification was made by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method, using melezitose as internal standard (IS). The results were expressed in mg per 100 g of fresh weight (fw).

Organic acids were analysed by ultra-fast liquid chromatography (UFLC) coupled to a photodiode array detector (PDA) according to Pereira *et al.* (2013). Briefly, fresh tissue (9 g) was ground and the grinding paste was subsequently extracted by stirring with 25 mL of meta-phosphoric acid (25 ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, samples were filtered through 0.2 µm nylon filters. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm or 245 nm (for ascorbic acid) with calibration curves obtained from commercial standards of each compound, *i.e.,* quinic, malic, oxalic, and ascorbic acids. The results were expressed in mg per 100 g of fresh weight (fw).

Lipophilic compounds

Fatty acids were analysed by gas chromatography with flame ionization detection (GC-FID)/capillary column according to Pereira *et al.* (2011). The identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

Tocopherols were determined by HPLC coupled to a fluorescence detector (FP-2020; Jasco) following the procedures previously described by Pereira *et al.* (2011). The identification was made by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method, using tocol as IS. The results were expressed in mg per 100 g of fresh weight (fw).

4.1.2.6. Bioactive properties

Four *in vitro* assays were performed to evaluate the extracts antioxidant activity (Pinela *et al.* 2015), which were prepared according to Pereira *et al.* (2011) using a mixture of methanol:water (80:20, v/v) as extraction solvent. Briefly, the DPPH$^{\bullet}$ scavenging activity and the reducing power assays were performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA). The reduction of DPPH$^{\bullet}$ was determined by measuring the absorbance at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH$^{\bullet}$ discoloration using the equation (4).

$$RSA\ (\%) = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100 \quad (4)$$

where A_{DPPH} is the absorbance of the DPPH$^{\bullet}$ solution and A_S is the absorbance of the solution containing the sample extract. The reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm. The β-carotene bleaching inhibition (CBI) was evaluated by measuring the capacity to neutralize linoleate free radicals, which was monitored at 470 nm in a Model 200 spectrophotometer (AnalytikJena, Jena, Germany), and calculated using the equation (5).

$$CBI\ (\%) = (A_{\beta T2}/A_{\beta T0}) \times 100 \quad (5)$$

where $A_{\beta T2}$ is the absorbance of the emulsion after 2 h of incubation at 50 ºC and $A_{\beta T0}$ is the initial absorbance. The thiobarbituric acid reactive substances (TBARS) formation inhibition capacity was evaluated in porcine brain homogenates. The colour intensity of the malondialdehyde-thiobarbituric acid complex formed during heating at 80 ºC was measured at 532 nm, and the inhibition ratio calculated using the equation (6).

$$Inhibition\ ratio\ (\%) = [(A - B)/A] \times 100 \quad (6)$$

where A and B correspond to the absorbance of the control and the sample solution, respectively. Results were expressed in EC_{50} values (mg/mL), *i.e.,* sample concentration

providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as a positive control.

Total phenolics (Wolfe *et al.* 2003) and flavonoids (Zhishen *et al.* 1999) were quantified in the hydromethanolic extracts concentrated at 0.625 or 1.25 mg/mL by reading the absorbance at 765 nm or 510 nm, respectively. The standard curves were calculated using gallic acid (for phenolics) and catechin (for flavonoids), and the results were respectively expressed as mg of gallic acid equivalents (GAE) or catechin equivalents (CE) per g of extract.

4.1.2.7. Statistical analysis

For each postharvest treatment, three independent samples were analysed. Data were expressed as mean±standard deviation. All statistical tests were performed at a 5% significance level using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

The differences among treatments were analysed using one-way analysis of variance (ANOVA). The fulfilment of ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Principal components analysis (PCA) was applied as pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as higher as possible) explained by the number of components selected. The number of plotted dimensions (two) was chosen in order to allow meaningful interpretations.

4.1.3. Results and discussion

4.1.3.1. Headspace gas composition

The initial levels of N_2 and Ar inside the packages reached values of above 95% and were lower at the end of the storage period. In both non-conventional MAP, the levels of CO_2 and O_2 were lower than 10 and 15%, respectively. The N_2 concentration inside the N_2-enriched MAP decreased approximately 25%, while this gas evolved within the Ar-enriched MAP (~35%). The final headspace gas composition of air-packaged buckler sorrel leaves (non-irradiated and irradiated) revealed comparable values of N_2 (>75%), while the percentages of

CO_2 and O_2 were ~5% and 20%, respectively. The observed changes can be attributed to the plant respiration process and diffusion of gases through the film.

4.1.3.2. Effect of the packaging atmosphere

Colour parameters

The colour is a very important quality parameter that plays a key role in establishing consumer acceptability of the product, being more important than flavour and texture in the initial food-selection process. Consequently, colour loss is one of the major external postharvest problems. Based on the one-way ANOVA *p*-values (**Table 24**), it was possible to conclude that the assayed packaging atmospheres induced significant changes in all colour parameters, except for greenness (*a**) registered in the abaxial surface of the leaves. Vacuum and non-conventional MAP increased the leaves lightness (*L**) in both surfaces, compared to the non-stored control and to air-packaged leaves. The lower *a** values were registered in the adaxial surface of the leaves stored under N_2-enriched atmospheres, but without statistical difference from those stored in air and Ar-enriched atmospheres. Furthermore, N_2-enriched MAP and air-packaging were the less effective treatments in preventing the leaves yellowing (higher *b** values) in the adaxial and abaxial surfaces, respectively. Additionally, the adaxial surface was more propitious for colour changes than the abaxial one, which could be related to the mesophyll structure and presence of cuticle.

Proximate composition

The proximate composition of the buckler sorrel leaves stored under different packaging atmospheres is presented in **Table 25**. The proximate composition of this species collected in the same geographical region was previously described by Pereira *et al.* (2011). The authors reported lower protein content (1.31 g/100 g fw) and higher amounts of carbohydrates (6.93 g/100 g fw). Despite this, the moisture (~90.29 g/100 g fw), ash (~1.07 g/100 g fw) and fat (~0.39 g/100 g fw) contents and the energetic contribution (~36.47 kcal/100 g fw) were similar to those described in this study. According to the present results, it can be concluded that some of the assayed packaging atmospheres induced significant changes on the protein, fat and ash contents ($p < 0.05$), while moisture, carbohydrates and the energetic contribution were not affected. Air-packaged buckler sorrel leaves presented slightly higher protein and ash levels, contrarily to the observed regarding fat content. In fact, when the plant metabolism is not slowed down by the applied postharvest treatment, the organic reserves of fat may be consumed faster and new compounds will be synthesized and accumulated in the leaves tissues.

Table 24 Colour parameters (indicated for adaxial and abaxial surface of leaves) for buckler sorrel leaves exposed to different postharvest treatments.[1]

		Harvest (day 0)	**12 days of storage at 4 °C**							*p*-values	
			Non-conventional MAP		**Conventional packaging**					1-way ANOVA[2]	
		Non-stored control	**N_2**	**Ar**	**Vacuum**	**Air**					
						0 kGy	**1 kGy**	**2 kGy**	**6 kGy**	PA	ID
Adaxial surface	*L**	39±1^{bD}	46±3^{a}	45±4^{a}	45±2^{a}	41±2^{bCD}	43±2^{BC}	46±4^{A}	46±2^{AB}	<0.001	<0.001
	*a**	-14±1^{aA}	-17±2^{b}	-15±2^{ab}	-14±2^{a}	-16±1^{abAB}	-16±1^{BC}	-16±1^{BC}	-17±1^{C}	0.002	<0.001
	*b**	19±1^{bcD}	25±5^{a}	20±4^{bc}	17±2^{c}	23±3^{abC}	24±2^{BC}	26±2^{AB}	27±4^{A}	<0.001	<0.001
Abaxial surface	*L**	50±2^{bC}	55±1^{a}	55±4^{a}	56±2^{a}	50±2^{bC}	51±1^{BC}	53±2^{AB}	54±2^{A}	<0.001	<0.001
	*a**	-14±1	-14±1	-13±1	-14±2	-14±1	-15±1	-14±1	-15±1	0.114	0.058
	*b**	20±2^{bB}	20±1^{b}	20±3^{ab}	22±4^{ab}	23±1^{aA}	25±3^{A}	23±3^{AB}	25±3^{A}	0.015	<0.001

[1]The results are presented as the mean±SD. [2]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one packaging atmosphere (PA) or irradiation dose (ID) differs from the others (in this case multiple comparison tests were performed). For each preservation treatment, means within a row with different letters (lower case for PA and capital letters for ID) differ significantly ($p<0.05$).

*L** (lightness ↔ darkness); *a** (redness ↔ greenness); *b** (yellowness ↔ blueness).

Table 25 Proximate composition (g/100 g), energy (kcal/100 g), and individual sugars (mg/100 g) and organic acids (mg/100 g) for buckler sorrel leaves exposed to different postharvest treatments.[1]

	Harvest (day 0)	**12 days of storage at 4 °C**							*p*-values	
		Non-conventional MAP		**Conventional packaging**					1-way ANOVA[2]	
	Non-stored control	**N_2**	**Ar**	**Vacuum**	**Air**					
					0 kGy	**1 kGy**	**2 kGy**	**6 kGy**	PA	ID
Moisture	90±1	91±1	91±1	91±1	90±1	91±1	91±1	91±1	0.135	0.103
Ash	0.91±0.02^{bB}	0.96±0.05^{ab}	0.91±0.05^{b}	0.94±0.05^{ab}	1.00±0.05^{aA}	0.99±0.05^{AB}	0.99±0.05^{AB}	0.98±0.05^{AB}	0.026	0.015
Proteins	2.9±0.1^{bBC}	2.9±0.1^{b}	2.9±0.1^{b}	2.9±0.1^{b}	3.3±0.1^{aA}	3.0±0.1^{B}	3.0±0.1^{BC}	2.9±0.1^{C}	<0.001	<0.001
Fat	0.39±0.02^{aA}	0.38±0.03^{ab}	0.39±0.03^{a}	0.36±0.02^{b}	0.35±0.02^{bB}	0.29±0.04^{C}	0.31±0.04^{BC}	0.30±0.03^{C}	0.001	<0.001
Carbohydrates	5.5±0.5	5.0±0.5	5.1±0.5	5.1±0.5	5.3±0.5	5.0±0.5	4.9±0.5	5.0±0.5	0.537	0.403
Calorific	36±3	34±2	34±2	34±3	36±2	33±3	33±3	33±3	0.170	0.054
Fructose	399±19^{aA}	269±9^{b}	234±12^{c}	240±9^{c}	165±7^{dC}	176±8^{C}	191±7^{B}	135±9^{D}	<0.001	<0.001
Glucose	385±17^{aA}	184±6^{b}	157±5^{c}	173±5^{b}	127±7^{dB}	113±6^{C}	124±7^{BC}	86±4^{D}	<0.001	<0.001
Sucrose	66±4^{cA}	26±2^{d}	72±4^{b}	94±4^{a}	24±2^{dC}	24±4^{C}	27±3^{C}	53±4^{B}	<0.001	<0.001
Trehalose	61±3^{aA}	37±3^{b}	34±4^{b}	29±3^{c}	17±2^{dB}	15±2^{B}	6±1^{C}	17±2^{B}	<0.001	<0.001
Total sugars	912±33^{aA}	516±17^{bc}	498±21^{c}	536±11^{b}	332±15^{dB}	328±12^{B}	348±15^{B}	291±14^{C}	<0.001	<0.001
Oxalic acid	0.70±0.04^{abB}	0.64±0.05^{bc}	0.69±0.05^{ab}	0.58±0.05^{c}	0.76±0.05^{aAB}	0.80±0.05^{A}	0.68±0.05^{B}	0.51±0.05^{C}	<0.001	<0.001
Quinic acid	2.0±0.2^{dC}	3.2±0.2^{b}	3.5±0.1^{a}	3.0±0.1^{c}	2.9±0.1^{cB}	3.0±0.1^{AB}	3.1±0.1^{A}	2.8±0.2^{B}	<0.001	<0.001
Malic acid	0.31±0.03^{cA}	0.43±0.05^{b}	0.42±0.05^{b}	0.56±0.05^{a}	0.26±0.04^{cB}	0.35±0.04^{A}	0.14±0.02^{C}	0.14±0.02^{C}	<0.001	<0.001
Ascorbic acid	0.020±0.002^{aA}	0.005±0.001^{d}	0.008±0.001^{c}	0.002±0.001^{e}	0.011±0.001^{bB}	0.009±0.002^{C}	0.008±0.002^{C}	0.007±0.002^{C}	<0.001	<0.001
Total organic acids	3.0±0.1^{dD}	4.3±0.3^{b}	4.6±0.2^{a}	4.1±0.2^{bc}	3.9±0.2^{cB}	4.2±0.2^{A}	3.9±0.2^{B}	3.5±0.2^{C}	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one packaging atmosphere (PA) or irradiation dose (ID) differs from the others (in this case multiple comparison tests were performed). For each preservation treatment, means within a row with different letters (lower case for PA and capital letters for ID) differ significantly ($p<0.05$).

Hydrophilic compounds

From the one-way ANOVA p-values, it can be concluded that the assayed packaging atmospheres induced significant changes in the free sugars content (**Table 25**). Fructose, glucose, sucrose and trehalose were identified in the buckler sorrel leaves, being fructose the most abundant. The non-stored control revealed the highest contents in free sugars, except for sucrose, which values decreased under air and N2-enriched atmospheres and were higher under vacuum and Ar-enriched atmospheres. Among treatments, air-packaged buckler sorrel leaves revealed the lowest sugars content, presenting a total sugars decrease of 63.6%. Vacuum-packaged and N2-enriched MAP samples presented the highest amounts of glucose and total sugars, while sucrose was particularly high in vacuum-packaged and Ar-enriched MAP samples, and fructose in N2-enriched MAP.

All fresh vegetables remain biologically living after harvest. Therefore, the decrease of fructose and glucose levels in stored buckler sorrel leaves can be related to their use by the plant to produce the energy required for metabolism, since reducing sugars are the main substrates in the respiration process (Heldt and Piechulla 2011). Thus, air-packaging, where the total sugars content was lower, can be associated to some incapacity in slowing down those physiological processes. Likewise, sugars are considered to be postharvest quality markers.

Besides these sugars, Pereira *et al.* (2011) also reported the presence of raffinose (~19 mg/100 g fw) in buckler sorrel leaves, as well as a total sugars content (~483 mg/100 g fw) ~47% lower than that found in our non-stored control leaves. Despite the higher levels of sucrose (~121 mg/100 g fw), lower amounts of fructose (166 mg/100 g fw), glucose (122 mg/100 g fw) and trehalose (53 mg/100 g fw) were reported. Differences in the specimens phenological stage at harvest time, as well as disparity on soil and annual weather conditions of the gathering sites, may affect the chemical composition (Nikolopoulou *et al.* 2007), justifying the observed variations.

The organic acids content was also affected by the different packaging atmospheres (**Table 25**). Oxalic, quinic, malic and ascorbic acids were detected, and quinic acid was the most abundant. The refrigerated storage decreased the ascorbic acid content, while quinic acid and the total organic acids contents were higher in packaged buckler sorrel leaves. Air-packaging preserved better the ascorbic acid but increased the oxalic acid content, while vacuum-packaging showed opposite results. Ar- and N2-enriched MAP also revealed higher amounts of malic and total organic acids compared to the non-stored control leaves. The oxalic acid was previously reported by Ferreres *et al.* (2006) in aqueous extracts of buckler sorrel leaves collected in the same region. After that, Guerra *et al.* (2008) revealed the additional presence of ascorbic, citric, malic and shikimic acids, and demonstrated that the growing conditions and phenological stage affect the total amount of organic acids.

Lipophilic compounds

The results for fatty acids composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and the ratios of PUFA/SFA and omega-6/omega-3 (n-6/n-3) are shown in **Table 26**. The assayed packaging atmospheres induced significant changes in the 22 detected fatty acids and given ratios ($p < 0.05$). In non-stored control buckler sorrel leaves, α-linolenic (C18:3n3), palmitic (C16:0) and linoleic (C18:2n6) acids were the 3 most abundant, in agreement to Pereira *et al.* (2011). C16:0 gave the lower values in the non-stored control leaves and increasing in the packaged ones especially under Ar-enriched MAP. In turn, the amounts of C18:2n6 and C18:3n3 were lower after the 12 days of refrigerated storage, being the air-packaging treatment the one which preserved better these fatty acids. In general, Ar-enriched MAP and vacuum-packaging induced negative effects, namely increasing the amounts of SFA (mainly due to the contribution of arachidic acid (C20:0)) and the n-6/n-3 ratios, and giving the low PUFA/SFA ratios. Despite MUFA also increased in packaged samples (except in the air-packaged ones), PUFA decreased under these conditions. Air-packaging was the most appropriate treatment to retain high values of PUFA and PUFA/SFA ratios, and low n-6/n-3 ratios as recommended for good nutritional quality.

The fatty acids content can be used as indicator that freshly harvested plants may have entered into stress or senescence (Yi *et al.* 2009). The onset of this process is connected with the loss of unsaturated fatty acids and accumulation of peroxidation products. As targets of free radicals and therefore substrates for peroxidation, elevated levels of PUFA are unfavourable and its uncoupling may be activated in mitochondria leading to an overall decrease in the double bond index (Yi *et al.* 2009). In addition, the elimination of reactive oxygen species is strongly linked to the activity of the enzymes that catalyze the peroxidation of unsaturated fatty acids from the cellular wall (Baysal and Demirdöven 2007). In our work, the increase in SFA and the decrease of PUFA under vacuum and Ar-enriched atmospheres can therefore be linked to unfavourable preservation conditions. The air-packaging treatment showed the most desired ratios and the higher values of PUFA, despite the initial headspace gas composition surrounding the buckler sorrel leaves in the package contained oxygen, which could have been caused by the protective effect of tocopherols.

Table 26 Fatty acids (relative %) and tocopherols (mg/100 g) for buckler sorrel leaves exposed to different postharvest treatments[1]

	Harvest (day 0)	12 days of storage at 4 °C						
		Non-conventional MAP		Conventional packaging				
	Non-stored control	N_2	Ar	Vacuum	Air			
					0 kGy	1 kGy	2 kGy	6 kGy
C6:0	0.056±0.004^{bcD}	0.32±0.05^{a}	0.032±0.005^{c}	0.073±0.005^{b}	0.083±0.005^{bC}	0.11±0.01^{B}	0.13±0.01^{A}	0.08±0.01^{C}
C8:0	0.10±0.01^{cBC}	0.48±0.05^{a}	0.22±0.04^{b}	0.06±0.01^{d}	0.11±0.02^{cB}	0.18±0.02^{A}	0.18±0.01^{A}	0.08±0.01^{C}
C10:0	0.31±0.04^{dD}	1.9±0.1^{b}	2.7±0.1^{a}	0.36±0.04^{d}	0.46±0.04^{cC}	0.78±0.05^{A}	0.61±0.04^{B}	0.33±0.01^{D}
C12:0	1.6±0.1^{dC}	3.0±0.1^{c}	4.6±0.1^{a}	3.6±0.1^{b}	1.4±0.2^{eD}	2.6±0.2^{A}	2.0±0.1^{B}	1.5±0.1^{CD}
C13:0	0.034±0.004^{dBC}	0.076±0.004^{b}	0.146±0.005^{a}	0.065±0.005^{c}	0.033±0.003^{dC}	0.071±0.005^{A}	0.041±0.005^{B}	0.023±0.004^{D}
C14:0	2.3±0.1^{dC}	5.2±0.2^{c}	12.0±0.4^{a}	6.9±0.2^{b}	1.9±0.1^{eD}	3.4±0.2^{A}	3.1±0.2^{B}	1.5±0.1^{E}
C15:0	0.29±0.01^{cBC}	0.45±0.04^{b}	0.96±0.05^{a}	0.41±0.04^{b}	0.25±0.04^{cBC}	0.43±0.05^{A}	0.31±0.05^{B}	0.23±0.04^{C}
C15:1	0.037±0.003^{cB}	0.057±0.003^{c}	0.18±0.03^{b}	0.26±0.03^{a}	0.030±0.003^{cC}	0.058±0.005^{a}	0.042±0.003^{B}	0.013±0.002^{D}
C16:0	19.5±0.4^{dC}	21.4±0.4^{c}	30.4±0.4^{a}	26.5±0.4^{b}	21.8±0.4^{cB}	24.8±0.5^{A}	16.8±0.4^{D}	13.7±0.4^{E}
C17:0	0.31±0.02^{dC}	0.48±0.04^{b}	0.86±0.05^{a}	0.53±0.05^{b}	0.38±0.04^{cB}	0.51±0.05^{A}	0.39±0.04^{B}	0.28±0.04^{C}
C18:0	5.2±0.2^{dC}	9.6±0.2^{c}	15.0±0.2^{a}	14.2±0.5^{b}	4.1±0.2^{eD}	7.3±0.4^{A}	5.9±0.4^{B}	3.9±0.2^{D}
C18:1n9	3.1±0.1^{dA}	6.2±0.2^{b}	12.2±0.3^{a}	3.5±0.3^{c}	2.9±0.2^{dA}	3.2±0.2^{A}	2.6±0.3^{B}	2.0±0.2^{C}
C18:2n6	14.7±0.4^{aA}	9.1±0.3^{c}	0.9±0.1^{e}	4.2±0.2^{d}	10.5±0.4^{bC}	9.0±0.4^{D}	14.2±0.2^{B}	13.9±0.4^{B}
C18:3n3	38.7±0.5^{aA}	23.9±0.5^{c}	1.9±0.1^{e}	6.3±0.2^{d}	33.7±0.5^{bC}	16.3±0.5^{D}	35.1±0.5^{B}	34.9±0.5^{B}
C20:0	4.7±0.2^{dD}	8.4±0.2^{b}	8.3±0.2^{b}	17.3±0.2^{a}	5.2±0.1^{cC}	10.3±0.3^{A}	7.7±0.1^{B}	7.5±0.2^{B}
C20:1	0.23±0.02^{cA}	0.03±0.01^{d}	0.42±0.05^{b}	0.63±0.05^{a}	0.08±0.01^{dC}	0.12±0.01^{B}	0.02±0.01^{D}	0.02±0.01^{D}
C20:2	0.84±0.05^{bBC}	0.63±0.05^{c}	0.23±0.01^{d}	0.85±0.05^{b}	1.1±0.1^{aA}	1.0±0.2^{AB}	0.5±0.1^{D}	0.8±0.1^{C}
C20:3n3	0.9±0.1^{bB}	0.53±0.04^{c}	0.19±0.02^{e}	0.37±0.05^{d}	1.2±0.1^{aA}	0.38±0.05^{D}	0.59±0.05^{C}	1.2±0.3^{A}
C20:5n3	0.41±0.05^{bC}	0.61±0.05^{a}	0.13±0.05^{c}	0.48±0.05^{b}	0.43±0.05^{bC}	0.29±0.03^{C}	1.6±0.1^{B}	5.7±03^{A}
C22:0	3.4±0.2^{eE}	4.8±0.2^{d}	5.1±0.2^{c}	8.5±0.1^{a}	8.1±0.4^{bB}	10.2±0.4^{A}	5.4±0.2^{D}	6.9±0.3^{C}
C22:2	0.41±0.05^{cC}	0.018±0.002^{d}	0.55±0.03^{b}	0.020±0.003^{d}	1.6±0.2^{aA}	0.75±0.04^{B}	0.39±0.04^{C}	0.045±0.004^{D}
C24:0	2.7±0.1^{dD}	3.0±0.1^{c}	2.9±0.2^{cd}	5.0±0.2^{a}	4.6±0.2^{bC}	8.2±0.5^{A}	2.3±0.2^{D}	5.3±0.3^{B}

	Harvest (day 0)	12 days of storage at 4 °C						
		Non-conventional MAP		Conventional packaging				
	Non-stored control	N_2	Ar	Vacuum	Air			
					0 kGy	1 kGy	2 kGy	6 kGy
Total SFA	40.6±04^{dE}	58.9±0.5^{b}	83.3±0.2^{a}	83.4±0.4^{a}	48.4±0.5^{cB}	70.0±0.5^{A}	45.0±0.5^{C}	41.4±0.5^{D}
Total MUFA	3.4±0.2^{dA}	6.3±0.2^{b}	12.8±0.2^{a}	4.4±0.2^{c}	3.0±0.2^{eB}	3.4±0.1^{A}	2.7±0.3^{C}	2.0±0.2^{D}
Total PUFA	56.0±0.4^{aA}	34.8±0.5^{c}	3.9±0.2^{e}	12.2±0.3^{d}	48.5±0.4^{bC}	27.7±0.5^{D}	52.3±0.5^{B}	56.6±0.5^{A}
PUFA/SFA	1.38±0.02^{aA}	0.59±0.02^{c}	0.05±0.01^{e}	0.15±0.01^{d}	1.00±0.02^{bC}	0.40±0.01^{D}	1.16±0.03^{B}	1.37±0.05^{A}
n-6/n-3	0.40±0.02^{cB}	0.39±0.02^{cd}	0.78±0.02^{a}	0.71±0.02^{b}	0.37±0.02^{dC}	0.63±0.03^{A}	0.41±0.01^{B}	0.35±0.02^{C}
α-tocopherol	3.8±0.2^{aA}	2.7±0.1^{b}	2.9±0.2^{b}	2.8±0.1^{b}	2.8±0.1^{bB}	1.3±0.1^{C}	1.1±0.1^{D}	0.9±0.1^{E}
β-tocopherol	0.10±0.02^{aA}	0.08±0.01^{b}	0.09±0.01^{b}	0.08±0.01^{b}	0.06±0.01^{cB}	0.03±0.01^{D}	0.04±0.01^{C}	0.03±0.01^{D}
γ-tocopherol	1.6±0.1^{dC}	2.2±0.1^{b}	2.2±0.1^{b}	1.8±0.1^{c}	3.2±0.2^{aA}	1.7±0.1^{B}	1.5±0.1^{C}	1.2±0.1^{D}
δ-tocopherol	0.33±0.04^{eE}	1.3±0.1^{c}	1.7±0.1^{a}	0.96±0.05^{d}	1.6±0.1^{bB}	1.5±0.1^{C}	1.8±0.1^{A}	1.1±0.1^{D}
Total tocopherols	5.8±0.3^{dB}	6.3±0.3^{c}	7.0±0.1^{b}	5.7±0.1^{d}	7.7±0.3^{aA}	4.5±0.2^{C}	4.5±0.2^{C}	3.2±0.1^{D}

[1]The results are presented as the mean±SD. The one-way ANOVA *p*-values were <0.001 in all cases, indicating that at least one packaging atmosphere (PA) or irradiation dose (ID) differs from the others (in this case multiple comparison tests were performed). For each preservation treatment, means within a row with different letters (lower case for PA and capital letters for ID) differ significantly ($p<0.05$).

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); *cis*-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); *cis*-11,14,17-Eicosatrienoic acid (C20:3n3); *cis*-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); *cis*-13,16-Docosadienoic acid (C22:2); Lignoceric acid (C24:0); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

The assayed treatments induced also significant changes in the tocopherols content (**Table 26**). The non-stored control leaves revealed the highest amounts of α- and β-tocopherols, but the lowest amounts of the γ- and δ- isoforms. While the assayed packaging atmospheres had no effect on the α- and β-tocopherols, γ- and δ-tocopherols were mainly increased under air-packaging and Ar-enriched MAP, respectively. The air-packaged buckler sorrel leaves showed the highest content in total tocopherols, being in agreement with the alterations in the PUFA contents. In fact, these strong lipophilic antioxidants might have preserved the amounts of PUFA.

Considering the literature, stress conditions, such as those induced by unfavourable packaging conditions, may cause an increase in the levels of total tocopherols (Munné-Bosch 2005). In these situations, tocopherols are synthesized to protect the plant tissue from the increased levels of reactive species and to inhibit lipid peroxidation, thus avoiding oxidative damage. Thus, the observed variations in the tocopherols content among the different packaging atmospheres can be related to their ability to provide an adequate protective condition from stressful situations. In this work, the air-packaged buckler sorrel leaves not only showed higher levels of tocopherols, but also low levels of total sugars and organic acids. Seemingly, the passive atmosphere originated inside the package was ineffective in reducing the metabolic activity, and therefore preventing compositional changes associated with senescence.

Bioactive properties

The values for antioxidant activity and total phenolics and flavonoids of the buckler sorrel leaves hydromethanolic extracts are presented in **Table 27**. The assayed packaging atmospheres induced significant changes ($p < 0.050$) in these parameters. In general, the antioxidant activity was lower in non-stored control buckler sorrel leaves (except for the reducing power), while the ones stored under vacuum exhibited the best antioxidant activity and high amounts of total phenolics and flavonoids. High levels of total phenolics and flavonoids were also detected in air-packaged and Ar-enriched MAP samples, respectively, but decreased under N_2-enriched MAP. Lower phenolic (117 mg GAE/g extract) and higher flavonoid (90 mg CE/g extract) contents and similar reduction power were reported by Pereira *et al.* (2011) in methanolic extracts. Despite this, these extracts exhibited better performances for the other *in vitro* assays. Also, the flavonoid synthesis is a plant strategy to withstand stress conditions (Pérez-Gregorio *et al.* 2011).

Table 27 Antioxidant activity (EC_{50} values, mg/mL) and total phenolic (mg GAE/g extract) and flavonoid (mg CE/g extract) content for buckler sorrel leaves exposed to different postharvest treatments[1]

	Harvest (day 0)	**12 days of storage at 4 °C**						
		Non-conventional MAP		**Conventional packaging**				
	Non-stored control	**N_2**	**Ar**	**Vacuum**	**Air**			
					0 kGy	**1 kGy**	**2 kGy**	**6 kGy**
DPPH$^{\bullet}$ scavenging activity	0.67±0.01^{aA}	0.60±0.01^{b}	0.60±0.01^{b}	0.53±0.01^{c}	0.59±0.01^{bC}	0.53±0.01^{E}	0.65±0.01^{B}	0.57±0.01^{D}
Reducing power	0.21±0.02^{cB}	0.28±0.03^{a}	0.30±0.02^{a}	0.24±0.02^{bc}	0.24±0.02^{bB}	0.31±0.03^{A}	0.32±0.04^{A}	0.32±0.03^{A}
β-Carotene blanching inhibition	0.38±0.01^{aC}	0.36±0.01^{b}	0.39±0.02^{a}	0.35±0.01^{b}	0.26±0.01^{cD}	0.40±0.02^{B}	0.39±0.01^{BC}	0.62±0.01^{A}
TBARS formation inhibition	0.29±0.01^{aA}	0.19±0.01^{c}	0.12±0.01^{d}	0.10±0.01^{e}	0.22±0.02^{bC}	0.26±0.01^{B}	0.18±0.01^{D}	0.15±0.01^{E}
Total phenolics	144±2^{bB}	135±2^{d}	140±4^{c}	146±4^{ab}	148±1^{aA}	135±2^{C}	124±1^{E}	127±1^{D}
Total flavonoids	40±1^{cA}	37±1^{d}	43±3^{b}	47±2^{a}	36±1^{dC}	35±2^{C}	37±1^{B}	36±1^{C}

[1]The results are presented as the mean±SD. The one-way ANOVA *p*-values were <0.001 in all cases, indicating that at least one packaging atmosphere (PA) or irradiation dose (ID) differs from the others (in this case multiple comparison tests were performed). For each preservation treatment, means within a row with different letters (lower case for PA and capital letters for ID) differ significantly ($p<0.05$).

GAE: gallic acid equivalents; CE: catechin equivalents.

4.1.3.3. Effect of the irradiation dose

The suitability of the γ-irradiation treatment for preserving the postharvest quality parameters of the buckler sorrel leaves during cold storage was investigated and the results are presented below. After harvest, fresh vegetables remain living organisms able to protect its tissues from adverse conditions due to the presence of a wide range of bioactive phytochemicals. Besides, the γ-irradiation treatment can also increase the extractability of certain compounds (Hussain *et al.* 2016). Therefore, nonlinear dose-response effects of the γ-irradiation treatment on the evaluated quality parameters can be expected after the 12 days of cold storage.

Colour parameters

Based on the one-way ANOVA p-values (**Table 24**), it was possible to conclude that the different γ-irradiation doses also induced significant changes in all colour parameters, except for greenness (a*) registered in the abaxial surface of the leaves. The applied doses increased the leaves lightness (L*) in both surfaces. The yellowness (b*) was also increased with the storage time. In adaxial surfaces, these values were higher with the consequent increase of the irradiation dose, while the greenness (a*) values were decreased (corresponding to a greener colour) with storage and higher irradiation doses.

Proximate composition

The proximate composition of non-irradiated and irradiated buckler sorrel leaves stored at 4 ºC for 12 days is presented in **Table 25**. The different doses induced significant changes ($p < 0.05$) on the protein, fat and ash contents, while moisture, carbohydrates and the energetic contribution were not affected, as observed for the different packaging atmospheres. The ash content was slightly increased during storage, mainly in non-irradiated leaves. In contrast, the fat amounts were lower at the end of the storage period and were better preserved in non-irradiated leaves. This reduction in fat levels could have been caused by a reduced activity of the enzymes involved in the de novo synthesis of fatty acids induced by the γ-irradiation treatment (Pérez *et al.* 2007) in combination with the other factors presented in the previous section. Besides, irradiation of high moisture content foods can cause fat oxidation (Cheng *et al.* 2011).

Hydrophilic compounds

Individual sugars and organic acids profiles are presented in **Table 25**, showing that γ-irradiation had a significant effect ($p < 0.05$) on these hydrophilic compounds. The non-stored control buckler sorrel leaves revealed the highest contents of the identified sugars, which were detected in a lesser extent after storage. In general, the 6 kGy dose led to lower

amounts of fructose, glucose and total sugars. Nevertheless, the other applied doses did not induced differences in the total amounts of sugars. Lower levels of fructose and glucose were also detected in bananas irradiated at 1, 1.5 and 2 kGy and stored at 16 ºC for 21 days (Gloria and Adão 2013). Regarding organic acids, the non-stored control leaves revealed higher values of ascorbic acid and lower values for quinic acid and total organic acids. Among stored samples, the 6 kGy dose induced negative effects on the oxalic, malic, ascorbic and total organic acids contents, and the 1 kGy dose demonstrated to be the able to increase the amount of these hydrophilic compounds. The decrease of these organic acids may be attributed to the direct effects of γ-rays or to the action of the free radicals possibly generated during radiolysis of water, which promote the conversion (oxidation) of ascorbic acid to dehydroascorbic acid. This phenomenon is supported by the work of Hussain *et al.* (2016) with fenugreek (*Trigonella foenum-graceum* L.) and spinach (*Spinacia oleracea* L.) leaves irradiated at 0.25 and 1.5 kGy and stored at 3 ºC for 4 days. The reduction of the ascorbic acid content may also be attributed to its interactive effect with other compounds, which react to protect them against the oxidative damage.

Lipophilic compounds

The results for fatty acids composition, SFA, MUFA, PUFA and the ratios of PUFA/SFA and n-6/n-3 are shown in **Table 26**. Similar to the assays under different atmospheres, buckler sorrel leaves suffer significant ($p<0.05$) changes in their fatty acids composition with the applied irradiation doses. Regarding the 3 main fatty acids, C16:0 decreased with the two highest doses and increased with the 1 kGy dose and in non-irradiated leaves, while C18:2n6 and C18:3n3 were better preserved with the two highest doses and negatively affected with the 1 kGy dose, in comparison with the non-stored control. The SFA values were higher in stored samples especially in those irradiated at 1 kGy. The same dose protected MUFA and increased the n-6/n-3 ratio, while decreasing PUFA and the PUFA/SFA ratio. Interestingly, the 6 kGy dose preserved PUFA and the PUFA/SFA and n-6/n-3 ratios during storage. These variations in the fatty acids profile may result from oxidative and radiolytic processes (Fernandes *et al.* 2016), as well as from variations in the levels of antioxidants (*e.g.,* ascorbic acid and tocopherols). γ-Irradiation can also affect the activity of enzymes involved in the desaturation of fatty acids (Pérez *et al.* 2007).

Concerning tocopherols profile, the non-stored control leaves presented the higher amounts of α- and β-tocopherols, and the lower ones of δ-tocopherol. The α-tocopherol values were reduced with the increase of the irradiation dose, as verified by Di Stefano *et al.* (2015) in raw unpeeled almond kernels (*Prunus dulcis* (Mill.) D. A. Webb) irradiated up to 10 kGy. Non-irradiated stored leaves reveal the higher total tocopherols content, which might have been stimulated by unfavourable storage condition (since tocopherols are known to be

effective lipid-soluble antioxidants involved in the repair of oxidative damage), as explained in the discussion regarding the effects of the different packaging atmospheres. Besides, the irradiation treatment delays physiological processes (ICGFI 1999).

Bioactive properties

The applied doses also induced significant changes ($p < 0.05$) in the antioxidant activity and total phenolics and flavonoids of the hydromethanolic extracts prepared from the buckler sorrel leaves (**Table 27**). Each of the performed antioxidant activity assays seemed to have been favoured by a specific γ-irradiation dose: the DPPH$^{\bullet}$ scavenging capacity was improved with the 1 kGy dose, the β-carotene bleaching inhibition capacity was higher in non-irradiated leaves (which showed also the highest phenolic and tocopherols contents), and the TBARS formation inhibition capacity was higher in those irradiated at 6 kGy. Comparable effects on the DPPH• scavenging capacity were observed by Hussain *et al.* (2016) in fenugreek and spinach leaves irradiated with doses above 0.75 kGy. Buckler sorrel leaves irradiated at 2 and 6 kGy revealed high TBARS formation inhibition capacity, which is in accordance with the higher amounts of PUFA (including C18:2n6 and C18:3n3). Furthermore, changes induced by oxidation and radiolytic processes, and variations in the structure and extractability of phenolic compounds may also affect the antioxidant capacity of the samples (Fernandes *et al.* 2016, Ito *et al.* 2016).

Comparative evaluation of the effects on the overall postharvest quality

In the former sections, the effects of packaging the buckler sorrel leaves under different atmospheres, or expose them to different γ-irradiation doses, were studied in several quality parameters. As it could be concluded, all the tested postharvest treatments induced significant changes, hindering the immediate selection of a single process able to maintain the wholesomeness of the fresh buckler sorrel leaves during shelf-life. Nevertheless, it would be useful to find the most suitable treatment when considering the contribution of all assayed quality parameters simultaneously, instead of verifying each parameter one by one. Accordingly, the results were evaluated considering data for all the studied packaging atmospheres and γ-irradiation doses through a categorical principal component analysis (CATPCA).

The plot of object scores (**Figure 29**) for the different postharvest treatments indicates that the first two dimensions (first: Cronbach's α, 0.968; eigenvalue, 20.932; second: Cronbach's α, 0.928; eigenvalue, 11.433) account for most of the variance of all quantified variables (44.9% and 29.1%, respectively). Groups corresponding to each treatment (air, N2, Ar, vacuum, 1 kGy, 2 kGy and 6 kGy) were completely individualized, indicating that the assayed postharvest treatments affect the studied quality parameters in a highly specific

manner. In fact, all of them had positive and negative effects. Air-packaged buckler sorrel leaves were mainly characterized for their low antioxidant activity and flavonoid content, but kept high amounts of PUFA (mainly due to C18:2n6 and C18:3n3) along the storage time. Objects corresponding to leaves stored under N2-enriched atmospheres were placed close to the origin of coordinates, thereby indicating that they were not characterized by particularly high (or low) levels of none of the assayed parameters. Leaves stored under vacuum, showed low levels of PUFA (especially C18:2n6 and C18:3n3), but high amounts of γ-tocopherol, MUFA (mainly oleic acid (C18:1n9)) and flavonoids. The results for the effects of Ar-enriched atmospheres were similar, with the additional advantages of their high DPPH• scavenging activity and TBARS formation inhibition capacity.

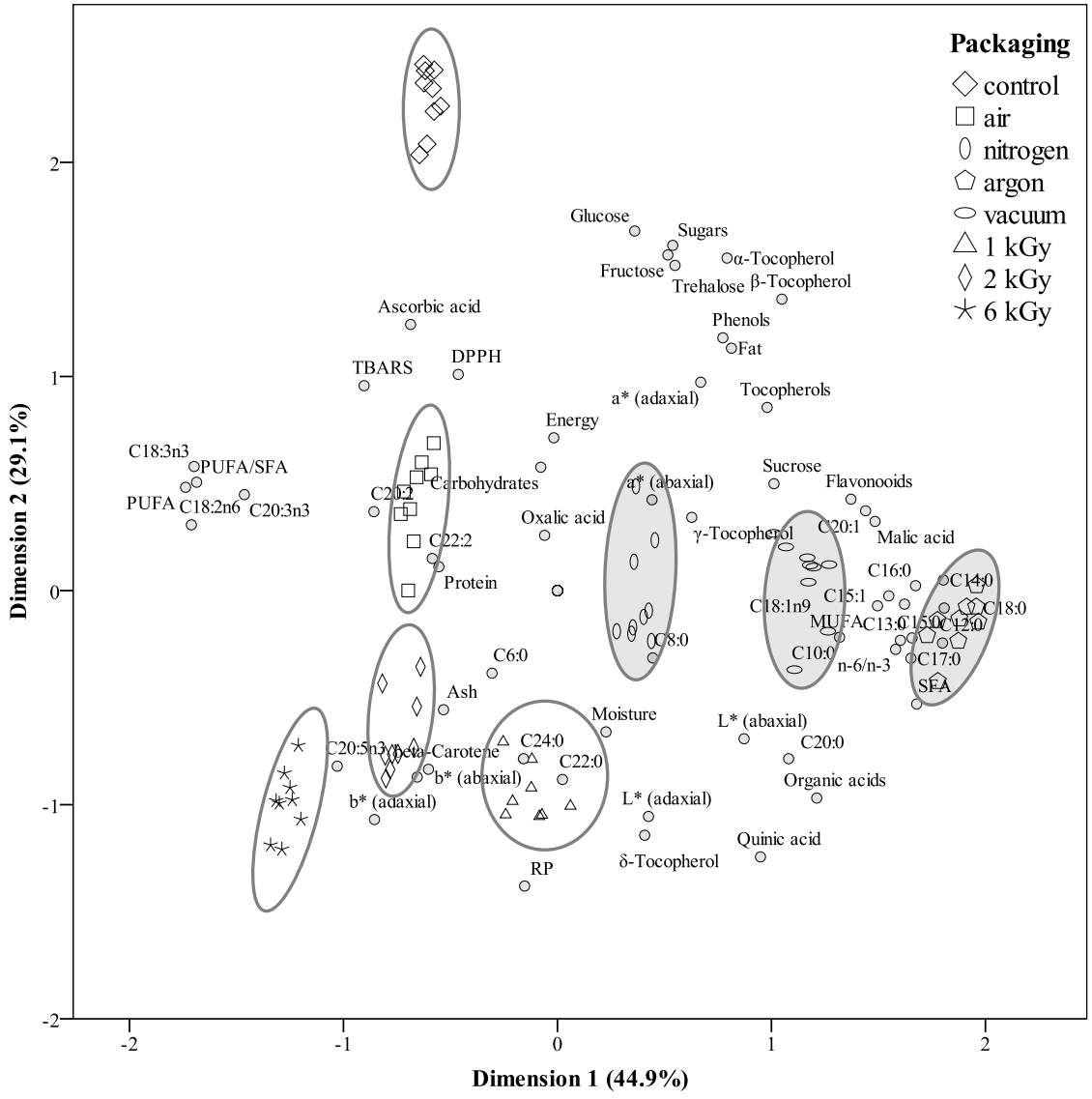

Figure 29 Biplot of object scores (postharvest treatments) and component loadings (evaluated quality parameters).

Regarding the effects of γ-irradiation, buckler sorrel leaves treated with the 1 kGy dose presented a low reducing power and β-carotene bleaching inhibition capacity and also low ascorbic acid levels; the major positive points were the high levels of δ-tocopherol. Results for the 2 and 6 kGy doses were somewhat similar, showing low levels of total phenolics, sugars and tocopherols (particularly the α- and β- isoforms); on the other hand, these leaves presented high L* values (the leaves showed higher lightness) and low a* values, *i.e.,* their colour was more green.

4.1.4. Conclusions

Overall, and considering the CATPCA results, it is suggested that buckler sorrel would be more suitably stored under an Ar-enriched atmosphere, since the treatment provided the maintenance of the most relevant postharvest quality attributes; while the 6 kGy dose was a good option to preserve PUFA and the ratios of PUFA/SFA and n-6/n-3 fatty acids. In addition to these findings, the present study highlighted the nutritional and antioxidant properties of buckler sorrel, as well as the interest of its inclusion in contemporary diets. Further studies are of interest to evaluate the effect of the studied preservation treatments on other quality attributes and physiological parameters, as well as different combinations between them and with other technologies.

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Watercress (*Nasturtium officinale* R. Br.)

4.2. Postharvest quality changes in fresh-cut watercress stored under conventional and inert gas-enriched atmospheres


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Postharvest quality changes in fresh-cut watercress stored under conventional and inert gas-enriched modified atmosphere packaging

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Abstract

The effect of modified atmosphere packaging (MAP) on the postharvest quality of fresh-cut watercress (*Nasturtium officinale* R. Br.) stored at 4 ºC for 7 d was studied. A portion of watercress was immediately analysed (non-stored control) and the remaining fresh material was stored packaged under atmospheres enriched with N_2, Ar, air, or vacuum. The analysed parameters included colour, total soluble solids, pH, macronutrients, the individual profiles of sugars, organic acids, tocopherols and fatty acids, and total phenolics and flavonoids. Furthermore, four *in vitro* assays were performed to evaluate the antioxidant activity. After assessing the effect on individual quality parameters, it was possible to conclude that air was the less efficient atmosphere in preserving quality attributes of the non-stored control samples during cold storage. In turn, Ar-enriched MAP was the most suitable choice to preserve the overall postharvest quality. The present study also highlighted the nutritional and antioxidant properties of watercress, as well as the interest of its inclusion in human diets.

Keywords: *Nasturtium officinale*; fresh-cut watercress; modified atmosphere packaging; refrigerated storage; postharvest quality; antioxidant activity.

4.2.1. Introduction

As a response to consumers' demand for fresh, healthy and easy-to-prepare food products, conjoint with consumer lifestyle changes, with little time to prepare a convenient meal and to have a balanced diet, a wide variety of minimally processed vegetables has been developed (Ramos *et al.* 2013). Among them, watercress (*Nasturtium officinale* R. Br.) stands out due to its consumption since ancient times. This perennial species of the Brassicaceae family grows in and around water and is highly appreciated in the Mediterranean cuisine, being eaten raw in salads, soups and other recipes (Carvalho and Morales 2010). Apart from its interesting nutritional value (Pereira *et al.* 2011, Manchali *et al.* 2012), this vegetable has medicinal and therapeutic properties (Hecht *et al.* 1995, Syed Alwi *et al.* 2010, Casanova and Carballo 2011, Manchali *et al.* 2012, Freitas *et al.* 2013, Sadeghi *et al.* 2014), mainly due to its high content in bioactive molecules.

A limiting factor that reduces watercress consumption is its perishable nature, characterized by a reduced shelf-life after harvest of approximately seven days (Cruz *et al.* 2009, Silveira *et al.* 2014). The main symptoms of quality loss are surface dehydration, softening of tissues and loss of green colour. Most conventional postharvest treatments can't control all parameters necessary to extend produce shelf-life, without compromising its quality (Pinela and Ferreira 2017). Additionally, consumers are looking for safe food products that suffer minimal processing with high quality retention (Ramos *et al.* 2013). To satisfy these requirements, it is necessary to design appropriate and more sustainable postharvest treatments, aiming to preserve the quality and extend the shelf-life of fresh vegetables including watercress. For this reason, novel postharvest technologies are being investigated by the food industry, such as modified atmosphere packaging (MAP) combined with cold storage (Pinela and Ferreira 2017).

MAP is an economical and effective technology that involves altering the air surrounding the product in the package to another composition. Using this method, the initial fresh state of the product may be prolonged by reducing the metabolic activity and chemical oxidation, thus retarding compositional changes associated with maturation and senescence, reducing microorganism growth and retaining all attributes that consumers consider as freshness markers (Murcia *et al.* 2009, Niemira and Fan 2014). Recently, the use of non-conventional argon (Ar)- and nitrogen (N_2)-enriched atmospheres has gained a considerable interest (Artés *et al.* 2009, Char *et al.* 2012). Ar is biochemically active, probably due to its enhanced solubility in water, and appears to interfere with enzymatic oxygen receptor sites, thus reducing metabolic activity of the food product (Char *et al.* 2012). This gas has also been reported to reduce microbial growth and to improve quality of fresh produce (Jamie and Saltveit 2002). Regarding N_2, it has a low solubility in water and other food constituents and does not support the growth of aerobic microbes, thereby inhibiting the growth of aerobic

spoilage (Sandhya 2010). When properly used, this technology may preserve and extend the quality of food, allowing a longer period for commercialisation. Even so, the application of MAP to a specific food product, such as watercress, requires further research.

A previous study demonstrated the effectiveness of non-conventional MAP in preserving some quality attributes of fresh-cut watercress (Silveira *et al.* 2014). However, no clear effect of the studied gases on colour, total polyphenols, microbial growth, or sensory parameters was verified. In this study we explored and compared the effects of conventional and non-conventional MAP enriched with inert gases on quality parameters of fresh-cut watercress stored at 4 ºC for 7 d.

4.2.2. Materials and methods

4.2.2.1. Standards and reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), other individual fatty acid isomers, tocopherols (α-, β-, and γ-isoforms), sugars ($_D$(–)-fructose, $_D$(+)-glucose anhydrous, $_D$(+)-melezitose hydrate, $_D$(+)-sucrose), organic acids (citric, malic, oxalic and fumaric acids), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and catechin standards were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 g L^{-1}, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) as obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

4.2.2.2. Sampling and samples preparation

Watercress (*Nasturtium officinale* R. Br.) is claimed to have nutritional and healing properties when gathered in the proper season and phenological stage (Carvalho 2010, Carvalho and Morales 2010). Therefore, wild specimens were gathered in February 2014 in a local stream in the Bragança region (Trás-os-Montes, North-eastern Portugal), considering local consumers' sites, criteria and preferences. Subsequently, healthy and undamaged aerial parts (stalk and leaves) were selected, rinsed in tap water and drained to eliminate excess water. A portion of watercress was immediately analysed (non-stored control), and the remaining fresh material was subjected to the treatments described below and analysed in the end of the storage period. A voucher specimen was deposited in the Herbarium of the School of Agriculture of Bragança.

4.2.2.3. Samples packaging and storage

Approximately 20 g of watercress were placed in 11.5 cm × 15 cm sterilized packages made of low-density polyethylene film (black LDPE resin, thickness of 63 µm, the O_2 transmission rate was 7.99 × 10^{-7} L m^{-2} s^{-1} at 25 ºC and standard pressure and the CO_2 transmission rate was 2.91 × 10^{-6} L m^{-2} s^{-1} at the same temperature and pressure conditions (VWR, Lisbon, Portugal); the headspace volume inside the packages was approximately 0.5 L) and packaged under four different atmospheres: (1) atmospheric air (control in passive MAP); (2) vacuum (no atmosphere); (3) N_2- enriched atmosphere; and (4) Ar-enriched atmosphere. Briefly, air-packaging consisted of sealing without eliminating the air in the package (20.8% O_2 and <0.1% CO_2) and vacuum-packaging was performed by eliminating the air with a domestic vacuum-packaging machine. For non-conventional MAP, the headspace air in the packages was first eliminated and then the target gas (100% N_2 or Ar) was injected.

A total of 40 packages were prepared, 10 for each treatment, and stored in the dark at 4 ºC for 7 d.

4.2.2.4. Headspace gas analysis

The O_2, CO_2, and N_2 concentrations inside the packages were monitored using a portable gas analyser (model Oxybaby 6.0, WITT, Denmark) previously calibrated by sampling atmospheric air. Ar concentration in the packages was calculated according to the equation: 100 - ([O_2] + [CO_2] + [N_2]). Values were expressed as a percentage. Measurements were performed after packaging and at the end of the storage period.

4.2.2.5. Physical and physicochemical analysis

For colour measurement, samples were placed on a black surface to reduce external interferences and data were collected on nine randomly selected leaves (adaxial surface) with a colorimeter (model CR-400; Konica Minolta Sensing Inc., Japan) previously calibrated using the standard white plate. Using illuminant C and the diaphragm opening of 8 mm, the CIE *L*a*b** colour space values were registered through the computerized system using a colour data software “Spectra Magic Nx” (version CM-S100W 2.03.0006). Average values were considered to determine the colour coordinates, where *L** represents lightness, *a** represents chromaticity on a green (–) to red (+) axis, and *b** represents chromaticity on a blue (–) to yellow (+) axis.

For total soluble solids (TSS) and pH determination, fresh tissue was ground and the grinding paste was subsequently filtered through Whatman No. 4 paper. The TSS content in the squeezed juice was measured with a digital hand refractometer (model HI 96801, Hanna Instruments, Woonsocket, RI, USA) and expressed as percentage (%). The pH was

measured with a digital pH-meter (model pH 211, Hanna Instruments, Woonsocket, RI, USA) in the same juice.

4.2.2.6. Chemical composition analysis

Samples were analysed for moisture, proteins, fat, ash and carbohydrates using the AOAC procedures (AOAC 2005). Briefly, the crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method, using an automatic distillation and titration unit (model UDK152; VELP Scientifica, Italy); the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC; and total carbohydrates were calculated by difference. The results were expressed as g per kg of fresh weight. The energy was calculated according to the equation: $4 \times (m_{proteins} + m_{carbohydrates}) + 9 \times (m_{fats})$ (Regulation (EU) No 1169/2011) and expressed as kJ per kg of fresh weight.

Free sugars and tocopherols were determined by high performance liquid chromatography (HPLC) coupled to a refraction index detector (RI) or to a fluorescence detector (FP-2020; Jasco), respectively. Procedures and equipment were previously described by Pereira *et al.* (2011). The identification was made by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method, wherein melezitose was used as internal standard for free sugars and tocol for tocopherols. The results were expressed in mg per kg of fresh weight.

Organic acids were analysed by ultra-fast liquid chromatography (UFLC) coupled to a photodiode array detector (PDA) according to Pereira *et al.* (2013). Briefly, fresh tissue (9 g) was ground and the grinding paste was subsequently extracted by stirring with 25 mL of meta-phosphoric acid (25 ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, samples were filtered through 0.2 µm nylon filters. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm or 245 nm (for ascorbic acid) with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per kg of fresh weight.

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column. Procedures and equipment were described by Pereira *et al.* (2011). The identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

4.2.2.7. In vitro bioactive properties evaluation

Watercress extracts were prepared according to Pereira *et al.* (2011), using a mixture of methanol:water (80:20, v/v) as extraction solvent. Briefly, a fine dried powder (20 mesh; ~1 g)

was stirred (150 rpm) with 50 mL of extraction solvent for 1 h at 25 ºC. The supernatant was filtered through Whatman No. 4 paper and the residue was re-extracted with an additional portion of solvent (50 mL). The combined extracts were then evaporated at 35 ºC under reduced pressure (Free Zone 4.5, Labconco; Kansas City, MO, USA), redissolved in the same solvent (final concentration 5 g L^{-1}) and successively diluted to different concentrations to evaluate their antioxidant activity and the total phenolic and flavonoid content.

Four different *in vitro* assays were performed to evaluate the hydromethanolic extracts antioxidant activity (Pinela *et al.* 2015): DPPH• scavenging activity, reducing power (measured by ferricyanide Prussian blue assay), β-carotene blanching inhibition, and thiobarbituric acid reactive substances (TBARS) formation inhibition. Briefly, the DPPH• scavenging activity and the reducing power assays were performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA). The reduction of DPPH• was determined by measuring the absorbance at 515 nm and calculated as a percentage of DPPH• discolouration using the equation: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_{DPPH} is the absorbance of the DPPH• solution and A_S is the absorbance of the solution containing the sample extract. The reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm. The β-carotene bleaching inhibition was evaluated by measuring the capacity to neutralize linoleate free radicals, which was monitored at 470 nm in a Model 200 spectrophotometer (AnalytikJena, Jena, Germany), and calculated using the equation: $(A_{\beta T2}/A_{\beta T0}) \times 100$, where $A_{\beta T2}$ is the absorbance of the emulsion after 2 h of incubation at 50 ºC and $A_{\beta T0}$ is the initial absorbance. The TBARS formation inhibition was evaluated in porcine (*Sus scrofa*) brain homogenates (brains were obtained from official slaughtered animals). The colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex formed during heating at 80 ºC for 20 min was measured at 532 nm, and the inhibition ratio calculated using the equation: $[(A - B)/A] \times 100\%$, where A and B correspond to the absorbance of the control and the sample solution, respectively. All results were expressed in EC_{50} values (g L^{-1}), *i.e.,* sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

Total phenolic (Wolfe *et al.* 2003) and flavonoid (Zhishen *et al.* 1999) contents were quantified according to the authors in the hydromethanolic extracts concentrated at 0.625 or 1.25 g L^{-1} by reading the absorbance at 765 nm or 510 nm, respectively. The standard curves were calculated using gallic acid (for phenolics) and catechin (for flavonoids), and the results were respectively expressed as g of gallic acid equivalents (GAE) or catechin equivalents (CE) per kg of extract.

4.2.2.8. Statistical analysis

Samples of the 10 replicates of each treatment were divided into three batches and independently analysed in triplicate for each quality parameter. Data were expressed as mean±standard deviation. All statistical tests were performed at a 5% significance level using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

The differences among treatments were analysed using one-way analysis of variance (ANOVA). The fulfilment the ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Furthermore, a linear discriminant analysis (LDA) was used to evaluate the effect of the different packaging atmospheres on the overall postharvest quality of watercress samples. A stepwise technique, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination processes, where the inclusion of a new variable is preceded by ensuring that all variables selected previously remain significant (Marôco 2007). With this approach, it is possible to determine which of the independent variables account most for the differences in the average score profiles of the different treatments. To verify the significance of canonical discriminant functions, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

4.2.3. Results and discussion

4.2.3.1. Headspace gas composition

The initial levels of N_2 and Ar inside the non-conventional packaging systems reached values of above 98 and 95%, respectively. After 7 d of refrigerated storage, the N_2 concentration inside the N_2-enriched MAP decreased approximately 23%, while this gas evolved within the Ar-enriched MAP (constituting 27% of the headspace composition). In both MAP, the final content of CO_2 and O_2 were less than 16 and 9% for N_2- and Ar-enriched MAP, respectively. The final headspace gas composition of air-packaged samples revealed comparable values of N_2 (~73%), while the percentages of CO_2 and O_2 were approximately 15 and 12%, respectively. The observed changes can be attributed to the plant respiration and diffusion of gases through the LDPE film (Belay *et al.* 2016).

4.2.3.2. Physical and physicochemical parameters

The green colour is an important attribute for the perception of watercress freshness. Based on the one-way ANOVA *p*-values it is possible to conclude that the assayed packaging atmospheres induced significant changes on *L** and *a** colour values, TSS and pH; but had no effect on *b** value (**Table 28**). Thus, the tested packaging atmospheres did not induce watercress yellowing. The *a** value was only significantly different in samples packaged in Ar-enriched atmospheres, which reveal the highest values, corresponding to an increased redness. Samples stored under Ar-enriched atmospheres showed also the highest lightness (*L**) values, followed by N_2-enriched MAP, air-packaging and vacuum-packaging, which showed similar values to the non-stored control. A slight increase of lightness in watercress samples stored under different non-conventional MAP was also reported by Silveira *et al.* (2014), but no clear effect of the tested non-conventional gases on colour parameters was reported. Slight increases were also found in fresh-cut red chard (*Beta vulgaris* var. cycla) baby leaves as a general trend for all treatments during storage at 5 ºC for 8 d (Tomás-Callejas *et al.* 2011). Similarly, Char *et al.* (2012) also recorded an increase in lightness with the storage time (8 d at 5 ºC) in ready-to-eat arugula (*Eruca vesicaria* Mill.) salads, suggesting incipient yellowing of leaves.

The TSS content in plant tissues is mainly related to the presence of soluble sugars and also smaller amounts of organic acids, vitamins and proteins. **Table 28** shows that the highest TSS values were found in control samples (day 0) and N_2-enriched MAP samples. On the other hand, a significant reduction in TSS index was verified in vacuum-packaged and Ar-enriched MAP samples, probably related with over-maturation or senescence. The utilization of free sugars in various metabolic processes could also cause the decrease of TSS contents.

A decrease in pH values was observed in packaged samples compared to non-stored control (**Table 28**). This acidification was more marked in N_2-enriched MAP samples, while air-packaging was the most appropriate treatment to maintain the initial pH value. These results could be related to the increase of organic acids, namely malic acid, and reduced glucose levels, probably due to fermentative processes.

Table 28 Colour parameters, TSS (%) and pH values of fresh-cut watercress samples stored under conventional and inert gas-enriched MAP.[1]

	Harvest (day 0)	**7 d of storage at 4 °C**				*p*-values		
	Non-stored control	**Conventional packaging**		**Non-conventional MAP**		Homoscedasticity[2]	Normal distribution[3]	One-way ANOVA[4]
		Air	**Vacuum**	**N_2**	**Ar**			
*L**	39 ± 3^{c}	45 ± 2^{b}	41 ± 2^{c}	46 ± 3^{ab}	49 ± 4^{a}	0.002	0.390	<0.001
*a**	-17 ± 2^{b}	-18 ± 1^{b}	-17 ± 2^{b}	-17 ± 1^{b}	-14 ± 2^{a}	0.720	0.033	<0.001
*b**	27 ± 2	31 ± 4	27 ± 3	30 ± 3	31 ± 5	0.204	0.016	0.050
TSS	3.4 ± 0.1^{a}	3.2 ± 0.1^{b}	2.9 ± 0.1^{c}	3.5 ± 0.1^{a}	3.0 ± 0.1^{c}	0.253	0.011	<0.001
pH	5.49 ± 0.04^{a}	5.40 ± 0.05^{b}	5.36 ± 0.04^{c}	5.23 ± 0.03^{d}	5.32 ± 0.04^{c}	0.575	0.484	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among packaging systems was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one packaging atmosphere differs from the others (in this case multiple comparison tests were performed). For each storage condition, means within a row with different letters differ significantly ($p<0.05$).

*L** (lightness ↔ darkness); *a** (redness ↔ greenness); *b** (yellowness ↔ blueness). TSS - total soluble solids.

4.2.3.3. Chemical composition

The nutritional value and the sugars profiles obtained for the different packaging atmospheres are presented in **Table 29**. As it can be concluded from the one-way ANOVA *p*-values, the assayed packaging atmospheres induced significant changes in all nutritional parameters and quantified sugars. The single component with similar values for all packaging atmospheres was the ash content. In general, the moisture content increased in packaged samples, especially in those under vacuum and Ar-enriched atmospheres (it should be noticed that before packaging the samples were sprayed with water). Non-conventional MAP was the best treatment to retain the protein levels, while N_2 and air atmospheres seem to be suitable choices to preserve fat content and energetic value along storage time.

The nutritional value of watercress was already described. Pereira *et al.* (2011) reported lower protein content (~9.2 g kg^{-1}) and a slightly higher ash (~11.4 g kg^{-1}), fat (2 g kg^{-1}) and carbohydrate (45.1 g kg^{-1}) contents in watercress samples collected in the same region. Despite this, the energetic contribution (~983 kJ kg^{-1}) was similar to that described in this study (~1023 kJ kg^{-1}) for non-stored control samples. Other work describes a higher protein (30 g kg^{-1}) and fat (10 g kg^{-1}) content, but much lower values for carbohydrates (3.5 g kg^{-1}) (Manchali *et al.* 2012). Nevertheless, the protein and fat contents described in this work for the non-stored control samples are very similar to those present in the USDA National Nutrient Database for Standard Reference (23 and 1 g kg^{-1}, respectively) (USDA 2011). These small differences in the reported values may be explained by the use of different analytical procedures and slight differences in the phenological stage of watercress in the collection period, as well as variations on soil characteristics and annual climatic conditions of the locations where the samples were collected, which may affect the plant composition during the growing season (Nikolopoulou *et al.* 2007).

Fructose, glucose and sucrose were identified in watercress samples, being fructose the most abundant. Fructose, glucose and total sugars gave the highest values in control samples, while sucrose was higher in packaged samples, especially in those stored under conventional packaging systems. Among treatments, vacuum-packaging retained the highest levels of fructose, sucrose and total sugars. No significant statistical differences were found among treatments for glucose. The decrease of fructose and glucose contents in stored samples can be related to its use by the plant to produce the energy required to its metabolism, once reducing sugars are the main substrates in the respiration process. Similar results were found by Workneh *et al.* (2012) which reported lower levels of glucose and an increase in the sucrose content on tomatoes during the first 8 days of storage at 13 °C, after disinfection treatments and packaging in micro-perforated packages. Besides these sugars, Pereira *et al.* (2011) also reported the presence of trehalose and raffinose in wild samples of

watercress; being sucrose the main sugar in those samples, followed by fructose and glucose. The total sugars content of our non-stored control (~2034 mg kg^{-1}), analysed immediately after harvest, was similar to that described by other authors, *i.e.,* 2099 mg kg^{-1} (Pereira *et al.* 2011) and 2000 mg kg^{-1} (USDA 2011).

Likewise, the organic acids content was also affected by the tested packaging atmospheres. Oxalic, malic, citric and fumaric acids were detected (**Table 29**), being oxalic acid the most abundant. Levels of this organic acid were higher in the non-stored control and samples stored under N_2-enriched atmospheres. Citric acid was also found in higher amounts in non-stored control samples. Regarding malic and fumaric acids, their values were higher in air-packaged samples. In general, air-packaging and N_2-enriched MAP increased the total organic acid levels while vacuum-packaging and Ar-enriched MAP showed lower values than the non-stored control.

Contrarily to some available results, reporting the presence of ascorbic acid in watercress (Cruz *et al.* 2008, 2009, Gonçalves *et al.* 2009, Pereira *et al.* 2011), this vitamin was not detected in our samples or in those previously analysed by Pereira *et al.* (2013).

The results for fatty acids composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and the ratios of PUFA/SFA and n-6/n-3 are shown in **Table 30**. From the one-way ANOVA *p*-values, we can conclude that the assayed packaging atmospheres induced significant changes in the detected fatty acids and given ratios. Up to 23 fatty acids were detected in most of the samples, being palmitic (C16:0) and α-linolenic (C18:3n3) acids the most abundant, in agreement to Pereira *et al.* (2011). Palmitic acid gave lower values in stored samples, while α-linolenic acid increased under the same conditions. Other abundant fatty acids were linoleic (C18:2n6), stearic (C18:0), lignoceric (C24:0) and eicosadienoic (C20:2) acids. Both palmitoleic (C16:1) and nervonic acid (C24:1) were only detected in samples submitted to refrigerated storage. SFA (~56.8%) levels were higher than MUFA (~1.03%) and PUFA (~42.2%) in control samples. All the assayed packaging atmospheres induced positive effects, namely an increase in MUFA and PUFA contents and PUFA/SFA ratios, and decreased SFA levels and n-6/n-3 ratios, as recommended for a “good nutritional quality” (Guil *et al.* 1996). N_2-packaged samples reveal the lowest SFA (~31.4%) content and the highest values of PUFA (~66.1%), as well as the highest PUFA/SFA ratios (~2.11) and lowest n-6/n-3 polyunsaturated fatty acid ratios (~0.19).

Table 29 Proximate composition (g kg^{-1}), energy (kJ kg^{-1}), individual sugars (mg kg^{-1}) and organic acids (mg kg^{-1}) of fresh-cut watercress samples stored under conventional and inert gas-enriched MAP.[1]

	Harvest (day 0)	**7 d of storage at 4 °C**				*p*-values		
	Non-stored control	**Conventional packaging**		**Non-conventional MAP**		Homoscedasticity[2]	Normal distribution[3]	One-way ANOVA[4]
		Air	**Vacuum**	**N_2**	**Ar**			
Moisture	931 ± 10^{b}	941 ± 10^{ab}	945 ± 10^{a}	940 ± 10^{ab}	943 ± 10^{a}	0.860	0.346	0.001
Ash	9.4 ± 0.9	8.8 ± 0.4	8.7 ± 0.7	8.7 ± 0.7	8.9 ± 0.8	0.027	0.465	0.299
Proteins	22.4 ± 0.7^{a}	19.4 ± 0.6^{c}	19.8 ± 0.7^{c}	21.4 ± 0.9^{ab}	21.3 ± 0.7^{b}	0.697	0.496	<0.001
Fat	1.43 ± 0.08^{a}	1.3 ± 0.1^{ab}	1.2 ± 0.1^{b}	1.41 ± 0.08^{a}	1.27 ± 0.09^{b}	0.607	0.272	<0.001
Carbohydrates	35.6 ± 0.9^{a}	30 ± 1^{b}	25 ± 1^{d}	28 ± 1^{c}	26 ± 1^{d}	0.954	0.001	<0.001
Energy	1023 ± 15^{a}	869 ± 28^{b}	799 ± 18^{d}	877 ± 20^{b}	841 ± 17^{c}	0.651	<0.001	<0.001
Fructose	1104 ± 31^{a}	681 ± 28^{c}	977 ± 28^{b}	628 ± 99^{cd}	584 ± 42^{d}	<0.001	<0.001	<0.001
Glucose	696 ± 20^{a}	320 ± 41^{b}	347 ± 79^{b}	349 ± 25^{b}	324 ± 34^{b}	0.006	<0.001	<0.001
Sucrose	233 ± 51^{c}	446 ± 37^{a}	495 ± 57^{a}	380 ± 58^{b}	440 ± 30^{ab}	0.113	0.008	<0.001
Total sugars	2034 ± 31^{a}	1447 ± 38^{c}	1818 ± 56^{b}	1357 ± 159^{c}	1347 ± 46^{c}	0.001	0.001	<0.001
Oxalic acid	7541 ± 244^{a}	6319 ± 219^{b}	5507 ± 260^{c}	7262 ± 215^{a}	6442 ± 322^{b}	0.465	0.189	<0.001
Malic acid	730 ± 17^{d}	2892 ± 143^{a}	2109 ± 133^{b}	1988 ± 193^{bc}	1849 ± 77^{c}	0.002	0.001	<0.001
Citric acid	1089 ± 39^{a}	1000 ± 60^{b}	718 ± 52^{c}	974 ± 78^{b}	624 ± 77^{d}	0.179	0.001	<0.001
Fumaric acid	3.5 ± 0.2^{d}	15.0 ± 0.8^{a}	8.7 ± 0.7^{b}	6.2 ± 0.7^{c}	7.0 ± 0.5^{c}	0.027	<0.001	<0.001
Total organic acids	9364 ± 216^{b}	10226 ± 365^{a}	8343 ± 209^{d}	10230 ± 190^{a}	8922 ± 387^{c}	0.407	0.044	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among packaging systems was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one packaging atmosphere differs from the others (in this case multiple comparison tests were performed). For each storage condition, means within a row with different letters differ significantly ($p<0.05$).

Table 30 Fatty acids (relative %) and tocopherols (mg kg^{-1}) of fresh-cut watercress samples stored under conventional and inert gas-enriched MAP.[1]

	Harvest (day 0)	**7 d of storage at 4 °C**				*p*-values		
	Non-stored control	**Conventional packaging**		**Non-conventional MAP**		Homoscedasticity[2]	Normal distribution[3]	One-way ANOVA[4]
		Air	**Vacuum**	**N_2**	**Ar**			
C6:0	0.014 ± 0.002^{d}	0.097 ± 0.002^{a}	0.030 ± 0.002^{c}	0.039 ± 0.003^{b}	0.040 ± 0.005^{b}	0.003	<0.001	<0.001
C8:0	0.048 ± 0.005^{c}	0.130 ± 0.001^{a}	0.062 ± 0.004^{b}	0.044 ± 0.003^{c}	0.066 ± 0.005^{b}	0.004	<0.001	<0.001
C10:0	0.063 ± 0.005^{c}	0.373 ± 0.005^{a}	0.068 ± 0.004^{c}	0.060 ± 0.004^{c}	0.09 ± 0.01^{b}	0.001	<0.001	<0.001
C12:0	0.225 ± 0.004^{b}	0.317 ± 0.004^{a}	0.17 ± 0.01^{c}	0.115 ± 0.007^{e}	0.154 ± 0.005^{d}	0.031	<0.001	<0.001
C14:0	1.7 ± 0.1^{b}	1.5 ± 0.1^{c}	1.22 ± 0.05^{e}	1.35 ± 0.05^{d}	1.89 ± 0.05^{a}	0.640	0.150	<0.001
C15:0	0.38 ± 0.04^{c}	0.41 ± 0.01^{bc}	0.46 ± 0.02^{b}	0.43 ± 0.04^{bc}	0.59 ± 0.03^{a}	0.048	0.004	<0.001
C16:0	45 ± 1^{a}	29 ± 1^{c}	25.5 ± 0.4^{d}	22.9 ± 0.5^{e}	32.2 ± 0.5^{b}	0.143	<0.001	<0.001
C16:1	nd	0.17 ± 0.01^{d}	0.37 ± 0.03^{a}	0.30 ± 0.01^{c}	0.33 ± 0.04^{b}	<0.001	<0.001	<0.001
C17:0	0.89 ± 0.04^{a}	0.38 ± 0.03^{d}	0.75 ± 0.04^{b}	0.32 ± 0.01^{e}	0.57 ± 0.04^{c}	0.105	<0.001	<0.001
C18:0	4.1 ± 0.4^{a}	4.4 ± 0.1^{a}	3.1 ± 0.3^{b}	2.9 ± 0.2^{b}	4.17 ± 0.1^{a}	0.088	0.020	<0.001
C18:1n9	0.61 ± 0.05^{d}	1.47 ± 0.01^{b}	1.43 ± 0.05^{b}	1.19 ± 0.05^{c}	1.6 ± 0.2^{a}	0.036	<0.001	<0.001
C18:2n6	8.4 ± 0.5^{c}	10.3 ± 0.1^{a}	9.3 ± 0.3^{b}	9.0 ± 0.4^{bc}	9.1 ± 0.2^{b}	0.068	0.041	<0.001
C18:3n3	27 ± 1^{e}	43 ± 1^{c}	48.4 ± 0.5^{b}	54.6 ± 0.4^{a}	38.2 ± 0.5^{d}	0.636	0.002	<0.001
C20:0	0.25 ± 0.04^{d}	0.50 ± 0.01^{b}	0.61 ± 0.04^{a}	0.38 ± 0.02^{c}	0.57 ± 0.05^{a}	0.022	0.081	<0.001
C20:1	0.42 ± 0.05^{a}	0.18 ± 0.01^{b}	0.12 ± 0.01^{c}	0.09 ± 0.01^{c}	0.19 ± 0.01^{b}	<0.001	<0.001	<0.001
C20:2	2.3 ± 0.3^{a}	1.2 ± 0.1^{c}	2.5 ± 0.2^{a}	1.0 ± 0.1^{c}	1.7 ± 0.1^{b}	0.015	0.002	<0.001
C20:4n6	1.2 ± 0.2^{a}	0.53 ± 0.02^{c}	0.56 ± 0.05^{c}	0.19 ± 0.02^{d}	1.02 ± 0.05^{b}	<0.001	0.006	<0.001
C20:3n3+C21:0	1.0 ± 0.1^{a}	0.68 ± 0.03^{c}	0.76 ± 0.05^{bc}	0.72 ± 0.05^{c}	0.84 ± 0.05^{b}	0.066	0.002	<0.001
C20:5n3	0.3 ± 0.1^{b}	0.2 ± 0.1^{b}	0.9 ± 0.1^{a}	0.13 ± 0.01^{b}	1.0 ± 0.1^{a}	0.001	<0.001	<0.001
C22:0	1.3 ± 0.1^{a}	0.9 ± 0.1^{b}	0.90 ± 0.05^{b}	0.85 ± 0.03^{b}	1.26 ± 0.05^{a}	0.006	<0.001	<0.001
C22:2	1.6 ± 0.1^{a}	0.57 ± 0.01^{b}	0.18 ± 0.01^{d}	0.34 ± 0.03^{c}	0.53 ± 0.05^{b}	<0.001	<0.001	<0.001
C24:0	2.8 ± 0.2^{a}	2.6 ± 0.1^{a}	1.7 ± 0.1^{b}	2.0 ± 0.1^{b}	2.7 ± 0.2^{a}	0.368	0.065	<0.001
C24:1	nd	0.8 ± 0.1^{c}	0.93 ± 0.05^{bc}	1.02 ± 0.05^{b}	1.21 ± 0.05^{a}	<0.001	<0.001	<0.001

	Harvest (day 0)	**7 d of storage at 4 °C**				*p*-values		
	Non-stored control	**Conventional packaging**		**Non-conventional MAP**		Homoscedasticity[2]	Normal distribution[3]	One-way ANOVA[4]
		Air	**Vacuum**	**N_2**	**Ar**			
SFA	57 ± 1^{a}	40 ± 1^{c}	34.6 ± 0.5^{d}	31.4 ± 0.3^{e}	44 ± 1^{b}	0.218	<0.001	<0.001
MUFA	1.0 ± 0.1^{d}	2.6 ± 0.2^{c}	2.85 ± 0.05^{b}	2.59 ± 0.05^{c}	3.3 ± 0.1^{a}	0.002	<0.001	<0.001
PUFA	42 ± 1^{e}	57 ± 1^{c}	63 ± 1^{b}	66 ± 1^{a}	52 ± 1^{d}	0.280	<0.001	<0.001
PUFA/SFA	0.74 ± 0.02^{e}	1.41 ± 0.05^{c}	1.81 ± 0.04^{b}	2.11 ± 0.03^{a}	1.18 ± 0.03^{d}	0.163	0.003	<0.001
n-6/n-3	0.47 ± 0.03^{a}	0.28 ± 0.01^{c}	0.25 ± 0.01^{d}	0.19 ± 0.01^{e}	0.31 ± 0.01^{b}	0.001	<0.001	<0.001
α-tocopherol	5.0 ± 0.3^{d}	12.4 ± 0.8^{a}	7.9 ± 0.1^{c}	11.2 ± 0.8^{b}	10.7 ± 0.1^{b}	<0.001	0.001	<0.001
β-tocopherol	0.11 ± 0.01^{b}	0.18 ± 0.02^{a}	0.10 ± 0.01^{bc}	0.09 ± 0.01^{c}	0.09 ± 0.01^{c}	0.073	<0.001	<0.001
γ-tocopherol	0.38 ± 0.03^{d}	0.63 ± 0.04^{b}	0.70 ± 0.05^{a}	0.51 ± 0.05^{c}	0.43 ± 0.03^{d}	0.306	0.017	<0.001
Total tocopherols	5.5 ± 0.4^{d}	13.2 ± 0.8^{a}	8.7 ± 0.1^{c}	11.8 ± 0.8^{b}	11.2 ± 0.1^{b}	<0.001	0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among packaging systems was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one storage atmosphere differs from the others (in this case multiple comparison tests were performed). For each storage condition, means within a row with different letters differ significantly ($p<0.05$).

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); Arachidonic acid (C20:4n6); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); *cis*-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); *cis*-13,16-Docosadienoic acid (C22:2); Lignoceric acid (C24:0); Nervonic acid (C24:1). SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids. nd - not detected.

The assayed treatments induced also significant changes in the tocopherols content (**Table 30**), with the lowest values being recorded in control samples. α-Tocopherol was always the most abundant isoform, followed by γ-tocopherol and β-tocopherol, in accordance with the results previously reported by Pereira *et al.* (2011). Air-packaged samples reveal the highest content of α- and β-tocopherol, and total tocopherols content. The highest values for γ-tocopherol were shown by the vacuum-packaged samples. In general, non-conventional MAP was able to maintain similar levels of β- and γ- isoforms, comparatively with the non-stored control, whilst vacuum-packaging presented the closest ones regarding the total tocopherols content. According to the literature, stress conditions can induce an increase in the total tocopherol levels (Munné-Bosch 2005, Yusuf *et al.* 2010). In these situations, there is an increase in tocopherol synthesis, followed by a net tocopherol loss. The initial enhanced tocopherol levels contribute to tissue protection by reducing reactive oxygen species levels and inhibiting lipid peroxidation, thus avoiding oxidative damage (Munné-Bosch 2005). Additionally, α-tocopherol plays a major role in the alleviation of stress, and its levels change significantly under stress (such as that induced by the different packaging conditions), as a result of the altered expression of pathway-related genes, degradation and recycling (Munné-Bosch 2005). Thus, tocopherol increases verified in this work can be related to the assayed packaging atmosphere incapacity to maintain favourable preservation conditions to watercress. Nevertheless, the observed increase contributes to a higher bioactivity.

4.2.3.4. In vitro bioactive properties

Table 31 presents the values for antioxidant activity and total phenolic and flavonoid contents of the watercress extracts. From the one-way ANOVA *p*-values, it can be concluded that the assayed packaging atmospheres induced significant changes in these parameters. Non-stored control samples showed the highest DPPH$^{\bullet}$ scavenging activity, reducing power and β-carotene blanching inhibition capacity. Regarding the assayed packaging atmospheres, Ar-enriched MAP were the best treatment to preserve the DPPH$^{\bullet}$ scavenging activity and β-carotene blanching inhibition capacity, showing also an increase in the total phenolics content. In turn, samples under N_2-enriched MAP present lower lipid peroxidation inhibition capacity, despite the high levels of total phenolics and flavonoids. The TBARS formation inhibition capacity was improved by conventional packaging relatively to the non-stored control samples. However, air-packaging gave the highest EC_{50} values (corresponding to the lowest antioxidant activity) in the DPPH$^{\bullet}$ and reducing power assays.

Table 31 Antioxidant activity (EC_{50} values, g L^{-1}) and total phenolics (g GAE kg^{-1} extract) and flavonoids (g CE kg^{-1} extract) for fresh-cut watercress samples stored under conventional and inert gas-enriched MAP.[1]

	Harvest (day 0)	**7 d of storage at 4 °C**				*p*-values		
	Non-stored control	**Conventional packaging**		**Non-conventional MAP**		Homoscedasticity[2]	Normal distribution[3]	One-way ANOVA[4]
		Air	**Vacuum**	**N_2**	**Ar**			
DPPH$^{\bullet}$ scavenging activity	0.49 ± 0.01^{e}	0.81 ± 0.01^{a}	0.65 ± 0.01^{c}	0.67 ± 0.02^{b}	0.61 ± 0.02^{d}	0.001	0.002	<0.001
Reducing power	0.38 ± 0.01^{e}	0.48 ± 0.01^{a}	0.43 ± 0.01^{d}	0.46 ± 0.01^{c}	0.47 ± 0.01^{b}	<0.001	<0.001	<0.001
β-carotene blanching inhibition	0.33 ± 0.02^{e}	0.62 ± 0.01^{b}	0.50 ± 0.02^{c}	0.75 ± 0.01^{a}	0.42 ± 0.01^{d}	0.008	0.007	<0.001
TBARS formation inhibition	0.27 ± 0.01^{b}	0.21 ± 0.01^{d}	0.24 ± 0.01^{c}	0.43 ± 0.01^{a}	0.26 ± 0.01^{b}	0.001	<0.001	<0.001
Total phenolics	87 ± 2^{c}	97 ± 2^{b}	99 ± 4^{b}	108 ± 2^{a}	105.8 ± 0.3^{a}	<0.001	0.008	<0.001
Total flavonoids	36 ± 1^{a}	25 ± 1^{c}	25 ± 1^{c}	28 ± 1^{b}	26 ± 1^{c}	<0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among packaging systems was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one packaging atmosphere differs from the others (in this case multiple comparison tests were performed). For each storage condition, means within a row with different letters differ significantly ($p<0.05$).

GAE: gallic acid equivalents; CE: catechin equivalents

Similar phenolic (96-97 g GAE kg^{-1} extract) and higher flavonoid (62-63 g CE kg^{-1} extract) contents were reported by Yazdanparast *et al.* (2008) and Bahramikia and Yazdanparast (2010) in hydro-ethanolic extracts of a watercress sample from Iran, as well as analogous TBARS inhibition properties (0.27 g L^{-1}). On the other hand, the study conducted by Pereira *et al.* (2011) reported lower values for phenolics (~50.42 g GAE kg^{-1} extract) but similar flavonoids content for the control samples (~35.17 g CE kg^{-1} extract) in methanolic extracts. Regarding its antioxidant performance, assessed using the same *in vitro* methodologies, only the $DPPH^{\bullet}$ scavenging capacity gave better results (~0.13 g L^{-1}) in comparison with our hydromethanolic extracts (except for the N_2-packaged sample which showed a higher EC_{50} value in the TBARS assay). Regarding the effect of non-conventional MAP on the total polyphenols content of watercress, Silveira *et al.* (2014) reported initial contents of ~2 g GAE kg^{-1} extract (obtained after homogenizing 1 g of frozen watercress with 3 mL of methanol/water (4:1, v/v)), where the lowest value corresponded to the samples packaged in N_2-enriched atmospheres. They also found maintenance on polyphenol levels during 13 days of storage, with the same trend observed at the beginning of the experiment, and higher levels in air-packaged and Ar-enriched MAP samples. An antioxidant activity increase was verified in He and N_2O packaged samples at the end of the storage period. Tomás-Callejas *et al.* (2011) reported an increase of 61% in the polyphenol content of red chard stored under N_2-enriched MAP for 6 d and attributed this behaviour to the wound-induced phenomenon in phenolic metabolism.

4.2.3.5. Overall postharvest quality assessment using LDA

After evaluating the effects on individual quality parameters, a LDA was applied to obtain a complete perspective about the effects of MAP on the overall postharvest quality of fresh-cut watercress. The basic purpose of this discriminant analysis was finding the packaging atmosphere (categorical dependent variable) that maintained physicochemical, nutritional and antioxidant profiles (set of quantitative independent variables) of the non-stored control samples throughout the storage time. The significant independent variables were selected following the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

The discriminant model selected 4 significant functions, which included 100.0% of the observed variance. The graph representation (**Figure 30**) of the three first functions (function 1: 67.7%, function 2: 19.0%, function 3: 8.0%) showed the complete individualization of markers corresponding to each packaging atmosphere. Function 1, mainly correlated with C18:3 and β-carotene bleaching inhibition, as deduced from the canonical discriminant functions standardized coefficients, separated primarily air-packaged and N_2-enriched MAP samples, which were also placed far from the non-stored control samples through function 2

(more correlated to capric acid (C10:0) and C18:3). Function 3, more powerfully correlated with TBARS formation inhibition and MUFA content, clarified that air-packaged samples were those with the most dissimilar chemical and antioxidant profiles, in comparison to the non-stored control samples. The complete individualization of the four packaging atmospheres (100.0% of the assayed species for the originally grouped cases as well as for the cross-validated cases) indicates their lack of effectiveness in maintaining the original quality attributes of the non-stored watercress samples during refrigerated storage.

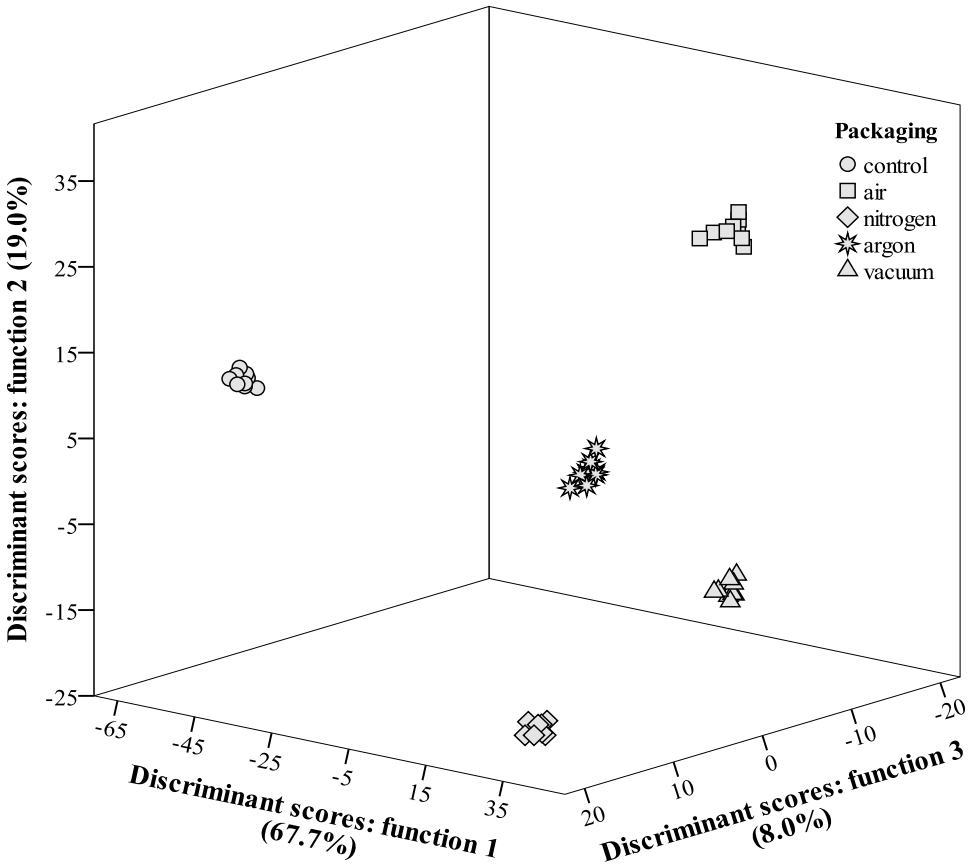

Figure 30 Biplot of object scores (packaging atmospheres) and component loadings (evaluated quality parameters).

4.2.4. Conclusions

None of the tested packaging atmospheres induced watercress yellowing. In general, sucrose levels increased with refrigerated storage, especially in samples under conventional packaging; while vacuum-packaging preserved the highest levels of fructose and total sugars. Tocopherols also increased in stored samples, being the highest levels of α-, β- and total tocopherols recorded in air-packaged samples, which may have been caused by stress conditions. All packaged samples showed higher MUFA and PUFA contents and PUFA/SFA ratios and lower SFA levels and n-6/n-3 ratios. Furthermore, Ar-enriched MAP was the best option to preserve the DPPH$^{\bullet}$ scavenging activity and β-carotene blanching inhibition

capacity, as well as to increase the total phenolic content. Based on the LDA, it was possible to conclude that air-packaged samples were those that presented the most dissimilar profiles from the non-stored control samples, followed by those stored under N_2-enriched MAP. In turn, Ar-enriched MAP was the most suitable choice to preserve the overall postharvest quality of fresh-cut watercress. The present study also highlights the nutritional and health-promoting composition of these wild watercress samples, as well as the interest of its inclusion in traditional and modern diets. Despite these findings, further studies are of interest to evaluate other quality attributes as well as physiological parameters.

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4.3. Suitability of gamma irradiation for preserving fresh-cut watercress quality during cold storage

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Suitability of gamma irradiation for preserving fresh-cut watercress quality during cold storage

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Abstract

The suitability of gamma irradiation (1, 2 and 5 kGy) for preserving quality parameters of fresh-cut watercress (*Nasturtium officinale* R. Br.) during storage at 4±1 ºC for 7 d was investigated. The storage time decreased the protein content and the main carbohydrates, and increased the levels of malic and fumaric acids, sucrose and mono- and polyunsaturated fatty acids (MUFA and PUFA). The different irradiation doses did not cause any significant colour change. In general, the 2 kGy dose favoured PUFA and was the most suitable to preserve the overall postharvest quality of fresh-cut watercress during cold storage. In turn, the 5 kGy dose better preserved the antioxidant activity and total flavonoids and favoured MUFA, tocopherols and total phenolics, thus originating a final product with enhanced functional properties. Therefore, the suitability of gamma irradiation for preserving fresh-cut watercress quality during cold storage was demonstrated.

Keywords: *Nasturtium officinale*; fresh-cut watercress; gamma irradiation; refrigerated storage; postharvest quality; antioxidant activity.

4.3.1. Introduction

Watercress (*Nasturtium officinale* R. Br.) is a rapidly growing, aquatic or semi-aquatic perennial plant of the Brassicaceae family widely consumed in salads. It features sharp, peppery and slightly tangy taste and contains health-promoting phytochemicals endowed with therapeutic properties (Rose, Yen, *et al.* 2005, Sadeghi *et al.* 2014). Its consumption as a fresh-cut product has increased in recent years, as well as the global market of minimally processed vegetables (Sillani and Nassivera 2015, Pinela and Ferreira 2017). This demand is driven by the growing interest in the role of food in promoting the human health and well-being and to meet consumer needs for fresh-like and more convenient foods. Besides, consumers are receptive to innovations that enhance the quality and nutritional value of food and critical of technologies that excessively modify the original product or could be dangerous to human health and the environment (Sillani and Nassivera 2015).

Minimally processed vegetables are highly perishable because of exposed internal tissues, lack of skin or cuticle for protection and elevated metabolism, so that the deterioration is faster than in intact ones (Francis *et al.* 2012). Meanwhile, there are some concerns surrounding efficacy of the commonly used sanitizing methods. Chemical agents do not provide an efficient microbial reduction, besides being perceived negatively by the consumers, dangerous for human health and harmful to the environment (Ramos *et al.* 2013). In turn, the conventional thermal treatments may negatively affect several quality parameters of these perishable foods (Pinela and Ferreira 2017). For this reasons, innovative and more sustainable postharvest treatments based on non-thermal physical technologies are being investigated.

The gamma irradiation treatment has become an effective means of improving both safety and shelf-life of food products. Its commercial use is steadily growing because it provides a safe solution to quarantines and is tolerated by more fresh commodities than any alternative treatment in use (Hallman 2017). A joint FAO/IAEA/WHO Expert Committee on the wholesomeness of irradiated foods has ruled that foods subjected to irradiation doses up to 10 kGy are safe and do not require toxicological testing (WHO 1999). For fresh horticultural products, irradiation treatments have been used to prevent sprouting and post-packaging contamination, delay postharvest ripening, maturation and senescence and thereby extend the shelf-life and reduce food losses (ICGFI 1999). Examples of minimally processed vegetables treated by gamma irradiation, aiming both quality preservation and shelf-life extension, include arugula (Nunes *et al.* 2013), spinach (Fan and Sokorai 2011), cauliflower (Vaishnav *et al.* 2015), ash gourd (Tripathi *et al.* 2013), bamboo shoots (Zeng *et al.* 2015), coriander, parsley, lettuce and watercress (Trigo *et al.* 2009). However, little information is still available regarding changes on physical, chemical or bioactive properties, as well as on the shelf-life of minimally processed vegetables. In this sense, this study was

carried out to evaluate the suitability of gamma irradiation at different doses (1, 2 and 5 kGy) for preserving visual, nutritional and antioxidant quality parameters of fresh-cut watercress during cold storage at 4±1 ºC for 7 d.

4.3.2. Materials and methods

4.3.2.1. Standards and reagents

Amber Perspex routine dosimeters, Batch X, were purchased from Harwell Company (Oxfordshire, UK). Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), other individual fatty acid isomers, tocopherols (α-, β-, and γ-isoforms), sugars ($_D$(–)-fructose, $_D$(+)-glucose anhydrous, $_D$(+)-melezitose hydrate, $_D$(+)-sucrose), organic acids (citric, malic, oxalic and fumaric acids), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and catechin standards were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) as obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

4.3.2.2. Sampling and samples preparation

Wild samples of watercress (*Nasturtium officinale* R. Br., synonym of *Rorippa nasturtium-aquaticum* (L.) Hayek) were hand-harvested in February 2014 in a local stream in the civil parish of Calvelhe, municipality of Bragança, Portugal, considering local consumers' sites, criteria and preferences, such as the season and phenological stage (Carvalho and Morales 2010). Taxonomic identification of the plant material was confirmed by the botanist Dr. Ana Maria Carvalho, from the Polytechnic Institute of Bragança, Portugal. A voucher specimen was deposited in the Herbarium of the School of Agriculture of Bragança. The use of wild-harvested samples instead of ones from commercial sources allowed having control over the postharvest age of the plant. In the same day, healthy and undamaged aerial parts (tender stems and leaves) were hand-picked (removing yellow leaves, thicker stems and roots), rinsed in tap water and drained to eliminate excess water. Then, a portion of watercress was immediately analysed (non-stored control) and the remaining fresh material was packaged in 11.5 cm × 15 cm sterilized packages made from low-density polyethylene film (thickness 63 μm) using the passive modification mode (air). Forty packages containing 20 g of sample were prepared.

4.3.2.3. Samples irradiation and storage

Packaged samples were divided into four groups of 10 packages and submitted to 1, 2 and 5 kGy of gamma-rays (predicted doses). The other group was a non-irradiated (0 kGy) control that followed all the experiment. The irradiation was performed one day after harvest in an cobalt-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) located at C2TN (Centre for Nuclear Sciences and Technologies), in Portugal, with four sources and a total activity of 177 TBq (4.78 kCi; February 2014). During the irradiation process, amber Perspex routine dosimeters were used to measure the distribution of the absorbed energy and to determine the maximum (D_{max}) and the minimum (D_{min}) dose absorbed by the samples following the procedure previously described by Fernandes *et al*. (2012). The measured average doses were 1.2 ± 0.1 kGy, 2.2 ± 0.2 kGy and 5.2 ± 0.4 kGy for the samples irradiated at the predicted doses of 1, 2 and 5 kGy, respectively. For simplicity, the predicted dose values were considered in the text. The estimated dose rate for the irradiation position was 1.6 kGy/h and the dose uniformity ratio (D_{max}/D_{min}) was 1.5. Irradiated and non-irradiated packaged samples were stored in the dark at 4 ± 1 ºC for 7 d.

4.3.2.4. Quality analysis

Colour parameters

Watercress samples were placed on a black surface to reduce external interferences and data were collected on the adaxial surface of nine randomly selected leaves with a colorimeter (model CR-400; Konica Minolta Sensing Inc., Japan) previously calibrated using the standard white plate (Pereira, Antonio, *et al.* 2015). Using illuminant C and the diaphragm opening of 8 mm, the CIE *L*a*b** colour space values were registered through the computerized system using a colour data software "Spectra Magic Nx" (version CM-S100W 2.03.0006). Average values were considered to determine the colour coordinates, where *L** represents lightness, *a** represents chromaticity on a green (–) to red (+) axis, and *b** represents chromaticity on a blue (–) to yellow (+) axis.

Total soluble solids and pH

Watercress samples were ground and the grinding paste was subsequently filtered through Whatman No. 4 paper. The total soluble solids (TSS) content (%) in the squeezed juice was measured with a digital hand refractometer (model HI 96801, Hanna Instruments, Woonsocket, RI, USA) and the pH with a digital pH-meter (model pH 211, Hanna Instruments, Woonsocket, RI, USA).

Proximate composition

Watercress samples were analysed for moisture, proteins, fat, ash and carbohydrates using the AOAC procedures (AOAC 2005). Samples were lyophilised (FreeZone 4.5, Labconco, Kansas City, MO, USA) and reduced to a fine powder (20 mesh) for analysis of proteins, fat and ash. Briefly, the crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method, using an automatic distillation and titration unit (model UDK152, VELP Scientifica, Italy); the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 ºC; and total carbohydrates were calculated by difference according to the equation: 100 - ($m_{moisture}$ + $m_{proteins}$ + m_{fats} + m_{ashes}). The results were expressed as g per 100 g of fresh weight (fw). The total energy was calculated according to the equation: 4 × ($m_{proteins}$ + $m_{carbohydrates}$) + 9 × (m_{fats}) (Regulation (EU) No 1169/2011) and expressed as kcal per 100 g of fresh weight (fw).

Free sugars

Free sugars were determined by high performance liquid chromatography (HPLC) coupled to a refraction index (RI) detector as described by Pereira *et al.* (2011). Briefly, lyophilized powdered samples (1 g) were spiked with melezitose (internal standard, 5 mg/mL) and extracted with 40 mL of 80% aqueous ethanol at 80 ºC for 30 min. The mixture was centrifuged at 15,000*g* (Centurion K24OR-2003 refrigerated centrifuge) for 10 min and the supernatant was concentrated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and defatted with ethyl ether. After concentration at 40 ºC, the residues were dissolved in 5 mL of water and filtered through 0.2 µm nylon filters for subsequent injection. The HPLC equipment consisted of an integrated system with a Smartline system 1000 pump (Knauer, Berlin, Germany), a Smartline Manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD, USA) and a Smartline 2300 RI detector. The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC in a 7971 R Grace oven. The mobile phase was acetonitrile/deionized water, 70:30 (v/v), at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards and quantified using the internal standard method. The results were expressed in mg per 100 g of fresh weight (fw).

Organic acids

Organic acids were analysed by ultra fast liquid chromatography (UFLC) coupled to a photodiode array (PDA) detector according to Pereira *et al.* (2013). Briefly, fresh tissue (9 g) was ground and the grinding paste was subsequently extracted by stirring with 25 mL of meta-phosphoric acid (25 ºC at 150 rpm) for 45 min and subsequently filtered through

Whatman No. 4 paper. Before injection, samples were filtered through 0.2 µm nylon filters. The analysis was carried out with a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). The chromatographic separation was achieved with a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 µm, 250 × 4.6 mm i.d) thermostated at 35 °C. The elution was performed with sulphuric acid 3.6 mM at a flow rate of 0.8 mL/min. Detection was carried out in a PDA detector, using 215 and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks with calibration curves obtained from commercial standards of each compound, *i.e.,* oxalic, malic, citric and fumaric acids. The results were expressed in mg per 100 g of fresh weight (fw).

Fatty acids

Fatty acids were analysed by gas chromatography with flame ionization detection (GC-FID)/capillary column as described previously by Barros *et al.* (2010). Briefly, the fatty acids obtained after Soxhlet extraction were methylated with methanol:sulfuric acid:toluene 2:1:1 (v:v:v) during at least 12 h in a bath at 50 ºC and 160 rpm. Then, deionised water was added to obtain a phase separation and the FAME were recovered with diethyl ether by shaking in vortex. The upper phase was dehydrated with sodium sulfate anhydrous and filtered through 0.2 µm nylon filters for subsequent injection. The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a FID detector at 260 °C and a Macherey-Nagel column (30 m × 0.32 mmID × 0.25 µm d_f). The oven temperature programme was as described by Pereira *et al.* (2011). The identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

Tocopherols

Tocopherols were determined by HPLC coupled to a fluorescence (FP-2020; Jasco) detector following the procedures previously described by Pereira *et al.* (2011). Briefly, lyophilized powdered samples (500 mg) were spiked with a BHT solution (10 mg/mL) and tocol (internal standard, 50 µg/mL) and homogenized with methanol (4 mL) by shaking in vortex (1 min) and then with hexane (4 mL). After that, a saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized, centrifuged (5 min, 4000*g*) and the clear upper layer was collected. The extraction was repeated twice with hexane. The obtained extracts were dried under a nitrogen stream, redissolved in 2 mL of n-hexane, dehydrated, and filtered through 0.22 µm disposable LC filters for subsequent injection. The analysis was carried out in the HPLC system described above connected to the FP-2020 detector programmed for

excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 30 °C. The mobile phase was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards and quantified using the internal standard method. The results were expressed in mg per 100 g of fresh weight (fw).

4.3.2.5. Antioxidant properties evaluation

General: Watercress extracts were prepared according to procedures previously described by Pereira *et al.* (2011). Briefly, a fine dried powder (20 mesh; ~1 g) was stirred (150 rpm) with 50 mL of methanol:water (80:20, v/v) for 1 h at 25 ºC. The supernatant was filtered through Whatman No. 4 paper and the residue was re-extracted with an additional portion of solvent (50 mL). The combined extracts were then evaporated at 35 ºC under reduced pressure, redissolved in the same solvent (final concentration of 5 mg/mL) and successively diluted to different concentrations.

Antioxidant activity: Four *in vitro* assays were performed to evaluate the hydromethanolic extracts antioxidant activity (Pereira *et al.* 2011): DPPH$^{\bullet}$ scavenging activity, reducing power (measured by ferricyanide Prussian blue assay), β-carotene blanching inhibition, and thiobarbituric acid reactive substances (TBARS) formation inhibition. Briefly, the DPPH$^{\bullet}$ scavenging activity and the reducing power assays were performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA). The reduction of DPPH$^{\bullet}$ was determined by measuring the absorbance at 515 nm and calculated as a percentage of DPPH$^{\bullet}$ discolouration using the equation: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_{DPPH} is the absorbance of the DPPH$^{\bullet}$ solution and A_S is the absorbance of the solution containing the sample extract. The reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm. The β-carotene bleaching inhibition was evaluated by measuring the capacity to neutralize linoleate free radicals, which was monitored at 470 nm in a Model 200 spectrophotometer (AnalytikJena, Jena, Germany), and calculated using the equation: $(A_{\beta T2}/A_{\beta T0}) \times 100$, where $A_{\beta T2}$ is the absorbance of the emulsion after 2 h of incubation at 50 ºC and $A_{\beta T0}$ is the initial absorbance. The TBARS formation inhibition was evaluated in porcine brain homogenates. The colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) complex formed during heating at 80 ºC for 20 min was measured at 532 nm, and the inhibition ratio calculated using the equation: $[(A - B)/A] \times 100\%$, where A and B correspond to the absorbance of the control and the sample solution, respectively. The results were expressed in EC_{50} values (mg/mL), *i.e.,* sample concentration

providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control.

Total phenolics and flavonoids: The total phenolic content was determined by the Folin-Ciocalteu method with slight modifications (Pereira *et al.* 2011) in the hydromethanolic extracts concentrated at 0.625 mg/mL. This assay is based on the formation of a blue-coloured complex between the molybdenum and tungsten present in the Folin-Ciocalteu reagent upon reaction with reducing agents, which is monitored at 765 nm. The standard curve was calculated using gallic acid and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract. The total flavonoid content was determined using the aluminum chloride colorimetric method as described by the authors (Barros *et al.* 2010) in the extracts concentrated at 1.25 mg/mL. This assay is based on the formation of a complex flavonoid-aluminum, which is monitored at 510 nm. The standard curve was calculated using catechin and the results were expressed as mg of catechin equivalents (CE) per g of extract.

4.3.2.6. Statistical analysis

Replicates of each treatment were divided into three batches and independently analysed. All the quality analyses were carried out in triplicates. Data were expressed as mean ± standard deviation. All statistical tests were performed at a 5% significance level using SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

The differences among treatments were analysed using the one-way analysis of variance (ANOVA). The fulfilment the ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Furthermore, a linear discriminant analysis (LDA) was used to evaluate the effect of gamma irradiation on the overall postharvest quality of watercress during cold storage. A stepwise technique, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination processes, where the inclusion of a new variable is preceded by ensuring that all variables selected previously remain significant. With this approach, it is possible to determine which of the independent variables account most for the differences in the average score profiles of the different treatments. To verify the significance of canonical discriminant functions, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

4.3.3. Results and discussion

The suitability of the irradiation treatment for preserving postharvest quality parameters depends on the applied dose and plant material under investigation (Trigo *et al.* 2009). During irradiation, free radicals and reactive species are generated due to the radiolysis of water, which are capable of breaking chemical bonds and modify various biomolecules (Fanaro *et al.* 2015). Besides, fresh-cut vegetables are still living organisms composed by a wide range of bioactive compounds able to interact and protect the plant tissues from adverse postharvest conditions. In addition, hormetic effects can also be induced by ionizing radiation (Shama and Alderson 2005). Therefore, nonlinear dose-response effects of the irradiation treatment on the evaluated quality parameters can be expected during shelf-life.

4.3.3.1. Effects on colour, TSS and pH

Colour plays a key role in food acceptability and is more important than flavour or texture in the initial food-selection process. Therefore, colour loss is one of the major external postharvest problems. Based on the one-way ANOVA *p*-values it was possible to conclude that the treatment induced significant changes on the *L** and *b** colour values, but had no effect on greenness (*a** value) (**Table 32**). The observed changes were attributed to the storage time, since those recorded in stored samples did not differ significantly from each other. The yellowing (*b**) just differed significantly between non-stored and non-irradiated stored samples. The increased lightness (*L**) of the stored samples, especially those non-irradiated and irradiated at 2 and 5 kGy, suggested incipient yellowing of the leaves. Silveira *et al.* (2014) also reported a slight increase in lightness of watercress leaves stored at 5 ºC under some non-conventional modified atmospheres. In contrast, a decrease was reported for watercress samples stored at 5 ºC for 14 d and previously sanitized using chemical agents (Hinojosa *et al.* 2013). Other study demonstrated that gamma irradiation doses up to 1 kGy had no effect on watercress colour (Trigo *et al.* 2009). The authors also reported an increase in *L** values of irradiated and non-irradiated lettuce samples stored at 4 ºC.

The TSS content and pH also allow the indirect evaluation of changes in sensory attributes (*i.e.,* flavour) that have significant influence in the consumers' evaluation of the product (Barrett *et al.* 2010). The TSS content decreased in non-irradiated samples and in those irradiated at 5 kGy (**Table 32**). The consumption of the sugars included in the TSS fraction during storage by the plant metabolic processes may justify this reduction (Heldt and Piechulla 2011). The 2 kGy dose preserved the initial TSS content, which was increased with the 1 kGy dose. Curiously, the last samples also revealed the sharpest increase of total organic acids (which will be discussed below). As it is known, TSS include mainly soluble sugars but also small amounts of organic acids. In turn, the initial pH values were only maintained in samples irradiated at 5 kGy. Nevertheless, these values were always higher

than 5.34. The evolution of these physicochemical parameters during storage may affect the sensorial properties of watercress, especially the sweetness and sourness sensation (Barrett *et al.* 2010). However, sensory analysis with a trained panel will be interesting to evaluate the samples acceptance.

4.3.3.2. Effects on the proximate composition

The nutritional composition of fresh and irradiated samples of watercress is presented in **Table 33**. From the one-way ANOVA *p*-values, it can be concluded that the treatment induced significant changes in all macronutrients, except for fat content. In general, the storage time decreased the protein and carbohydrates content and, consequently, the energetic contribution, independently of the irradiation dose. Just slight variations of moisture, ash and proteins were found among the irradiated samples, which did not differ in the carbohydrates content. A previous study of Pereira *et al.* (2011) reported a similar energetic contribution (~23.5 kcal/100 g fw) to that described in this study for control samples, but lower amounts of proteins (~0.92 g/100 g fw) and slightly higher levels of ash (~1.14 g/100 g fw), fat (0.20 g/100 g fw) and carbohydrate (4.51 g/100 g fw) in watercress samples collected in the same region. This small difference in the reported values may be linked to variations on edafoclimatic conditions of the locations where the samples were collected, which may affect the plant composition during the growing season (Nikolopoulou *et al.* 2007).

Table 32 Combined effects of gamma irradiation and cold storage on colour parameters, TSS (%) and pH values of fresh-cut watercress samples.[1]

	Fresh samples	**Irradiated samples stored at 4 ± 1 ºC for 7 d**				*p*-values		
	Non-stored control	**0 kGy**	**1 kGy**	**2 kGy**	**5 kGy**	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
*L**	39±3^{b}	45±2^{a}	42±4^{ab}	44±5^{a}	45±3^{a}	0.267	0.272	0.003
*a**	-17±2	-18±1	-17±2	-18±2	-17±1	0.324	0.058	0.408
*b**	27±2^{b}	31±4^{a}	29±3^{ab}	31±3^{ab}	29±2^{ab}	0.104	0.156	0.038
TSS	3.4±0.1^{b}	3.2±0.1^{c}	3.7±0.1^{a}	3.5±0.1^{b}	3.2±0.1^{c}	0.092	0.038	<0.001
pH	5.49±0.04^{a}	5.40±0.05^{b}	5.34±0.03^{b}	5.36±0.03^{b}	5.56±0.03^{a}	<0.001	0.031	<0.001

[1]The results are presented as the mean ± standard deviation.

[2]Homoscedasticity among different doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$.

[3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test.

[4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed).

For each treatment, means within a row with different letters differ significantly ($p<0.05$).

*L**: darkness $^{(0)}$ ↔ lightness $^{(100)}$; *a**: redness $^{(+)}$ ↔ greenness $^{(-)}$; *b**: yellowness $^{(+)}$ ↔ blueness $^{(-)}$.

Table 33 Combined effects of gamma irradiation and cold storage on the proximate composition (g/100 g fw), energetic value (kcal/100 g fw), individual sugars (mg/100 g fw) and organic acids (mg/100 g fw) of fresh-cut watercress samples.[1]

	Fresh samples	**Irradiated samples stored at 4 ± 1 ºC for 7 d**				*p*-values		
	Non-stored control	**0 kGy**	**1 kGy**	**2 kGy**	**5 kGy**	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
Moisture	93±1^{b}	94±1^{a}	94±1^{ab}	94±1^{ab}	94±1^{ab}	0.300	0.051	0.010
Ash	0.94±0.05^{a}	0.88±0.05^{ab}	0.87±0.03^{ab}	0.84±0.02^{b}	0.90±0.05^{ab}	<0.001	0.006	0.016
Proteins	2.2±0.1^{a}	1.9±0.1^{bc}	1.9±0.1^{c}	2.0±0.1^{b}	2.0±0.1^{bc}	0.859	0.026	<0.001
Fat	0.14±0.05	0.13±0.02	0.12±0.01	0.14±0.05	0.13±0.01	<0.001	<0.001	0.261
Carbohydrates	3.6±0.1^{a}	3.0±0.1^{b}	3.0±0.2^{b}	3.0±0.1^{b}	2.9±0.1^{b}	0.068	0.001	<0.001
Energy	24.5±0.3^{a}	20.8±0.5^{bc}	20.7±0.5^{c}	21.5±0.5^{b}	20.8±0.3^{bc}	0.180	<0.001	<0.001
Fructose	110±3^{a}	69±3^{b}	43±4^{c}	71±5^{b}	42±2^{c}	0.316	<0.001	<0.001
Glucose	70±2^{a}	31±4^{c}	17±5^{d}	39±9^{b}	39±4^{b}	0.005	0.003	<0.001
Sucrose	23±5^{c}	46±4^{ab}	42±3^{b}	42±5^{b}	50±5^{a}	0.368	0.003	<0.001
Total sugars	203±3^{a}	145±4^{b}	102±6^{d}	153±11^{b}	132±5^{c}	0.005	0.002	<0.001
Oxalic acid	754±24^{b}	630±9^{d}	850±25^{a}	714±38^{c}	656±36^{d}	0.007	0.010	<0.001
Malic acid	73±2^{d}	286±8^{c}	365±25^{b}	445±23^{a}	325±68^{bc}	<0.001	<0.001	<0.001
Citric acid	109±4^{a}	98±8^{b}	66±4^{c}	44±4^{d}	38±4^{e}	0.088	<0.001	<0.001
Fumaric acid	0.35±0.02^{d}	1.50±0.05^{b}	1.25±0.05^{c}	1.24±0.05^{c}	1.63±0.05^{a}	0.088	<0.001	<0.001
Total organic acids	936±22^{d}	1015±22^{c}	1282±12^{a}	1205±33^{b}	1021±92^{c}	<0.001	0.001	<0.001

[1]The results are presented as the mean ± standard deviation.

[2]Homoscedasticity among different doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$.

[3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test.

[4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed).

For each treatment, means within a row with different letters differ significantly ($p<0.05$).

4.3.3.3. Effects on sugars and organic acids

The individual sugars and organic acid profiles are presented in **Table 33**. As it can be concluded from the one-way ANOVA *p*-values, the different irradiation doses induced significant changes in these hydrophilic compounds. In fresh samples, fructose was the most abundant sugar, followed by glucose and sucrose. The storage time decreased the amounts of fructose, glucose and total sugars, but increased the sucrose levels. This increase in sucrose was more marked in samples irradiated at 5 kGy, which also showed the lowest levels of fructose. The 2 kGy dose was suitable to preserve high levels of fructose, glucose and total sugars, samples that revealed comparable amounts of fructose, sucrose and total sugars to those irradiated at 1 kGy. The observed decrease of fructose and glucose (reducing sugars) levels during storage can be attributed to the plant metabolic activity, which is higher in wounded tissues (Uritani and Asahi 1980). However, it is also known that irradiation may decrease the melting point and optical rotation of sugars, or even cause its degradation due to the direct action of gamma-rays and interaction with radiolytic products (Molins 2001). The glycosidic bond of disaccharide can also be broken by irradiation (Molins 2001), but higher levels of sucrose were found in stored samples. Most probably there was a mobilization of carbohydrates, hence its decline. Comparable results were found by Workneh *et al.* (2012) in tomato samples stored at 13 ºC for 8 d, in particular the decrease in glucose levels and the increase in sucrose. In addition to the sugars described herein, trehalose and raffinose were already detected in other watercress samples (Pereira *et al.* 2011); in which sucrose was the main sugar. Additionally, the total sugars content of our fresh samples was similar to that described by Pereira *et al.* (2011), *i.e.,* 210 mg/100 g fw.

Oxalic, malic, citric and fumaric acids were detected (**Table 33**), being oxalic acid the most abundant. The cold storage decreased the amounts of oxalic (except for the 1 kGy dose) and citric acids, and increased the levels of malic, fumaric and total organic acids. The different irradiation doses favoured different organic acids: the highest values of oxalic, malic and fumaric acids were registered in samples irradiated at 1, 2 and 5 kGy, respectively. Regarding citric acid, a dose dependent effect was observed, *i.e.,* increasing the irradiation dose decreases the content in citric acid. This decrease may be attributed to the direct impact of gamma-rays or to the reaction with reactive intermediates of water radiolysis. The radiolytic decomposition of this organic acid in aqueous solutions was shown to increase steadily with the absorbed dose (Semelová *et al.* 2008). In our study, this trend was also verified for total organic acids. Moreover, malic acid may result from the radiolysis of citric acid (Apelblat 2014). Curiously, the ascorbic acid was not detected in these samples or in those previously analysed by Pereira *et al.* (2013).

4.3.3.4. Effects on fatty acids and tocopherols

Lipids are one of the main targets of the attack of the free radicals generated during radiolysis. This attack is called lipid peroxidation and promotes the production of different reactive species (Ferreira *et al.* 2009). If not stopped by the plant antioxidants defences, this phenomenon can lead to much superior damage than the reactive species that initiate the reaction. Therefore, detailed analysis of fatty acids is important in irradiated foods. The results for the fatty acids composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and the ratios of PUFA/SFA and n-6/n-3 are shown in **Table 34**. Up to 23 fatty acids were detected, being palmitic (C16:0) and α-linolenic (C18:3n3) acids the most abundant, in agreement to Pereira *et al.* (2011). Looking at the one-way ANOVA *p*-values, it is clear that the induced changes were significant. C16:0 decreased during storage while C18:3n3 increased under the same conditions, except for the results achieved with the 1 kGy dose. Other abundant fatty acids were linoleic (C18:2n6), stearic (C18:0), lignoceric (C24:0) and eicosadienoic (C20:2) acids. Both palmitoleic (C16:1) and nervonic (C24:1) acid were not detected in fresh samples or in those irradiated at 1 kGy. Fresh samples revealed higher levels of SFA (~57%) than the sum of MUFA (~1%) and PUFA (~42%). Generally, the storage time induced positive effects increasing the levels of MUFA and PUFA and the PUFA/SFA ratio, and decreasing the levels of SFA and the n-6/n-3 ratio. Regarding the effects of gamma irradiation, the 5 kGy dose favoured MUFA (*e.g.,* C16:1, oleic acid (C18:1n9) and C24:1), while the 2 kGy dose was especially effective in favouring PUFA (*e.g.,* C18:3n3 and eicosapentaenoic acid (C20:5n3)) and the PUFA/SFA ratio. In either case, the irradiation treatment allowed obtaining watercress samples with a healthier fatty acids profile. According to some studies, a lower ratio of n-6/n-3 fatty acids is desirable in reducing the risk of many of the chronic diseases of high prevalence in Western societies, as well as in the developing countries (Simopoulos 2008).

Concerning the tocopherols profile, α-tocopherol was the most abundant isoform, followed by γ-tocopherol and β-tocopherol, in accordance with the results previously reported by Pereira *et al.* (2011). The most relevant result was the effect achieved with the 5 kGy dose, particularly owing the contributions of α-, γ-tocopherols and the total tocopherols content. The storage time also increased the total tocopherols content in non-irradiated samples, mainly due to the contribution of α- and β-tocopherols. It is known that tocopherols levels can increase in response to stress conditions (Munné-Bosch 2005, Yusuf *et al.* 2010). In these situations, the production of free radicals and reactive species, stimulated in wounded tissues and resulting from the radiolysis of water, can lead to oxidative damage. The high levels of tocopherols enhance the oxidative stability of tissues owing to its ability to protect polyunsaturated fatty acids from peroxidation and to scavenge free radicals. Additionally, α-tocopherol plays a major role in the alleviation of stress (Munné-Bosch 2005).

Therefore, the tocopherols increase verified in this work can be related to the inaptness of some of the applied doses in conferring suitable preservation conditions to the samples during storage. Nevertheless, the observed increase confers a higher bioactivity. The observed phenomenon can also be attributed to a hormetic effect induced by the applied doses, which promote beneficial effects through mild stress (Shama and Alderson 2005).

4.3.3.5. Effects on the antioxidant activity and total phenolics and flavonoids

The antioxidant capacity of vegetables is conferred by high levels of phenolic compounds, and also of vitamin C (ascorbic acid), vitamin E (tocopherols), and carotenoids, among other compounds (Carocho and Ferreira 2013). The results for the antioxidant properties and total phenolics and flavonoids are presented in **Table 35**. Again, the one-way ANOVA *p*-values validated the statistically significant differences among irradiated samples. Fresh samples, analysed immediately after harvest, revealed the lowest EC_{50} values (higher antioxidant activity) for the three *in vitro* assays of DPPH$^{\bullet}$ scavenging activity, reducing power and β-carotene blanching inhibition capacity. The decline in antioxidant activity during storage could probably be due to the degradation of antioxidants as a result of oxidation. It is quite clear that the 5 kGy allowed the best results (except for TBARS formation inhibition) in maintaining the antioxidant activity, as well as in the total phenolics and flavonoids, measured in fresh samples. Note that these samples have demonstrated the highest levels of α- and γ-tocopherols and the total tocopherols content, which are antioxidants capable to donate phenolic hydrogen and, therefore, halt the oxidative chain reaction. Moreover, differences among the results achieved with the applied assays could be related to the dissimilar mechanisms of action involved, *e.g.,* the β-carotene blanching inhibition assay is based on a lipophilic reaction; in the DPPH$^{\bullet}$ scavenging assay both electron and hydrogen atom transfer mechanisms are possible, while the reducing power assay evaluates the ability of an antioxidant to donate an electron (Carocho and Ferreira 2013).

Overall, the total phenolic content was higher after the 7 d of cold storage, while flavonoids decreased under the same conditions. Similar amounts of phenolics (96-97 mg GAE/g extract) and higher levels of flavonoids (62-63 mg CE/g extract) were reported by Yazdanparast *et al.* (2008) and Bahramikia and Yazdanparast (2010) in hydro-ethanolic extracts of watercress samples from Iran. These samples also revealed a comparable TBARS inhibition capacity (0.27 mg/mL). In contrast, Pereira *et al.* (2011) described lower values for phenolics (~50.42 mg GAE/g extract) but a similar content in flavonoids (~35.17 mg CE/g extract) in methanolic extracts, comparing to our fresh samples and those irradiated at 5 kGy.

Table 34 Combined effects of gamma irradiation and cold storage on fatty acids (relative percentage) and tocopherols (mg/100 g fw) composition of fresh-cut watercress samples.[1]

	Fresh samples	**Irradiated samples stored at 4 ± 1 ºC for 7 d**				*p*-values		
	Non-stored control	**0 kGy**	**1 kGy**	**2 kGy**	**5 kGy**	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
C6:0	0.014±0.002^{c}	0.097±0.002^{a}	0.014±0.002^{c}	0.030±0.003^{b}	0.018±0.003^{c}	0.011	<0.001	<0.001
C8:0	0.048±0.005^{c}	0.130±0.001^{a}	0.063±0.005^{b}	0.017±0.002^{d}	0.047±0.005^{c}	<0.001	<0.001	<0.001
C10:0	0.063±0.005^{c}	0.373±0.005^{a}	0.063±0.005^{c}	0.038±0.005^{d}	0.098±0.005^{b}	0.014	<0.001	<0.001
C12:0	0.225±0.004^{b}	0.317±0.004^{a}	0.204±0.005^{c}	0.087±0.005^{e}	0.161±0.005^{d}	<0.001	0.003	<0.001
C14:0	1.7±0.1^{b}	1.5±0.1^{c}	2.1±0.1^{a}	1.1±0.1^{d}	1.8±0.1^{b}	0.003	0.233	<0.001
C15:0	0.38±0.04^{b}	0.41±0.01^{b}	0.65±0.05^{a}	0.37±0.05^{b}	0.41±0.05^{b}	0.001	<0.001	<0.001
C16:0	45±1^{b}	29±1^{d}	49±1^{a}	27±1^{e}	37±1^{c}	0039	<0.001	<0.001
C16:1	nd	0.17±0.01^{b}	nd	0.12±0.01^{c}	0.22±0.01^{a}	<0.001	<0.001	<0.001
C17:0	0.89±0.04^{b}	0.38±0.03^{d}	1.00±0.05^{a}	0.36±0.04^{d}	0.64±0.05^{c}	0006	<0.001	<0.001
C18:0	4.1±0.4^{b}	4.4±0.1^{ab}	4.5±0.3^{a}	2.7±0.3^{c}	4.1±0.3^{b}	0.002	<0.001	<0.001
C18:1n9	0.61±0.05^{d}	1.47±0.01^{c}	2.23±0.05^{b}	1.37±0.05^{c}	2.54±0.05^{a}	<0.001	0.015	<0.001
C18:2n6	8.4±0.5^{b}	10.3±0.1^{a}	6.9±0.2^{d}	9.0±0.3^{b}	7.8±0.5^{c}	<0.001	0.006	<0.001
C18:3n3	27±1^{d}	43±1^{b}	21±1^{e}	46±1^{a}	32±1^{c}	<0.001	<0.001	<0.001
C20:0	0.25±0.04^{d}	0.50±0.01^{b}	0.42±0.02^{bc}	0.38±0.02^{c}	0.99±0.05^{a}	<0.001	<0.001	<0.001
C20:1	0.42±0.05^{a}	0.18±0.01^{c}	0.41±0.03^{a}	0.19±0.03^{c}	0.31±0.04^{b}	<0.001	0.002	<0.001
C20:2	2.3±0.3^{a}	1.2±0.1^{d}	1.9±0.1^{b}	1.4±0.1^{c}	1.9±0.2^{b}	0.009	0.031	<0.001
C20:4n6	1.2±0.2^{b}	0.53±0.02^{c}	1.33±0.05^{a}	0.33±0.05^{d}	1.09±0.05^{b}	0.001	<0.001	<0.001
C20:3n3+C21:0	1.0±0.1^{a}	0.68±0.03^{b}	0.77±0.05^{b}	0.75±0.05^{b}	1.02±0.05^{a}	0.082	0.007	<0.001
C20:5n3	0.3±0.1^{c}	0.2±0.1^{c}	0.9±0.1^{b}	5.1±0.4^{a}	nd	<0.001	<0.001	<0.001
C22:0	1.3±0.1^{b}	0.9±0.1^{c}	1.6±01^{a}	0.7±0.1^{d}	1.5±01^{ab}	0.037	0.016	<0.001
C22:2	1.6±0.1^{a}	0.57±0.01^{d}	1.3±0.1^{b}	0.44±0.05^{e}	1.1±0.1^{c}	<0.001	0.002	<0.001
C24:0	2.8±0.2^{bc}	2.6±0.1^{c}	3.6±0.3^{a}	2.0±0.1^{d}	3.2±0.5^{b}	0.003	0.134	<0.001
C24:1	nd	0.8±0.1^{b}	nd	0.54±0.05^{c}	1.7±0.1^{a}	<0.001	<0.001	<0.001

	Fresh samples	**Irradiated samples stored at 4 ± 1 ºC for 7 d**				*p*-values		
	Non-stored control	**0 kGy**	**1 kGy**	**2 kGy**	**5 kGy**	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
Total SFA	57±1[b]	40±1[d]	63±1[a]	35±1[e]	50±1[c]	0.010	0.001	<0.001
Total MUFA	1.0±0.1[d]	2.6±0.2[b]	2.6±0.1[b]	2.2±0.2[c]	4.8±0.4[a]	<0.001	<0.001	<0.001
Total PUFA	42±1[d]	57±1[b]	34±1[e]	63±1[a]	45±1[c]	0.001	<0.001	<0.001
PUFA/SFA	0.74±0.02[d]	1.41±0.05[b]	0.55±0.01[e]	1.80±0.05[a]	0.90±0.04[c]	<0.001	<0.001	<0.001
n-6/n-3	0.47±0.03[a]	0.28±0.01[c]	0.50±0.02[a]	0.22±0.01[d]	0.36±0.01[b]	<0.001	0.001	<0.001
α-tocopherol	0.50±0.03[c]	1.25±0.05[a]	0.96±0.05[b]	0.26±0.01[d]	1.21±0.05[a]	0.001	<0.001	<0.001
β-tocopherol	0.011±0.001[b]	0.021±0.004[a]	0.011±0.002[b]	0.005±0.001[c]	0.012±0.001[b]	<0.001	0.001	<0.001
γ-tocopherol	0.038±0.003[c]	0.067±0.005[b]	0.070±0.003[b]	0.041±0.001[c]	0.22±0.01[a]	0.001	<0.001	<0.001
Total tocopherols	0.55±0.04[d]	1.34±0.05[b]	1.04±0.05[c]	0.31±0.01[e]	1.44±0.05[a]	0.003	<0.001	<0.001

[1]The results are presented as the mean ± standard deviation.

[2]Homoscedasticity among different doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$.

[3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test.

[4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed).

For each treatment, means within a row with different letters differ significantly ($p<0.05$).

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); Arachidonic acid (C20:4n6); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); *cis*-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); *cis*-13,16-Docosadienoic acid (C22:2); Lignoceric acid (C24:0); Nervonic acid (C24:1).

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; nd: not detected

Table 35 Combined effects of gamma irradiation and cold storage on the antioxidant activity (EC_{50} values, mg/mL) and total phenolics (mg GAE/g extract) and flavonoids (mg CE/g extract) of fresh-cut watercress samples.[1]

	Fresh samples	**Irradiated samples stored at 4 ± 1 ºC for 7 d**				*p*-values		
	Non-stored control	**0 kGy**	**1 kGy**	**2 kGy**	**5 kGy**	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
DPPH$^{\bullet}$ scavenging activity	0.49±0.01^{e}	0.81±0.01^{c}	0.85±0.01^{b}	1.01±0.02^{a}	0.58±0.01^{d}	0.007	<0.001	<0.001
Reducing power	0.38±0.01^{e}	0.48±0.01^{a}	0.45±0.01^{c}	0.46±0.01^{b}	0.42±0.01^{d}	0.049	0.001	<0.001
β-carotene blanching inhibition	0.33±0.02^{e}	0.62±0.01^{c}	1.27±0.02^{a}	0.95±0.02^{b}	0.53±0.01^{d}	0.036	<0.001	<0.001
TBARS formation inhibition	0.27±0.01^{b}	0.21±0.01^{c}	0.28±0.01^{b}	0.27±0.01^{b}	0.50±0.01^{a}	0.183	<0.001	<0.001
Total phenolics	87±2^{c}	97±2^{a}	93±1^{b}	92±3^{b}	98±1^{a}	<0.001	0.010	<0.001
Total flavonoids	36±1^{a}	25±1^{c}	26±2^{c}	21±2^{d}	34±2^{b}	<0.001	<0.001	<0.001

[1]The results are presented as the mean ± standard deviation.

[2]Homoscedasticity among different doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$.

[3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test.

[4]$p<0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed).

For each treatment, means within a row with different letters differ significantly ($p<0.05$).

GAE: gallic acid equivalents; CE: catechin equivalents.

The production of phenolic compounds during storage can be stimulated by the wounding-induced stress caused on the watercress tissues, which is a plant defence mechanism to withstand the stress conditions (Heredia and Cisneros-Zevallos 2009, Pérez-Gregorio *et al.* 2011). Besides, and as mentioned above, the free radicals and reactive species generated during the irradiation process may act as stress signals and may trigger stress responses in vegetables, resulting in increased antioxidant synthesis (*e.g.,* tocopherols), which enforces the hormesis effect idea. Vaishnav *et al.* (2015) observed that irradiated samples of minimally processed cauliflower preserved the total phenolic content over the course of storage, while the non-irradiated samples suffered a reduction of these compounds. The same authors also observed a no significant change in the flavonoid content due to radiation processing; however, as verified in our study, a time dependent reduction was observed during storage. In other work conducted by Tripathi *et al.* (2013) it was found an increase in the total phenolic content of irradiated minimally processed ash gourd cubes, compared to the non-irradiated control samples, at all stages of storage. The increase in total phenolics of irradiated plants has also been attributed to depolymerisation and dissolution of the cell wall polysaccharides, which facilitates higher extractability (Bhat *et al.* 2007).

4.3.3.6. Overall effect on quality

The effects of gamma irradiation on the overall quality of fresh-cut watercress during cold storage were accessed through a LDA. The basic purpose of this discriminant analysis was finding the irradiation dose (categorical dependent variable) that maintained quality profiles (set of quantitative independent variables) of the fresh samples throughout the cold storage time. The significant independent variables were selected following the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

The discriminant model selected 4 significant functions, which included 100.0% of the observed variance. **Figure 31** represents the three first functions (function 1: 47.6%, function 2: 25.7%, function 3: 15.6%), which allowed the complete individualization of each gamma irradiation dose from the non-stored control. From the 58 studied variables, the discriminant model considered fructose, capric acid (C10:0), pentadecanoic acid (C15:0), C16:1, C18:3n3, C20:5n3, γ-tocopherol and β-carotene bleaching inhibition as those with significant discriminant ability. From the collective contribution of the three discriminant functions it is obvious that markers corresponding to the 2 kGy dose were those placed nearest to the fresh control ones, indicating that this treatment would be the preferable choice to preserve the postharvest quality of these samples. In agreement with our results, a report of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (FAO/IAEA 2006),

recommends irradiation doses up to 2 kGy for processing watercress as well as for other green vegetables such as endive, chicory, lettuce, arugula, spinach and cabbage. Nevertheless, the result obtained in this study for the 5 kGy dose, which gave a high antioxidant activity and detachable levels of C18:1n3, C18:3n3 and total tocopherols, should also be considered when aiming to obtain a final product with an enhanced health-promoting composition.

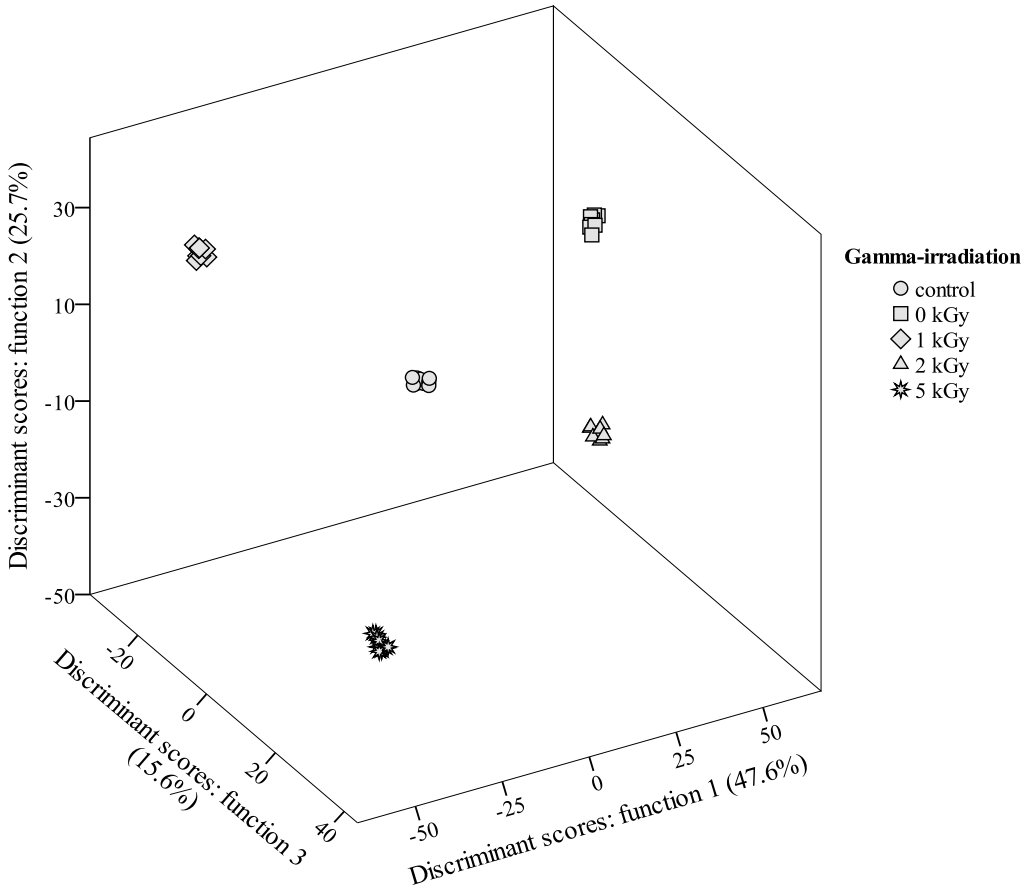

Figure 31 Biplot of object scores (gamma irradiation doses) and component loadings (evaluated quality parameters).

4.3.4. Conclusions

The colour parameters were not affected by the applied irradiation doses. The storage time decreased the amounts of proteins, carbohydrates, fructose and glucose. Contrariwise, the contents of malic and fumaric acids, MUFA, PUFA and total phenolics were higher past 7 d of cold storage. The 2 kGy dose preserved high levels of reducing sugars and favoured PUFA; while samples irradiated at 5 kGy revealed high contents of sucrose and MUFA. In either case, watercress samples with a healthier fatty acids profile were obtained. Concerning tocopherols, the most relevant result was achieved with the 5 kGy dose, particularly owing the contributions of α- (the major isoform) and γ-tocopherols. This dose

better preserved the antioxidant activity and total flavonoids. Overall, samples irradiated at 2 kGy were those with the most similar quality profiles to the non-stored control samples. In further studies, the effects of gamma irradiation on physiological and other quality parameters of fresh-cut watercress during cold storage, as well as the combination with other preservation technologies will be interesting.

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4.4. Postharvest changes in the phenolic profile of watercress induced by post-packaging irradiation and modified atmosphere packaging

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Abstract

The effects of γ-ray irradiation and modified atmosphere packaging (MAP) on watercress (*Nasturtium officinale* R. Br.) phenolic compounds were evaluated after 7-day storage at 4 ºC. Irradiation doses of 1, 2 and 5 kGy were tested, as well as vacuum-packaging and MAP enriched with 100 % N_2 and Ar. A non-irradiated, air-packaged control followed all the experiment. *p*-Coumaric acid was the most abundant compound in fresh watercress, followed by quercetin-3-*O*-sophoroside and isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside. Four kaempferol glycoside derivatives were identified for the first time in this specie. In general, flavonoids predominated over phenolic acids. Samples stored under vacuum and irradiated at 2 kGy revealed lower phenolic levels. Ar-enriched MAP and control conditions preserved the initial phenolic content. The 5 kGy dose also maintained the concentrations of flavonoids and total phenolic compounds, but increased the phenolic acids content. Additionally, flavonoids were found strongly correlated to $DPPH^{\bullet}$ scavenging activity and β-carotene bleaching inhibition capacity.

Keywords: *Nasturtium officinale*; phenolic compounds; HPLC-DAD-ESI/MS; modified atmosphere packaging; post-packaging irradiation; refrigerated storage.

4.4.1. Introduction

Fresh-cut vegetables are more and more popular in consumers' market baskets due to their convenience, nutritional and flavour properties, and health benefits (Baselice *et al.* 2017).

The global interest in minimally processed packaged foods promoted the exponential growth of this industrial sector, which is showing positive figures and innovation in product quality and safety attributes (Baselice *et al.* 2017, Ma *et al.* 2017). In the past few years, watercress (*Nasturtium officinale* R. Br.) has become popular as a fresh-cut product due to the sharp, peppery and slightly tangy taste. This fast-growing semi-aquatic plant of the Brassicaceae family is frequently classed as a superfood or functional food due to the high level of bioactive phytochemicals and health-promoting effects (Zeb 2015, Spínola *et al.* 2016). Watercress supplementation in diet has been linked to a reduced risk of cancer in healthy adults, via decreased damage to DNA and possible modulation of antioxidant status (Boyd *et al.* 2006, Gill *et al.* 2007), and might be useful in modulating breast cancer progression and disease recurrence (Ravasco, João, Jorge, *et al.* 2015, Ravasco, João, Rowland, *et al.* 2015). However, like most fresh-cut vegetables, watercress has a reduced shelf-life of approximately 7 days (Silveira *et al.* 2014).

After harvest, fresh vegetables begin to lose quality due to various chemical and/or enzymatic reactions (Sandhya 2010). In addition, the damage caused by the cutting and processing operations promotes a faster deterioration of the fresh-cut vegetables than the unprocessed ones (Francis *et al.* 2012). The wound-induced stress activates plant defence mechanisms involved in the synthesis and/or degradation of antioxidants, such as phenolic compounds (Han *et al.* 2016), whose accumulation in wounded tissues has been associated with an increase in antioxidant activity (Reyes *et al.* 2007). Therefore, as the popularity of watercress increases, there is a need to understand how the phenolic profile of this cruciferous vegetable is affected by different postharvest treatments and how evolves during storage.

The fresh-cut sector is constantly evolving and innovating in order to enhance the quality and safety of the marketed products. For this reason, the impact of non-thermal preservation technologies on quality attributes of fresh vegetables has been widely investigated so as to find suitable postharvest treatments for shelf-life extension and microbiological safety assurance, as well as to replace the communally used chemical sanitizers (Pinela and Ferreira 2017). In this way, the consumers' expectations for convenient foods with fresh-like properties, free of chemical residues, and treated in a more sustainable way may be attended. These requisites can be achieved by using post-packaging irradiation and modified atmosphere packaging (MAP) in combination with refrigerated storage. γ-Ray irradiation is a clean technology capable of reducing the microbial load, control insect pests, and delay ripening and senescence on different plants (Barkai-Golan and Follett 2017). This physical treatment can be applied to packaged foods, thus preventing the occurrence of recontaminations during storage. However, suitable doses for shelf-life extension need to be established for each food commodity always aiming at a minimal impact on quality

parameters. On the other hand, MAP consists of changing the headspace gas composition in the package with the aim of reducing physiological and oxidation processes, as well as the microbial grow (Sandhya 2010, Silveira *et al.* 2014, Pinela, Barreira, Barros, Antonio, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* 2016). Using these non-thermal preservation techniques in combination with refrigerated storage, the food shelf-life can be extended, postharvest losses can be reduced, and new markets can be reached.

In previous studies, we evaluated how important quality attributes of watercress (*i.e.,* colour, pH, total soluble solids, proximate composition, free sugars, organic acids, fatty acids, tocopherols, and antioxidant properties) are affected by post-packaging γ-ray irradiation (Pinela, Barreira, Barros, Cabo Verde, Antonio, Carvalho, *et al.* 2016) and MAP (Pinela, Barreira, Barros, Antonio, *et al.* 2016). However, the impact of these postharvest treatments on individual phenolic compounds was not investigated and, as far as we know, only the effects of a 10-day storage period at 3±1 ºC were studied until now (Santos *et al.* 2014). Therefore, the present study was carried out to ascertain the effects of irradiation (0, 1, 2 and 5 kGy) and MAP (vacuum, 100 % N_2 and 100 % Ar) on the phenolic profile of fresh-cut watercress during 7-day storage at 4 ºC. A possible correlation between phenolic compounds and antioxidant activity was also investigated.

4.4.2. Materials and methods

4.4.2.1. Standards and reagents

HPLC-grade acetonitrile was purchased from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (ferulic, sinapic, *p*-coumaric and caffeic acids, and kaempferol-3-*O*-rutinoside, quercetin-3-*O*-rutinoside, and quercetin-3-*O*-glucoside) were purchased from Extrasynthese (Genay, France). All other chemicals were of analytical grade and were purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

4.4.2.2. Sampling, samples preparation and postharvest preservation treatments

Watercress (*Nasturtium officinale* R. Br.) specimens were wild harvested in February 2014 in a stream located in the civil parish of Calvelhe, Bragança municipality, in Portugal, considering local consumers' sites and preferences (Carvalho and Morales 2010). After sampling, undamaged watercress parts were selected, rinsed in tap water, and a portion was immediately analysed (non-stored control). The remaining fresh material was packaged in 11.5 cm × 15 cm sterilized bags (0.5 L headspace volume) of low-density polyethylene (63 μm thickness) and subjected to different postharvest preservation treatments (Pinela, Barreira, Barros, Antonio, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio,

Carvalho, *et al.* 2016). In brief, 70 bags containing 20 g of watercress were prepared and then divided into 7 groups: i) 3 groups were γ-ray irradiated at 1, 2 and 5 kGy (predicted doses) in an cobalt-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) located in the Centre for Nuclear Sciences and Technologies (C2TN) in Bobadela, Portugal; ii) one group was a non-irradiated (0 kGy) control in conventional atmosphere (air) that followed all the experiment; iii) another group was vacuum-packaged; and iv) two groups were packaged in modified atmosphere enriched with 100 % N_2 and Ar. The evolution of the headspace gas composition in each bag was monitored as previously reported (Pinela, Barreira, Barros, Antonio, *et al.* 2016). All bags were stored at 4 ºC for 7 days.

4.4.2.3. Preparation of extracts

The watercress samples were lyophilised (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to powder (20 mesh), and subjected to a solid-liquid extraction as described by the authors (Pinela, Barreira, Barros, Antonio, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio, Carvalho, *et al.* 2016). In brief, 1 g of dry powder was stirred (30 *g*) with 50 mL of a methanol:water mixture (80:20, v/v) for 1 h at room temperature (~25 ºC). The supernatant was then filtered through filter paper (Whatman No. 4) and the residue was re-extracted with an additional 50 mL of solvent. After extracts concentration under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland), the aqueous phase was lyophilized to obtain dry extracts.

4.4.2.4. Analysis of phenolic compounds by HPLC-DAD-ESI/MS

The dry extracts (~10 mg) were dissolved in a methanol:water mixture (20:80 v/v) and then filtered through 0.22 µm disposable LC filter disks for subsequent analysis in a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a diode array detector (DAD) coupled to an electrospray ionization mass detector (ESI-MS) (ThermoFinnigan, San Jose, CA, USA). The system and analytical procedures were described by Bessada *et al.* (2016). The compounds were tentatively identified by comparing their retention time and UV-vis and mass spectra with those obtained from commercial standards, when available (280 and 370 nm were the selected wavelengths). The external standards mentioned above were used for quantification. The results were expressed in g per kg of extract.

4.4.2.5. Statistical analysis

All analyses were performed in triplicate. Data were expressed as mean±standard deviation. All statistical tests were performed at a 5 % significance level using the SPSS Statistics software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The

one-way analysis of variance (ANOVA) was used to assess the differences among treatments and a linear discriminant analysis (LDA) was performed to evaluate the overall effects of the different postharvest treatments on the phenolic profile of watercress. These statistical procedures were previously described by the authors (Pinela, Barreira, Barros, Antonio, *et al.* 2016, Pinela, Barreira, Barros, Cabo Verde, Antonio, Carvalho, *et al.* 2016).

4.4.3. Results and discussion

4.4.3.1. Characterization of phenolic compounds

The phenolic chromatographic profile of watercress recorded at 370 nm is shown in **Figure 32**. Compound characteristics, tentative identities and quantitative results are presented in **Table 36** and **Table 37**. Compounds were identified based on their chromatographic, UV-vis and mass spectra characteristics. Up to twenty-six compounds were detected (**Table 36**), seven of which were phenolic acid derivatives, eighteen flavonoids glycoside derivatives, and one sesquiterpenoid (roseoside). Many of these compounds have already been previously reported in watercress (Martínez-Sánchez *et al.* 2008, Santos *et al.* 2014, Spínola *et al.* 2016), so that their identities were assumed; in some of them the fragmentation patterns given by Martínez-Sánchez *et al.* (2008), Santos *et al.* (2014) and Spínola *et al.* (2016) were also used to establish the position of location of the substituents (*e.g.,* peaks 4 and 8). Nonetheless, to the best of our knowledge, compounds 17 and 23-26 were not previously reported for this species. Their identities were tentatively assigned from their masses and MS^2 fragments based on the compositional patterns of the previously described molecules, and assuming the presence of kaempferol as aglycon, as concluded from the fragment at *m/z* 285. Thus, compound 25 ($[M-H]^-$ at *m/z* 901) was assigned as kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside by comparison with peak 21, although in this case bearing a hydroxyferuloylglucuronide substituent instead of hydroxyferuloylhexoside. Peaks 23 ($[M-H]^-$ at *m/z* 931), 24 ($[M-H]^-$ at *m/z* 785) and 26 ($[M-H]^-$ at *m/z* 871) presented in their fragmentation pattern a common product ion at *m/z* 623, which can be associated to kaempferol-*O*-feruloylhexoside, and different fragments derived from the losses of feruloylhexoside (338 u), rutinoside (308 u), malonylhexoside (248 u) or hexoside (162 u) residues. According to the distinct losses observed in each case these compounds were tentatively identified as kaempferol-*O*-feruloylhexoside-*O*-rutinoside, kaempferol-*O*-feruloylhexoside-*O*-hexoside and kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside, respectively.

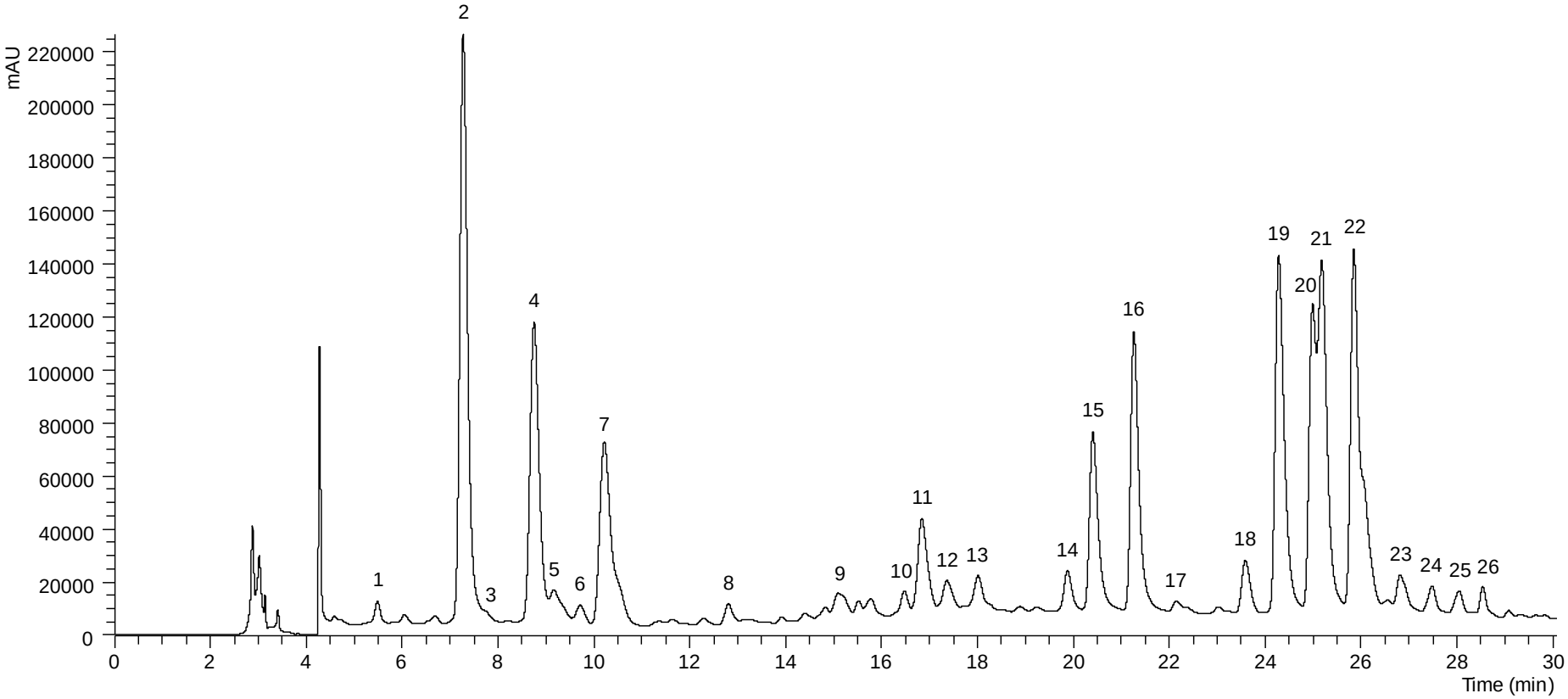

Figure 32 HPLC profile of phenolic compounds of the non-stored fresh-cut watercress sample recorded at 370 nm. See **Table 36** for peak identification.

Table 36 Retention time (Rt), wavelengths of maximum absorption (λ_{max}), mass spectral data, relative abundances of fragment ions and tentative identification of phenolic compounds in fresh-cut watercress extracts.

Compound	R_t (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Tentative identification	Type of identification	References
1	5.5	326	341	179 (100)	Caffeic acid hexoside	DAD-MS	Zeb (2015)
2	7.3	354	625	463(100),301(13)	Quercetin-3-*O*-sophoroside	DAD-MS	Santos *et al.* (2014)
3	7.8	314	325	163(100)	*p*-Coumaric acid hexoside	DAD-MS	-
4	8.8	354	711	667(50),625(8),505(100),463(40),301(15)	Quercetin-3-*O*-manolylglucoside-7-*O*-glucoside	DAD-MS	Martínez-Sánchez *et al.* (2008), Spínola *et al.* (2016)
5	9.2	338	355	193(46),175(100),159(48),134(44)	Ferulic acid hexoside	DAD-MS	Spínola *et al.* (2016)
6	9.7	358	431	385(20),223(100),163(3)	Roseoside	DAD-MS	Spínola *et al.* (2016)
7	10.2	330	179	119(100)	Caffeic acid	DAD-MS, standard	-
8	12.8	348	695	651(86),489(64),447(79),285(100)	Kaempferol-3-*O*-manolylhexoside-7-*O*-hexoside	DAD-MS,	Martínez-Sánchez *et al.* (2008)
9	15.1	312	163	133(56),119(100)	*p*-Coumaric acid	DAD-MS, standard	Santos *et al.* (2014), Spínola *et al.* (2016)
10	16.4	350	771	609(43),301(100)	Quercetin-3-*O*-rutinoside-7-*O*-glucoside	DAD-MS	Santos *et al.* (2014), Spínola *et al.* (2016)
11	16.8	330	193	149(8),134(100),115(11)	Ferulic acid	DAD-MS, standard	Santos *et al.* (2014), Spínola *et al.* (2016)
12	17.4	332	339	223(70),208(100),179(54),164(95)	Sinapoylmalic acid	DAD-MS	Spínola *et al.* (2016)
13	18.0	356	609	301(100)	Quercetin-3-*O*-rutinoside (rutin)	DAD-MS, standard	Spínola *et al.* (2016)
14	19.9	350	933	625(100),609(24),301(18)	Quercetin-*O*-sophoroside-*O*-rutinoside	DAD-MS	Spínola *et al.* (2016)
15	20.4	338	787	625(100),463(10),301(14)	Quercetin-*O*-coumaroylsophoroside	DAD-MS	Spínola *et al.* (2016)
16	21.3	338	873	829(50),787(22),625(100),505(20),301(10)	Quercetin-*O*-sophoroside-*O*-malonylhexoside	DAD-MS	Spínola *et al.* (2016)
17	22.2	334	873	829(100),787(5),625(8),505(47),301(19)	Quercetin-O-dihexosyl-O-malonylhexoside	DAD-MS	-
18	23.6	340	977	669(100),609(31),301(8)	Quercetin-*O*-sinapoylhexoside-*O*-rutinoside	DAD-MS,	Spínola *et al.* (2016)
19	24.3	334	831	669(6),477(11),463(46),315(18)	Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside	DAD-MS	Spínola *et al.* (2016)
20	25.0	336	801	639(48),463(43),315(27)	Isorhamnetin-*O*-sophoroside-*O*-hexoside	DAD-MS	Spínola *et al.* (2016)
21	25.2	340	917	873(50),669(100),505(13),315(10)	Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside	DAD-MS	Spínola *et al.* (2016)
22	25.9	336	887	843(100),639(28),505(36),315(13)	Isorhamnetin-*O*-sophoroside-*O*-malonylhexoside	DAD-MS	Spínola *et al.* (2016)

Compound	R_t (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Tentative identification	Type of identification	References
23	26.8	332	931	623(100),593(32),285(12)	Kaempferol-*O*-feruloylhexoside-*O*-rutinoside	DAD-MS	-
24	27.5	328	785	623(100),285(45)	Kaempferol-*O*-feruloylhexoside-*O*-hexoside	DAD-MS	-
25	28.0	328	901	857(50),653(100),489(11),285(5)	Kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside	DAD-MS	-
26	28.5	331	871	827(46),623(100),489(8),285(5)	Kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside	DAD-MS	-

Finally, peak 17 ([M-H]$^-$ at *m/z* 873) was identified as quercetin-*O*-dihexosyl-*O*-malonylhexoside. Santos *et al.* (2014) presented a compound with the same pseudomolecular ion, which was identified as quercetin-3-caffeolyglucoside-6´´-malonylglucoside; however, the UV-vis characteristics of our compound did not present an additional shoulder with a maximum of 320 nm, which is expected to be observed with the allocation of a caffeoyl residue. Moreover, if all the substituents would be expected to be positioned on the 3-*O* of the aglycon, the fragment with *m/z* 301 should present a higher abundance.

4.4.3.2. Effect of the postharvest treatments on the phenolic profile

The results of the quantification of phenolic compounds in fresh (non-stored control) and minimally processed watercress stored at 4 ºC for 7 days are shown in **Table 37**. Interestingly, the characterized profile in the minimally processed samples is not dominated by a low number of compounds, as it is often observed in plants (*e.g.,* Koike *et al.* (2015a, 2015b)), but most compounds are preserved in similar levels. *p*-Coumaric acid was identified as the most abundant phenolic compound in the non-stored watercress sample, followed by quercetin-3-*O*-sophoroside, isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside, isorhamnetin-O-hydroxyferuloylhexoside-*O*-malonylhexoside, and isorhamnetin-*O*-sophoroside-*O*-malonylhexoside. The latter compound was also reported as being dominant in watercress juice by Spínola *et al.* (2016), although those authors identified caffeoylmalic acid, disinapoylgentibiose and ferulic acid as the most abundant phenolics. Grouped flavonoids predominated over phenolic acids and derivatives due to the relatively high levels of isorhamnetin and quercetin glycosides and, in lesser extent, of kaempferol. Martínez-Sánchez *et al.* (2008) also described high contents of quercetin and kaempferol derivatives in extract of baby-leaf watercress. Different quercetin and isorhamnetin derivatives were also identified in watercress extracts obtained by pressurized liquid extraction (Santos *et al.* 2014).

Nevertheless, besides characterizing the phenolic profile of watercress, the main purpose of this work was verifying how that profile is affected by non-thermal preservation methods, namely post-packaging γ-ray irradiation and MAP. As deduced from **Table 37**, most individual phenolic compounds (except some quercetin and all kaempferol derivatives) suffer minimal but statistically significant changes after 7 days of storage at 4 ºC, either in just refrigerated samples (air-packaging, 0 KGy) or submitted to the different preservation treatment. Only in a few cases (*i.e.,* compounds 2, 4, 5 and 6), the levels measured in the non-stored control sample were not maintained by any of the applied postharvest treatments. In turn, the quantified levels of some phenolics (compounds 9, 10, 11 and 12) were at least as high as or, in some cases, even higher than those determined in the non-stored control

sample. Despite the lack of an unequivocal tendency, samples submitted to post-packaging irradiation at 5 kGy reveal higher concentration of phenolic acids and preserved the amounts of flavonoids and total phenolic compounds, after the 7-day storage in comparison to the non-stored control. Also, higher levels of phenolic acids were also determined in the samples submitted to 1 kGy dose. This effect induced by the low dose of 1 kGy had already been observed in edible flowers of *Viola tricolor* L. (Koike *et al.* 2015b). By contrast, 2 kGy had a clear deleterious effect over many of the quantified phenolic compounds. Samples stored under vacuum also revealed lower levels of grouped phenolic acids and total phenolic compounds compared to the non-stored control. However, the initial levels of grouped phenolic acids, flavonoids and total phenolic compounds did not suffer statistically significant changes when Ar-enriched MAP or just refrigeration (air-packaged non-irradiated) conditions were used. Santos *et al.* (2014) also found that the total phenolic content of ready-to-eat baby-leaf watercress was stable during a 10-day storage period at 3±1 ºC.

4.4.3.3. Overall effects of the applied postharvest treatments on phenolic compounds

Despite the effects induced by each postharvest treatment over individual phenolic compounds were significant in multiple occasions (**Table 37**), it was necessary to clarify the correlations among identified phenolic compounds and applied postharvest preservation treatments, in order to conclude the best strategy to be used in watercress preservation. Statistical classification techniques, such as linear discriminant analysis (LDA), allow comparing all phenolic profile changes and postharvest treatment conditions simultaneously, thereby permitting finding the best possible solution.

Herein, the significant independent variables (quantified phenolic compounds) were selected by the stepwise method considering their statistically significant classification performance ($p < 0.05$), based on the Wilks' λ test. Among the 27 entered variables (24 individual phenolic compounds plus grouped phenolic acids, flavonoids and total phenolic compounds), those corresponding to compounds 3 (*p*-coumaric acid hexoside), 4 (quercetin-3-*O*-malonylglucoside-7-*O*-glucoside), 5 (ferulic acid hexoside), 6 (roseoside), 9 (*p*-coumaric acid), 10 (quercetin-3-*O*-rutinoside-7-*O*-glucoside), 12 (sinapoylmalic acid), 22 (isorhamnetin-*O*-sophoroside-*O*-malonylhexoside) and phenolic acids group were selected as having ability to discriminate each applied preservation treatment. These variables contributed to define seven discriminant functions, among which the first five showed statistical significance (eigenvalue higher than 1). The first three discriminant functions, which cumulatively explained 98.7 % (first: 68.3 %; second, 26.0 %; third, 4.4 %) of the observed variance, are plotted in **Figure 33**.

Table 37 Quantification[1] of phenolic compounds in fresh-cut watercress samples submitted to different postharvest preservation treatments.

Compound	Day 0	7 days of storage at 4 ºC							*p*-value (n = 72)
	Non-stored control	Air-packaging				Vacuum-packaging	N_2-enriched MAP	Ar-enriched MAP	
		0 kGy	1 kGy	2 kGy	5 kGy				
1	tr	tr	tr	tr	tr	tr	tr	tr	-
2	2.2 ± 0.1^{a}	1.7 ± 0.2^{bc}	1.7 ± 0.2^{bc}	1.6 ± 0.1^{cd}	1.9 ± 0.2^{b}	1.5 ± 0.1^{d}	1.7 ± 0.1^{bcd}	1.7 ± 0.2^{bcd}	<0.001
3	0.22 ± 0.02^{c}	0.24 ± 0.01^{c}	0.63 ± 0.04^{a}	0.33 ± 0.03^{b}	0.23 ± 0.02^{c}	0.09 ± 0.01^{d}	0.005 ± 0.002^{e}	0.005 ± 0.002^{e}	<0.001
4	1.7 ± 0.1^{a}	1.3 ± 0.2^{bc}	1.3 ± 0.2^{bc}	1.1 ± 0.1^{d}	1.1 ± 0.1^{cd}	1.2 ± 0.1^{bcd}	1.2 ± 0.1^{bcd}	1.4 ± 0.1^{bc}	<0.001
5	0.097 ± 0.003^{a}	0.076 ± 0.002^{b}	0.072 ± 0.002^{c}	0.065 ± 0.002^{e}	0.076 ± 0.002^{b}	0.069 ± 0.002^{d}	0.066 ± 0.002^{de}	0.065 ± 0.002^{e}	<0.001
6	0.051 ± 0.002^{a}	0.021 ± 0.001^{b}	0.012 ± 0.001^{d}	0.004 ± 0.001^{f}	0.014 ± 0.002^{c}	0.011 ± 0.001^{d}	0.012 ± 0.001^{d}	0.007 ± 0.001^{e}	<0.001
7	1.5 ± 0.2^{cd}	1.7 ± 0.2^{ab}	1.7 ± 0.2^{a}	0.8 ± 0.1^{e}	1.5 ± 0.2^{bc}	1.0 ± 0.1^{e}	1.3 ± 0.1^{d}	1.3 ± 0.2^{cd}	<0.001
8	tr	tr	tr	tr	tr	tr	tr	tr	-
9	2.9 ± 0.4^{b}	3.0 ± 0.5^{b}	3.0 ± 0.4^{b}	3.1 ± 0.3^{b}	4.6 ± 0.4^{a}	2.6 ± 0.4^{b}	2.9 ± 0.4^{b}	3.2 ± 0.4^{b}	<0.001
10	0.9 ± 0.1^{ab}	1.0 ± 0.1^{ab}	1.0 ± 0.1^{a}	0.9 ± 0.1^{b}	0.9 ± 0.1^{b}	0.9 ± 0.1^{b}	0.9 ± 0.1^{ab}	0.9 ± 0.1^{ab}	0.003
11	0.7 ± 0.1^{bc}	0.9 ± 0.2^{ab}	1.0 ± 0.2^{a}	0.8 ± 0.1^{abc}	0.9 ± 0.2^{abc}	0.7 ± 0.1^{bc}	1.0 ± 0.2^{a}	0.9 ± 0.1^{abc}	0.001
12	0.17 ± 0.02^{d}	0.38 ± 0.02^{a}	0.32 ± 0.02^{b}	0.15 ± 0.02^{d}	0.36 ± 0.02^{a}	0.25 ± 0.02^{c}	0.30 ± 0.02^{b}	0.39 ± 0.02^{a}	<0.001
13	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.339
14	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	1.0 ± 02	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	0.676
15	1.4 ± 0.1^{a}	1.1 ± 0.1^{bc}	1.2 ± 0.2^{abc}	1.0 ± 0.1^{c}	1.4 ± 0.1^{a}	1.2 ± 0.1^{ab}	1.2 ± 0.1^{abc}	1.2 ± 0.1^{ab}	<0.001
16	1.5 ± 0.1^{a}	1.3 ± 0.2^{bc}	1.2 ± 0.1^{cd}	0.9 ± 0.1^{d}	1.3 ± 0.1^{bc}	1.4 ± 0.2^{ab}	1.2 ± 0.1^{bc}	1.4 ± 0.1^{ab}	<0.001
17	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.520
18	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	0.680
19	1.9 ± 0.1^{ab}	1.7 ± 0.1^{bc}	1.7 ± 0.2^{bc}	1.3 ± 0.1^{d}	2.0 ± 0.2^{a}	1.6 ± 0.1^{c}	1.6 ± 0.1^{c}	1.8 ± 0.2^{bc}	<0.001
20	1.5 ± 0.1^{b}	1.3 ± 0.1^{bc}	1.4 ± 0.1^{b}	1.2 ± 0.1^{c}	1.8 ± 0.2^{a}	1.3 ± 0.2^{bc}	1.4 ± 0.1^{bc}	1.5 ± 0.1^{b}	<0.001
21	1.9 ± 0.1^{a}	1.4 ± 0.2^{c}	1.4 ± 0.1^{c}	1.1 ± 0.1^{d}	1.5 ± 0.2^{bc}	1.7 ± 0.2^{ab}	1.4 ± 0.1^{c}	1.7 ± 0.2^{ab}	<0.001
22	1.7 ± 0.2^{ab}	1.4 ± 0.2^{bc}	1.4 ± 0.1^{c}	1.0 ± 0.1^{d}	1.3 ± 0.1^{c}	1.6 ± 0.2^{ab}	1.4 ± 0.2^{c}	1.7 ± 0.2^{a}	<0.001

Compound	Day 0	7 days of storage at 4 ºC							*p*-value (n = 72)
	Non-stored control	Air-packaging				Vacuum-packaging	N_2-enriched MAP	Ar-enriched MAP	
		0 kGy	1 kGy	2 kGy	5 kGy				
23	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.527
24	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.940
25	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.770
26	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.981
Total phenolic acids	5.6 ± 0.5^{cd}	6.3 ± 0.5^{bc}	6.7 ± 0.5^{b}	5.2 ± 0.5^{de}	7.6 ± 0.5^{a}	4.7 ± 0.5^{e}	5.5 ± 0.4^{cde}	5.8 ± 0.5^{bcd}	<0.001
Total flavonoids	22 ± 1^{a}	20 ± 1^{ab}	20 ± 2^{b}	17 ± 1^{c}	21 ± 2^{ab}	20 ± 1^{ab}	20 ± 1^{bc}	21 ± 2^{ab}	<0.001
Total phenolic compounds	28 ± 2^{ab}	26 ± 2^{abc}	26 ± 2^{abc}	23 ± 1^{d}	28 ± 2^{a}	24 ± 2^{cd}	25 ± 2^{bcd}	27 ± 2^{abc}	<0.001

[1] Results expressed in g kg-1 of extract and presented as mean ± standard deviation. In each row, different letters indicate statistically significant differences between samples ($p < 0.05$).

tr: traces

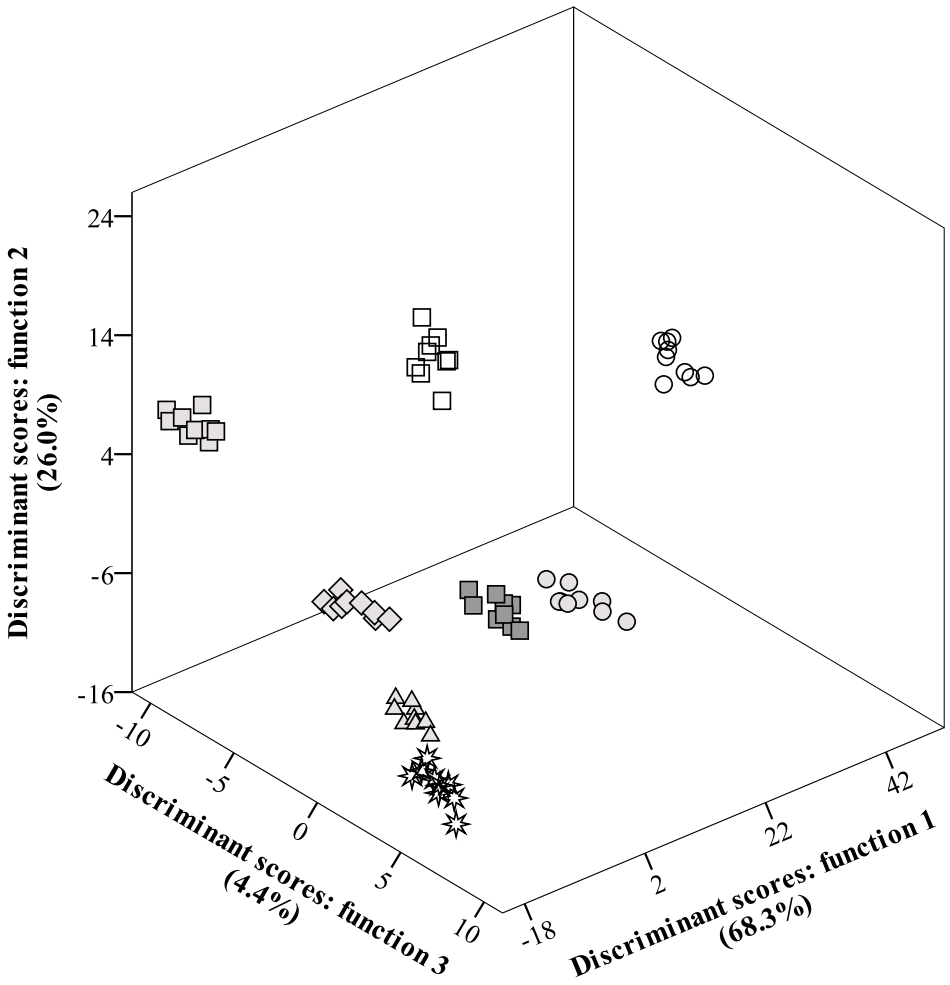

Figure 33 3D biplot of object scores (postharvest preservation treatments) and component loadings (quantified phenolic compounds). Non-stored control (○); non-irradiated, air-packaged control (●); 1 kGy (□); 2 kGy (▣); 5 kGy (■); vacuum-packaging (◇); N_2-enriched MAP (△); and Ar-enriched MAP (✡).

A clear separation of markers corresponding to the non-stored control and samples stored under different MAP or initially submitted to different γ-ray irradiation doses is noticeable. According to function 1, which established the highest correlation with roseoside (a non-phenolic sesquiterpenoid compound), the most notorious result was the obvious separation of markers corresponding to the non-stored control sample (with significantly higher quantities of roseoside), as also confirmed by the means of canonical variance (MCV) values (non-stored control: 45.341, stored control: 5.788, 1 kGy: -13.332, 2 kGy: -17.734, 5 kGy: -1.882, vacuum-packaging: -4.516, N_2-enriched MAP: -3.762, Ar-enriched MAP: -9.902). This result is, therefore, a strong indicator that the variables with highest correlations (especially roseoside) with function 1 could not be improved or maintained by any of the applied treatments. Function 2, on the other hand, was particularly effective in separating samples stored under MAP and those irradiated at 1 kGy (MCV, non-stored control: 4.715, stored control: 0.013, 1 kGy: 22.919, 2 kGy: 7.532, 5 kGy: -0.896, vacuum-packaging: -

6.515, N_2-enriched MAP: -13.162, Ar-enriched MAP: -14.606), especially due to differences in *p*-coumaric acid hexoside contents (lower values in MAP samples and particularly high in samples irradiated at 1 kGy). Likewise, this indicates that all compounds more related to function 2 (with special relevance to *p*-coumaric acid hexoside) were specially favoured in samples irradiated at 1 kGy dose. Finally, function 3 was not particularly effective in separating any of the assayed samples, which is in line with the lower percentage of explained variation (4.4 %). Nevertheless, this function contributed to increase the resolution of sample clusters separated by functions 1 and 2. In terms of classification performance, all samples were correctly classified, either for original grouped cases, as well as for cross-validated ones.

In previous studies, the suitability of post-packaging γ-ray irradiation (Pinela, Barreira, Barros, Cabo Verde, Antonio, Carvalho, *et al.* 2016) and inert gas-enriched MAP (Pinela, Barreira, Barros, Antonio, *et al.* 2016) for preserving quality parameters of fresh-cut watercress was evaluated. In general, it was concluded that Ar was an appropriate gas and 2 kGy a suitable dose for preserving the overall postharvest quality of fresh-cut watercress during refrigerated storage. In addition, the 5 kGy dose originated a final product with enhanced health-promoting properties, characterized by an interesting antioxidant activity and levels of linoleic and α-linolenic acids, monounsaturated fatty acids such as oleic acid, total tocopherols, and total phenolic compounds. Interestingly, the same irradiation dose better preserved or led to an increase in the contents of some individual phenolic compounds, namely quercetin-3-*O*-sophoroside, *p*-coumaric acid, quercetin-*O*-coumaroylsophoroside, isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside and isorhamnetin-*O*-sophoroside-*O*-hexoside, as also of grouped phenolic acids and total phenolic compounds. Therefore, these results support the previous findings regarding the suitability of the irradiation treatment at 5 kGy as also of Ar-enriched MAP preservation.

4.4.3.4. Correlation between phenolic compounds and antioxidant activity

Based on the Pearson correlation coefficients (*R*) presented in **Annexe 8**, it was concluded that total flavonoids were strongly correlated to the DPPH free-radical scavenging activity and β-carotene bleaching inhibition capacity of the watercress extracts (antioxidant activity data previously reported by Pinela, Barreira, Barros, Antonio, *et al.* (2016) and Pinela, Barreira, Barros, Cabo Verde, Antonio, Carvalho, *et al.* (2016)), while total phenolic compounds were found strongly and moderately correlated, respectively. Quercetin-derived compounds 15 and 18 were strongly correlated with the DPPH free-radical scavenging activity, while compounds 16, 17 (quercetin derivatives) and 21 (an isorhamnetin derivative) were found strongly correlated with the β-carotene bleaching inhibition capacity. All these

correlations were negative, but the antioxidant activity results were expressed in EC_{50} values, and the lower the EC_{50} value the higher the antioxidant capacity of the extract.

4.4.4. Conclusions

As far as we know, this is the first report of the effects of post-packaging γ-ray irradiation and MAP on the phenolic profile of watercress. *p*-Coumaric acid was identified as the most abundant phenolic compound in watercress, although the group of flavonoids predominated over phenolic acids. Four kaempferol derivatives (kaempferol-*O*-rutinoside-*O*-feruloylhexoside, kaempferol-*O*-feruloylhexoside-*O*-hexoside, kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside and kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside) were identified for the first time in this cruciferous vegetable. Samples stored under vacuum and irradiated at 2 kGy revealed a lower phenolic content due to a decrease in both total phenolic acids and flavonoids. Ar-enriched MAP and simple refrigeration at 4 ºC preserved well the initial phenolic content, while irradiation at 5 kGy increased the concentrations of phenolic acids also maintaining those of flavonoids and total phenolic compounds. Moreover, flavonoids were found strongly correlated to DPPH free-radical scavenging activity and β-carotene bleaching inhibition capacity.

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4.5. Cold extraction of phenolic compounds from watercress by high hydrostatic pressure: Process modelling and optimization

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Abstract

High hydrostatic pressure (HHP) was applied to the extraction of phenolic compounds from watercress (*Nasturtium officinale*). The process was optimized by response surface methodology using a five-level central composite design combining the independent variables of processing time (*t*, 1.5-33.5 min), pressure (*P*, 0.1-600 MPa) and solvent (*S*, 0-100 % of ethanol, v/v). The individual and grouped phenolic compounds, analysed by HPLC-DAD-ESI/MS, and the extraction yield were used as response variables. The theoretical models were fitted to the experimental data, statistically validated, and used in the prediction and optimization steps. The optimal HHP conditions for the extraction of phenolic compounds were: *t*= 3.1 min, *P*= 600 MPa and *S*= 100%, and originated 64.68±2.97 mg/g of extract. This study highlighted the HHP as a promising emerging technology to cold extract phenolic compounds (phenolic acids and flavonoids) from watercress in a selective way using a green solvent and reduced extraction times.

Keywords: *Nasturtium officinale*; Phenolic acids; Flavonoids; High hydrostatic pressure; Extraction optimization.

4.5.1. Introduction

The recovery of bioactive compounds from natural sources and their further incorporation into foods (Caleja *et al.* 2016), dietary supplements (Pereira, Barros, *et al.* 2015) and cosmeceuticals (Taofiq *et al.* 2016), either in isolated form or in enriched extracts, is a

current hot topic that involves many research fields. Phenolic compounds are among the most desired plant secondary metabolites because of their recognized bioactivities and capacity to protect against free radical-mediated diseases (Ferreira *et al.* 2017). Several studies have been carried out in recent years to improve the extraction of these compounds from plant materials (Pinela, Prieto, *et al.* 2016, Caleja *et al.* 2017, Vieira *et al.* 2017), but more efficient and sustainable methods need to be developed to achieve higher yields and superior quality products at lower processing costs.

Watercress (*Nasturtium officinale* R. Br.) is a semi-aquatic fast-growing plant of the Brassicaceae family with recognized health-promoting effects. Its consumption in a daily diet has been linked with a reduced risk of chronic diseases including different types of cancer (Gill *et al.* 2007, Higdon *et al.* 2007, Ravasco, João, Jorge, *et al.* 2015, Ravasco, João, Rowland, *et al.* 2015). This species is an interesting source of pharmacologically active phytochemicals (Pinela n.d., Zeb 2015, Spínola *et al.* 2016) whose involvement in antigenotoxic and anticancer processes has been demonstrated in both *in vivo* and *in vitro* assays (Rose *et al.* 2000, Rose, Huang, *et al.* 2005, Boyd *et al.* 2006, Gill *et al.* 2007). A previous study reported *p*-coumaric acid, quercetin-3-*O*-sophoroside and isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside as the most abundant phenolic compound in wild watercress (Pinela n.d.). Higher concentrations of flavonoids than phenolic acids were reported in these extracts due to the high contents of isorhamnetin and quercetin glycosides and, in lesser extent, of kaempferol (Pinela n.d.). In turn, a dimer of caffeoylmalic acid, disinapoylgentibiose and ferulic acid were identified as the predominant polyphenols in watercress juice, which demonstrated capacity to inhibit digestive enzymes relevant to type 2 diabetes and obesity (Spínola *et al.* 2016). Despite the great potential of these compounds in various industrial sectors, the development of innovative processes for their recovery from watercress and other natural sources remains challenging.

High hydrostatic pressure (HHP) is an emerging technology increasingly used in the food industry as a cold pasteurization method (Mújica-Paz *et al.* 2011, Castro and Saraiva 2014, Hernández-Carrión *et al.* 2014, Pinela and Ferreira 2017). It consists on subjecting packaged or in bulk foods to pressures up to 1000 MPa inside a vessel filled with water, fluid that acts as pressure-transmitting medium (Mújica-Paz *et al.* 2011, Castro and Saraiva 2014). During processing, the pressure is transmitted in an isostatic and quasi-instantaneous manner throughout the sample, which makes the processing time independent of the sample shape or size. In addition, the temperature increase with increasing pressure is minimal (~3 °C/100 MPa) (Wang 2014, Strati *et al.* 2015), thus being a good alternative to heat-based treatments. Its application for extraction of high added-value compounds from plant materials is recent and very promising. The applied pressure promotes the rupture of the plant tissues, cell walls and organelles, a phenomenon that enhances the mass transfer of the solvent into

the sample and of compounds to the solvent (Huang *et al.* 2013). In addition, the higher the hydrostatic pressure is, the more solvent can enter cells and the more compounds can permeate out to the solvent (Prasad *et al.* 2009, Jun *et al.* 2010).

Previous studies reported that HHP is a good alternative to conventional extraction methods since it avoids the degradation of thermosensitive molecules, reduces the extraction time and solvent consumption, and improves the extraction efficiency in terms of yield, quality and selectivity (Shouqin *et al.* 2005, Prasad *et al.* 2009, Jun *et al.* 2010, Huang *et al.* 2013, Briones-Labarca *et al.* 2015, Alexandre *et al.* 2017a). Moreover, a low-energy input is required by this eco-friendly technology to compress a sample to 500 MPa as compared to heating to 100 ºC (Pereira and Vicente 2010). As examples, HHP was successfully applied to extract bioactive compounds from pomegranate (Alexandre *et al.* 2017b) and citrus (Casquete *et al.* 2014) peels and fig by-products (Alexandre *et al.* 2017a), flavonoids from propolis (Shouqin *et al.* 2005), anthocyanins from grape skins (Corrales *et al.* 2009), catechins (Jun *et al.* 2010) and caffeine (Jun 2009) from green tea leaves, ginsenosides from ginseng (*Panax ginseng* C.A. Meyer) (Lee *et al.* 2011), ferulic acid from Radix *Angelica sinensis* (Xi and Luo 2016), and carotenoids from tomato wastes (Strati *et al.* 2015). However, the performance of this extraction method can be affected by a number of independent variables such as processing time, pressure and solvent (Alexandre *et al.* 2017a, 2017b), whose effect on one or more dependent (response) variables can be evaluated using the response surface methodology (RSM). This is a time- and reagent-saving statistical tool increasingly used in process optimization since one-factor-at-a-time experiments cannot predict optimal conditions and neglect interactions between variables.

The present study was carried out to optimize the HHP extraction of phenolic compounds from watercress using RSM. The response variables used in the development of mathematical models describing the extraction process (namely individual and grouped phenolic compounds) were obtained by high-performance liquid chromatography coupled to mass spectrometry (HPLC-DAD-ESI/MS).

4.5.2. Material and methods

4.5.2.1. Standards and reagents

HPLC-grade acetonitrile was purchased from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (ferulic, sinapic, *p*-coumaric and caffeic acids, and kaempferol-3-*O*-rutinoside, quercetin-3-*O*-rutinoside, and quercetin-3-*O*-glucoside) were purchased from Extrasynthese (Genay, France). All other chemicals were of analytical grade and were purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

4.5.2.2. Plant material

Fresh samples of watercress (*Nasturtium officinale* R. Br.) were commercially obtained from a local supermarket in Bragança, Portugal. The taxonomic identification of the plant material was confirmed by the botanist Dr. Ana Maria Carvalho from the Polytechnic Institute of Bragança, Portugal. The samples were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine powder (~20 mesh), and kept at -20 ºC until processing.

4.5.2.3. High hydrostatic pressure extraction

The extractions were carried out on a pilot-scale high-pressure equipment (Model 55, Hyperbaric, Burgos, Spain) with a pressure vessel of 55 L, connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) to control the temperature of the input water used as pressure-transmitting fluid. Heat-sealed plastic bags containing 0.6 g of dry powder sample and 20 mL of solvent were placed in the pressure vessel and then subjected to different conditions of processing time (1.5-33.5 min), pressure (0.1-600 MPa) and solvent (0-100 % of ethanol, v/v) as defined in the circumscribed central composite design (CCCD) presented in **Table 38**. Ethanol:water mixtures were used since ethanol has low toxicity and GRAS (generally recognized as safe) status. The solid/liquid ratio was maintained at 30 g/L. All extractions were performed at 20 ºC (cold extraction). However, since the pressure increases the temperature by ~3 ºC/100 MPa (Wang 2014, Strati *et al.* 2015), processing at 600 MPa resulted in an adiabatic temperature increase from 20 ºC to ~38 ºC, which should still be not enough to promote the thermal degradation of bioactive compounds. After HHP processing, the mixture was filtered through filter paper (Whatman No. 4) and the filtrate was collected and kept at -80 ºC until analysis.

4.5.2.4. Calculation of the extraction yield

The extraction yields (%) were calculated based on the dry weight (crude extract) obtained after evaporation of the solvent. First, the filtrates were concentrated at 35 ºC under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and the aqueous phase was then lyophilised to obtain a dried extract.

Table 38 Experimental design (independent variables and their coded and natural values) and values for phenolic compounds (mg/g of extract) and extraction yield (%) achieved under the 20 runs involved in the HHP extraction optimization by RSM.

Five-level CCCD experimental design																						
	Runs		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Coded values	X_1 : Time (t)		-1	1	-1	1	-1	1	-1	1	-1.68	1.68	0	0	0	0	0	0	0	0	0	0
	X_2 : Pressure (P)		-1	-1	1	1	-1	-1	1	1	0	0	-1.68	1.68	0	0	0	0	0	0	0	0
	X_3 : Solvent (S)		-1	-1	-1	-1	1	1	1	1	0	0	0	0	-1.68	1.68	0	0	0	0	0	0
Natural values	X_1 : t (min)		8	27	8	27	8	27	8	27	1.5	33.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5
	X_2 : P (MPa)		122	122	478	478	122	122	478	478	300	300	0.1	600	300	300	300	300	300	300	300	300
	X_3 : S (% of ethanol, v/v)		20	20	20	20	80	80	80	80	50	50	50	50	0	100	50	50	50	50	50	50
Response variables for RSM application																						
Quercetin-3-*O*-sophoroside		P1	1.30	1.24	0.99	1.06	1.13	1.10	1.21	1.25	1.17	1.16	1.17	1.12	1.01	0.99	1.13	1.13	1.14	1.13	1.13	1.16
Quercetin-3-*O*-manolylglucoside-7-*O*-glucoside		P3	1.89	1.80	1.18	1.32	1.27	1.14	2.13	1.93	1.59	1.53	1.54	1.51	1.20	1.04	1.44	1.44	1.45	1.45	1.45	1.47
Quercetin-3-*O*-rutinoside-7-*O*-glucoside		P7	0.00	0.01	0.01	0.01	0.88	0.91	0.93	0.92	0.91	0.90	0.90	0.89	0.88	0.92	0.89	0.90	0.90	0.90	0.91	0.91
Quercetin-3-*O*-rutinoside (rutin)		P10	1.05	1.03	0.99	1.06	1.09	1.11	1.05	1.07	1.11	1.10	1.13	1.15	1.06	0.96	1.19	1.20	1.21	1.20	1.20	1.20
Quercetin-*O*-sophoroside-*O*-rutinoside		P11	1.00	0.97	0.95	1.03	1.01	1.01	1.11	1.13	1.07	1.05	1.07	1.13	0.91	0.93	1.15	1.15	1.16	1.15	1.15	1.15
Quercetin-*O*-coumaroylsophoroside		P12	1.39	1.33	1.11	1.22	1.03	0.97	1.32	1.30	1.17	1.13	1.19	1.38	1.71	1.00	1.48	1.47	1.46	1.48	1.30	1.51
Quercetin-*O*-sophoroside-*O*-malonylhexoside		P13	2.14	1.96	1.57	1.99	2.24	1.94	6.09	5.13	2.98	2.74	2.73	3.33	1.39	0.93	3.29	3.15	3.22	3.30	2.82	3.29
Quercetin-*O*-dihexosyl-*O*-malonylhexoside		P14	0.95	0.89	0.87	0.89	0.92	0.91	1.05	1.01	0.94	0.92	0.93	0.93	0.86	0.00	0.94	0.95	0.95	0.94	0.93	0.99
Quercetin-*O*-sinapoylhexoside-*O*-rutinoside		P15	1.17	1.13	1.04	1.18	1.19	1.16	1.32	1.35	1.25	1.23	1.24	1.29	0.99	0.99	1.32	1.30	1.32	1.31	1.26	1.30
Total quercetin glycoside derivatives (Qgd)		--	10.88	10.36	8.70	9.76	10.77	10.24	16.20	15.09	12.20	11.76	11.90	12.72	10.01	13.76	12.83	12.68	12.79	12.85	12.15	12.96
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside		P16	1.58	1.53	1.38	1.83	1.73	1.61	2.79	2.72	2.08	1.98	2.06	2.43	1.12	1.05	2.54	2.47	2.51	2.52	2.47	2.54
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside		P17	2.97	2.89	1.96	3.01	3.60	3.00	9.22	7.88	4.31	3.99	4.01	4.70	1.77	0.98	4.80	4.59	4.78	4.73	4.73	4.73
Isorhamnetin-*O*-sophoroside-*O*-malonylhexoside		P18	2.31	2.25	1.67	2.49	2.94	2.43	7.35	6.25	3.44	3.13	3.09	3.52	1.47	1.03	3.53	3.47	3.53	3.51	3.51	3.53
Total isorhamnetin glycoside derivatives (Igd)		--	6.86	6.67	5.01	7.33	8.27	7.03	19.36	16.85	9.83	9.11	9.15	10.65	4.36	8.06	10.88	10.54	10.82	10.76	10.71	10.81
Kaempferol-*O*-feruloylhexoside-*O*-rutinoside		P19	1.04	1.00	0.95	1.02	1.03	1.01	1.21	1.20	1.08	1.06	1.07	1.13	0.92	0.96	1.14	1.14	1.14	1.14	1.14	1.14

Five-level CCCD experimental design

Runs		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Kaempferol-*O*-feruloylhexoside-*O*-hexoside	P20	0.99	0.97	0.93	1.00	0.99	0.98	1.14	1.13	1.05	1.03	1.04	1.10	0.91	0.93	1.11	1.12	1.12	1.11	1.11	1.11
Kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside	P21	1.27	1.24	1.06	1.26	1.42	1.31	2.24	2.12	1.48	1.43	1.43	1.52	1.03	0.93	1.53	1.51	1.54	1.52	1.51	1.54
Kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside	P22	1.20	1.21	1.03	1.18	1.27	1.18	1.92	1.81	1.42	1.39	1.33	1.48	0.90	1.10	1.43	1.42	1.43	1.42	1.42	1.43
Total kaempferol glycoside derivatives (Kgd)	--	4.51	4.42	3.97	4.46	4.72	4.49	6.51	6.27	5.04	4.91	4.88	5.23	3.76	5.92	5.22	5.19	5.23	5.19	5.18	5.21
Total flavonoids	--	22.3	21.4	17.7	21.6	23.8	21.8	42.1	38.2	27.1	25.8	25.9	28.6	18.1	24.7	28.9	28.4	28.8	28.8	28.0	29.0
p-Coumaric acid hexoside	P2	0.00	0.00	0.00	0.00	0.06	0.07	0.03	0.03	0.04	0.04	0.04	0.04	0.02	0.05	0.04	0.05	0.04	0.04	0.04	0.04
Ferulic acid hexoside	P4	0.00	0.01	0.01	0.08	0.11	0.11	0.09	0.10	0.09	0.09	0.09	0.09	0.02	0.08	0.18	0.18	0.18	0.18	0.18	0.18
Caffeic acid	P5	2.63	2.62	1.39	2.15	2.21	1.91	3.32	3.13	2.68	2.58	2.64	2.44	2.00	2.05	2.56	2.40	2.51	2.51	2.50	2.48
p-Coumaric acid	P6	3.91	3.72	2.05	3.40	4.25	4.07	4.82	4.91	4.54	4.38	4.51	4.07	3.06	2.88	4.30	4.11	4.24	4.12	4.16	4.12
Ferulic acid	P8	1.07	1.12	0.59	0.96	1.33	1.28	1.80	1.72	1.39	1.28	1.31	1.19	0.95	0.17	1.23	1.18	1.21	1.18	1.17	1.22
Sinapoylmalic acid	P9	0.33	0.32	0.16	0.27	0.49	0.43	0.89	0.78	0.51	0.46	0.51	0.45	0.24	0.18	0.43	0.43	0.43	0.44	0.45	0.46
Total phenolic acids	--	7.9	7.8	4.2	6.9	8.4	7.9	11.0	10.7	9.2	8.8	9.1	8.3	6.3	10.4	8.7	8.3	8.6	8.5	8.5	8.5
Total phenolic compounds	--	30.2	29.2	21.9	28.4	32.2	29.6	53.0	48.9	36.3	34.6	35.0	36.9	24.4	33.2	37.7	36.7	37.5	37.3	36.5	37.5
Extraction yield (crude extract)	--	19.4	21.5	27.5	26.8	14.9	16.9	16.1	19.5	23.8	26.2	18.9	21.0	26.0	12.0	22.6	24.2	21.8	24.5	22.1	23.9

4.5.2.5. Chromatographic analysis of phenolic compounds

The dried extracts (~10 mg) were dissolved in a methanol:water mixture (20:80 v/v) and filtered through 0.22 µm disposable LC filter disks. The chromatographic analysis was performed in a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system equipped with a diode array detector (DAD) coupled to an electrospray ionization mass detector (ESI-MS) (ThermoFinnigan, San Jose, CA, USA) as described by Bessada *et al.* (2016). The phenolic compounds were identified using 280 nm and 370 nm as preferred wavelengths and by comparing their retention time and UV-vis and mass spectra with those obtained from authentic standards, when available. For quantitative analysis, a baseline to valley integration with baseline projection mode was used to calculate the peak areas and the external standards mentioned above were used for quantification. The results were expressed in mg per g of extract.

4.5.2.6. Experimental design, modelling and optimization

Experimental design

A five-level CCCD (Box-Behnken design) coupled with RSM was implemented to optimize the HHP conditions for the extraction of phenolic compounds from watercress. The coded and natural values of the independent variables X_1 (processing time (*t*), min), X_2 (pressure (*P*), MPa) and X_3 (solvent (*S*), % of ethanol, v/v) are presented in **Table 38**. This CCCD includes 6 replicated centre points and a group of axial points chosen to allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the design centre. The experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

Mathematical modelling

The response surface models were fitted by means of least-squares calculation using the following Box-Behnken design equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \qquad \text{Eq. (1)}$$

In this equation, *Y* represents the dependent variable (response variable) to be modelled, X_i and X_j are the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} is the coefficient of quadratic effect, and *n* is the number of variables. The extraction yield and the individual and grouped phenolic compounds (22 compounds and 6 groups) were used as dependent variables.

Procedure to optimize the variables to a maximum response

A simplex method was used to optimize the predictive model by solving nonlinear problems in order to maximize the extraction yield and the recovery of phenolic compounds (Pinela, Prieto, *et al.* 2016). Certain limitations were imposed (*i.e.,* times lower than 0) to avoid variables with unnatural and unrealistic physical conditions.

4.5.2.7. Cluster analysis

A cluster analysis was performed to group the phenolic compounds according to the extraction conditions that maximize their response values using the "XLSTAT 2016", a Microsoft Excel add-in. A comparative agglomerative hierarchical clustering analysis (HCA) with Pearson correlation coefficient was used for clustering (similarity analysis). The algorithm used was a complete linkage with automatic truncation based on entropy.

4.5.2.8. Fitting procedures and statistical analysis

Fitting procedures, coefficient estimates and statistical calculations were performed as previously described by Pinela, Prieto, *et al.* (2016). In brief, a) the coefficient measurement was performed using the nonlinear least-square (quasi-Newton) method provided by the macro "Solver" in Microsoft Excel, which allows minimizing the sum of the quadratic differences between the observed and model-predicted values; b) the coefficient significance was evaluated using the 'SolverAid' to determine the parametric confidence intervals. The not statistically significant terms (p-value > 0.05) were dropped to simplify the model; and c) the model reliability was verified using the following criteria: i) the Fisher F-test (α=0.05) was used to determine whether the constructed models were adequate to describe the observed data; ii) the 'SolverStat' macro was used for the assessment of parameter and model prediction uncertainties; iii) the R^2 was interpreted as the proportion of variability of the dependent variable explained by the model.

4.5.3. Results and discussion

4.5.3.1. Response criteria for the RSM analysis

The experimental values of the 20 experimental runs of the CCCD design are presented in **Table 38**. The HPLC phenolic profile (recorded at 370 nm) of the watercress extract obtained under the experimental run no. 20 is shown in **Figure 34** (see HHP extraction conditions in **Table 38**). This profile is concordant with that previously characterized by Pinela *et al.* (n.d.) for wild watercress. Up to twenty-two compounds were identified (**Table 38**) based on their chromatographic, UV-vis and mass spectra characteristics, six of which were phenolic acid derivatives (hydroxycinnamic acids) and sixteen were flavonoid glycoside derivatives. Many of these compounds were also reported by other authors in this species (Martínez-Sánchez

et al. 2008, Santos *et al.* 2014, Spínola *et al.* 2016). Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside, *p*-coumaric acid, isorhamnetin-*O*-sophoroside-*O*-malonylhexoside, quercetin-*O*-sophoroside-*O*-malonylhexoside and caffeic acid were identified as the most abundant compounds. Flavonoids predominated over phenolic acids and, in general, more quercetin and isorhamnetin glycoside derivatives were quantified than phenolic acids.

For optimization purposes, the quantified phenolic compounds (**Table 38**) were grouped in total phenolic acids (compounds 2, 4, 5, 6, 8 and 9), total flavonoids, comprising the subgroups of quercetin glycoside derivatives (*Qgd*: compounds 1, 3, 7, 10, 11, 12, 13, 14 and 15), isorhamnetin glycoside derivatives (*Igd*: compounds 16, 17 and 18) and kaempferol glycoside derivatives (*Kgd*: compounds 19, 20, 21 and 22), and total phenolic compounds (including all quantified phenolics). The individual and grouped compounds were used as response criteria to optimize the HHP conditions for their extraction from watercress using RSM. The values of the extraction yield were also considered, which ranged from 12 to 27.5% with the experimental runs nº 14 and 3, respectively (**Table 38**). Therefore, a total of 29 response variables were computed and used as optimization criteria.

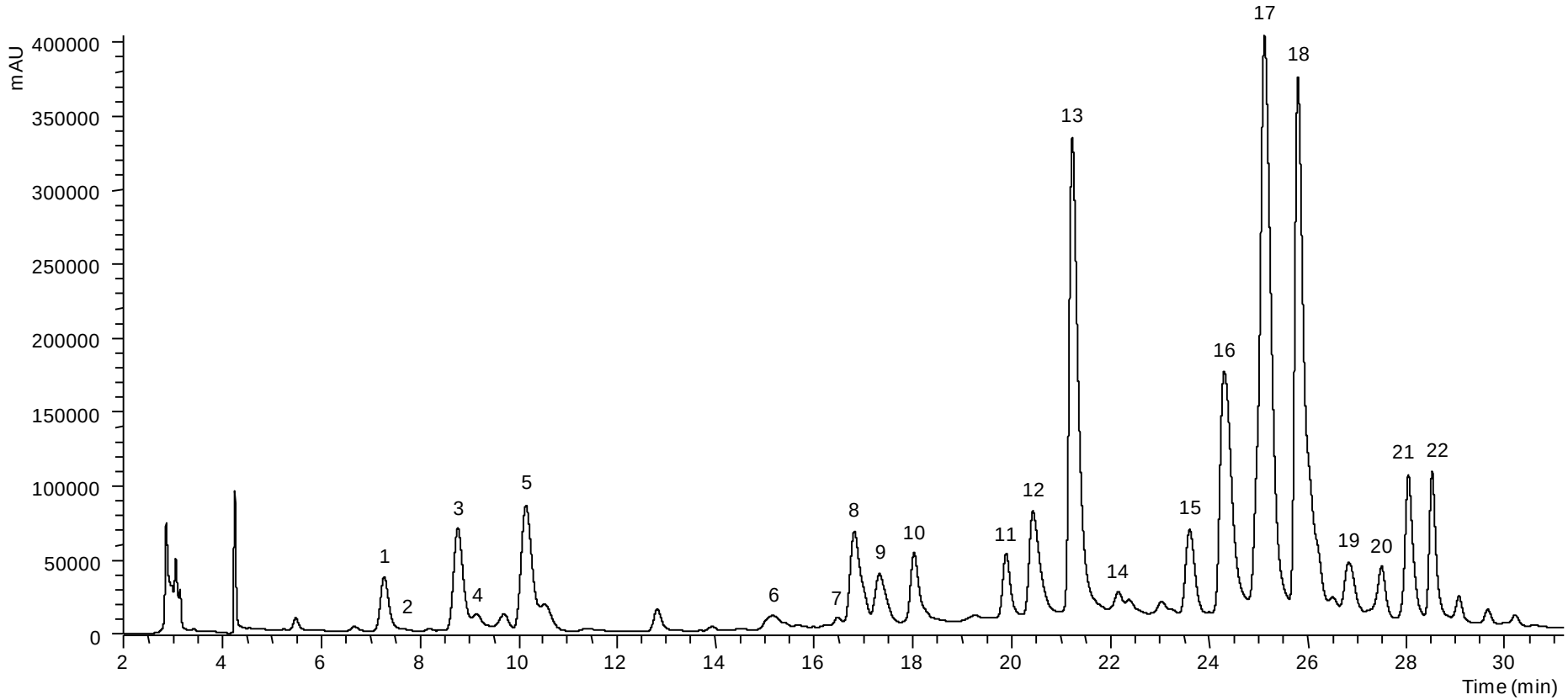

Figure 34 HPLC profile of phenolic compounds of the watercress extract obtained under the experimental run no. 20, recorded at 370 nm. See **Table 38** for peak identification.

4.5.3.2. Theoretical response surface models

As in many research fields, when trying to develop theoretical models to predict and comprehend the effects of independent variables on certain response variables, it is necessary to evaluate its precision by fitting these models to the experimental values. In this study, the response values (**Table 38**) were fitted to a second-order polynomial model using a nonlinear algorithm (least-squares estimations) to develop mathematical models for each response criteria (**Table 39**). **Table 40** shows the estimated coefficient values obtained from the polynomial model of Eq. (1) and the coefficient of correlation (R^2) for each parametric response of the extraction process. These parametric values translate the response patterns and show the complexity of the possible interactions between variables. However, not all the parameters of Eq. (1) were used for building the model since some coefficients were non-significant (*ns*). The significant ones were assessed at a 95% confidence level ($\alpha = 0.05$). The statistic lack of fit, used to test the adequacy of the obtained models, demonstrated that no considerable improvement was achieved by the inclusion of the statistically *ns* parametric values. The resulting models for each of the 29 assessed responses are presented in **Table 39**. In all cases, R^2 coefficients higher than 0.79 were obtained (**Table 40**), which indicates that the percentage of variability of each response can be explained by the model. These workable models were applied in the subsequent prediction and optimization steps, showing a good agreement between the experimental and predicted values, which indicates that the variation is explained by the independent variables.

The obtained model coefficients (**Table 40**) are empirical and cannot be associated with physical or chemical significance. However, they are useful for predicting the results of untested extraction conditions (Ranic *et al.* 2014). The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level, and when a factor has a negative effect, the response is lower at the high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable. Based on the mathematical expressions (**Table 39**), no associations were found between the response variables of phenolic acids, flavonoids, quercetin glycoside derivatives (*Qgd*), isorhamnetin glycoside derivatives (*Igd*) and kaempferol glycoside derivatives (*Kgd*). However, certain features regarding the general effects of the variables are displayed. The relevance of the significant parametric values can be order as a function of the variables involved in a decreasing form as *S*>*P*>>*t*. Alexandre *et al.* (2017b) also found *S* as the most relevant variable on the HHP extraction of bioactive compounds from pomegranate (*Punica granatum* L.) peels. Regarding the linear, quadratic, and interactive parametric effects of the developed equations, it was found that they play an important and significant role in all evaluated responses. For the linear effect, the variables *P* and *S* had strong values; meanwhile, the effect of *t* was negligible in almost all cases. All

independent variables had moderate quadratic or nonlinear effects. Regarding the interactive effects, the interactions of the variable *t* with the other variables (*tP* and *tS*) were of minor relevance; meanwhile, the *PS* interaction had a strong significance in describing the behaviour of almost all responses (with the exception of compound 10). The interactive parametric values of *PS* were accentuated in the responses of flavonoids, *Qgd*, *Igd*, phenolic acids, and total phenolic compounds. To make the combined effects more explicit and to visually describe the extraction trends, the results were presented in the response surface plots discussed below.

Table 39 Mathematical models of the extraction process derived from the second-order polynomial model of Eq. (1).

Quercetin-3-*O*-sophoroside	$Y_{P1} = 1.14 - 0.02P + 0.02t^2 + 0.02P^2 - 0.04S^2 + 0.09tP + 0.09PS$	Eq. (2)
Quercetin-3-*O*-manolylglucoside-7-*O*-glucoside	$Y_{P3} = 1.51 - 0.03t + 0.06P^2 - 0.09S^2 - 0.05tS + 0.36PS$	Eq. (3)
Quercetin-3-*O*-rutinoside-7-*O*-glucoside	$Y_{P7} = 0.90 + 0.02P + 0.02S^2 - 0.02tP + 0.02tS + 0.02PS$	Eq. (4)
Quercetin-3-*O*-rutinoside (rutin)	$Y_{P10} = 1.20 - 0.04t^2 - 0.03P^2 - 0.07S^2$	Eq. (5)
Quercetin-*O*-sophoroside-*O*-rutinoside	$Y_{P11} = 1.15 + 0.02P + 0.03S - 0.03t^2 - 0.23P^2 - 0.02S^2 - 0.08tP + 0.02tS$	Eq. (6)
Quercetin-*O*-coumaroylsophoroside	$Y_{P12} = 1.45 + 0.04P - 0.12S - 0.12t^2 - 0.07P^2 - 0.04S^2 + 0.12PS$	Eq. (7)
Quercetin-*O*-sophoroside-*O*-malonylhexoside	$Y_{P13} = 3.00 - 0.12t + 0.57P + 0.95S$	Eq. (8)
Quercetin-*O*-dihexosyl-*O*-malonylhexoside	$Y_{P14} = 0.94 - 0.01t + 0.01P + 0.04S - 0.01P^2 + 0.04PS$	Eq. (9)
Quercetin-*O*-sinapoylhexoside-*O*-rutinoside	$Y_{P15} = 1.29 + 0.02P + 0.04S - 0.01t^2 - 0.10S^2 - 0.03tP + 0.05PS$	Eq. (10)
Total quercetin glycoside derivatives (Qgd)	$Y_{Qgd} = 12.52 + 0.65P + 1.39S - 0.35t^2 - 0.37S^2 + 1.63PS$	Eq. (11)
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside	$Y_{P16} = 2.51 + 0.21P + 0.18S - 0.14t^2 - 0.06P^2 - 0.47S^2 + 0.26PS$	Eq. (12)
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside	$Y_{P17} = 4.46 - 0.16t + 0.85P + 1.53S + 1.26PS$	Eq. (13)
Isorhamnetin-*O*-sophoroside-*O*-malonylhexoside	$Y_{P18} = 3.44 + 0.63P + 1.28S - 0.30tS + 1.08PS$	Eq. (14)
Total isorhamnetin glycoside derivatives (Igd)	$Y_{Igd} = 10.74 - 0.15t + 1.72P + 1.62S - 1.84S^2 - 0.82tS + 2.24PS$	Eq. (15)
Kaempferol-*O*-feruloylhexoside-*O*-rutinoside	$Y_{P19} = 1.13 + 0.03P + 0.04S - 0.02t^2 - 0.07S^2 + 0.02tP + 0.05PS$	Eq. (16)
Kaempferol-*O*-feruloylhexoside-*O*-hexoside	$Y_{P20} = 1.11 + 0.03P + 0.03S - 0.02t^2 - 0.01P^2 - 0.07S^2 + 0.02tP + 0.04PS$	Eq. (17)
Kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside	$Y_{P21} = 1.50 + 0.12P + 0.28S - 0.05tS + 0.23PS$	Eq. (18)
Kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside	$Y_{P22} = 1.44 + 0.10P + 0.14S - 0.13S^2 - 0.04tS + 0.19PS$	Eq. (19)
Total kaempferol glycoside derivatives (Kgd)	$Y_{Kgd} = 5.20 + 0.27P + 0.58S - 0.08t^2 - 0.05P^2 - 0.16S^2 + 0.07tP - 0.11tS + 0.51PS$	Eq. (20)
Total flavonoids	$Y_{Fl} = 28.68 + 2.55P + 5.23S - 0.91t^2 - 0.61P^2 - 0.85S^2 - 1.11tS + 4.90PS$	Eq. (21)
p-Coumaric acid hexoside	$Y_{P2} = 0.04 - 0.01P + 0.02S + 0.02t^2 - 0.01S^2 - 0.02PS$	Eq. (22)
Ferulic acid hexoside	$Y_{P4} = 0.18 + 0.01t + 0.03S - 0.03t^2 - 0.04P^2 - 0.05S^2 + 0.02tP - 0.01tS - 0.01PS$	Eq. (23)
Caffeic acid	$Y_{P5} = 2.54 + 0.13S - 015S^2 + 0.08tP - 0.12tS + 0.47PS$	Eq. (24)
p-Coumaric acid	$Y_{P6} = 4.19 - 0.11P + 0.55S - 0.19S^2 + 0.23tP - 0.16tS + 0.45PS$	Eq. (25)
Ferulic acid	$Y_{P8} = 1.20 + 0.28S - 0.03t^2 - 0.04S^2 - 0.03tP - 0.07tS + 0.19PS$	Eq. (26)
Sinapoylmalic acid	$Y_{P9} = 0.45 + 0.04P + 0.17S - 0.03S^2 - 0.01tS + 0.10PS$	Eq. (27)
Total phenolic acids	$Y_{Pa} = 8.56 + 1.27S - 0.26S^2 + 0.39tP - 0.42tS + 1.25PS$	Eq. (28)
Total phenolic compounds	$Y_{Ph} = 37.23 + 2.49P + 6.49S - 0.85t^2 - 0.66P^2 - 1.11S^2 - 1.53tS + 6.15PS$	Eq. (29)
Extraction yield (crude extract)	$Y_{EY} = 23.90 + 0.79t + 1.52P - 4.01S - 0.97P^2 - 3.45S^2 + 1.21PS$	Eq. (30)

Table 40 Fitting coefficients and R^2 determined for the models obtained for individual and grouped phenolic compounds and extraction yield (**Table 39**), and optimal HHP conditions and response values.

Response variables	Fitting coefficients obtained after applying the Box-Behnken design equation										R^2	Optimal processing conditions and response values			
	Intercept	Linear effect			Quadratic effect			Interactive effect				*t* (min)	*P* (MPa)	*S* (%)	Optimum
	b_0	b_1 (*t*)	b_2 (*P*)	b_3 (*S*)	b_{11} (t^2)	b_{22} (P^2)	b_{33} (S^2)	b_{12} (*tP*)	b_{13} (*tS*)	b_{23} (*PS*)					
P1	1.14±0.01	ns	-0.02±0.01	ns	0.02±0.01	0.02±0.01	-0.04±0.01	0.03±0.01	ns	0.09±0.01	0.8649	1.5±0.1	0.0±6.7	0.0±9.7	1.50±0.52
P3	1.51±0.05	-0.03±0.00	ns	ns	ns	0.06±0.04	-0.09±0.04	ns	-0.05±0.05	0.36±0.05	0.8073	1.5±0.1	600.0±3.0	100.0±5.1	2.61±0.87
P7	0.90±0.07	ns	0.02±0.01	0.02±0.01	ns	ns	ns	-0.02±0.01	0.02±0.01	0.02±0.01	0.9093	1.5±0.1	600.0±1.5	100.0±0.2	0.98±0.32
P10	1.20±0.01	ns	ns	ns	-0.04±0.01	-0.03±0.01	-0.07±0.01	ns	ns	ns	0.8310	17.5±0.3	300.0±4.5	50.0±0.7	1.20±0.17
P11	1.15±0.01	ns	0.02±0.01	0.03±0.01	-0.03±0.01	-0.02±0.01	-0.08±0.01	0.02±0.01	ns	0.03±0.01	0.9490	21.1±0.2	521.4±4.8	61.4±0.6	1.17±0.39
P12	1.45±0.04	ns	0.04±0.03	-0.12±0.03	-0.12±0.03	-0.07±0.03	-0.04±0.03	ns	ns	0.12±0.04	0.7933	17.5±0.8	75.3±3.3	0.0±0.0	1.64±0.55
P13	3.00±0.10	-0.12±0.12	0.57±0.12	0.95±0.13	ns	ns	ns	ns	ns	0.91±0.16	0.8956	1.5±0.2	600.0±6.3	100.0±10.4	8.31±2.35
P14	0.94±0.01	-0.01±0.01	0.01±0.01	0.04±0.01	ns	-0.01±0.01	ns	ns	ns	0.04±0.01	0.8187	1.5±0.1	600.0±3.8	100.0±0.6	1.13±0.58
P15	1.29±0.02	ns	0.02±0.01	0.04±0.01	-0.01±0.01	ns	-0.10±0.01	0.03±0.02	ns	0.05±0.02	0.8682	33.5±0.6	600.0±9.1	67.3±1.1	1.41±0.51
Qgd	12.52±0.26	ns	0.65±0.19	1.39±0.23	-0.35±0.03	ns	-0.37±0.24	ns	ns	1.63±0.24	0.8993	17.5±0.9	600.0±13.4	100.0±5.6	19.53±2.18
P16	2.51±0.09	ns	0.21±0.06	0.18±0.06	-0.14±0.06	-0.06±0.06	-0.47±0.06	ns	ns	0.26±0.08	0.8736	17.5±1.7	600.0±5.7	69.3±6.5	2.88±0.73
P17	4.46±0.16	-0.16±0.13	0.85±0.19	1.53±0.21	ns	ns	ns	ns	ns	1.26±0.24	0.8952	1.5±0.3	600.0±9.8	100.0±6.4	12.30±2.86
P18	3.44±0.14	ns	0.63±0.15	1.28±0.20	ns	ns	ns	ns	-0.30±0.20	1.08±0.20	0.9031	1.5±0.2	600.0±8.6	100.0±4.3	10.54±2.65
Igd	10.74±0.27	-0.15±0.03	1.72±0.27	1.62±0.27	ns	ns	-1.84±0.27	ns	-0.82±0.14	2.24±0.27	0.8926	1.5±0.1	600.0±11.8	97.1±24.3	16.89±2.26
P19	1.13±0.01	ns	0.03±0.01	0.04±0.01	-0.02±0.01	ns	-0.07±0.01	0.01±0.01	ns	0.05±0.01	0.8928	24.5±0.3	600.0±6.9	79.1±0.9	1.26±0.48
P20	1.11±0.01	ns	0.03±0.01	0.03±0.01	-0.02±0.01	-0.01±0.01	-0.07±0.01	0.01±0.01	ns	0.04±0.01	0.9213	22.2±0.3	600.0±6.3	71.8±0.8	1.16±0.39
P21	1.50±0.02	ns	0.12±0.03	0.28±0.03	ns	ns	ns	ns	-0.05±0.03	0.23±0.03	0.9300	1.5±0.1	600.0±9.2	100.0±2.2	2.95±1.40
P22	1.44±0.03	ns	0.10±0.03	0.14±0.03	ns	ns	-0.13±0.03	ns	-0.04±0.04	0.19±0.04	0.8466	1.5±0.1	600.0±9.3	100.0±3.2	2.11±0.79
Kgd	5.20±0.03	ns	0.27±0.03	0.58±0.03	-0.08±0.01	-0.05±0.03	-0.16±0.03	0.07±0.01	-0.11±0.04	0.51±0.03	0.9603	13.7±0.4	600.0±16.4	100.0±2.7	7.49±0.88
FLAV	28.68±0.84	ns	2.55±0.51	5.23±0.63	-0.91±0.52	-0.61±0.52	-0.85±0.65	ns	-1.11±0.66	4.90±0.66	0.9357	7.8±0.5	600.0±5.0	100.0±8.6	52.45±2.63
P2	0.04±0.01	ns	-0.01±0.00	0.02±0.00	0.02±0.01	ns	-0.01±0.01	ns	ns	-0.02±0.01	0.8848	17.5±0.1	298.0±1.1	97.3±0.4	0.06±0.08

Response variables	Fitting coefficients obtained after applying the Box-Behnken design equation										R^2	Optimal processing conditions and response values			
	Intercept	Linear effect			Quadratic effect			Interactive effect				*t* (min)	*P* (MPa)	*S* (%)	Optimum
	b_0	b_1 (*t*)	b_2 (*P*)	b_3 (*S*)	b_{11} (t^2)	b_{22} (P^2)	b_{33} (S^2)	b_{12} (*tP*)	b_{13} (*tS*)	b_{23} (*PS*)					
P4	0.18±0.01	0.01±0.00	ns	0.03±0.00	-0.03±0.01	-0.04±0.01	-0.05±0.00	0.02±0.01	-0.01±0.01	-0.01±0.01	0.9659	18.0±0.1	289.9±1.7	59.5±0.3	0.19±0.14
P5	2.54±0.05	ns	ns	0.13±0.05	ns	ns	-0.15±0.05	0.08±0.06	-0.12±0.06	0.47±0.06	0.8725	1.5±0.1	600.0±3.6	100.0±5.1	3.79±0.60
P6	4.19±0.07	ns	-0.11±0.07	0.55±0.07	ns	ns	-0.19±0.07	0.23±0.09	-0.16±0.09	0.45±0.09	0.8825	1.5±0.1	0.0±9.7	54.7±4.1	5.02±0.96
P8	1.20±0.03	ns	ns	0.28±0.02	0.03±0.02	ns	0.04±0.02	0.03±0.01	-0.07±0.03	0.19±0.03	0.9176	1.5±0.1	600.0±8.3	100.0±3.1	2.51±0.68
P9	0.45±0.02	ns	0.04±0.02	0.17±0.02	ns	ns	0.03±0.01	ns	-0.02±0.01	0.10±0.02	0.9026	1.5±0.1	600.0±9.6	100.0±1.8	1.21±0.60
TPA	8.56±0.16	ns	ns	1.27±0.17	ns	ns	-0.26±0.18	0.39±0.18	-0.42±0.18	1.25±0.18	0.9107	1.5±0.3	600.0±9.7	100.0±6.2	13.58±1.99
TPC	37.23±1.02	ns	2.49±0.62	6.49±0.76	-0.85±0.13	-0.66±0.63	-1.11±0.80	ns	-1.53±0.81	6.15±0.81	0.9350	3.0±3.2	600.0±6.3	100.0±2.2	64.68±2.97
Yield	23.90±0.62	0.79±0.49	1.52±0.49	-4.00±0.49	ns	-0.97±0.47	-3.45±0.47	ns	ns	-1.21±0.63	0.9142	33.5±0.8	530.6±3.3	26.1±6.2	27.82 ±2.26

See **Table 38** for compounds identification. Qgd: Total quercetin glycoside derivatives; Igd: Total isorhamnetin glycoside derivatives; Kgd: Total kaempferol glycoside derivatives; FLAV: total flavonoids; TPA: total phenolic acids; TPC: total phenolic compounds; Yield: extraction yield. ns: non-significant coefficient; R^2: Correlation coefficient.

4.5.3.3. Effect of the independent variables on the target responses and optimal extraction conditions

Although parametric results can depict the patterns of the responses, 3D and 2D graphical representations may aid on their comprehension. **Figure 35** shows the response surface plots of extraction yield and grouped phenolic compounds (total phenolic acids, total flavonoids and total phenolic compounds) as well as their statistical analysis. **Figure 36** illustrates in a similar way the results for *Qgd*, *Igs* and *Kgd*. Both **Figure 35** and **Figure 36** are divided in three subsections: i) the subsection A illustrates the 3D response surface plots, whose grid surfaces were predicted with the respective second-order polynomial model described by Eq. (1) using the theoretical values presented in **Table 40**. For representation of these binary combinations, the excluded variable was positioned at the optimum of their experimental domain (**Table 40**); ii) the subsection B illustrates the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable; and iii) the subsection C shows the individual 2D responses and the optimum values (⊙). In each plot, each independent variable was positioned at the optimal value of the other two variables.

Observing the response surface plots of the extraction yield (**Figure 35**), it is possible to verify that the amount of extracted material increases to an optimum value and then, in most cases, it decreases as a function of the involved independent variable. Consequently, the optimum value can be found as being a single point in almost all combinations, which allows computing the extraction conditions that lead to an absolute maximum. **Figure 35C** simplifies the interpretation of the effects of the independent variables on the extraction process and highlights the optimum value of each variable. The extraction yield was maximal (27.82±2.26%) when the optimal HHP conditions (t= 33.5 min, P= 530.6 MPa and S= 26.1 % of ethanol, v/v) presented in **Table 40** were applied for extraction. Zhang *et al*. (2007) have shown that the crude extract obtained from *Rhodiola sachalinensis* is greater when HHP is used than when the extraction is done by the conventional methods of reflux or Soxhlet. High extraction yields were also achieved by Prasad *et al*. (2009) when processing longan fruit (*Dimpcarpus longan* Lour.) pericarps under pressures up to 500 MPa and using lower extraction times than those required in a conventional extraction.

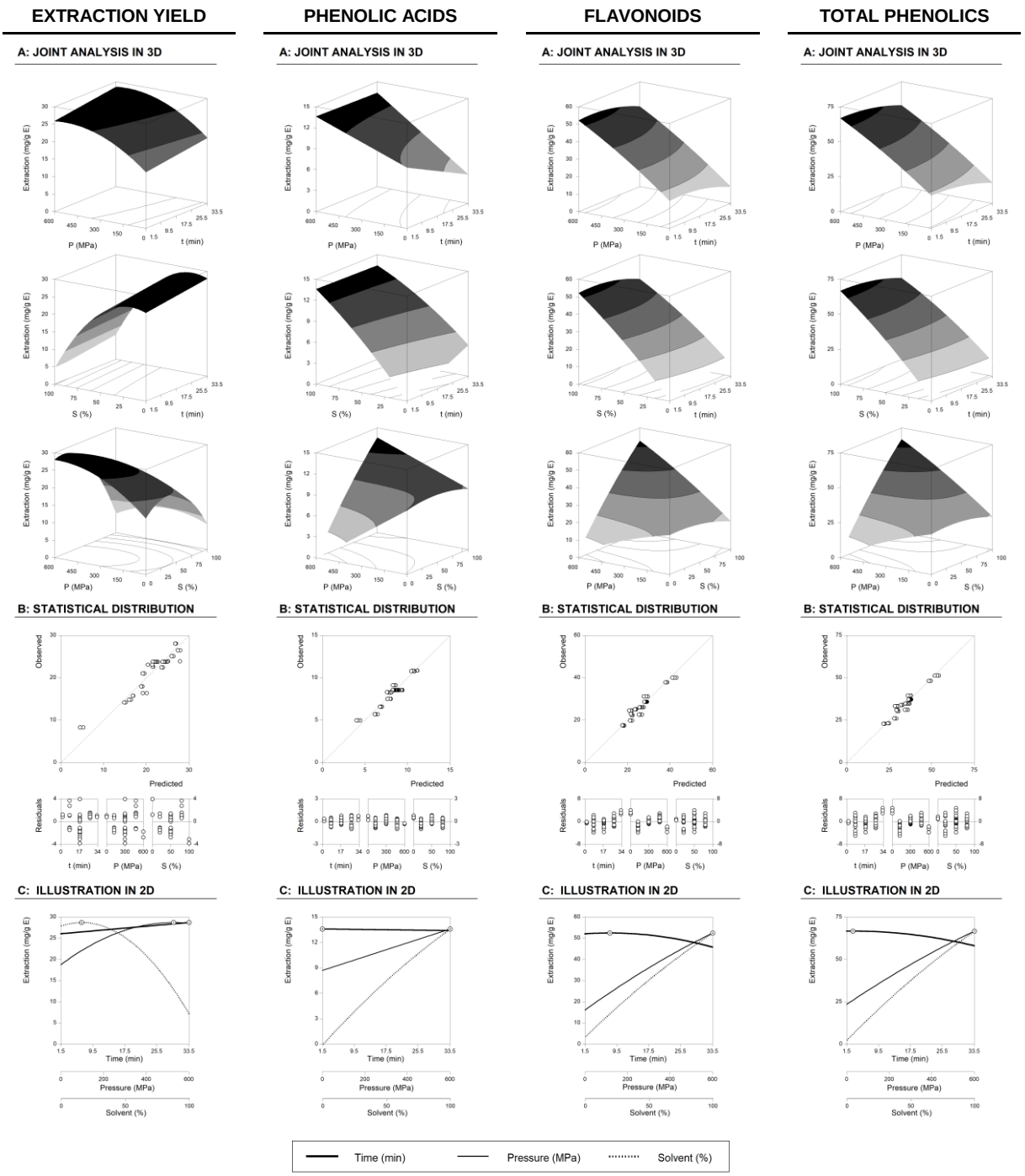

Figure 35 Response surface plots of extraction yield and grouped phenolic compounds (total phenolic acids, total flavonoids and total phenolic compounds). Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 40**) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (**Table 40**). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable. Part C: individual 2D responses and optimum values (⊙). Each independent variable was positioned at the optimal value of the other two variables.

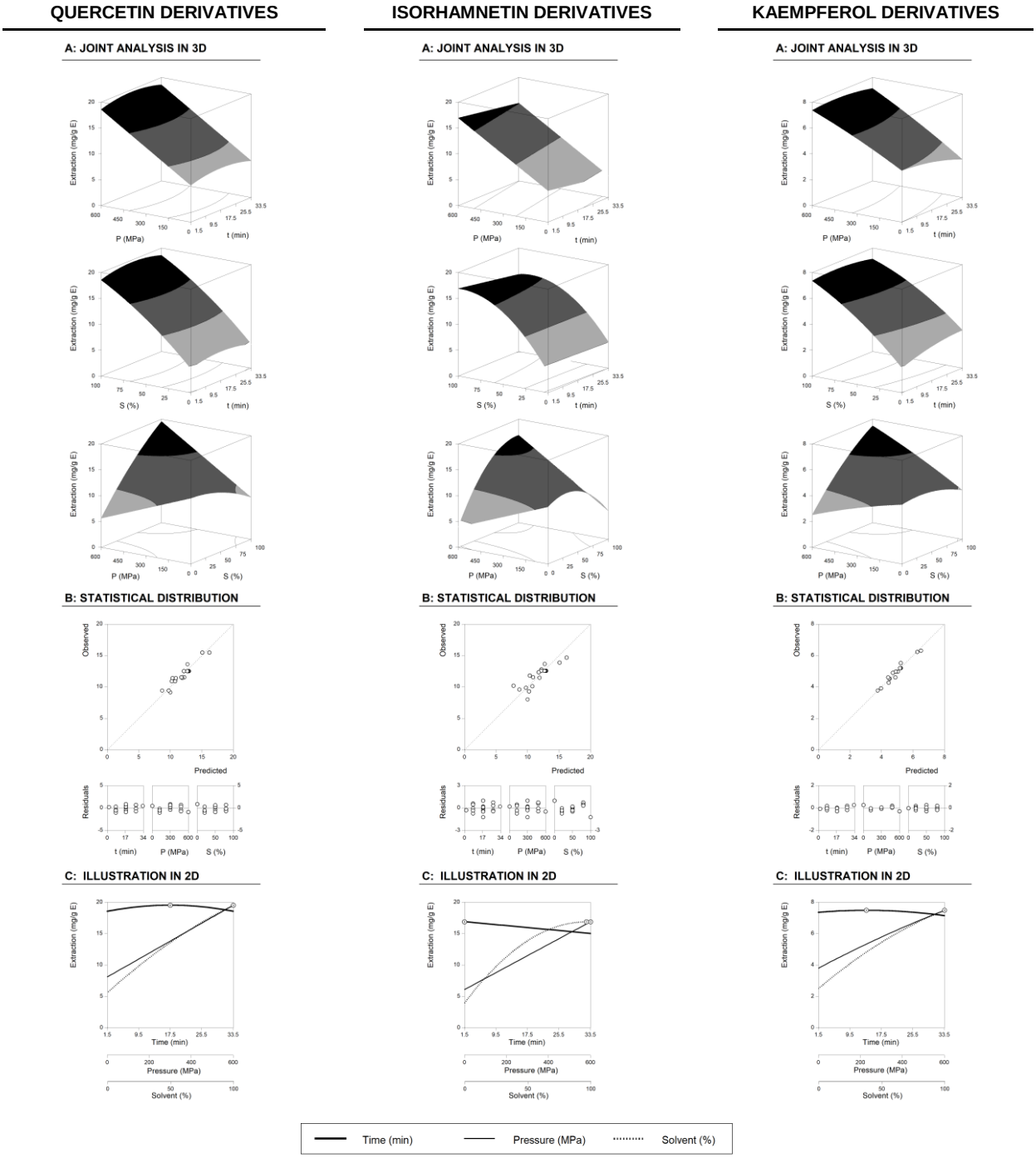

Figure 36 Response surface plots of the flavonoid subgroups of quercetin, isorhamnetin and kaempferol glycoside derivatives. Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 40**) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (**Table 40**). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable. Part C: individual 2D responses and optimum values (⊙). Each independent variable was positioned at the optimal value of the other two variables.

The response surface plots of grouped phenolic acids and flavonoids and total phenolic compounds are showed in **Figure 35** and the optimal HHP conditions that maximize their recovery from watercress are presented in **Table 40**. These responses were similarly affected by the screened variables; they were favoured by high values of *P* and *S* and short values of *t* as summarized below:

- For phenolic acids, the optimal HHP conditions were: *t*= 1.5±0.3 min, *P*= 600.0±5.0 MPa and *S*= 100.0±8.6% of ethanol (v/v), and originated 13.58±1.99 mg/g of extract.
- For flavonoids, the optimal HHP conditions were: *t*= 7.8±0.5 min, *P*= 600.0±5.0 MPa and *S*= 100.0±8.6% of ethanol (v/v), and originated 52.45±2.63 mg/g of extract.
- For total phenolic compounds, the optimal HHP conditions were: *t*= 3.1±3.2 min, *P*= 600.0±6.3 MPa and *S*= 100.0±2.2 of ethanol (v/v), and originated 64.68±2.97 mg/g of extract.

The optimum extraction values for the flavonoid derivatives *Qgd*, *Igs* and *Kgd* were achieved using very similar HHP conditions (**Figure 36** and **Table 40**), probably due to structural similarities between these compounds. Once more, the extraction was favoured by high values of *P* and *S* and short values of *t*, as summarized below:

- For *Qgd*, the optimal HHP conditions were: *t*= 17.5±1.0 min, *P*= 600.0±13.4 MPa and 100.0±5.6% of ethanol (v/v), and originated 19.53±2.18 mg/g of extract.
- For *Igd*, the optimal HHP conditions were: *t*= 1.5±0.1 min, *P*= 600.0±11.8 MPa and 97.1±24.3% of ethanol, and originated 16.89±2.26 mg/g of extract.
- For *Qgd*, the optimal HHP conditions were: *t*= 13.7±0.4 min, *P*= 600.0±16.4 MPa and *S*=100.0±2.7% of ethanol (v/v), and originated 7.49±0.88 mg/g of extract.

According to the literature, the use of high pressures increases the extraction of bioactive compounds from plants matrices (Alexandre *et al.* 2017a). Briones-Labarca *et al*. (2015) demonstrated that HHP is more effective than ultrasound-assisted extraction or conventional extraction (2 h) to recover bioactive compounds from Chilean papaya (*Vasconcellea pubescens*) seeds. In addition, HHP was a time-saving extraction method. The lower energy consumption is another advantage of HHP comparatively to conventional methods (Jun 2009). In our study, it is also interesting to note that the HHP conditions that maximize the yield of crude extract and the recovery of phenolic compounds differ mostly in the required processing time and ethanol concentration. In this way, the extracts obtained under the optimal conditions established for phenolic compounds (**Table 40**) will contain a lower quantity of compounds other than phenolics, thus making the recovery process more selective for the target compounds.

The effects of the independent variables on the extraction of individual phenolic compounds from watercress are 2D represented in **Figure 37**. The processing conditions that generated optimal response values (⊙) are numerically described in **Table 40**. The

identified flavonoids were organized as a function of the maximum amount achieved (mg/g of extract) in a decreasing order as follows: P17 (12.3±2.86) > P18 (10.54±2.65) > P13 (8.31±2.35) >> P21 (2.95±1.40) > P16 (2.88±0.73) > P3 (2.61±0.87) > P22 (2.11±0.79) > P12 (1.64±0.55) > P1 (1.5±0.52) > P15 (1.41±0.51) > P19 (1.26±0.48) > P10 (1.2±0.17) > P11 (1.17±0.39) > P20 (1.16±0.39) > P14 (1.13±0.58) > P7 (0.98±0.32). Meanwhile, the phenolic acids were organized as follows: P6 (5.02±0.96) > P5 (3.79±0.60) >> P8 (2.51±0.68) > P9 (1.21±0.60) > P4 (0.19±0.14) > P2 (0.06±0.08). Pinela *et al*. (n.d.) reported lower quantities of phenolic acids (5.6±0.5 mg/g of extract), flavonoids (22±1 mg/g of extract) and total phenolic compounds (28±2 mg/g of extract) in an extract of wild watercress obtained by a conventional solid-liquid extraction of 2 h and using a methanol:water mixture (80:20, v/v) as a extraction solvent. These differences highlight the suitability of HHP as an innovative extraction technique to recover a greater amount of phenolic compounds from watercress using shorter processing times and greener solvents.

4.5.3.4. Clustering of phenolic compounds according to the HHP conditions that maximize their extraction

Table 41 shows the maximum response values of each phenolic compound and their values if extracted under the optimal HHP conditions of the other compounds (**Table 40**). These values presented in part B were calculated dividing the optimum value of each compound by the maximum of the others compounds. Therefore, when two compounds display the value 1 (corresponding to values of 100%), the optimum response value of both compounds is achieved under the same HHP conditions. This is the case of compounds 3, 5, 7, 8, 9, 13, 14, 17, 18, 21 and 22, which were clustered in C3a under the same HHP conditions (**Figure 38**). In turn, when a 0 is display, it means that the conditions that maximize the extraction of a certain compound (compounds 1, 3, 0, 7, 8, 9, 12, 13, 14, 17, 18, 21 and 22) do not favour at all the extraction of the other one (compounds 2 and 4).

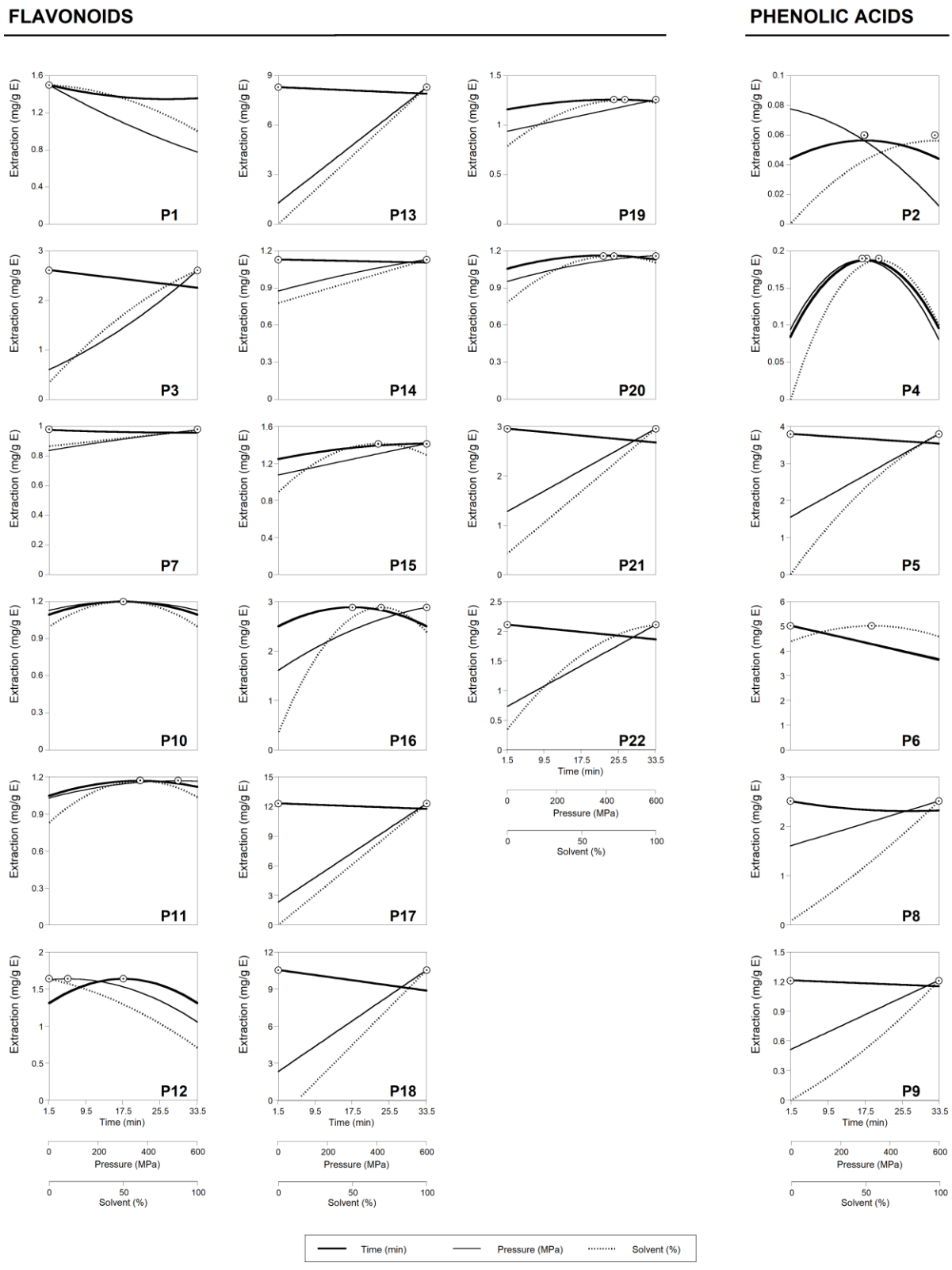

Figure 37 2D graphical response of the effects of the independent variables on the extraction of phenolic compounds from watercress (see **Table 38** for peak identification). Dots (⊙) represent the optimal values. In each plot, each independent variable was positioned at the optimal value of the other two variables (**Table 40**).

Table 41 Maximum response values of each phenolic compound and their values at the optimal processing conditions of the other compounds presented in **Table 40**.

A) Maximum response values (mg/g of extract) of the individual phenolic compounds

Peak:	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22
Optimum:	1.50	0.06	2.61	0.19	3.79	5.02	0.98	2.51	1.21	1.20	1.17	1.64	8.31	1.13	1.41	2.88	12.30	10.54	1.26	1.16	2.95	2.11

B) Values of each phenolic compound (%) at the optimal conditions of the other compounds

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22
P1	*1*	0.69	0.85	0.75	0.85	0.89	0.85	0.85	0.85	0.76	0.79	0.85	0.85	0.85	0.90	0.81	0.85	0.85	0.86	0.84	0.85	0.85
P2	0	*1*	0	0.86	0	0.59	0	0	0	0.76	0.54	0	0	0	0.16	0.38	0	0	0.30	0.35	0	0
P3	0.90	0.49	*1*	0.57	1	0.62	1	1	1	0.58	0.66	0.81	1	1	0.73	0.77	1	1	0.81	0.78	1	1
P4	0	0.59	0	*1*	0	0.05	0	0	0	0.97	0.67	0	0	0	0.01	0.35	0	0	0.19	0.33	0	0
P5	0.82	0.62	1	0.67	*1*	0.71	1	1	1	0.67	0.74	0.77	1	1	0.82	0.81	1	1	0.87	0.83	1	1
P6	0.87	0.91	1	0.87	1	*1*	1	1	1	0.84	0.90	0.76	1	1	1	0.95	1	1	1	1	1	1
P7	0.97	0.94	1	0.93	1	0.91	*1*	1	1	0.92	0.92	0.95	1	1	0.92	0.94	1	1	0.95	0.94	1	1
P8	0.55	0.69	1	0.52	1	0.56	1	*1*	1	0.48	0.57	0.50	1	1	0.67	0.64	1	1	0.74	0.67	1	1
P9	0.35	0.66	1	0.41	1	0.32	1	1	*1*	0.37	0.51	0.33	1	1	0.59	0.62	1	1	0.72	0.64	1	1
P10	0.68	0.85	0.68	0.99	0.68	0.85	0.68	0.68	0.68	*1*	0.95	0.80	0.68	0.68	0.83	0.91	0.68	0.68	0.87	0.90	0.68	0.68
P11	0.73	0.85	0.78	0.98	0.78	0.88	0.78	0.78	0.78	0.98	*1*	0.76	0.78	0.78	0.96	0.99	0.78	0.78	0.98	0.99	0.78	0.78
P12	0.79	0.70	0.63	0.86	0.63	0.50	0.63	0.63	0.63	0.89	0.85	*1*	0.63	0.63	0.63	0.84	0.63	0.63	0.80	0.82	0.63	0.63
P13	0.39	0.54	1	0.39	1	0.26	1	1	1	0.36	0.54	0.31	*1*	1	0.63	0.67	1	1	0.76	0.69	1	1
P14	0.85	0.89	1	0.84	1	0.81	1	1	1	0.83	0.86	0.83	1	*1*	0.88	0.89	1	1	0.92	0.90	1	1
P15	0.77	0.77	0.79	0.92	0.79	0.92	0.79	0.79	0.79	0.92	0.96	0.72	0.79	0.79	*1*	0.97	0.79	0.79	0.98	0.98	0.79	0.79
P16	0.24	0.55	0.69	0.87	0.69	0.53	0.69	0.69	0.69	0.87	0.96	0.37	0.69	0.69	0.87	*1*	0.69	0.69	0.96	0.99	0.69	0.69
P17	0.35	0.56	1	0.40	1	0.26	1	1	1	0.36	0.54	0.28	1	1	0.63	0.67	*1*	1	0.76	0.69	1	1
P18	0.23	0.52	1	0.36	1	0.23	1	1	1	0.33	0.49	0.26	1	1	0.57	0.62	1	*1*	0.69	0.63	1	1
P19	0.78	0.82	0.90	0.90	0.90	0.85	0.90	0.90	0.90	0.90	0.96	0.77	0.90	0.90	0.98	0.99	0.90	0.90	*1*	1	0.90	0.90
P20	0.76	0.85	0.86	0.96	0.86	0.86	0.86	0.86	0.86	0.96	0.99	0.78	0.86	0.86	0.97	0.99	0.86	0.86	1	*1*	0.86	0.86
P21	0.45	0.66	1	0.53	1	0.44	1	1	1	0.51	0.63	0.46	1	1	0.69	0.72	1	1	0.78	0.73	*1*	1
P22	0.51	0.63	1	0.69	1	0.60	1	1	1	0.68	0.79	0.52	1	1	0.84	0.87	1	1	0.89	0.88	1	*1*

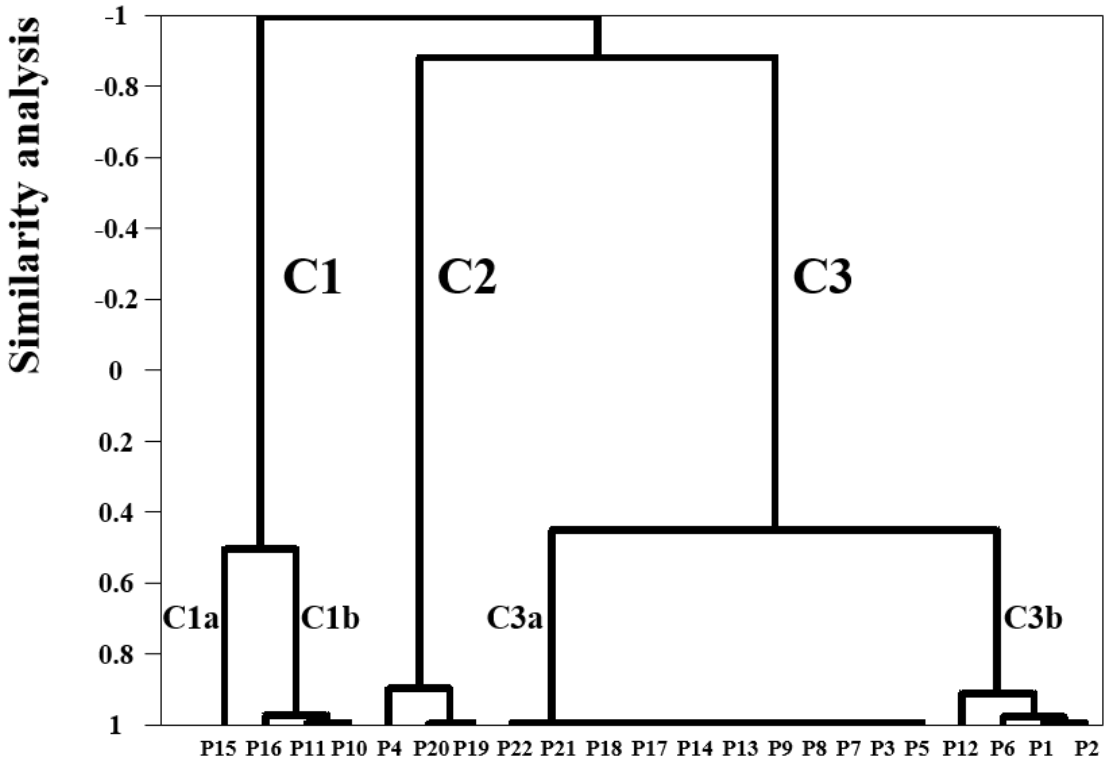

Figure 38 HCA dendrogram of phenolic compounds according to the HHP conditions that maximize their extraction from watercress.

Using the complete dataset of **Table 41** and performing a multi-objective optimization problem using an appropriate clustering algorithm, different clusters of phenolic compounds whose maximum response values are obtained under similar HHP extraction conditions were created. The results of HCA are presented in **Figure 38**. In the HCA dendrogram, the shorter distance between compounds indicates a higher similarity in terms of conditions that favour their extraction and the compounds clustered into the same group are better extracted under similar HHP conditions. Three significant clusters (C1, C2 and C3) were generated. C1 and C3 were also be divided in two (a and b) pertinent subgroups. Additionally, other less relevant subgroups were created in C2, C1b and C3b, but they can be considered as a residual noise produced by the algorithm.

- Cluster C1 included the compounds 15, 16, 11 and 10. Meanwhile, compound 15 was subdivided in C1a and compounds 16, 11 and 10 were grouped in C1b. The extraction of these compounds was maximized by medium *t*, high *P* and medium *S* (**Table 40**). The subgroups C1a and C1b were mainly differentiated by the *t* values.
- Cluster C2 included the compounds 4, 20 and 19. No significant subgroups were created. The extraction of these compounds was favoured by medium *t*, high *P* and medium-large *S* values.

- Cluster C3 included the compounds 22, 21, 18, 17, 14, 13, 9, 8, 7, 3, 5, 12, 6, 1 and 2, which were subdivided in C3a e C3b. The extraction of the compounds in C3a was maximized when using low *t*, high *P* and high *S*. On the other hand, the compounds in C3b exhibited a broad set of conditions with no clear interconnections between each other.

Although it was expected that compounds with similar chemical characteristics would exhibit comparable optimal extraction conditions, no clear similarity was detected between the created groups of compounds and the conditions that maximize their extraction. However, this HCA analysis was an interesting and innovative approach in the field of extraction of high added-value compound from natural sources since this analysis highlighted suitable HHP conditions for maximize the simultaneous recovery of specific groups of compounds from watercress.

4.5.4. Conclusions

The suitability of HHP for extracting phenolic compounds from watercress was demonstrated in this study. The variables of *t*, *P* and *S* were combined in a five-level CCCD design coupled to RSM for optimization. The developed polynomial response models were expressed as 2D and 3D surface plots to better visualize the effects on the response variables (extraction yield and individual and grouped phenolic compounds). A good agreement between experimental and theoretical results was observed. In general, the recovery of phenolic compounds was maximized when high pressures, high ethanol concentrations and short extraction times were applied, which validate this cold extraction method as a very promising technique compared to the time-consuming conventional methods. This study also highlighted watercress as being an interesting source of phytochemicals, namely phenolic acids and flavonoids.

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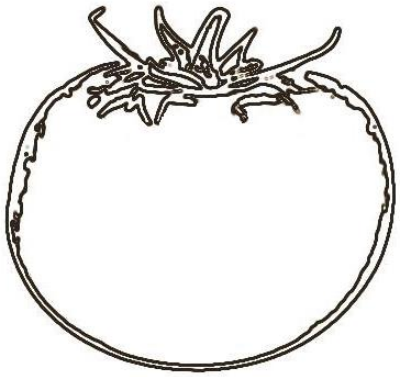
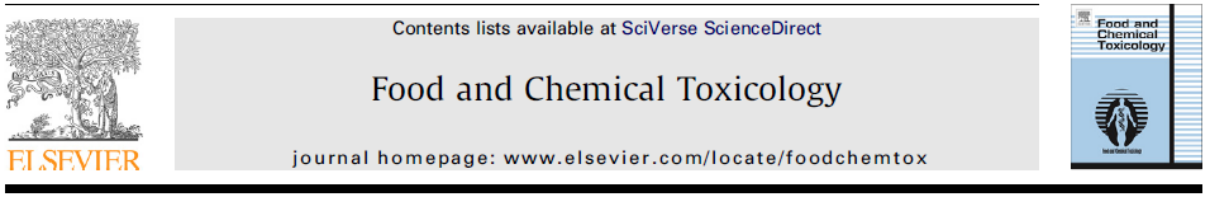
5. Tomato Farmers' Varieties



This **5th chapter** describes the nutritional value, chemical composition and antioxidant activity of four tomato (*Lycopersicon esculentum* Mill.) farmers' varieties in North-eastern Portugal homegardens. Optimization studies performed using the response surface methodology regarding the microwave-assisted extraction of antioxidants and phenolic compounds from tomatoes are also presented.

Tomato (*Lycopersicon esculentum* Mill.)

5.1. Nutritional composition and antioxidant activity of four tomato farmers' varieties in North-eastern Portugal homegardens



Nutritional composition and antioxidant activity of four tomato (*Lycopersicon esculentum* L.) farmer' varieties in Northeastern Portugal homegardens

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Abstract

The nutritional and antioxidant composition of four tomato Portuguese farmer' varieties widely cultivated in homegardens was determined. The analysed components included macronutrients, individual profiles of sugars and fatty acids by chromatographic techniques, hydrophilic antioxidants such as vitamin C, phenolics, flavonols and anthocyanins, and lipophilic antioxidants such as tocopherols, β-carotene and lycopene. Furthermore, the antioxidant activity was evaluated through DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition. One of the four varieties, which is locally known as round tomato or potato tomato, proved to be the most powerful in antioxidant activity (EC_{50} values ≤ 1.63 mg/ml), phenolic compounds (phenolics 31.23 mg ClAE/g extract, flavonols 6.36 mg QE/g extract and anthocyanins 3.45 mg ME/g extract) and carotenoids (β-carotene 0.51 mg/100 g and lycopene 9.49 mg/100 g), while the so-called yellow tomato variety revealed interesting nutritional composition, including higher fructose (3.42 g/100 g), glucose (3.18 g/100 g), α-linolenic acid (15.53%) and total tocopherols (1.44 mg/100 g) levels. Overall, these farmer' varieties of garden tomato cultivated in the North-eastern Portuguese region could contribute as sources of important antioxidants related to the prevention of chronic diseases associated to oxidative stress, such as cancer and coronary artery disease.

Keywords: tomato; *Lycopersicon esculentum*; farmers' varieties; nutrients; antioxidants; antioxidant activity.

5.1.1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely consumed vegetables, being the second most important vegetable crop worldwide. It is a key component in the so-called "Mediterranean diet", which is strongly associated with a reduced risk of chronic degenerative diseases (Rao and Agarwal 1998, Agarwal and Rao 2000).

Tomato is a major source of antioxidants contributing to the daily intake of a significant amount of these molecules. It is consumed fresh or as processed products such as canned tomato, sauce, juice ketchup, stews and soup (Lenucci *et al.* 2006). In fact, epidemiological studies have shown that consumption of raw tomato and its tomato-based products is associated with a reduced risk of cancer and cardiovascular diseases (Clinton 1998, Giovannucci *et al.* 2002). This protective effect has been mainly attributed to its valuable bioactive components with antioxidant properties (Borguini and Torres 2009).

Tomato antioxidants include carotenoids such as β-carotene, a precursor of vitamin A, and mainly lycopene, which is largely responsible for the red colour of the fruit, vitamins such as ascorbic acid and tocopherols, and phenolic compounds such as flavonoids and hydroxycinnamic acid derivatives (Clinton 1998, Moco *et al.* 2006, Borguini and Torres 2009, Kotíková *et al.* 2009, 2011, Vallverdú-Queralt *et al.* 2011).

These compounds may play an important role inhibiting reactive oxygen species responsible for many important diseases, through free-radical scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways (Clinton 1998, Crozier *et al.* 2009).

At present, there is a large number of tomato cultivars with a wide range of morphological and sensorial characteristics which determine their use. There are studies on nutritional value and antioxidant properties of tomato from different origins such as Czech Republic (Kotíková *et al.* 2009, 2011), France (Gautier *et al.* 2008), Italy (Ilahy *et al.* 2011), Spain (Guil-Guerrero and Rebolloso-Fuentes 2009, Vallverdú-Queralt *et al.* 2011) and Taiwan (Chang *et al.* 2006). Nevertheless, still now there are no reports on Portuguese varieties, moreover on Portuguese local varieties grown for a long time in homegardens.

In the past 30 years, significant changes in farming systems and crop diversity have taken place in several rural areas of Portugal, particularly in the most north-eastern region, known as Trás-os-Montes (Frazão-Moreira *et al.* 2009, Carvalho 2010). New trends in rural lifestyles have highlighted the importance of a wide range of greens, particularly wild greens (Carvalho and Morales 2010), and of local farmers' varieties grown since a long time, such as beans, cabbages, pimento and tomato.

At the same time, cultivation and consumption of vegetables have increased in the Iberian Peninsula which is due to the generalized use of greenhouses, allowing better control of nutrients available to plants and a global supply of these products. However, local populations from Trás-os-Montes still prefer to consume traditional vegetables (*e.g.,* different farmer' varieties of tomato) which they find very tasty and healthy food, as they are grown using extensive farming techniques.

These farmers' varieties of tomato are thus being cultivated, but their nutritional composition has remained unreported until now. The main purpose of this study was to describe the nutritional value and the antioxidant activity of four non- analysed tomato farmer 'varieties from Trás-os-Montes, North-eastern Portugal.

5.1.2. Materials and methods

5.1.2.1. Samples

Four common farmer' varieties of tomato widely cultivated in rural communities from Miranda do Douro, Trás-os-Montes, North-eastern Portugal, were chosen according morphological and sensorial characteristics which determine their use, as defined by our informants (**Table 42**). These farmer' varieties are shown in **Figure 39**.

Table 42 Several morphologic characteristics and description of four different tomato Portuguese farmer' varieties: yellow tomato (Amarelo), round tomato (Batateiro), long tomato (Comprido) and heart tomato (Coração) (**Figure 39**).

	Amarelo	**Batateiro**	**Comprido**	**Coração**
Predominant fruit shape	flattened (oblate)	high rounded potato-shaped	ellipsoid (plum-shaped)	heart-shaped
Fruit size	intermediate (5.1-8 cm)	small (3-5 cm)	small (3-5 cm)	very large (>10 cm)
Fruit weight (average)	190 g	116 g	132 g	465 g
Exterior colour of mature fruit	yellow	red	orange/red	red/pink
Flesh colour of pericarp	yellow	red	orange/green	pink/red
Fruit cross-sectional shape	irregular	irregular	angular	irregular
Fruit blossom end shape	indented	flat	pointed	pointed
Fruit firmness	firm	intermediate	firm	soft
Fruit shoulder shape	strongly depressed	moderately depressed	flat	slightly depressed
Jointed pedicel	present	present	present	present
Number of locules	multilocular	multilocular	trilocular	multilocular
Seeds number	intermediate	hight	hight	small

Average of 10 fruits from different plants

Figure 39 Morphologic characteristics of four different Portuguese farmer' varieties of tomato: yellow tomato (Amarelo), round tomato (Batateiro), long tomato (Comprido) and heart tomato (Coração).

Such varieties are known by their local vernacular name and used differently: “tomate amarelo” (yellow tomato), of intense yellow colour even when ripened, is consumed raw in salads; “tomate redondo or batateiro” (round tomato) is round-shaped like a potato and eaten raw or stewed with fish and meat or made in sauce; “tomate comprido” (long tomato) is similar to plum tomatoes and is mainly frozen and stored, to be available for use in cooking during winter; “tomate coração” (heart tomato), is a big, fleshy, juicy heart-shaped tomato that is mostly used for cooking and for preparing a traditional marmalade.

Tomato fruits at the red-ripe stage were hand harvested randomly in September 2010 from the middle of six plants of each of the four varieties, in selected homegardens of two villages in the studied area. All plants from each tomato variety were grown under the same soil and climatic conditions and similar agricultural practices. The seeds were selected and kept by local farmers. The ripening stage for all samples was selected according to local consumers’ criteria. The edible portion of six fruits of each variety was prepared and used for analysis. The specimens of each variety were then lyophilised (FreeZone 4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh), mixed to obtain a homogenate sample and kept at -20 ºC until further analysis.

5.1.2.2. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), other individual fatty acid isomers, L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(-)-fructose, D(+)-glucose anhydrous, D(+)-melezitose hydrate, D(+)-sucrose), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), chlorogenic acid, malvidin 3-glucoside and quercetin dehydrate standards were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

5.1.2.3. Nutritional composition

Nutritional value: The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC 2005). The crude protein content (N × 6.25) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15 ºC. Total carbohydrates were calculated by difference. The energy was calculated according to the equation: $4 \times (m_{proteins} + m_{carbohydrates}) + 9 \times (m_{fats})$ (Regulation (EU) No 1169/2011) and expressed as kcal per 100 g of fresh weight (fw).

Sugars: Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI) as described by Pinela *et al.* (2011), using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of fresh weight (fw).

Fatty Acids: Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Pinela *et al.* 2011). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey-Nagel column (30 m × 0.32 mm ID × 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30ºC/min ramp to 125 ºC, 5ºC/min ramp to 160 ºC, 20ºC/min ramp to 180 ºC, 3ºC/min ramp to 200 ºC, 20ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 ml/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

5.1.2.4. Antioxidants composition

Tocopherols: Tocopherols content was determined following a procedure previously described by Barros, Carvalho and Ferreira (2010), using tocol as IS. The analysis was carried out in the HPLC system described above connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250 × 4.6 mm; YMC Waters) operating at 30ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method, and tocopherols contents were further expressed in mg per 100 g of dry fresh (fw).

Ascorbic acid: Ascorbic acid was determined following a procedure previously described by the authors (Barros *et al.* 2010) with 2,6-dichloroindophenol, and measuring the absorbance at 515 nm (spectrophotometer Analytik Jena, Germany). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic $_L$-ascorbic acid (0.006-0.1 mg/ml), and the results were expressed as mg of ascorbic acid per 100 g of fresh weight (fw).

Carotenoids: β-carotene and lycopene were determined following a procedure previously described by the authors (Barros *et al.* 2010), measuring the absorbance at 453, 505, 645, and 663 nm. Contents were calculated according to the following equations: β-carotene (mg/100 mL) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$; lycopene (mg/100

mL) = – 0.0458 × A_{663} + 0.204 × A_{645} - 0.304 × A_{505} + 0.452 × A_{453}, and further expressed in mg per 100 g of dry weight (dw).

Phenolics: A fine dried powder (20 mesh; ~1 g) stirring with 50 mL of methanol at 25 ºC at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with one additional 50 mL portion of methanol. The combined methanolic extracts were evaporated at 35ºC under reduced pressure (rotary evaporator Büchi R-210), re-dissolved in methanol at 50 mg/mL, and stored at 4 ºC for further analysis of phenolics and antioxidant properties.

The extract sample concentrated at 0.625 mg/mL (250 µL) was mixed with HCl 0.1% in 95% ethanol (250 µL) and HCl 2% (4550 µL). After 15 min the absorbance was measured at 280, 360 and 520 nm. The absorbance (A) at 280 nm was used to estimate total phenolic content, $A_{360\ nm}$ was used to estimate flavonols, and $A_{520\ nm}$ was used to estimate anthocyanins (Mazza *et al.* 1999). Chlorogenic acid was used to calculate the standard curve (0.2-3.2 mM) and the results were expressed as mg of chlorogenic acid equivalents (ClAE) per g of extract. Quercetin was used to calculate the standard curve (0.2-3.2 mM) and the results were expressed as mg of quercetin equivalents (QE) per g of extract. Malvidin 3-glucoside was used to calculate the standard curve (0.1-2.3 mM) and the results were expressed as mg of malvidin 3-glucoside equivalents (ME) per g of extract.

5.1.2.5. Evaluation of antioxidant activity

DPPH radical-scavenging activity: This methodology was performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the extracts (30 µL) and aqueous methanolic solution (80:20 v/v, 270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Pinela *et al.* 2011). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = [(A_{DPPH}-A_S)/A_{DPPH}] × 100, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as standard.

Reducing power: This methodology was performed using the Microplate Reader described above. The different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL).

The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm (Pinela *et al.* 2011). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

Inhibition of β-carotene bleaching: A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into different test tubes containing different concentrations of the extracts (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (Pinela *et al.* 2011). β-Carotene bleaching inhibition was calculated using the following equation: (β-carotene content after 2h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS): Brains were obtained from porcine (*Sus scrofa*), dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000*g* (Centorion K24OR refrigerated centrifuge) for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of the extracts (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm (Pinela *et al.* 2011). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A – B)/A] x 100%, where A and B were the absorbance of the control and the compound solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated from the graph of TBARS inhibition percentage against extract concentration. Trolox was used as standard.

5.1.2.6. Statistical analysis

For each sample three extracts were obtained and all the assays were carried out in triplicates. The results are expressed as mean values and standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α = 0.05. This treatment was carried out using SPSS v. 16.0 program.

5.1.3. Results and discussion

5.1.3.1. Nutritional composition

The results of the macronutrients composition and energetic value obtained for the studied tomato varieties are shown in **Table 43**. Moisture ranges between 90.63 g/100 g fw in the yellow tomato sample and 93.70 g/100 g fw in the long tomato. The highest levels of protein and ash were found in the yellow tomato (0.61 and 0.74 g/100 g fw, respectively). Otherwise, this sample gave the lowest fat levels (0.03 g/100 g fw). Carbohydrates were the most abundant macronutrients and the highest levels were also found in the yellow variety (7.99 g/100 g fw). This sample also gave the highest energetic value (34.67 kcal/100 g fw). Tomato varieties have high moisture, proteins and carbohydrates contents, in contrast to low fat levels, which make them suitable to incorporate low caloric diets. These proportions are in agreement to the proximate composition of Spanish tomato varieties reported by Guil-Guerrero and Rebolloso-Fuentes (2009). Nevertheless, those samples revealed higher fat levels but lower carbohydrates content and energetic value than the Portuguese samples herein studied.

Table 43 Macronutrients, energetic value and individual sugars composition of four different tomato Portuguese farmer'varieties: yellow tomato (Amarelo), round tomato (Batateiro), long tomato (Comprido) and heart tomato (Coração).

	Amarelo	**Batateiro**	**Comprido**	**Coração**
Moisture (g/100 g fw)	90.63 ± 0.46	92.21 ± 0.77	93.70 ± 1.02	92.76 ± 1.54
Ash (g/100 g fw)	0.74 ± 0.02 a	0.63 ± 0.03 b	0.59 ± 0.03 b	0.54 ± 0.00 c
Proteins (g/100 g fw)	0.61 ± 0.01 a	0.41 ± 0.00 b	0.40 ± 0.01 b	0.42 ± 0.00 b
Fat (g/100 g fw)	0.03 ± 0.00 d	0.11 ± 0.01 c	0.17 ± 0.01 a	0.13± 0.02 b
Carbohydrates (g/100 g fw)	7.99 ± 0.01 a	6.63 ± 0.02 b	5.14 ± 0.02 d	6.14 ± 0.01 c
Energy (kcal/100 g fw)	34.67 ± 0.09 a	29.17 ± 0.12 b	23.72 ± 0.10 d	27.44 ± 0.05 c
Fructose	3.42 ± 0.20 a	3.13 ± 0.30 ba	2.15 ± 0.01 c	2.71 ± 0.00 b
Glucose	3.18 ± 0.22 a	2.69 ± 0.27 b	1.74 ± 0.01 d	2.22 ± 0.01 c
Sucrose	0.02 ± 0.00 a	0.01 ± 0.00 b	0.02 ± 0.00 a	0.02 ± 0.00 a
Total sugars (g/100 g fw)	6.62 ± 0.41 a	5.83 ± 0.57 ba	3.91 ± 0.02 c	4.95 ± 0.01 b

In each row, different letters mean significant differences ($p<0.05$).

Sugars are abundant carbohydrates in the samples and followed the order fructose > glucose >> sucrose (**Table 43**). Once more, the yellow tomato revealed the highest total sugars content (6.62 g/100 g fw), with the highest levels of fructose (3.42 g/100 g fw), glucose (3.18 g/100 g fw) and sucrose (0.02 g/100 g fw). These sugars are the major source of energy for metabolism (Bernal *et al.* 2011).

The results of the main fatty acids found in the studied tomato varieties, as also their saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) percentages are shown in **Table 44**. Up to twenty-four fatty acids were detected in most of the samples. The major fatty acid found was linoleic acid (C18:2n6c). Other abundant PUFA was α-linolenic acid (C18:3n3), and PUFA levels were higher than MUFA and SFA in all the samples. The studied varieties also revealed the SFA palmitic acid (C16:0) and the MUFA oleic acid (C18:1n9) as major fatty acids. This profile is similar to the one described for Spanish tomato varieties, but with higher C18:3n3 levels (Guil-Guerrero and Rebolloso-Fuentes 2009). The long tomato gave the highest PUFA (58%) and MUFA (18%) percentages, with the highest levels C18:2n6c (52%) and C18:1n9 (17%). Otherwise, the yellow tomato showed the highest levels of SFA (33%) mainly C16:0 (21%), but also the highest levels of C18:3n3 (16%).

Fatty acids are important as nutritional substances in living organisms. Long-chain PUFA, especially those of the n-3 series, such as 18:3n3, are essential for human metabolism and have many beneficial effects including the prevention of a number of diseases, such as coronary heart diseases, inflammation, autoimmune disorders, hypertension, hypotriglyceridemic effect, etc. (Bernal *et al.* 2011).

5.1.3.2. Antioxidants composition

Antioxidants such as vitamins, carotenoids and phenolics were determined and the results are provided in **Table 45**. Ascorbic acid was the most abundant antioxidant in all the samples, and the highest concentration was found in the sample of the so-called heart tomato (18.56 mg/100 g fw). The values found in the present study were similar to the ones reported on varieties from Italy (Ilahy *et al.* 2011) and Taiwan (Juroszek *et al.* 2009) (both in the order of 20 mg/100 g fw), but lower than the values found in Czech Republic (21.7-25.8 mg/100 g fw; Kotíková *et al.* 2011) and Spanish (39-163 mg/100 g fw; Guil-Guerrero and Rebolloso-Fuentes 2009) varieties. The role of ascorbic acid in the prevention of diseases related to oxidative damage occurs due to its ability to neutralize the action of free radicals in the biological systems (Borguini and Torres 2009). This hydrophilic antioxidant is abundant in many fruits and is the most common in the majority of them, when compared with the presence of lipophilic antioxidants such as vitamin E (tocopherols).

Table 44 Fatty acids composition (relative %) of four different tomato Portuguese farmer' varieties: yellow tomato (Amarelo), round tomato (Batateiro), long tomato (Comprido) and heart tomato (Coração).

	Amarelo	**Batateiro**	**Comprido**	**Coração**
C6:0	0.01 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
C8:0	0.10 ± 0.01	0.09 ± 0.01	0.03 ± 0.00	0.02 ± 0.00
C10:0	0.07 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.01 ± 0.00
C12:0	0.15 ± 0.02	0.11 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
C14:0	0.93 ± 0.10	0.62 ± 0.01	0.32 ± 0.01	0.57 ± 0.01
C15:0	0.15 ± 0.00	0.11 ± 0.00	0.08 ± 0.01	0.11 ± 0.01
C16:0	20.53 ± 0.91	19.31 ± 0.18	15.96 ± 0.10	19.05 ± 0.04
C16:1	0.25 ± 0.00	0.32 ± 0.01	0.29 ± 0.00	0.28 ± 0.00
C17:0	0.33 ± 0.03	0.25 ± 0.01	0.18 ± 0.01	0.27 ± 0.00
C18:0	6.34 ± 0.03	5.47 ± 0.07	6.36 ± 0.31	5.39 ± 0.11
C18:1n9	10.60 ± 1.24	12.61 ± 0.23	17.45 ± 0.86	12.97 ± 0.36
C18:2n6	39.80 ± 1.85	46.33 ± 0.40	52.05 ± 0.64	48.19 ± 0.15
C18:3n3	15.53 ± 1.41	11.41 ± 0.42	5.55 ± 0.50	10.08 ± 0.34
C20:0	1.26 ± 0.11	0.80 ± 0.02	0.61 ± 0.02	0.83 ± 0.01
C20:1	0.12 ± 0.00	0.12 ± 0.01	0.05 ± 0.00	0.09 ± 0.01
C20:2	0.06 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00
C20:4n6	0.06 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.03 ± 0.00
C20:3n3+C21:0	0.20 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.14 ± 0.02
C20:5n3	0.03 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
C22:0	0.82 ± 0.12	0.55 ± 0.00	0.31 ± 0.00	0.66 ± 0.01
C22:1n9	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C22:2	0.10 ± 0.00	0.07 ± 0.00	0.03 ± 0.00	0.08 ± 0.01
C23:0	1.52 ± 0.19	0.78 ± 0.02	0.16 ± 0.01	0.24 ± 0.03
C24:0	1.01 ± 0.07	0.73 ± 0.01	0.45 ± 0.01	0.76 ± 0.02
Total SFA	33.22 ± 1.68 a	28.91 ± 0.26 b	24.57 ± 0.29 c	27.93 ± 0.09 b
Total MUFA	11.00 ± 1.24 c	13.08 ± 0.28 b	17.66 ± 0.86 a	13.45 ± 0.37 b
Total PUFA	55.78 ± 0.43 b	58.01 ± 0.02 a	57.77 ± 1.14 a	58.63 ± 0.46 a

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1c); *cis*-11,14-Eicosadienoic acid (C20:2c); Arachidonic acid (C20:4n6); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3+C21:0); cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3); Erucic acid (C22:1n9); *cis*-13,16-Docosadienoic acid (C22:2); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0).

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. In each row different letters mean significant differences ($p<0.05$).

The yellow tomato variety presented the highest content of tocopherols (1.44 mg/100 g fw) with the highest levels of α- (0.88 mg/100 g fw) and γ- (0.53 mg/100 g fw) isoforms. β-Carotene was found in lower amounts than tocopherols, while lycopene was present in

higher concentrations; the highest levels of carotenoids were observed in the sample from round tomato (0.51 and 9.49 mg/100 g fw for β-carotene and lycopene, respectively). The β-carotene levels found in the studied Portuguese farmer' varieties were similar to the concentration reported on varieties from Italy (Ilahy *et al.* 2011) and Taiwan (Juroszek *et al.* 2009) (~0.5 mg/100 g fw). Nevertheless, lycopene values observed in the studied samples were higher than the ones described for fresh and lyophilized samples from Taiwan (3 and 2 mg/100 g fw, respectively; Chang *et al.* 2006), but slightly lower than in Italian varieties (~10 mg/100 g fw; Ilahy *et al.* 2011). Lycopene is a carotenoid compound wildly present in tomato and dietary intake of food containing lycopene has been shown to be related to decreased risk of chronic diseases, such as cancer and cardiovascular disease (Agarwal and Rao 2000). The potential as an antioxidant is related to its capacity to scavenge singlet oxygen and radical peroxyl (Borguini and Torres 2009).

Table 45 Antioxidants composition of four different tomato Portuguese farmer' varieties: yellow tomato (Amarelo), round tomato (Batateiro), long tomato (Comprido) and heart tomato (Coração).

	Amarelo	**Batateiro**	**Comprido**	**Coração**
α-tocopherol	0.88 ± 0.03 a	0.68 ± 0.01 b	0.59 ± 0.01 c	0.68 ± 0.02 b
β-tocopherol	0.02 ± 0.00 b	0.03 ± 0.00 a	0.03 ± 0.00 a	0.03 ± 0.00 a
γ-tocopherol	0.53 ± 0.04 a	0.43 ± 0.01 b	0.40 ± 0.01 b	0.45 ± 0.04 b
δ-tocopherol	0.01 ± 0.00 b	0.02 ± 0.00 a	0.01 ± 0.00 b	0.02 ± 0.01 a
Total tocopherols (mg/100 g fw)	1.44 ± 0.07 a	1.16 ± 0.02 b	1.02 ± 0.01 c	1.18 ± 0.04 b
Vitamin C (mg/100 g fw)	16.03 ± 0.38 c	10.86 ± 0.09 d	16.50 ± 0.03 b	18.56 ± 0.04 a
β-carotene (mg/100 g fw)	0.42 ± 0.02 b	0.51 ± 0.03 a	0.30 ± 0.01 c	0.43 ± 0.02 b
Lycopene (mg/100 g fw)	5.02 ± 0.09 c	9.49 ± 0.18 a	8.10 ± 0.10 b	9.22 ± 0.15 a
Phenolics (mg ClAE/g extract)	21.34 ± 1.16 c	31.23 ± 1.15 a	24.48 ± 1.67 b	24.92 ± 3.04 b
Flavanols (mg QE/g extract)	3.06 ± 0.84 c	6.36 ± 0.28 a	4.05 ± 0.28 b	3.44 ± 0.45 cb
Anthocyanins (mg ME/g extract)	0.23 ± 0.08 d	3.45 ± 0.23 a	1.36 ± 0.26 b	1.02 ± 0.13 c

nd- not detected. In each row different letters mean significant differences ($p<0.05$).

All the differences observed in the antioxidant contents of tomato varieties are related to genotype, but also to several factors such as the ripening stage, cultivation practices (water availability, mineral nutrients), and climatic environment (mostly light and temperature) (Dumas *et al.* 2003).

Some non-essential dietary compounds such as phenolics, flavonols and anthocyanins were also determined and the highest levels were found in the farmer' variety known as long tomato (31.23 mg ClAE/g extract, 6.36 mg QE/g extract and 3.45 mg ME/g extract, respectively; **Table 45**). The main phenolic compounds found in tomato are the flavonols quercetin and kaempferol (mainly in conjugated form attached to sugar molecules) and the hydroxycinnamic acids, particularly the caffeic and chlorogenic acids (Vallverdú-

Queralt *et al.* 2011). Phenolic compounds have been associated with the inhibition of atherosclerosis and cancer due to their ability to chelate metals, inhibit lipid peroxidation and scavenge free radicals (Borguini and Torres 2009).

5.1.3.3. Antioxidant activity

The studied tomato Portuguese farmer' varieties demonstrated capacity to scavenge free radicals such as DPPH, high reducing power and capacity to inhibit lipid peroxidation in a β-carotene-linoleate system, after neutralization of the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models, and in brain cells homogenates avoiding the formation of TBARS.

The round tomato gave the best results in all the antioxidant activity assays (DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS inhibition) with EC_{50} values ≤ 1.63 mg/mL (**Table 46**). This is in agreement to its highest levels of antioxidants such as phenolics, flavonols, anthocyanins, β-carotene, lycopene, β-tocopherol and δ-tocopherol. Otherwise, the farmer' variety yellow tomato revealed the lowest antioxidant properties (EC_{50} values ≤ 2.11 mg/ml) and also the lowest phenolics, flavonols, anthocyanins and lycopene.

Table 46 Antioxidant properties (EC_{50} values, mg/mL) of four different tomato Portuguese farmer' varieties: yellow tomato (Amarelo), round tomato (Batateiro), long tomato (Comprido) and heart tomato (Coração).

	Amarelo	**Batateiro**	**Comprido**	**Coração**
DPPH scavenging activity	0.75 ± 0.01 a	0.55 ± 0.02 c	0.69 ± 0.01 b	0.65 ± 0.04 b
Reducing power	2.04 ± 0.01 a	1.63 ± 0.06 d	1.82 ± 0.04 c	1.91 ± 0.05 b
β-carotene bleaching inhibition	2.11 ± 0.24 a	0.89 ± 0.11 c	1.60 ± 0.16 b	1.49 ± 0.15 b
TBARS inhibition	1.82 ± 0.35 a	1.34 ± 0.45 b	1.58 ± 0.17 ba	1.71 ± 0.41 ba

In each row different letters mean significant differences ($p<0.05$).

As far as we know, the antioxidant potential of the studied varieties was not previously reported.

5.1.4. Conclusions

Current dietary guidelines to combat chronic diseases, including cancer and coronary artery disease, recommend increased intake of plant foods, including fruits and vegetables, which are rich sources of antioxidants, and many studies have shown that a close relation exists between the intake of vegetables and cancer prevention (Chang *et al.* 2006). Therefore, tomato as one of the most versatile and widely-used food plants could play an important role in human diet. Portuguese tomato farmers' varieties are rich sources in antioxidant compounds such as ascorbic acid, carotenoids, in particular lycopene, and phenolic

compounds. One of the studied varieties, the so-called round tomato proved to be the most powerful in antioxidant activity, phenolic compounds and carotenoids, while the variety locally known as yellow tomato revealed interesting nutritional composition, including higher fructose, glucose, α-linolenic acid and total tocopherols levels.

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5.2. Characterization and quantification of phenolic compounds in four tomato farmers' varieties in North-eastern Portugal homegardens

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ORIGINAL PAPER

Characterization and Quantification of Phenolic Compounds in Four Tomato (*Lycopersicon esculentum* L.) Farmers' Varieties in Northeastern Portugal Homegardens

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Abstract

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely consumed fresh and processed vegetables in the world, and contains bioactive key components. Phenolic compounds are one of those components and, according to the present study, farmer' varieties of tomato cultivated in homegardens from the north-eastern Portuguese region are a source of phenolic compounds, mainly phenolic acid derivatives. Using HPLC-DAD-ESI/MS, it was concluded that a *cis p*-coumaric acid derivative was the most abundant compound in yellow ("Amarelo") and round ("Batateiro") tomato varieties, while 4-*O*-caffeolyquinic acid was the most abundant one in long ("Comprido") and heart ("Coração") varieties. The most abundant flavonoid was quercetin pentosylrutinoside in the four tomato varieties. Yellow tomato presented the highest levels of phenolic compounds (54.23 µg/g fw), including phenolic acids (43.30 µg/g fw) and flavonoids (10.93 µg/g fw). The phenolic compounds profile obtained for the studied varieties is different from other tomato varieties available in different countries, which is certainly related to genetic features, cultivation conditions, and handling and storage methods associated to each sample.

Keywords: Tomato; *Lycopersicon esculentum*; farmers' varieties; phenolic compounds; HPLC-DAD-ESI/MS.

5.2.1. Introduction

Phenolic compounds are one of the main groups of dietary phytochemicals found in fruits, vegetables and grains. They include a range of plant secondary metabolites that can be divided in different groups, *i.e.,* flavonoids (*e.g.,* anthocyanins, flavanols, flavones, or isoflavones), phenolic acids, tannins, stilbenes and lignans. Several of these compounds are found in nature as glycosides and/or as esters and/or methyl ethers (Garcia-Salas *et al.* 2010).

In plants, they tend to accumulate in dermal tissues where they play a potential role in protection against UV radiation, as attractants in fruit dispersal or as defense chemicals against pathogens and predators (Tsao and McCallum 2009). They also exhibit a wide-range of physiological properties in animals, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, anti-thrombotic, cardioprotective, and vasodilatory effects (Balasundram *et al.* 2006). In recent years, dietary phenolics have attracted considerable attention for their putative effects on human health, which have been associated to their antioxidant and free-radical-scavenging activities (Heim *et al.* 2002, Fernandez-Panchon *et al.* 2008).

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely consumed fresh and processed vegetables in the world and contains bioactive components such as phenolics, carotenoids and vitamins C and E. Carotenoids consumption has been associated with a lower risk of several types of cancer and a lower incidence of coronary heart disease (Stahl and Sies 2005, Dolwick Grieb *et al.* 2009, Zhang *et al.* 2009). Lycopene is the major carotenoid present in tomato and shows strong antioxidant activity (Clinton 1998, Agarwal and Rao 2000). However, lycopene alone does not account for tomato's health benefits. Phenolics have been found to act synergistically with lycopene in preventing cell damage (Shen *et al.* 2007).

Phenolic compounds have been extensively characterized in tomato varieties from different countries (Periago *et al.* 2002, Bahorun *et al.* 2004, Slimestad *et al.* 2008, Gómez-Romero *et al.* 2010, Vallverdú-Queralt *et al.* 2010), including genetically modified tomatoes (Le Gall *et al.* 2003, Niggeweg *et al.* 2004). However, the chemical composition of tomatoes can vary among tissues of a single fruit (Moco *et al.* 2007, Peng *et al.* 2008) and type of tomatoes, according to the cultivar, cultivation conditions, and handling and storage methods (Davies and Hobson 1981, Dumas *et al.* 2003).

There are a large number of tomato cultivars with a wide range of morphological and sensory characteristics that determine their use. In Trás-os-Montes, North-eastern Portugal,

local population's lifestyles have highlighted the importance of local tomato farmers' varieties, which are grown using extensive farming techniques and considered very tasty and healthy food (Carvalho and Morales 2010). We had previously reported the nutritional composition and antioxidant activity of four farmers' varieties (Pinela *et al.* 2012), but their phenolic composition was not studied. Therefore, the present work aims to characterize the phenolic profiles of these tomato farmer' varieties from Trás-os-Montes.

5.2.2. Materials and Methods

5.2.2.1. Samples

Four common farmer' varieties of tomato widely cultivated in rural communities from Miranda do Douro, Trás-os-Montes, North-eastern Portugal, were chosen according to morphological, sensory and usage characteristics such as size and exterior colour of mature fruits (Pinela *et al.* 2012): "tomate amarelo" (yellow tomato; Royal Horticultural Society Colour Chart (RHS), yellow-orange group 14), "tomate redondo or batateiro" (round tomato; RHS, Red group 42), "tomate comprido" (long tomato; RHS, Red group 34) and "tomate coração" (heart tomato; RHS, Red group 47). Tomato fruits at the ripe stage were hand harvested randomly in September 2010 from the middle of six plants of each of the four varieties, in selected homegardens of two villages in the studied area. The seeds were selected and kept by local farmers. The ripening stage for all samples was selected according to local consumers' criteria. The edible portion (pericarps without jointed pedicels) of six fruits of each variety was prepared and used for analysis. The samples were lyophilised (4.5 model 7750031, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and kept at -20 ºC until analysis.

5.2.2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards were from Extrasynthese (Genay, France). All the other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

5.2.2.3. Phenolic compounds extraction procedure

Each sample (1 g) was extracted with 30 mL of methanol:water 80:20 (*v/v)* at room temperature, with agitation (150 rpm) for 1h. The extract was filtered through Whatman nº 4 paper. The residue was re-extracted twice with additional 30 mL portions of the same solvent. The combined extracts were evaporated at 35 ºC (rotary evaporator Büchi R-210) to remove methanol. For purification, the extract solution was deposited onto a C-18 SepPak®

Vac 3 cc cartridge (Phenomenex), wetted and activated with methanol followed by water; sugars and more polar substances were removed with 10 mL of water, and phenolic compounds were further eluted with 5 mL of methanol. The methanolic extract was concentrated under vacuum, re-dissolved in 1 mL of water:methanol 80:20 (*v/v*) and filtered through a 0.22-μm disposable LC filter disk for HPLC analysis.

5.2.2.4. HPLC-DAD-ESI/MS analysis

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors (Barros, Dueñas, Carvalho, *et al.* 2012). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standard compounds: caffeic acid (y=617.91x-691.51; R^2=0.9991); chlorogenic acid (y=600.27x-763.62; R^2=0.9998); *p*-coumaric acid (y=447.12x-1580.7; R^2=0.9962); ferulic acid (y=779.11x-869.22; R^2=0.9987); kaempferol-3-*O*-rutinoside (y=175.02x-43.877; R^2=0.9999); quercetin 3-*O*-glucoside (y=316.48x-2.9142; R^2=1); quercetin-3-*O*-rutinoside (y=222.79x-243.11; R^2=0.9998); and syringic acid (y=641.76x+246.82; R^2=0.9988). The results were expressed in μg per g of fresh weight (fw).

5.2.2.5. Statistical analysis

For each sample three extracts were obtained and all the assays were carried out in triplicate. The results are expressed as mean values with standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with α = 0.05. This treatment was carried out using SPSS v. 18.0 program.

5.2.3. Results and discussion

Figure 40A shows the phenolic profile of one of the studied tomato farmer' varieties: yellow tomato (Amarelo). Phenolic acid derivatives, mainly hydroxycinnamoyl derivatives, were the most abundant compounds in all tomato varieties (**Table 47**).

Compounds 12 and 17 corresponded to caffeic acid and *trans*-*p*-coumaric acid respectively, identified by comparison of their UV and mass characteristics and retention time with those of commercial standards. Compounds 6 and 7 showed the same pseudomolecular ion $[M-H]^-$ at *m/z* 353 consistent with caffeoylquinic acid isomers. Compound 7 was positively identified as 5-*O*-caffeoylquinic acid by comparison with an

authentic standard, and also its MS fragmentation pattern (Clifford *et al.* 2003, 2005). Compound 6 was identified as 4-*O*-caffeoylquinic acid based on the fragmentation pattern described by Clifford *et al*. (2003, 2005) for these compounds, with a base peak at *m/z* 173 ([quinic acid-H-H_2O]$^-$) accompanied by a secondary fragment ion at *m/z* 179 with approximately 65% abundance of base peak. Similar reasoning was applied for the identification of compounds 11 and 13 as 4-*O*-*p*-coumaroylquinic acid and 5-*O*-*p*-coumaroylquinic acid, respectively.

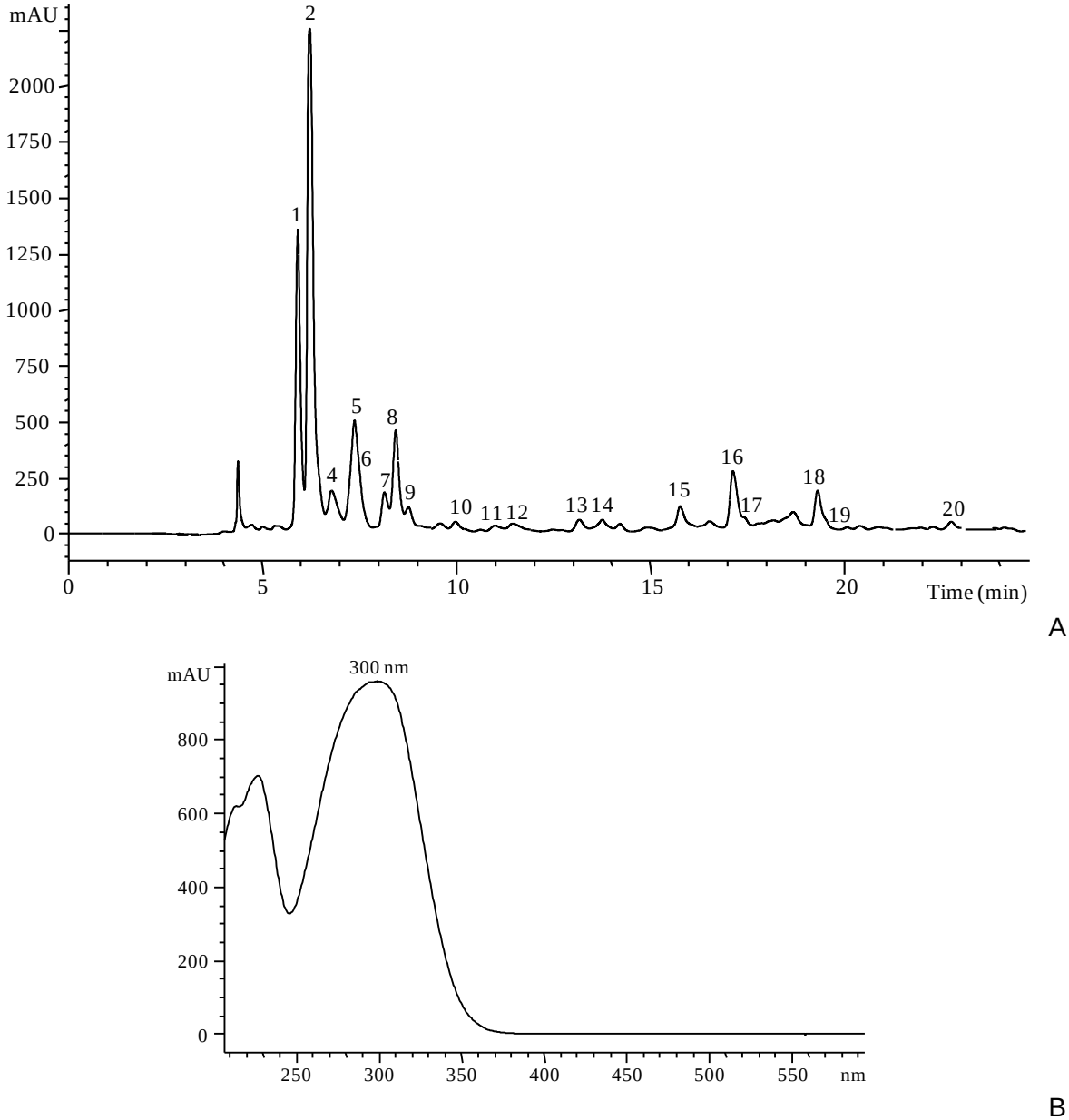

Figure 40 Individual chromatogram of yellow tomato variety (Amarelo) recorded at 280 nm (A) and UV spectrum of compounds 2 and 9 (B).

Compounds 3 and 5 presented the same pseudomolecular ion [M-H]$^-$ at *m/z* 341 and similar fragmentation pattern with the loss of 162 mu (hexosyl moiety) yielding a base peak

at *m/z* 179 mu ([caffeic acid-H]$^-$) and other two fragments at *m/z* 161 ([caffeic acid-H-H_2O]$^-$) and 135 ([caffeic acid-H-CO_2]$^-$), which allowed assigning them as caffeoyl hexosides I and II, respectively. Similarly, compounds 8 and 9 with MS^2 fragments at *m/z* 145 (base peak; [coumaric acid-H-H_2O]$^-$) and 163 (-162 mu; [coumaric acid-H]$^-$) could identified as *p*-coumaroyl hexosides. To confirm the existence of *cis* and *trans* isomers, a commercial standard of (*trans*) *p*-coumaric acid was submitted to UV irradiation (366 nm, 24h). Partial transformation was observed with the appearance of a new peak at earlier retention time in the HPLC chromatogram and a different UV spectrum with λ_{max} at 300 nm, which was attributed to the corresponding *cis* isomer (**Figure 40**). Therefore, compound 9 could be assigned as *cis p*-coumaroyl hexoside based on its UV spectrum with λ_{max} at 300 nm. Compound 8 might be the corresponding *trans* isomer, although it could be expected to elute later than the *cis* isomer if the pattern observed for *trans* and *cis p*-coumaric acid was maintained. The fact that both compounds eluted close to each other might explain the interchange in their elution order, although we cannot discard that a different hexosyl substituent could exist in each compound, either. Thus, the compound was tentatively identified as *trans p*-coumaroyl hexoside. Furthermore, peak 10 with MS^2 fragments at *m/z* 193 (-162 mu; [ferulic acid-H]$^-$) and 176 ([ferulic acid-H-H_2O]$^-$) was tentatively assigned as ferulic acid glucoside. The MS^2 fragmentation of compound 2 presented a base peak corresponding to the ion at *m/z* 163, corresponding to *p*-coumaric acid. The observation of a loss of 162 mu (hexosyl moiety), the base peak at *m/z* 163 ([coumaric acid-H]$^-$) and the presence of the ion at *m/z* 325 (coumaroyl hexose) in the MS^2 fragmentation of the compound pointed to that it could be a derivative of a *p*-coumaroyl hexose. Furthermore, the UV spectra showing λ_{max} at 300 nm, as mentioned above, suggested it as a possible *cis* isomer.

Compound 15 presented a pseudomolecular ion [M-H]$^-$ at *m/z* 359, yielding MS^2 fragments at *m/z* 197 (loss of a hexosyl moiety; [syringic acid-H]$^-$) and 153 (base peak; [syringic acid-H-CO_2]$^-$), suggesting that it could be a syringic acid hexoside. The UV spectrum with λ_{max} at 274 nm was also coherent with a syringic acid derivative. Compound 14 presented similar UV spectrum and more 44 mu (CO_2, carboxyl moiety) than compound 15. The observation of an MS^2 base peak at *m/z* 197 ([syringic acid-H]$^-$) and another fragment at *m/z* 241 from the loss of a hexose pointed to it was a syringic acid hexoside derivative, although no final structure could be assigned.

Table 47 Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and concentration of phenolic acids and flavonoids in four different tomato Portuguese farmer' varieties.

Peak	Compounds identification					Concentration of the identified compounds (μg/g fw)			
	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Amarelo	Batateiro	Comprido	Coração
1	5.93	286/320sh	431	341(2), 269(38), 179(4), 161(52), 113(15)	Benzyl alcohol dihexose**	12.03 ± 0.84^a	7.46 ± 0.28^b	2.37 ± 0.07^c	0.84 ± 0.03^d
2	6.24	300	651	489(5), 325(4), 205(2), 163(100), 119(72)	*cis p*-Coumaric acid derivative	17.96 ± 1.21^a	7.34 ± 2.19^b	0.68 ± 0.02^c	0.40 ± 0.01^c
3	6.48	328	341	179(100), 161(58), 135(57)	Caffeic acid hexoside I	nd	nd	1.29 ± 0.01^a	0.76 ± 0.10^b
4	6.80	258	411	249(24), 161(24), 113(7)	(Iso)pentyl dihexose	1.97 ± 0.06^a	1.48 ± 0.27^b	0.22 ± 0.00^c	0.01 ± 0.00^c
5	7.39	320	341	179(100), 161(13), 135(62)	Caffeic acid hexoside II	6.57 ± 0.15^a	5.21 ± 1.63^a	0.53 ± 0.03^b	0.25 ± 0.03^b
6	7.52	314	353	191(47)179(65), 173(100), 161(6), 135(45)	4-*O*-Caffeolyquinic acid**	4.38 ± 0.21^a	3.29 ± 0.91^a	3.81 ± 0.77^a	0.81 ± 0.19^b
7	8.16	326	353	191(100), 179(11), 173(7), 161(15), 135(6)	5-*O*-Caffeolyquinic acid	3.83 ±0.34^a	1.92 ± 0.22^b	0.20 ± 0.03^c	0.03 ± 0.00^c
8	8.44	316	325	163(40), 145(100),119(26)	*trans p*-Coumaric acid hexoside	3.90 ± 0.08^a	1.60 ± 0.51^b	0.02 ± 0.00^c	0.02 ± 0.00^c
9	8.77	300	325	163(29), 145(100),119(17)	*cis p*-Coumaric acid hexoside	0.61 ± 0.05^a	0.44 ± 0.14^b	0.16 ± 0.01^c	0.04 ± 0.00^c
10	9.97	330	355	193(30), 175(100), 161(43), 135*	Ferulic acid hexoside	0.97 ± 0.07^a	0.27 ± 0.06^b	0.04 ± 0.00^c	0.03 ± 0.00^c
11	11.00	312	337	191*, 173(100), 163(20), 155(8), 137(8)	4-*O*-*p*-Coumarolyquinic acid	0.04 ± 0.00^a	0.01 ± 0.00^b	0.01 ± 0.00^b	tr
12	11.45	328	179	135(100)	Caffeic acid	0.46 ± 0.07^a	0.20 ± 0.06^b	0.03 ± 0.00^c	0.02 ± 0.00^c
13	13.16	312	337	191(100), 173(12), 163(16), 155*	5-*O*-*p*-Coumarolyquinic acid	0.41 ± 0.08^a	0.33 ± 0.00^b	0.02 ± 0.00^c	0.01 ± 0.00^c
14	13.75	274	403	241(80), 197(100), 179(10), 137(10)	Syringic acid hexoside derivative	0.35 ± 0.00^a	0.83 ± 0.00^b	0.17 ± 0.01^c	0.11 ± 0.03^d
15	15.75	274	359	197(34),153(100), 135(8)	Syringic acid hexoside	1.11 ± 0.01^a	1.21 ± 0.01^b	0.62 ± 0.08^c	0.39 ± 0.03^d
16	17.08	352	741	609*, 301(28)	Quercetin pentosylrutinoside	4.76 ± 0.11^a	2.81 ± 0.05^b	0.34 ± 0.07^c	0.60 ± 0.02^d
17	17.26	312	163	119(100)	*trans*-*p*-Coumaric acid	2.70 ± 0.10^a	0.67 ± 0.04^b	0.13 ± 0.01^c	0.09 ± 0.00^c
18	19.32	330	609	301(100)	Quercetin-3-*O*-rutinoside	4.68 ± 0.49^a	2.62 ± 0.80^b	0.39 ± 0.06^c	0.09 ± 0.00^c
19	19.50	334	725	593*, 285(23)	Kaempferol pentosylrutinoside	1.25 ± 0.11^a	0.57 ± 0.14^b	0.03 ± 0.01^c	0.04 ± 0.00^c
20	22.79	318	593	285(100)	Kaempferol-3-*O*-rutinoside	0.24 ± 0.02^a	0.10 ± 0.03^b	0.05 ± 0.00^c	0.03 ± 0.00^c
					Total phenolic acids	43.30 ± 2.03^a	23.32 ± 1.25^b	7.69 ± 0.70^c	2.96 ± 0.27^d
					Total flavonoids	10.93 ± 0.52^a	6.10 ± 1.01^b	0.81 ± 0.13^c	0.76 ± 0.02^c
					Total phenolic compounds	54.23 ± 2.55^a	29.42 ± 2.26^b	8.50 ± 0.58^c	3.72 ± 0.25^d
					Total non-phenolic compounds	14.00 ± 0.90^a	8.94 ± 0.55^b	2.59 ± 0.07^c	0.85 ± 0.04^d

Figures in brackets after MS^2 fragment ions refer to their relative abundances. *Relative abundance < 2%. nd: not detected; tr: traces. **Concentrations of compound 1 and 4 were expressed as equivalents of caffeic acid and syringic acid, respectively. In each row different letters mean significant differences ($p<0.05$).

Peaks 1 and 4 presented pseudomolecular ions identical to two non-phenolic compounds reported by Gomez-Romero *et al.* (2010) to occur in tomato samples, *i.e.,* benzyl alcohol dihexose and (iso)pentyl dihexose. Those authors did not present a fragmentation pattern for the first one, but the fragmentation pattern reported for (iso)pentyl dihexose was similar to the one obtained in our study. Furthermore, the early retention time and the elution order of both compounds was coherent with the proposed identities. Therefore, compounds 1 and 4 were tentatively assigned as benzyl alcohol dihexose and (iso)pentyl dihexose, respectively.

The rest of detected compounds (peaks 16, 18, 19 and 20) were identified as flavonol derivatives derived from kaempferol and quercetin. Compounds 18 and 20 were positively identified as quecetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside, respectively, by comparison of MS fragmentation pattern and UV spectra with authentic standards. Compound 16 and 19 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 741 and 725, and similar MS^2 fragmentation patterns releasing two fragments from the successive losses of pentosyl ($[M-H-132]^-$; *m/z* at 609 and 593, respectively) and rutinosyl moieties ($[M-H-132-308]^-$; *m/z* at 301 and 285). Thus, these compounds were tentatively identified as quercetin pentosylrutinoside and kaempferol pentosylrutinoside, respectively.

Yellow tomato (Amarelo) was the variety that presented the highest levels of phenolic compounds (54.23 µg/g fw) followed by round tomato (Batateiro, 29.42 µg/g), long tomato (Comprido, 8.50 µg/g) and heart tomato (Coração, 3.72 µg/g) (**Table 47**). Phenolic acids were the most abundant group, being compound 2 (*cis p*-coumaric acid derivative) predominating in Amarelo and Batateiro tomato varieties, and 4-*O*-caffeolyquinic acid the most abundant compound in Comprido and Coração varieties. The non-phenolic compound, benzyl alcohol dihexose, was also predominant in all tomato varieties. The most abundant flavonoid was quercetin pentosylrutinoside in all the studied tomato varieties.

According to literature, chlorogenic acid (*i.e.,* 5-*O*-caffeoylquinic acid) was the main phenolic compound in tomato and the most extensively studied (Periago *et al.* 2002, Niggeweg *et al.* 2004, Slimestad *et al.* 2008, Vallverdú-Queralt *et al.* 2010), whereas flavonoids are represented by flavanones (naringenin glycosylated derivatives) and flavonols (quercetin, rutin and kaempferol glycosylated derivatives) (Le Gall *et al.* 2003, Bahorun *et al.* 2004, Slimestad *et al.* 2008, Gómez-Romero *et al.* 2010). In the samples studied herein, main phenolics also corresponded to hydroxycinnamoyl derivatives, although 5-*O*-caffeoylquinic acid was not the majority compound; furthermore, neither naringenin nor naringenin glycosylated derivatives were found; which can be interpreted as composition characteristics of the studied tomato samples, as related to genetic features, cultivation conditions, and/or handling and storage methods associated to each sample (Davies and Hobson 1981, Dumas *et al.* 2003). In fact, phenolic compounds have been reported as

cultivar- and variety-distinguishing factors in some plant products (Klepacka *et al.* 2011), being dependent on genotype and environmental factors (Beato *et al.* 2011).

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5.3. Optimization of microwave-assisted extraction of hydrophilic and lipophilic antioxidants from a surplus tomato crop by response surface methodology

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Optimization of microwave-assisted extraction of hydrophilic and lipophilic antioxidants from a surplus tomato crop by response surface methodology

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Abstract

Tomato is the second most important vegetable crop worldwide and a rich source of industrially interesting antioxidants. Hence, the microwave-assisted extraction of hydrophilic (*H*) and lipophilic (*L*) antioxidants from a surplus tomato crop was optimized using response surface methodology. The relevant independent variables were temperature (*T*), extraction time (*t*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*). The concentration-time

response methods of crocin and β-carotene bleaching were applied, since they are suitable *in vitro* assays to evaluate the antioxidant activity of *H* and *L* matrices, respectively. The optimum operating conditions that maximized the extraction were as follows: *t*, 2.25 min; *T*, 149.2 ºC; *Et*, 99.1 %; and *S/L*, 45.0 g/L for *H* antioxidants; and *t*, 15.4 min; *T*, 60.0 ºC; *Et*, 33.0 %; and *S/L*, 15.0 g/L for *L* antioxidants. This industrial approach indicated that surplus tomatoes possess a high content of antioxidants, offering an alternative source for obtaining natural value-added compounds. Additionally, by testing the relationship between the polarity of the extraction solvent and the antioxidant activity of the extracts in *H* and *L* media (polarity-activity relationship), useful information for the study of complex natural extracts containing components with variable degrees of polarity was obtained.

Keywords: *Lycopersicon esculentum*; microwave-assisted extraction; β-carotene/crocin bleaching inhibition assay; concentration-time response modelling; response surface methodology.

5.3.1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop worldwide after potato and is consumed either fresh or in the form of processed products. In 2013, about 164 million of tones were produced in the world, having been registered an increase of 2.6 million of tones over 2012 (FAOSTAT 2015). Apart from the large amounts of solid wastes produced by the processing industry, sometimes there is also a surplus production that leads to glut in the market, distress sale and low profit to the growers (Oliveira 2006, Sashimatsung *et al.* 2011). One solution for the problem of this glut may be its sustainable use for the recovery of value-added antioxidant compounds with applications in food, pharmaceutical and cosmeceutical industries. In fact, tomato is a rich source of hydrophilic and lipophilic antioxidants (Pinela *et al.* 2012). The hydrophilic fraction is constituted mainly by ascorbic acid and soluble phenolic compounds, while the lipophilic fraction contains carotenoids (mostly lycopene), tocopherols, sterols and lipophilic phenolics. Each of these compounds has their own function in the human organism, acting at different locations, but also working conjunctly, having the ability to offer protection against oxidative stress and various degenerative diseases (Carocho and Ferreira 2013a, 2013b). Besides, according to some reports, antioxidants belonging to the hydrophilic fraction have a far more significant impact on total antioxidant activity than does antioxidants of the lipophilic fraction (Kotíková *et al.* 2011, García-Valverde *et al.* 2013).

The antioxidant activity can be monitored using a large variety of assays, each one based on a specific mechanism of action, including hydrogen atom transfer, single electron transfer, reducing power, and metal chelation, among others (Carocho and Ferreira 2013a,

Shahidi and Zhong 2015). For this reason, it is important to understand the mechanisms behind the selected assay for a suitable evaluation of the antioxidant potential. Crocin and β-carotene bleaching reactions are two *in vitro* assays appropriate for the antioxidant activity evaluation of hydrophilic (*H*) and lipophilic (*L*) matrices, respectively, and can provide useful information in the study of complex natural extracts containing components with variable degrees of polarity (Prieto *et al.* 2013, Prieto and Vázquez 2014). Both assays are reproducible, especially accurate, and yields a low experimental error (Prieto, Murado, *et al.* 2014).

To recover antioxidants from plant-based products is necessary to follow suitable extraction methods that ensure and preserve its integrity and bioactivity. That's why the industry is looking for more efficient processes based on enhanced innovation capacity. Among them, microwave-assisted extraction (MAE) has gained significance due to its shortened extraction time, higher extraction rate, reduced solvent consumption and superior product's quality at lower cost (Gallo *et al.* 2010, Dahmoune *et al.* 2015), being one of the dominant trends of the "green chemistry" movement (Michel *et al.*, 2011). However, the extraction process efficiency depends on some variables and operating conditions (Bhuyan *et al.* 2015, Dahmoune *et al.* 2015), which may not be generalized for all plant materials due to the diverse nature of existing bioactive phytochemicals. Therefore, selection and optimization of variables and operating conditions for the MAE of antioxidants from tomato is necessary.

One-factor-at-a-time approaches are commonly used to optimize extraction processes; but it is well-known that optimal operating conditions or interactions between variables cannot be predicted with this methodology. Both problems may be overcome by employing the response surface methodology (RSM), a powerful statistical tool used to predict the optimum experimental conditions to maximize or minimize various independent variables. Indeed, RSM describes the relationship between independent variables and one or more responses, enabling process optimization such as the extraction of bioactive molecules from natural sources with a reduced number of experimental trials.

This study aimed at determining the optimal extraction conditions for *H* and *L* antioxidants from a tomato surplus. Four independent variables (temperature, extraction time, ethanol concentration and solid/liquid ratio) were studied and the extraction process was optimized by RSM. The concentration-time response methods of β-carotene and crocin bleaching were applied, which are appropriate for the evaluation of antioxidant properties of *L* and *H* fractions, respectively.

5.3.2. Material and methods

5.3.2.1. Equipment and reagents

Equipments: Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) using closed high precision glass vials. Multiskan Spectrum Microplate Photometer using 96-well polypropylene microplates.

Reagents: Linoleic acid (CID 5280450); β-Carotene (CID 5280489); Crocin (CID 5281233); 2,2'-Azobis(2-amidinopropane) (AAPH or ABAP, CID 1969). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

5.3.2.2. Plant material

A common tomato farmers' variety known as "tomate redondo or batateiro" (round tomato), and widely cultivated in rural communities from Miranda do Douro, North-eastern Portugal, was chosen for this study. Surplus tomatoes at the ripe stage were hand-harvested randomly from the middle of six plants, in selected homegardens of two villages in the studied area. The ripening stage was established according to local consumers' criteria based in morphological descriptors such as size, texture, and pericarp colour. Six tomatoes (pericarps without jointed pedicels and seeds) were frozen and lyophilized (Free Zone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) using a grinding machine and kept at -20 °C until analysis.

5.3.2.3. Microwave-assisted extraction of H and L antioxidants

The MAE process was performed using a Biotage Initiator Microwave apparatus in closed vials. The dried powdered samples were extracted at different time (*t*), temperature (*T*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) ranging as defined by the RSM design (**Figure 41**). The solvent volume was fixed at 20 mL. During extraction, samples were stirred at 600 rpm using a magnetic stirring bar and irradiated at 200 W. After that, the reaction mixture in the closed vial was quickly cooled in the processing chamber and then centrifuged at 6000 rpm for 10 min. The pellet was discarded and the supernatant was carefully collected, evaporated under reduced pressure to remove the solvent and finally re-suspended in distilled water for further analysis. The dry weight (dw) of the suspended solids in the supernatant of each solution was determined to calculate the extraction yield (g extract/g sample).

5.3.2.4. Determination of the concentration-time dependency of L and H antioxidants

β-Carotene (Marco 1968) and crocin (Bors *et al.* 1984) methods (βCM and CM, respectively) are widely used to evaluate the antioxidant activity of different matrices. Both *in vitro* assays share some analytical similarities as depicted in the next points.

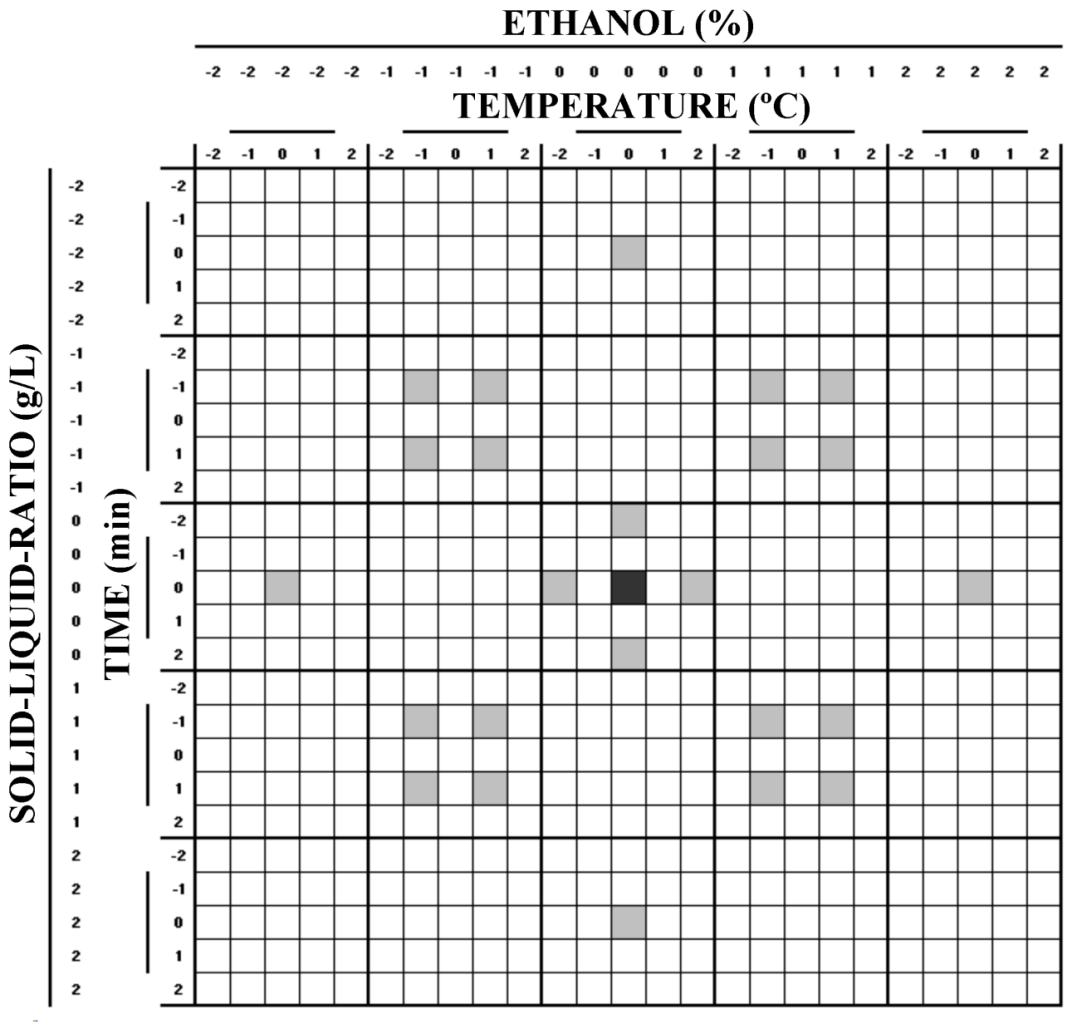

Figure 41 Visual representation of the applied experimental RSM design. Four independent variables (extraction time (X_1), temperature (X_2), ethanol concentration (X_3), and solid/liquid ratio (X_4)) were combined in a five-level full factorial design of 25 independent variable combinations (grey grid) and 7 replicates in the centre of the experimental domain (dark grid). Coded values (-2, -1, 0, +1, +2) are in natural values X_1 (*t, min*: 0, 5, 10, 15, 20), X_2 (*T*, ºC: 60, 90, 120, 150, 180), X_3 (*Et*, %: 0, 25, 50, 75, 100) and X_4 (*S/L*, g/L: 5, 15, 25, 35, 45).

Reaction conditions

βCM conditions (Prieto *et al.* 2012): 2 mg of β-carotene (βC, 1 μM in the final reaction), 0.25 mL of linoleic acid and 2 g of Tween-40 were dissolved in 20 mL of chloroform, vigorously mixed, followed by chloroform evaporation (45 °C/~15 min). To the resulting oily residue were added 300 mL of buffered Mili-Q water (100 mM Briton, pH=6.5) at 45 °C. The absorbance at 470 nm of the prepared reagent was ~1.40.

CM conditions (Prieto, Vázquez, *et al.* 2015): 4 mg of crocin (Cr, 100 µM in the final reaction) and 75 mg of AAPH (7.68 mM in the final reaction) were dissolved in 30 mL of a 100 mM Briton buffer, pH=5.5, in Mili-Q water. The absorbance at 450 nm of the prepared reagent was ~1.40.

Procedure

The procedure was performed by adding 50 µL of sample extract and 250 µL of reagent into the wells (350 µL) of the microplate. The microplate-reader was programmed at intervals of 3, 5 and 10 min (initiation, propagation and asymptotic phases), during a period of 200 min (total of 30 measures). The sample extracts were analysed kinetically for eight different concentrations obtained by serial dilution (1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and the control) in distilled water.

Quantification

The area under the curve (*AUC*) (Eq. 1), computed by any numerical integration method such as the trapezoidal rule, proved to be a highly robust criterion, able to summarize in a single and direct value the global feature of any kinetic profile.

$$AUC = \frac{R_1 \Delta t_1}{2} + \sum_{i=2}^{i=n-1} R_i \Delta t_i + \frac{R_n \Delta t_n}{2} \quad (1)$$

where *i* is the number of data measured over time *t*, R_i are the responses along an arbitrary time series, and Δt is the interval of each measurement.

The *AUC* of a concentration-response of an antioxidant was normalized against to the *AUC* obtained with the control, leading to the formulation of the relative area units or protected substrate ($\bar{P}$) in percentage (Eq. 2), as defined similarly by other authors (Naguib 2000, Huang *et al.* 2002, Dávalos 2004) for antioxidant responses.

$$\bar{P}(A) = S_0 \left(\frac{AUC_C - AUC_A}{AUC_C} \right) \frac{100}{S_0} \quad (2)$$

where AUC_C and AUC_A are the area units corresponding to the kinetic profiles found in the absence (control, *C*) and presence of an antioxidant concentration *A*, respectively, and S_0 is the initial substrate in the reaction (for the CM, the substrate is equivalent to 100 µM of Cr, and for the βCM it is equivalent to 1 µM of βC).

The relationship in Eq. (2) establishes that AUC_C (control) is also the maximum response achievable; consequently, the values obtained were also standardized in percentage. In addition, by normalizing the response, the results obtained are less dependent on the experimental conditions, which, in practice, is one of the common problems when analyzing the efficacy of response factors.

The asymptotic variation of $\bar{P}$ as function of an antioxidant compound suggests that some radical-generating property of the system can be saturated (Gieseg and Esterbauer 1994). This type of concentration-response patterns should behave in a similarly accumulative way with a number of different antioxidant compounds found in the extract material. Therefore, in general, this patterns can be adjusted by a group of mathematical expressions (mechanistic or not) that translates the pattern of the response into parameters that allow to deduce the meaning and/or quantify the effect of the dependent variable in a simple and global mode. Previous researchers discussed the applicability of different mathematical expressions (Prieto, Vázquez, *et al.* 2014); therefore, following their views, the Weibull cumulative distribution function was selected (Weibull and Sweden 1951). Thus, the variation of $\bar{P}$ as function of increasing concentrations of an antioxidant (*A*) can be described satisfactorily using the Weibull model rearranged for our own purposes as follows in Eq. (3).

$$\bar{P}(t,A) = P_m \left\{ 1 - \exp\left[-\ln(2)^{1-a} \left(\frac{2V_m}{P_m a} A \right)^a \right] \right\} \quad (3)$$

The parameter P_m is the averaged maximum protected substrate, asymptotic value of the response (% μM of βC or Cr), which is specific of each *A* agent. The parameter V_m corresponds to the average amount of protected molecules per gram of extracted material (% μM of protected substrate/g extract). The parameter *a* is a shape parameter related to the slope that can produce potential profiles (*a*<1), first order kinetic ones (*a*=1) and a variety of sigmoidal profiles (*a*>1).

In addition, the concentration needed to reach 50% of the maximum protective effect (the so called IC_{50}) can be determined according to Eq. (4).

$$IC_{50} = \frac{Ka \ln 2}{2V_m} \text{ ; therefore } \bar{P}(t,A) = P_m \left\{ 1 - \exp\left[-\ln(2)\left(t/IC_{50}\right)^a \right] \right\} \quad (4)$$

where IC_{50} is the concentration producing the half-maximal response and all other notations remain with the same meaning as above.

5.3.2.5. Response surface methodology

The RSM family designs are used for modelling and analyzing problems in which a response of interest is influenced by a set of variables. RSM was applied to optimize the MAE process with the purpose of finding the favourable processing conditions that would result in a higher extraction rate of *H* or *L* antioxidants.

Response criteria for evaluating the antioxidant capacity

The responses used in the RSM analysis were based in the numerical values of the parametric coefficients P_m, V_m and IC_{50} of Eqs. (3) and (4). The information provided by the combination of the values of the three response criteria represents a robust tool to compare the activity of different antioxidant agents based on the parametric concentration-time estimations.

Preliminary experiments

Preliminary single-factor experiments were conducted in order to select the significant variables and/or collateral factors in extraction process and to determine the preliminary range of the optimum level of each factor for an appropriate experimental RSM design. In this primary screening trial, the following variables and factors were considered:

- Internal independent variables of the microwave equipment: Pressure (1-30 bar), stirring rate (0-1000 rpm), microwave power (0-400 W), temperature (40-300 ºC) and extraction time (no limits).
- Internal factors of the instrument software: Absorption level (*very low*, *low*, *normal*, *high*, or *very high*), fixed hold time (if *on* the time countdown starts when the target temperature or pressure is reached, *i.e.,* the initial time taken to reach the set conditions is not included in the heating time; if *off* the time countdown starts when the heating starts), cooling (*on* or *off*), pre-stirring (during the fixed hold time, if selected; *on* or *off*) and vial type (2-5 mL or 10-20 mL).
- External independent variables and factors: Solid/liquid ratio and ethanol concentration. The type of solvent used in the extraction.

Experimental design

From the preliminary study, the independent variables X_1 (extraction time, min), X_2 (temperature, ºC), X_3 (ethanol concentration, %) and X_4 (solid/liquid ratio, g/L) were selected. Then, the combined effects of these variables on the extraction yield of *H* and *L* antioxidant were studied using *central composite design* as proposed by Box and Hunter (1957). In this design, the points of experiments are generated on a sphere around the centre point. The centre point is supposed to be an optimum position for the response and is repeated in order to maximize the prediction precision (Box *et al.* 2005). This design also requires five levels for each factor. The number of repetitions n_0 of the centre point is calculated by the formulas present in Eq. (5) for *k* factors based on the uniform precision.

$$\gamma = \frac{(k+3)+\sqrt{9k^2+14k-7}}{4(k+2)} \quad ; \quad \text{where:} \quad n_0 = floor\left(\gamma\left(\sqrt{2^k}+2\right)^2 - 2^k - 2k\right) \qquad (5)$$

where *floor* designates the highest integer value smaller than the argument. The number of experiments *n* for *k* factors is given as:

$$n = 2^k + 2k + 1 \quad (6)$$

Experimental runs were randomized, to minimize the effects of unexpected variability in the observed responses. Independent variables coded values and natural ones of the factorial design are coded and decoded by the expressions in Eq. (7).

$$v_c = \left(v_n - v_0\right) / \Delta v_n \quad \text{and} \quad v_n = v_0 + \Delta v_n \times v_c \quad (7)$$

where v_n and v_c is the natural (*n*) and coded (*c*) value in the centre of the experimental domain, v_0 is the initial value and Δv_n is the increment of v_n for unit of v_c.

Box-Behnken mathematical model

Response surface models were fitted by means of least-squares calculation using the following Box-Behnken equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (8)$$

where *Y* is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and *n* is the number of variables. As pointed out, three different response formats based in the parametric estimations (P_m, V_m and IC_{50}) of Eqs. (3) and (4) were used as the dependent variable for each *H* and *L* antioxidant analytical reaction (Y^H_{Pm}; $Y^H_{V\tau}$; $Y^H_{IC_{50}}$; Y^L_{Pm}; $Y^L_{V\tau}$; and $Y^L_{IC_{50}}$).

5.3.2.6. Numerical methods and statistical analysis

All fitting procedures, coefficient estimates and statistical calculations were performed on a Microsoft Excel spreadsheet. Fitting and statistical analysis of the experimental results to the proposed equations were carried out in four phases:

1) Coefficients determination: Parametric estimates were obtained by minimization of the sum of the quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* 2003 (Kemmer and Keller 2010), which allows a quick testing of a hypotheses and its consequences (Murado and Prieto 2013).

2) Coefficients significance: The determination of the parametric confidence intervals was calculated using the '*SolverAid*' (Prikler 2009). The model was simplified by dropping terms, which were not statistically significant *p*-value > 0.05.

3) Model consistency: The Fisher *F*-test (α=0.05) was used to determine whether the constructed models were adequate to describe the observed data (Shi and Tsai 2002).
4) Other statistical assessment criteria: To re-check the uniformity of the model the following criteria were applied: a) The 'SolverStat' macro was used for the assessment of the parameter and model prediction uncertainties (Comuzzi *et al.* 2003); b) The R^2 was interpreted as the proportion of variability of the dependent variable explained by the model; c) The adjusted coefficient of determination (R^2_{adj}) was a correction to R^2 taking into account the number of variables used in the model; d) Bias and accuracy factors of all equations were calculated to evaluate the fittings to experimental data, such as the Mean Squared Error (MSE), the Root Mean Square of the Errors (RMSE) and the Mean Absolute Percentage Error (MAPE); e) The Durbin-Watson coefficient (DW) was used to check if the residuals of the model are not autocorrelated; and f) The analysis of variance table (ANOVA) was used to evaluate the explanatory power of the variables.

5.3.3. Results and discussion

5.3.3.1. Preliminary study

As listed in the material and methods section, the MAE efficiency may be affected by five internal (pressure, stirring rate, microwave power, extraction time, and temperature) and two external (solid/liquid ratio and ethanol concentration) independent variables and five instrument/software factors (absorption level, fixed hold time, vial type, cooling option, and solvent type). Although there are previous research examples (Bhuyan *et al.* 2015, Dahmoune *et al.* 2015), the results are not generalizable for all plant materials due to the diverse nature of existing bioactive phytochemicals. Therefore, this preliminary study allowed screening of appropriate independent variables and determining their optimum experimental domain for an appropriate experimental RSM design. Variables/factors were investigated by testing a broad range, keeping other ones constant and analyzing their antioxidant responses.

- Type of extracting solvent is the key for separating *H* and *L* compounds. Water is the polar solvent with greater interest in biological processes than any other solvent. Ethanol has a polar hydroxyl group and dissolves many ionic compounds, but also has a non-polar end, which will contribute to dissolve non-polar substances. In this study, binary interactions of ethanol-water mixtures were selected due to their straight *H* affinity, but also because ethanol increases the *L* character of the aqueous-ethanolic mixture. All the tested ethanol concentrations give rise to significant differences; thus, the range from 0 to 100 % was selected.
- Pressure and temperature were correlated. The selected irradiation power was applied in the early stage of the extraction process to reach the selected

temperature/pressure in a short period of time. After that, it was automatically applied in less intensity (estimated by the microwave system) to keep constant the solution temperature/pressure. In consequence, the microwaves power was set at 200 W and the temperature was selected as the main controlled variable, since relevant differences were found within the range 60 to 180 ºC.

- Lower solid/liquid ratios can lead to a more efficient dissolution of constituents, but also to a waste of solvent. At an industrial scale, higher ratios are desirable since it is important to maximize the extraction yield (thus productivity) with a minimal solvent consumption (more sustainable process). Significant differences were found for all tested ratios in this preliminary study, being the range from 5 to 45 g/L selected for RSM analysis.
- The temperature caused strong decomposition phases of antioxidants for extraction times higher than 20 min, but this effect depended on the other variables that remained constant. Therefore, extraction times ranging from 0 to 20 min were selected.
- The cooling option showed relevant effects. It is used at the end of the MAE process to cool the sample. When *off*, the cooling time of the solution after processing was longer, affecting the extraction of antioxidants. Therefore, it was used *on* to quickly cool the solution and stop faster the extraction process, making the process more accurate.
- When testing the effects of other factors, such as the absorption level, fixed hold time and vial type, no significant changes were found. Therefore, a *normal* absorption level and vials of 10-20 mL were selected for further analysis. The fixed hold time was turned *off*, since the initial time taken to reach the set temperature or pressure was negligible.

Therefore, the RSM experiment was designed based on these preliminary results, using five variation levels of extraction time (0-20 min), temperature (60-180 ºC), ethanol concentration (0-100%) and solid/liquid ratio (5-45 g/L) as independent variables to optimize efficiently the MAE process, regarding the *H* and *L* antioxidant properties of the extracts. The coded values and their natural values are presented in **Figure 41**. Note that, for simplification reasons, the RSM design reduces the number of experimental trials. When studying 5 levels of 4 independent variables, the response would imply 625 possible combinations, but using RSM the experiment could be solved in 25 independent combinations and 7 replicates at the centre of the experimental domain.

5.3.3.2. Concentration-time antioxidant responses for RSM

Several reviews have discussed the numerous *in vitro* methods developed to evaluate the antioxidant activity of plant extracts and their controversial aspects regarding differences in the generated radicals, variables (mainly pH and temperature), reagents, and quantification procedures (Frankel and Meyer 2000, Jiménez-Escrig *et al.* 2000). However, when determining the bioactivity of a sample, the final activity response also depends on the degree of polarity of the intermediate reaction components of the applied method. For example, when evaluating the bioactivity of extracts obtained with *H* and *L* extraction solvents using the oxidative hemolysis inhibition assay (OxHLIA) (method in which the thermal decomposition of AAPH generates *H* radicals and the lipid peroxidation of the erythrocytes membranes generates *L* radicals (Niki *et al.* 1988), no clear conclusions can be made because both *H* or *L* antioxidants delay the haemolysis and, therefore, produce an antioxidant response. To the best of our knowledge only few articles have addressed the *H* and *L* intermediate components activity (Arnao *et al.* 2001, Prior *et al.* 2003, Prieto *et al.* 2013). Therefore, the lack of selective methods to differentiate the activity of *H* and *L* antioxidants is a current issue in the evaluation of antioxidant responses that need to be outlined in the following years.

In order to reduce the variability of experimental conditions, allowing meaningful comparisons, and to quantify the power of the antioxidants in function of the degree of polarity, the response models of CM and βCM were selected because they provide a microsystem for *H* and *L* oxidation processes, respectively (Prieto *et al.* 2013). The βC is an *L* oxidizable substrate that can join the system of lipid micelles in which the oxidation reaction is accomplished. The method is especially sensitive to antioxidants in a lipidic environment, producing a very low response to *H* antioxidants, even to powerful ones. In turn, Cr is an *H* oxidizable substrate and *L* antioxidants produce very low responses in the reaction system.

Figure 42 and **Figure 43** show an illustration of the antioxidant responses obtained for the tomato extracts produced under the experimental RSM design presented in **Figure 41** for each *H* and *L* antioxidant reaction (CM and βCM), respectively. In both figures, two well differentiated sections are presented at the left- and right-hand sides, showing the visual variable distribution of the 25 genuine combinations. The left-hand side shows the combinations of the concentration-time responses of seven serial dilutions (○:1/1, ▲: 1/2, △: 1/4, ■: 1/8, □: 1/16, ◆: 1/32, ◇: 1/64) and the control (●) for the remaining substrates (% μM Cr and βC). Meanwhile, the right-hand side shows the concentration-time transformation into the concentration-response values of the *AUC* computed by the numerical integration method in Eq. (1) and standardized in percentage of protected substrate (Cr or βC) by Eq. (2). The dots (●) are the raw values and lines (—) the fitted responses to the mathematical model of Eq. (3) or (4). The parametric fitting values of Eqs. (3) and (4) are presented in

Table 48. The estimated numerical values of P_m, V_m and IC_{50} were the three meaningful ways considered to evaluate the effectiveness of the antioxidant response by RSM.

5.3.3.3. Development of the theoretical response surface models and statistical verification

Table 48 shows the results of the parametric fitting coefficients (data presented in **Figure 42** and **Figure 43**) for each *H* and *L* reaction obtained after running 32 trials (25 genuine combinations and 7 replicates) following the experimental RSM design. Estimated coefficient values of Eq. (8), parametric intervals and numerical statistical criteria are shown in **Table 49**, for each coefficient used as response criteria and for *H* and *L* reactions. The coefficients that showed effects with *p-values* higher than 0.05 are not significant (*ns*) at the 95% confidence level and consequently were discarded for model development.

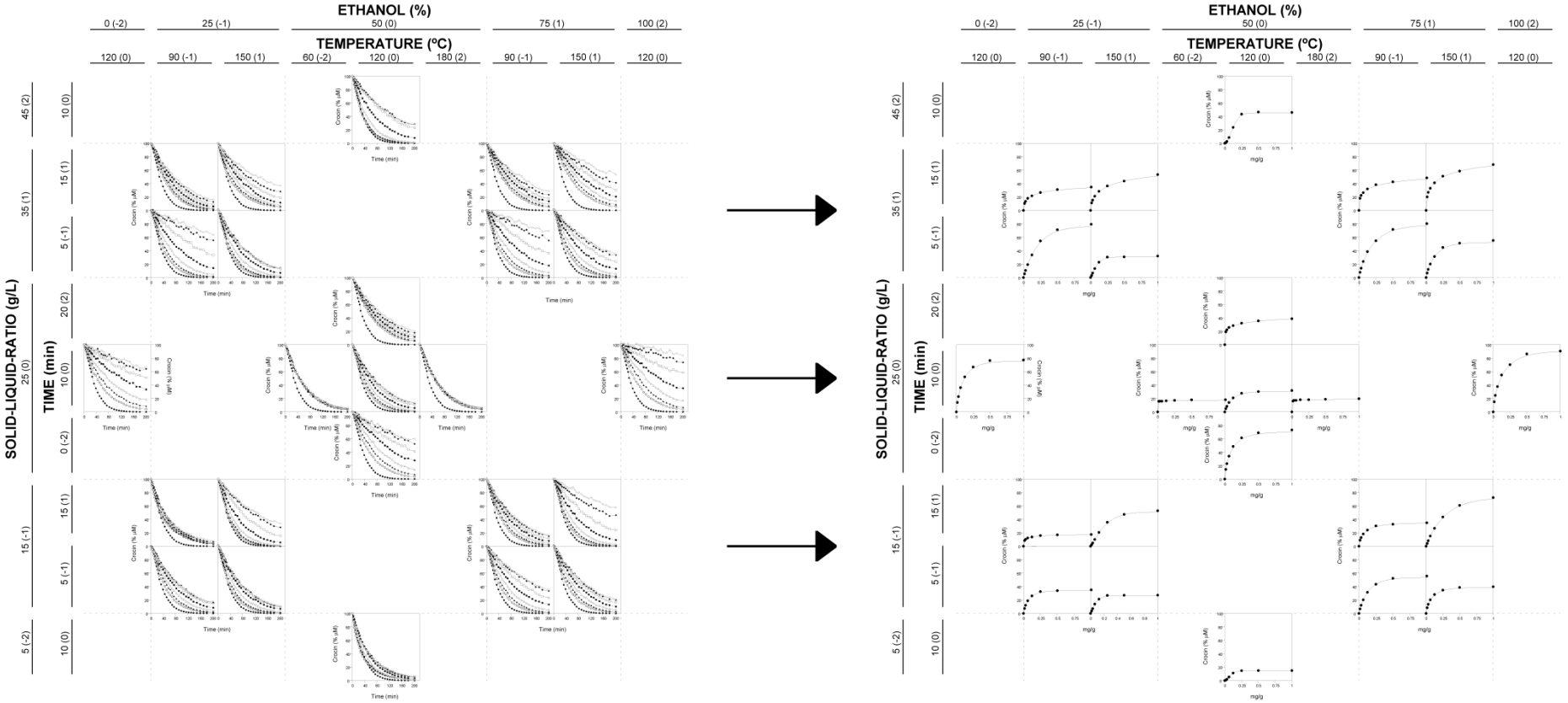

Figure 42 Illustration of the *H* responses obtained for the CM under the experimental RSM design presented in **Figure 41**. On the left-hand side, each graph illustrates one of the 25 independent variable combinations and inside each graph can be seem the concentration-time responses of seven serial dilutions (○:1/1, ▲: 1/2, △: 1/4, ■: 1/8, □: 1/16, ◆: 1/32, ◇: 1/64) and the control (●) of the extracted material. On the right-hand side, each graph shows: 1) Dots (●), which represents the standardized $\overline{P}$ (protected percentage of Cr) values in a concentration-response format obtained by applying Eq. (2) to the concentration-time responses presented in the left-hand side; and 2) Lines (—), fitted responses to the mathematical model of Eq. (3). The obtained parametric fitting values are presented in **Table 48**.

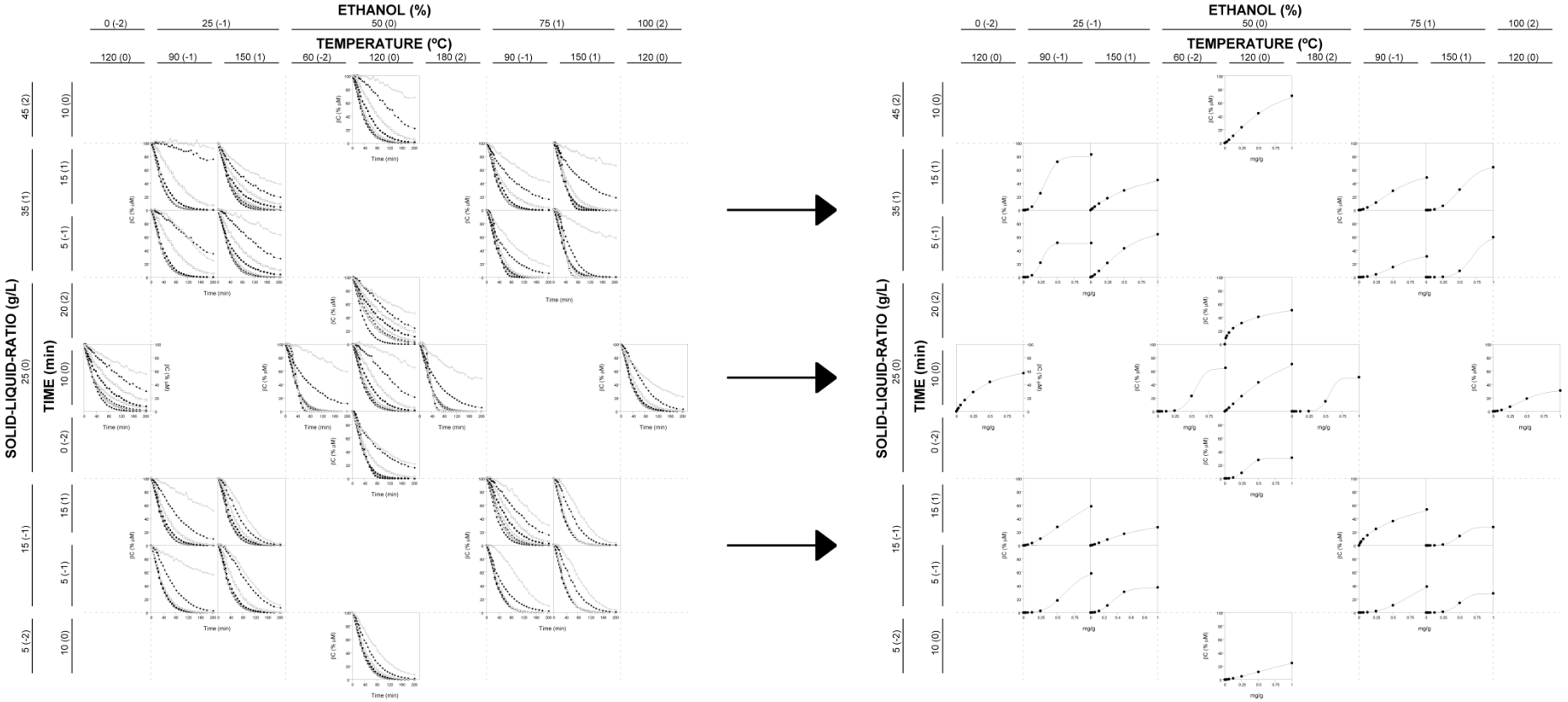

Figure 43 Illustration of the *L* responses obtained for the βCM under the experimental RSM design presented in **Figure 41**. On the left-hand side, each graph illustrates one of the 25 independent variable combinations and inside each graph can be seem the concentration-time responses of seven serial dilutions (○:1/1, ▲: 1/2, △: 1/4, ■: 1/8, □: 1/16, ◆: 1/32, ◇: 1/64) and the control (●) of the extracted material. On the right-hand side, each graph shows: 1) Dots (●), which represents the standardized $\bar{P}$ (protected percentage of βC) values in a concentration-response format obtained by applying Eq. (2) to the concentration-time responses presented in the left-hand side; and 2) Lines (—), fitted responses to the mathematical model of Eq. (3). The obtained parametric fitting values are presented in **Table 48**.

Table 48 Estimated numerical values of the parameters (P_m, V_m and IC_{50}) of Eqs. (3) and (4), after fitting the concentration-response values presented in the right-hand side of **Figure 42** and **Figure 43** (for CM and βCM, respectively) of the tomato extracts obtained under the experimental RSM design presented in **Figure 41**.

Run	Experimental domain				Parametric antioxidant responses								Statistics	
					CM (hydrophilic reaction)				βCM (lipophilic reaction)				R^2_{adj}	
	X_1: t	X_2: T	X_3: Et	X_4: S/L	P_m	IC_{50}	a	v_m	P_m	IC_{50}	a	v_m	CM	βCM
	min	°C	%	g/L	% μM Cr	g extract	--	% μM Cr/g extract	% μM βC	g extract	--	% μM βC/g extract		
1	-1(5)	-1(90)	-1(25)	-1(15)	34.08±0.47	0.17±0.01	0.84±0.14	58.24±4.75	59.64±1.52	1.88±0.17	3.06±0.32	33.69±1.39	0.9884	0.9569
2	1(15)	-1(90)	-1(25)	-1(15)	17.50±1.42	0.06±0.00	0.42±0.03	44.24±2.52	94.39±2.35	2.43±0.22	1.56±0.13	20.99±3.93	0.9567	0.9906
3	-1(5)	1(150)	-1(25)	-1(15)	26.63±1.81	0.19±0.02	1.16±0.03	56.07±0.36	36.28±1.70	1.02±0.03	2.35±0.00	28.87±4.35	0.9587	0.9727
4	1(15)	1(150)	-1(25)	-1(15)	51.12±3.09	0.51±0.04	1.18±0.16	41.33±4.78	31.79±1.05	1.41±0.04	1.25±0.04	9.79±1.25	0.9760	0.9896
5	-1(5)	-1(90)	1(75)	-1(15)	53.46±0.31	0.29±0.03	0.90±0.01	57.66±6.18	58.77±1.60	2.56±0.05	2.38±0.41	18.96±1.15	0.9744	0.9641
6	1(15)	-1(90)	1(75)	-1(15)	35.08±3.07	0.18±0.02	0.64±0.11	43.38±0.02	86.41±3.34	2.09±0.02	0.75±0.15	10.73±1.64	0.9706	0.9766
7	-1(5)	1(150)	1(75)	-1(15)	38.52±1.12	0.19±0.00	0.80±0.09	57.32±9.27	28.00±1.27	1.50±0.07	4.02±0.09	26.00±1.72	0.9549	0.9827
8	1(15)	1(150)	1(75)	-1(15)	72.90±5.31	0.59±0.01	0.98±0.09	41.77±4.87	27.31±1.19	1.50±0.10	3.81±0.23	24.01±4.50	0.9752	0.9847
9	-1(5)	-1(90)	-1(25)	1(35)	76.55±3.65	1.06±0.04	0.99±0.09	24.92±4.07	49.85±2.10	1.90±0.14	3.22±0.25	29.27±4.71	0.9726	0.9654
10	1(15)	-1(90)	-1(25)	1(35)	37.83±2.73	0.59±0.02	0.47±0.01	10.34±1.78	79.68±2.70	2.24±0.16	2.52±0.03	31.09±4.89	0.9921	0.9787
11	-1(5)	1(150)	-1(25)	1(35)	30.50±2.12	0.53±0.03	1.23±0.18	24.75±1.05	69.58±3.48	2.84±0.09	1.36±0.19	11.52±1.89	0.9828	0.9787
12	1(15)	1(150)	-1(25)	1(35)	61.51±5.82	1.18±0.12	0.53±0.09	9.55±1.57	57.48±3.64	3.44±0.06	0.98±0.07	5.70±0.25	0.9787	0.9531
13	-1(5)	-1(90)	1(75)	1(35)	78.81±2.66	0.97±0.07	0.88±0.13	24.92±4.79	33.10±2.59	3.81±0.27	2.15±0.08	6.48±0.24	0.9886	0.9919
14	1(15)	-1(90)	1(75)	1(35)	58.61±2.29	0.68±0.05	0.36±0.06	10.65±0.27	53.69±1.90	3.42±0.09	1.63±0.20	8.87±0.38	0.9677	0.9802
15	-1(5)	1(150)	1(75)	1(35)	52.40±4.11	0.67±0.04	0.91±0.02	24.62±1.73	57.62±5.40	4.76±0.10	4.54±0.18	19.03±3.35	0.9621	0.9740
16	1(15)	1(150)	1(75)	1(35)	80.03±7.95	0.82±0.01	0.44±0.01	14.79±2.66	64.42±4.82	3.65±0.14	2.65±0.26	16.21±1.22	0.9884	0.9920
17	-2(0)	0(120)	0(50)	0(25)	70.12±4.78	0.35±0.04	0.77±0.15	53.75±2.98	30.00±2.69	1.65±0.03	2.85±0.31	17.97±0.72	0.9742	0.9818
18	2(20)	0(120)	0(50)	0(25)	51.04±1.47	0.37±0.03	0.27±0.03	12.73±1.61	75.06±5.76	2.01±0.15	0.52±0.08	6.74±1.17	0.9840	0.9787
19	0(10)	-2(60)	0(50)	0(25)	32.22±2.89	0.48±0.03	0.05±0.01	1.20±0.10	63.15±4.18	2.77±0.05	4.68±0.57	36.97±5.78	0.9570	0.9858
20	0(10)	2(180)	0(50)	0(25)	34.89±0.90	0.63±0.04	0.06±0.01	1.15±0.15	49.13±3.50	2.78±0.11	6.15±0.14	37.60±1.55	0.9832	0.9728

Run	Experimental domain				Parametric antioxidant responses								Statistics	
					CM (hydrophilic reaction)				βCM (lipophilic reaction)				R^2_{adj}	
	X_1: t	X_2: T	X_3: Et	X_4: S/L	P_m	IC_{50}	a	v_m	P_m	IC_{50}	a	v_m	CM	βCM
	min	ºC	%	g/L	% μM Cr	g extract	--	% μM Cr/g extract	% μM βC	g extract	--	% μM βC/g extract		
21	0(10)	0(120)	-2(0)	0(25)	75.52±6.22	0.35±0.03	0.82±0.10	60.82±6.17	64.81±5.05	1.57±0.05	0.94±0.09	13.44±0.53	0.9586	0.9685
22	0(10)	0(120)	2(100)	0(25)	91.54±3.46	0.47±0.02	0.75±0.06	50.61±1.06	31.65±0.18	2.21±0.04	1.90±0.18	9.46±1.02	0.9710	0.9626
23	0(10)	0(120)	0(50)	-2(5)	14.31±0.87	0.08±0.01	1.67±0.29	104.79±7.95	41.18±3.07	0.84±0.08	1.44±0.03	24.35±0.89	0.9569	0.9628
24	0(10)	0(120)	0(50)	2(45)	45.20±0.57	1.11±0.03	1.82±0.33	25.84±0.25	80.94±0.64	4.13±0.51	1.23±0.04	8.37±0.10	0.9551	0.9755
25	0(10)	0(120)	0(50)	0(25)	30.39±1.56	0.38±0.04	0.91±0.02	25.43±0.89	97.33±6.29	3.03±0.31	1.11±0.18	12.35±0.56	0.9728	0.9592
26	0(10)	0(120)	0(50)	0(25)	30.39±2.04	0.38±0.02	0.91±0.00	25.43±4.30	97.33±8.12	3.13±0.11	1.26±0.11	13.63±1.08	0.9585	0.9870
27	0(10)	0(120)	0(50)	0(25)	30.39±0.50	0.38±0.04	0.91±0.01	25.43±2.09	97.33±2.22	3.05±0.16	1.14±0.14	12.58±1.01	0.9700	0.9843
28	0(10)	0(120)	0(50)	0(25)	30.39±1.15	0.38±0.03	0.91±0.03	25.43±0.14	97.33±4.47	3.05±0.16	1.12±0.01	12.34±0.38	0.9637	0.9834
29	0(10)	0(120)	0(50)	0(25)	30.39±1.50	0.38±0.01	0.91±0.08	25.43±0.27	97.33±0.94	3.04±0.28	1.10±0.15	12.21±1.68	0.9725	0.9575
30	0(10)	0(120)	0(50)	0(25)	30.39±2.12	0.38±0.04	0.91±0.11	25.43±5.02	97.33±3.58	2.80±0.28	1.08±0.04	13.03±0.14	0.9888	0.9782
31	0(10)	0(120)	0(50)	0(25)	30.39±1.83	0.38±0.02	0.91±0.00	25.43±2.82	87.46±3.60	2.66±0.30	1.43±0.17	16.30±2.64	0.9886	0.9823
32	0(10)	0(120)	0(50)	0(25)	30.39±0.57	0.38±0.01	0.91±0.13	25.43±1.54	97.33±8.41	3.00±0.18	1.16±0.19	13.02±2.59	0.9827	0.9906

Table 49 Estimated coefficient values of Eq. (8), parametric intervals and numerical statistical criteria for each parametric response criteria of the *H* and *L* reactions.

		Hydrophilic reaction			Lipophilic reaction		
		P_m	IC_{50}	V_m	P_m	IC_{50}	V_m
Fitting coefficients obtained from Eq. (8) and showed in Eqs. (9)-(14)							
Intercept	b_0	30.50±2.74	0.40±0.05	26.49±3.10	96.10±0.05	2.97±0.37	13.09±0.01
Linear effect	b_1	ns	ns	-8.10±1.79	8.02±0.05	ns	-2.87±0.01
	b_2	ns	0.04±0.04	ns	-7.13±0.03	ns	ns
	b_3	8.70±2.09	0.27±0.04	-17.22±1.79	5.10±0.03	0.76±0.17	-3.20±0.01
	b_4	6.92±2.09	ns	ns	-5.65±0.04	0.31±0.11	-2.02±0.01
Quadratic effect	b_{11}	7.29±1.87	ns	ns	-10.72±0.07	-0.24±0.10	ns
	b_{22}	ns	0.05±0.03	-6.96±1.79	-9.82±0.03	ns	5.94±0.01
	b_{33}	ns	0.06±0.03	9.08±1.79	-8.59±0.06	-0.07±0.03	ns
	b_{44}	13.02±1.87	ns	6.68±1.61	-11.80±0.08	-0.22±0.10	ns
Interactive effect	b_{12}	13.21±2.56	0.16±0.05	ns	-7.70±0.03	ns	ns
	b_{13}	ns	ns	ns	ns	ns	2.35±0.02
	b_{14}	ns	ns	ns	ns	-0.24±0.02	1.57±0.02
	b_{23}	-4.78±2.56	-0.05±0.05	ns	13.04±0.02	0.43±0.12	-1.72±0.02
	b_{24}	ns	ns	ns	ns	ns	6.21±0.02
	b_{34}	ns	ns	ns	ns	0.27±0.07	ns
Statistical information of the fitting analysis							
Observations		32	32	32	32	32	32
R^2		0.9526	0.9236	0.9743	0.9422	0.9437	0.9223
R^2adj		0.9331	0.9136	0.9612	0.9067	0.9058	0.9019
MSE		767.23	0.15	854.45	1127.20	1.50	152.70
RMSE		27.70	0.38	29.23	33.57	1.22	12.35
MAPE		5.99	12.63	22.22	8.14	7.97	9.72
DW		2.39	2.20	1.43	2.32	1.36	2.32

ns: no significant coefficient; R^2: Correlation coefficient; R^2adj: The adjusted coefficient of determination for the model; MSE: The mean squared error; RMSE: The root mean square of the errors; MAPE: The mean absolute percentage error; and DW: The Durbin-Watson statistic.

Mathematical models were built through nonlinear least-squares estimations based on the coded experimental plan and the response results, obtaining the following second-order polynomial Eq. (8):

when the hydrophilic *CM* was considered:

$$Y_{Pm}^{H} = 30.5 + 8.7x_3 + 6.9x_4 + 7.3x_1^2 + 13.1x_4^2 + 13.1x_1x_2 - 4.5x_2x_3 \quad (9)$$

$$Y_{V\tau}^{H} = 26.5 - 8.1x_1 - 17.2x_3 - 6.9x_2^2 + 9.1x_3^2 + 7.0x_4^2 \quad (10)$$

$$Y_{IC_{50}}^{H} = 0.40 + 0.04x_2 + 0.27x_3 + 0.05x_2^2 + 0.05x_3^2 + 0.16x_1x_2 - 0.05x_2x_3 \quad (11)$$

when the lipophilic *βCM* was considered:

$$Y_{Pm}^{L} = 96.1 + 8.1x_1 - 7.1x_2 + 5.1x_3 - 5.6x_4 - 10.7x_1^2 - 9.8x_2^2 - 8.6x_3^2 - 11.8x_4^2 - 7.7x_1x_2 + 13.1x_2x_3 \quad (12)$$

$$Y_{Vr}^{L} = 13.1 - 2.9x_1 - 3.2x_3 - 2.0x_4 + 5.9x_2^2 + 2.3x_1x_3 + 1.6x_1x_4 - 1.7x_2x_3 + 6.2x_2x_4 \quad (13)$$

$$Y_{IC_{50}}^{L} = 2.97 + 0.76x_3 + 0.31x_4 - 0.24x_1^2 - 0.07x_3^2 - 0.22x_4^2 - 0.24x_1x_4 + 0.43x_2x_3 + 0.27x_3x_4 \quad (14)$$

where X_1 (extraction time), X_2 (temperature), X_3 (ethanol concentration), X_4 (solid/liquid ratio), Y is the response, sub-indices indicates the coefficient criteria (P_m, V_m and IC_{50}) used as responses for RSM and super-indices *H* and *L* accounts for the *H* (CM) and *L* (βCM) reactions.

The multivariable characterization of the Box-Behnken second-order polynomial model is especially robust, minimizing the experimental errors, allowing explain the utmost of the results. In addition, once a model is designed, if the experimental data obtained do not span the full range and some of them fail to provide information about one or more of the parameters of the equation, the multivariable application describes simply and accurately all the areas. As explained, not all the parameters of Eq. (8) were used for building the model, since some terms were non-significant (**Table 49**). Model coefficients obtained are empirical and cannot be associated with physical or chemical significance. However, they are useful to predict the results of untested operation conditions (Ranic *et al.* 2014). The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level and when a factor has a negative effect, the response is lower at high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable. Based in the mathematical expressions, it was found that the responses in the *L* environment were much more complex than those found in the *H* one.

The statistic lack of fit, used to test the adequacy of the obtained models, demonstrated that no considerable improvement was achieved by the inclusion of the statistically non-significant effects (**Table 49**). This was also verified by the high R^2 and R^2_{adj} values indicating the percentage of variability of each response that is explained by the model (**Table 49**). The distribution of residuals always randomly scattered around zero and grouped data and autocorrelations were not observed. This means that these models are workable and can be applied in the subsequent prediction and optimization stages. Finally, the analysis of variance (ANOVA) was computed for the regression equations. The lack of fit was used to verify the adequacy of the model and was not significant ($p > 0.05$), indicating that Eqs. (12) to (14) adequately fit the experimental data.

5.3.3.4. Effect of Et and T variables as representative case of the typical H and L trends

The three response criteria (P_m, V_m and IC_{50}) characterize singular features of the response. Previous to the complete analysis of the *H* and *L* antioxidant extraction trends, the information provided by each parametric response criteria, which were used in the RSM design, was individually analysed. As an illustrative case study, it was selected the effect of the variables *Et* and *T*, meanwhile the variables *t* and *S/L* were positioned at the centre of their experimental domain (*t*=10 min and *S/L*=25 g/L). Graphical 3D representations are displayed in **Figure 44** and the parametric fitting values are present in **Table 49**. In general, it can be observed that the *H* and *L* antioxidant activity of the tomato extracts have opposite trends for *Et* and *T*. In more specific terms, for each criterion it can be concluded that:

a) The parameter P_m of Eq. (3) shows the maximum specific capability of the antioxidant agent to protect the substrate (% µM of Cr or βC) and, the higher the P_m value, the more powerful the protective capability of the antioxidant. In general, we can speculate that the more complex the content in antioxidant molecules in the extract (which act at different *H* or *L* oxidation levels), higher the parameter P_m. These types of extracts are usually obtained with longer extraction times. The conditions that favour the *H* activity of the P_m value were at high ranges (↑) of *Et* and low ranges (↓) of *T* or, in a much less active manner, at ↓ *Et* and ↑ *T*. In contrast, the *L* activity was found at intermediate ranges (↔) of *Et* and *T*, leading to a clear optimum at 50 % *Et* and 120 ºC. The inversion effect of the polarity-activity relationship proposed by the polar paradox theory is visible in these results (Porter, 1993). Actually, the speculated effect of the non-polar end of ethanol on the activity of the tomato extracts was not observed; they even showed an improvement of the *H* antioxidant activity at ↑ *Et*, while the *L* antioxidant activity decreased sharply at both ends of *Et*.
b) The parameter V_m of Eq. (3) corresponds to the average amount of protected molecules of Cr or βC per gram of extracted material (% µM of protected substrate/g extract), which is a value of maximal predictability. The higher the V_m value, the more powerful the antioxidant. There are a diverse number of compounds that would present a high specific protection, but only few would be present in an enough amount to show its activity. Therefore, the highest values should appear when an extraction peak of an antioxidant with a high specific protection would be found. The conditions that maximize the V_m response of the *H* activity were at ↓ *Et* and ↔ *T*; while for the *L* activity were found at ↓ *Et* and ↑ *T*. The effect of the inversion of the polar activity on the optimal response was not as evident as for the parameter P_m, but the opposite trends remain present as can be seen in each 3D surface.

c) The parameter IC_{50} of Eq. (4) provides directly the classical IC_{50} (g of extract), which will effectively summarize all effects of the other two responses. It provides the amount of extract needed to achieve a very specific response (50%). The lower the IC_{50} value, the more powerful the antioxidant. The lowest values should be found in an intermediate position between those speculated in the previous criteria. For the IC_{50} criteria, the conditions that maximize the response for the *H* antioxidant activity were at ↓ *Et* and ↓ *T*, while for the *L* activity were at ↓ *Et* and ↑ *T*.

The data published in literature often focus on only one response parameter, but each of them describes different intrinsic characteristics of the response. The information provided by the combination of the three values represents a robust tool to compare the activities of different antioxidant agents based on the parametric concentration-time estimations. By analyzing all parametric nonlinear values for the experimental RSM design, a more rigorous evaluation of the extraction efficiency of *H* and *L* antioxidants is accomplished.

5.3.3.5. Nonlinear relationship between extraction solvent polarity and antioxidant activity

Matrix combination of the 3D responses for the *H* and *L* environmental reactions obtained for the P_m, V_m and IC_{50} are presented in **Figure 45**, **Figure 46** and **Figure 47**, respectively. In addition, a simplified way to present the results in a 2D format for all responses is presented in **Annexe 9**. Eqs. (12) to (14) were used to simulate the surfaces. In each graphical illustration, the top diagonal part presents the response surfaces for *L* reactions and the bottom diagonal part presents the response surfaces for *H* reactions. The variables excluded in each 3D graph were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L).

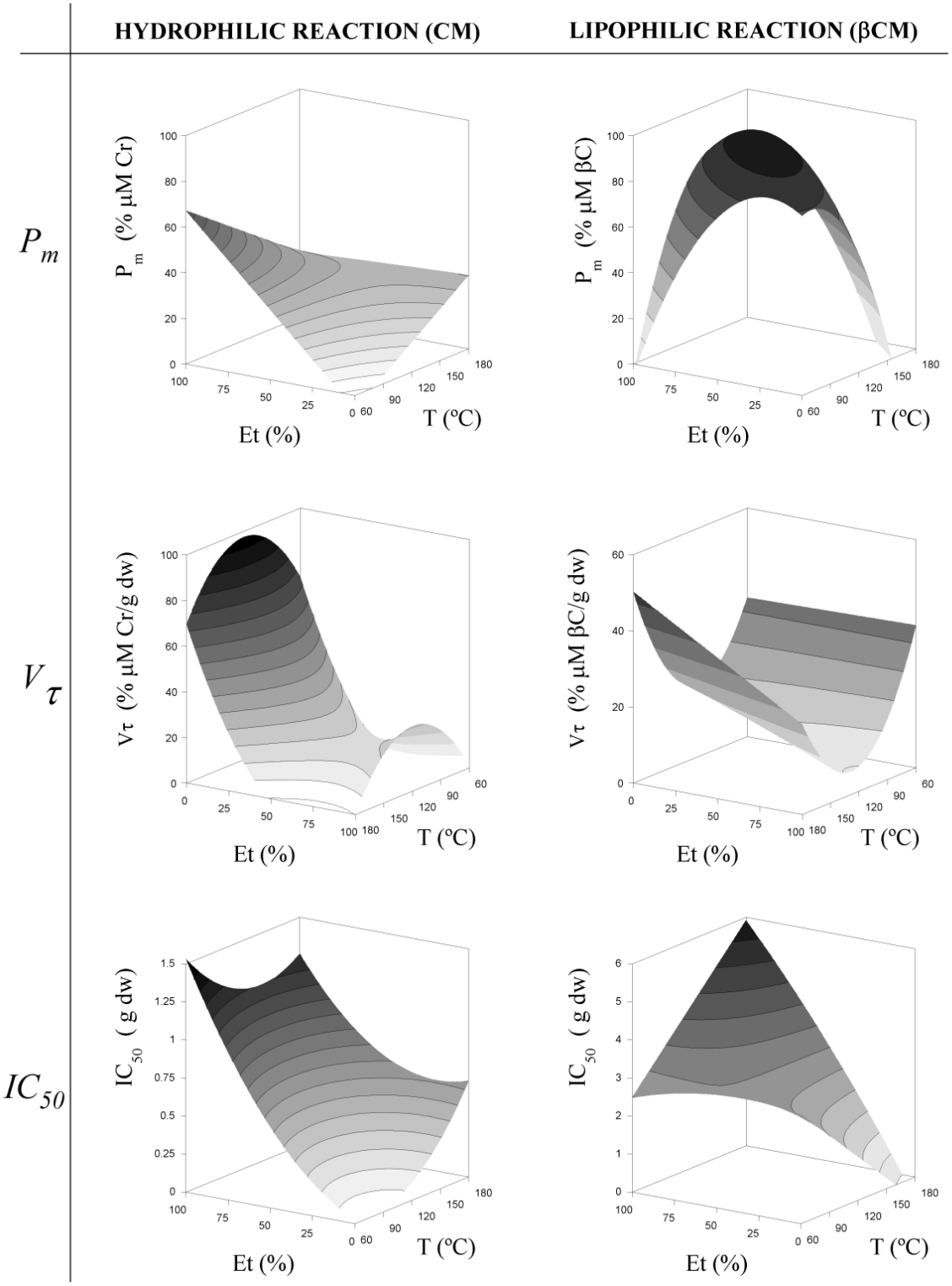

Figure 44 Response surfaces of the effects of ethanol concentration (*Et*) *vs* temperature (*T*) on *H* and *L* antioxidant reactions for the parametric response criteria P_m, V_m and IC_{50}. For representation purposes, the variables *t* and *S/L* were positioned at the centre of their experimental domain (*t*=10 min and *S/L*=25 g/L). The obtained parametric fitting values are presented in **Table 49**.

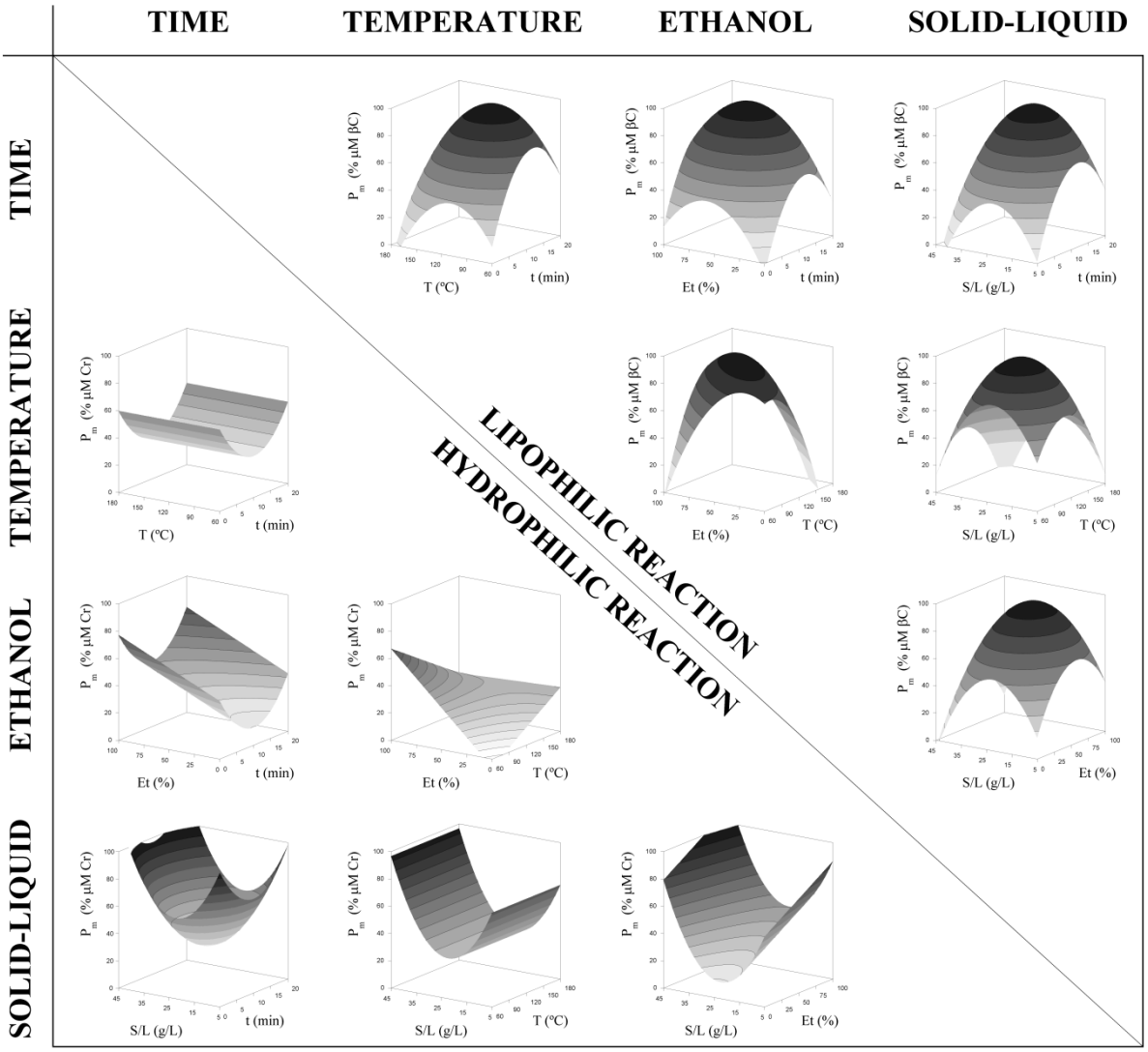

Figure 45 Matrix combination of the response surfaces of the *H* and *L* antioxidant reactions obtained for the parametric coefficient P_m (% µM of Cr or βC), which is organized as follows: a) in the top diagonal part is presented the response surface of the *L* reaction; and b) in the bottom diagonal part is presented the response surface of the *H* reaction. For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The obtained parametric fitting values are presented in **Table 49**.

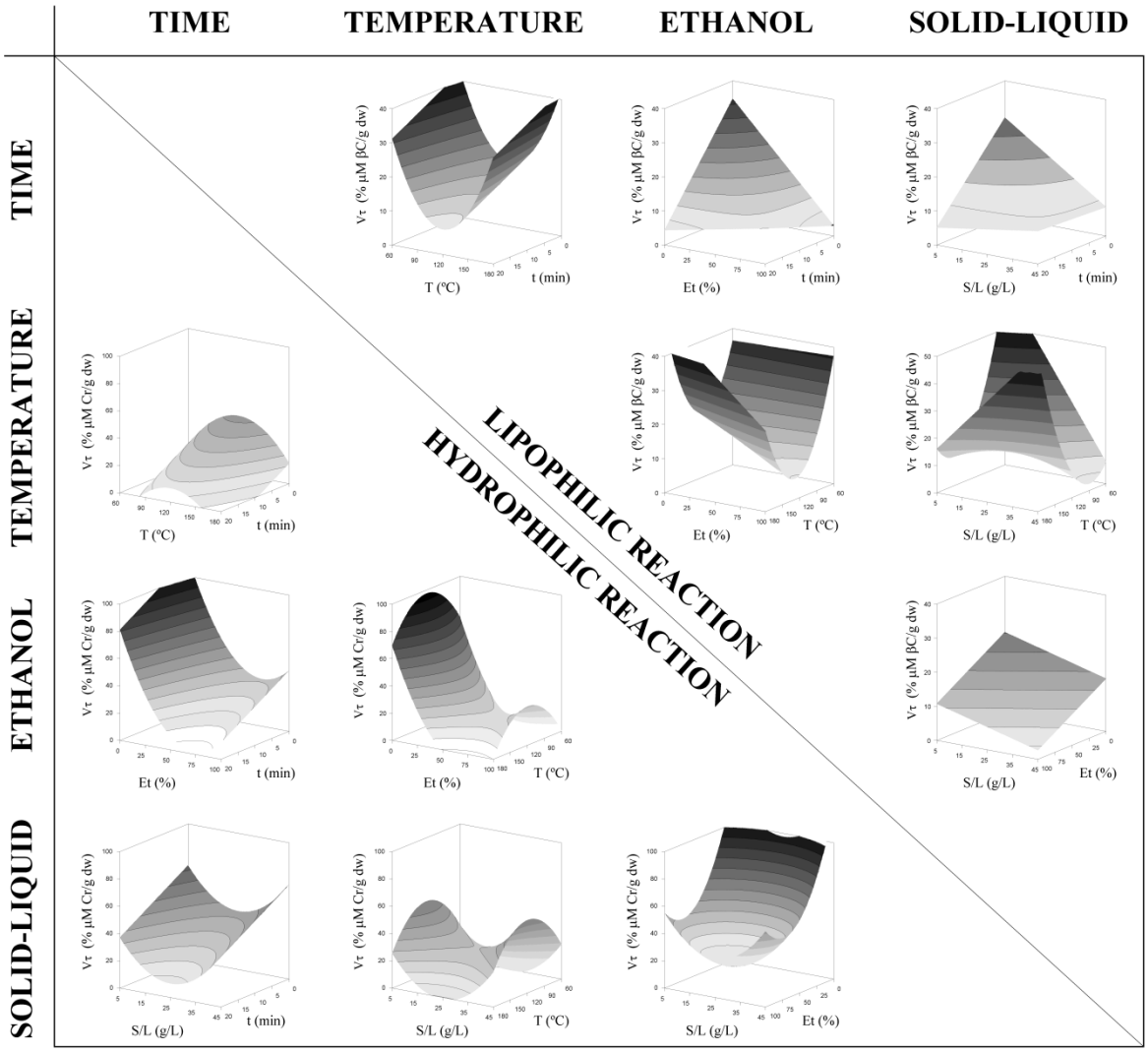

Figure 46 Matrix combination of the response surfaces of the *H* and *L* antioxidant reactions obtained for the parametric coefficient V_m (% µM of Cr or βC/g extract), which is organized as follows: a) in the top diagonal part is presented the response surface of the *L* reaction; and b) in the bottom diagonal part is presented the response surface of the *H* reaction. For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The obtained parametric fitting values are presented in **Table 49**.

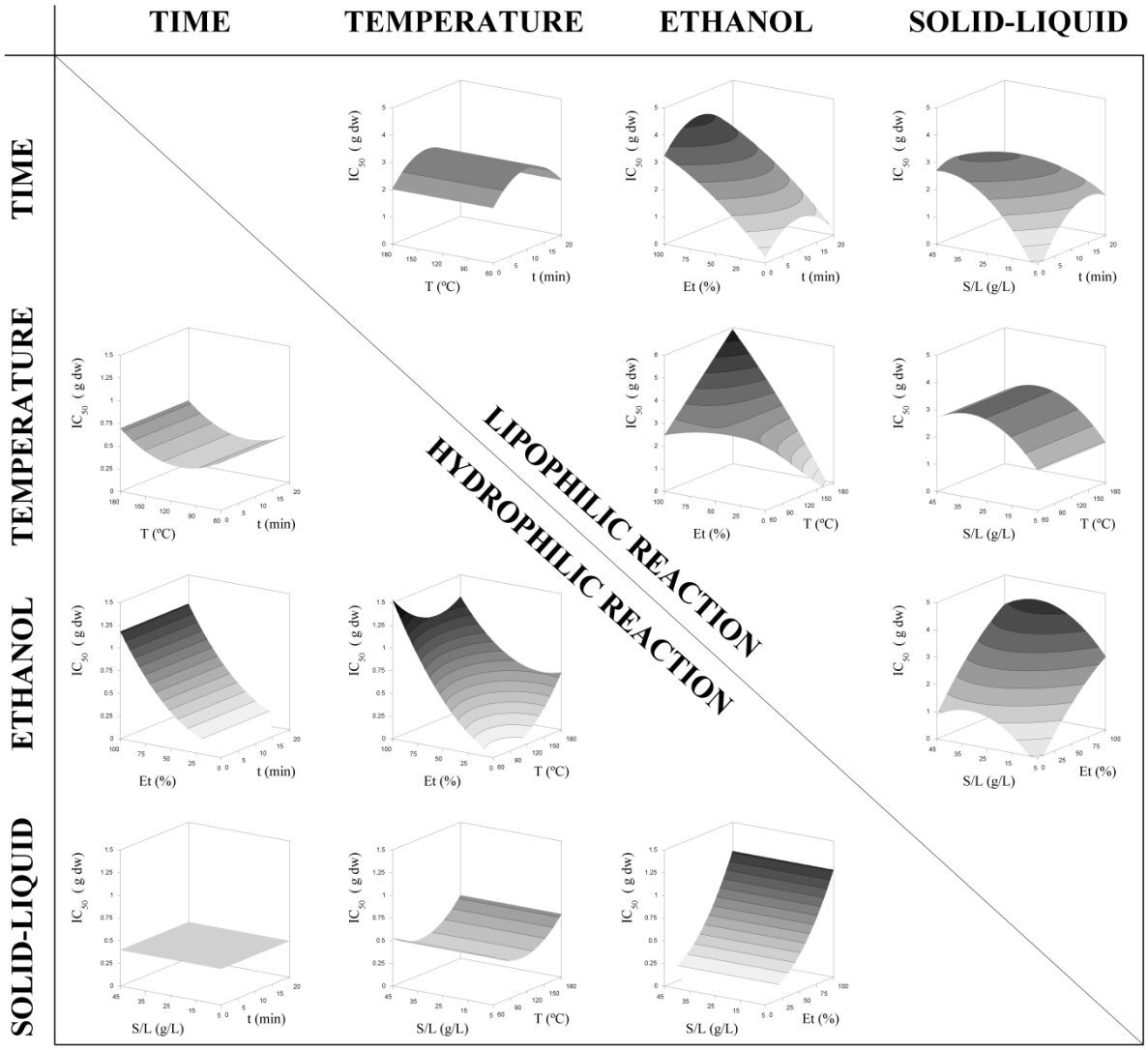

Figure 47 Matrix combination of the response surfaces of the *H* and *L* antioxidant reactions obtained for the parametric coefficient IC_{50} (g extract), which is organized as follows: a) in the top diagonal part is presented the response surface of the *L* reaction; and b) in the bottom diagonal part is presented the response surface of the *H* reaction. For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The obtained parametric fitting values are presented in **Table 49**.

In general, the inversion effect of the polarity-activity relationship was observed in almost all responses. The effects accounted between the *t*, *T* and *S/L* variables would describe the conditions that optimize the *H* and *L* antioxidant responses of the tomato extracts. However, the variable *Et* did not perform as theoretically expect. Such fuzziness between the polarity of extraction solvent and the antioxidant activity of the extracts in *H* and *L* environments (the so-called polarity-activity relationship) was found interesting enough to be considered. Generally, the extraction ability of solvents can be grouped in three main types: non-polar, polar aprotic and polar protic solvents (Kislik 2011, Huffman *et al.* 2012). The choice of the extracting solvent is the first crucial step towards the optimization of any extraction method (Sultana *et al.* 2009), which has a strong impact on the type of molecules

that would be separated. In turn, antioxidants are classified into two broad divisions (Arnao *et al.* 2001), depending on whether they are soluble in water (*H*, such as ascorbic acid) or in lipids (*L*, such as α-tocopherol). When performing an extraction, it is well known that *L* antioxidant molecules are mostly extracted in non-polar solvents (*i.e., n*-hexane) and *H* antioxidant molecules in polar ones (*i.e.,* water), according to the "like dissolves like" principle, as confirmed by several authors that separated effectively the molecular *H* and *L* character of molecules by applying different solvent combinations in conjunction with different extraction procedures (Watanabe *et al.* 2014). However, based on the achieved results, the separation of extracts according to their molecular polarity character does not guarantee that their polar or non-polar target activity can be separated as well. As stated before (Prieto *et al.* 2013), when testing the activity of *H* and *L* antioxidant extracts (hexane and methanol solvents, respectively), it was confirmed that *H* and *L* antioxidant extracts, normally in a much lesser extent, remain active in the opposite environment. In addition to that complex scenery, there are amphiphilic molecules presenting an affinity with solvents of various polarities (Taresco *et al.* 2015).

Thus, if their polarity-activity is not totally related with their distribution in the extracting solvents as defined by the polarity index (*i.e.,* dielectric constant), we may be using extracts for *L* environments (*i.e.,* oils) with a high content in molecules with a *H* antioxidant activity and vice versa. Nonetheless, it is recognized that antioxidants with a clear *H* and *L* character can cause the opposite effect when applied in the opposite environment (*i.e.,* ascorbic acid can initiate lipid oxidation in conjunction with metal cations) (Zhang and Omaye 2001). Actually, according to the polar paradox theory (Porter, 1993), polar antioxidants are more effective in less polar media, while non-polar or amphiphilic antioxidants tend to be more effective in a media of relatively higher polarity. The higher efficiency of *L* antioxidants in oil-in-water emulsions would be due to their tendency to concentrate at the interfacial membrane where the oxidation is supposed to occur, while more *H* antioxidants would tend to segregate into the aqueous phase where they would be much less effective (Frankel *et al.*, 1994). Our results support this phenomenon.

A possible hypothetical foundation behind the mechanisms that caused this effect could be the microwave absorbing properties of the solvent (Dahmoune *et al.* 2015). Polar molecules strongly absorb microwave energy because of the permanent dipole moment, and the degree of absorption increases with the dielectric constant. A simple comparison between water and ethanol shows that ethanol has a lesser ability to obstruct the microwaves as they pass through, but has a greater ability to dissipate the microwave energy into heat. This strong absorption provides an increase of the temperature inside the sample, leading to the rupture of cells by the *in situ* water. In some cases, it can promote the

degradation of the target antioxidants and, in other cases, can increase the diffusivity of the target antioxidants in the matrix.

Knowing all that, when describing the antioxidant activity of components of a complex natural extract as a function of the degree of polarity, scientific studies typically involve a first extraction step with solvents with different polarity index, followed by testing their activity by different analytical procedures. However, such a link between polarity-activity cannot be straightforward performed and further analyses are need. In the literature there are few reports addressing the previously mentioned associated issues (Jayasinghe *et al.*, 2013; Li *et al.*, 2015). However, it would be interesting to perform studies considering the following issues: a) a well-defined group of *in vitro* methods that could separate the polar activity of compounds in *H* and *L* antioxidants; b) a representative set of natural materials sources extracted with a set of solvents demonstratives of the different polarity index; c) a complex optimization of variable conditions that affect the extraction of *H* and *L* antioxidants to ensure and preserve its integrity and bioactivity; and d) clear target applications with a marked *H* and *L* character to prove in an *in vivo* form, whether or not the relation between the aspects stated in a), b) and c) are validated. The combination of all these requirements seems to be a labour-intensive approach, being out the context of this work.

5.3.3.6. Optimal extraction conditions for H and L antioxidants

The fitting results (**Table 49**) obtained by applying Eq. (8) to all the response criteria (P_m, V_m and IC_{50}) are presented in Eqs. (9) to (11) for the *H* reaction and in Eqs. (12) to (14) for the *L* reaction. By finding the partial derivatives of these regression equations, equating them to zero (**Annexe 10**) and solving the equations system, the coded values that optimize the response criteria were obtained. Then, the coded variable values were introduced in the original Eqs. (9)-(14) and the optimal response values were found. Finally, by decoding the coded values, the conditions that maximize the response were transformed into natural values.

The operating conditions that maximize the extraction of the tomato antioxidants and the optimal response values are presented in **Table 50** for each parametric estimation criteria (P_m, V_m and IC_{50}) and analytical reaction (*H* and *L*). For *H* antioxidants, the optimal conditions for P_m were at 180.0 ºC, with 56.8 % ethanol and 45.0 g/L of sample, during 18.7 min; for V_m were at 120.0 ºC, with 0.0 % ethanol and 5.0 g/L of sample, during 2.5 min; and for IC_{50} were at 90.0 ºC, with 44.0 % ethanol and 17.0 g/L of sample, during 14.5 min; and for *L* antioxidants, the optimal conditions for P_m were at 93.6 ºC, with 44.0 % ethanol and 21.3 g/L of sample, during 13.4 min; for V_m were at 180.0 ºC, with 100 % ethanol and 5.0 g/L of sample, during 2.2 min; and for IC_{50} were at 169.1 ºC, with 91.7 % ethanol and 10.9 g/L of sample, during 2.6 min. Optimal extraction conditions based on all the response criteria (P_m,

V_m and IC_{50}) were also determined for *H* and *L* antioxidants. Based on these values, it was found that the extraction of *L* antioxidants demands a longer *t* (15.4 min) but a lower *T* (60.0 ºC), *Et* (33.0 %) and *S/L* (15.0 g/L), comparing to the operating conditions outlined for *H* antioxidants (*i.e.,* t, 2.25 min; T, 149.2 ºC; Et, 99.1 %; and *S/L*, 45.0 g/L). These intermediate extraction conditions, and others that were optimized for each response criteria (P_m, V_m and IC_{50}) of both *H* and *L* antioxidants, were depicted using a simplex method tool to solve linear problem. Restrictions were made to the variable coded values that did not allowed the set of equations consider unnatural conditions (*i.e.,* lower times than 0). Additionally, optimal extraction conditions for both *H* and *L* antioxidants based on all the response criteria were determined (*i.e., t*, 12.1 min; *T*, 122.3 ºC; *Et*, 100 %; and *S/L*, 27.2 g/L), which allow to obtain the maximum extraction yield of both antioxidants simultaneously.

5.3.4. Conclusions

Optimal MAE conditions for *H* and *L* antioxidants from a surplus tomato crop were determined in this study. A five-level full factorial Box-Behnken design was successfully implemented and RSM used for analysis. The independent variables of *t*, *T*, *Et* and *S/L* had significant effects on MAE. To predict the optimal extraction conditions, a second-order polynomial model assuming interactive effects was fitted to each response and the regression coefficients were determined using the least-squares method. Optimal MAE conditions for *H*, *L* and both antioxidants were determined based on the parametric response criteria P_m, V_m and IC_{50}. Overall, MAE proved to be a powerful and efficient innovative methodology to extract the tomato antioxidants. In statistical terms, the high values of the adjusted coefficient of determination ($R^2_{adj} > 0.90$) and the non-significant difference between predicted and experimental values demonstrated the validity of the proposed optimization model. The results also indicated that the antioxidant capacity of the *H* fraction was much higher than the *L* one. Additionally, a discussion on the relationship between the extraction capacity of the solvent in function of its polarity and the antioxidant activity of the extracts in *H* and *L* media (the so-called polarity-activity relationship) was initiated, providing useful information in the study of complex natural extracts containing ingredients with opposite degrees of polarity.

Table 50 Operating conditions that maximize the extraction of *H* and *L* antioxidants from tomato and optimal response values for the parametric response criteria (P_m, V_m and IC_{50}) and antioxidant reactions (*H* or *L*). The independent variables *t*, *T*, *Et* and *S/L* are presented in natural values.

	Optimal extraction conditions				**Response optimum**
	X_1: t (min)	X_2: T (ºC)	X_3: Et (%)	X_4: S/L (g/L)	
For H antioxidants					
P_m (*H*)	18.7	180.0	56.8	45.0	100% *μM Cr*
V_m (*H*)	2.5	120.0	0.0	5.0	136.11% *μM Cr/g extract*
IC_{50} (*H*)	14.5	90.0	44.0	17.0	0.051*g extract*
For L antioxidants					
P_m (*L*)	13.4	93.6	44.0	21.3	100% *μM βC*
V_m (*L*)	2.2	180.0	100.0	5.0	78.70% *μM βC/g extract*
IC_{50} (*L*)	2.6	169.1	91.7	10.9	0.025*g extract*
For each response criteria of both H and L antioxidants					
P_m (*H*)	15.4	127.6	93.2	33.8	100.0% *μM Cr*
P_m (*L*)					43.4% *μM βC*
V_m (*H*)	3.9	63.3	0.0	5.0	108.93% *μM Cr/g extract*
V_m (*L*)					74.79% *μM βC/g extract*
IC_{50} (*H*)	13.9	112.7	89.0	5.3	0.06*g extract*
IC_{50} (*L*)					0.05*g extract*
For H and L antioxidants based on all the response criteria					
P_m (*H*)	2.25	149.2	99.1	45.0	100% *μM Cr*
V_m (*H*)					60.2% *μM Cr/g extract*
IC_{50} (*H*)					0.09*g extract*
P_m (*L*)	15.4	60.0	33.0	15.0	92.4% *μM βC*
V_m (*L*)					42.9% *μM βC/g extract*
IC_{50} (*L*)					0.38*g extract*
For both H and L antioxidants based on all the response criteria					
P_m (*H*)	12.1	122.3	100.0	27.2	100.0% *μM Cr*
P_m (*L*)					39.1% *μM βC*
V_m (*H*)					46.39% *μM Cr/g extract*
V_m (*L*)					9.64% *μM βC/g extract*
IC_{50} (*H*)					0.47*g extract*
IC_{50} (*L*)					0.47*g extract*

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5.4. Microwave-assisted extraction of phenolic acids and flavonoids and production of antioxidant ingredients from tomato: A nutraceutical-oriented optimization study

Microwave-assisted extraction of phenolic acids and flavonoids and production of antioxidant ingredients from tomato: A nutraceutical-oriented optimization study

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Abstract

The production of natural extracts requires suitable processing conditions to maximize the preservation of the bioactive ingredients. Herein, a microwave-assisted extraction (MAE) process was optimized, by means of response surface methodology (RSM), to maximize the recovery of phenolic acids and flavonoids and obtain antioxidant ingredients from tomato. A 5-level full factorial Box-Behnken design was successfully implemented for MAE optimization, in which the processing time (*t*), temperature (*T*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) were relevant independent variables. The proposed model was validated based on the high values of the adjusted coefficient of determination and on the non-significant differences between experimental and predicted values. The global optimum

processing conditions (*t*=20 min; *T*=180 ºC; *Et*=0 %; and *S/L*=45 g/L) provided tomato extracts with high potential as nutraceuticals or as active ingredients in the design of functional foods. Additionally, the round tomato variety was highlighted as a source of added-value phenolic acids and flavonoids.

Keywords: Microwave-assisted extraction; phenolic compounds; antioxidant activity; central composite design; *Lycopersicon esculentum*.

5.4.1. Introduction

Phenolic compounds are a group of secondary metabolites widely spread throughout the plant kingdom. Tomato (*Lycopersicon esculentum* Mill.) fruits, apart from being a functional food rich in carotenoids, vitamins and minerals (Pinela *et al.* 2012, Martínez-Huélamo *et al.* 2015), is also an important source of phenolic compounds, including phenolic acids and flavonoids (Barros, Dueñas, Pinela, *et al.* 2012). As antioxidants, these functional molecules play an important role in the prevention of human pathologies (Carocho and Ferreira 2013b, Friedman 2013) and found many applications in nutraceutical, pharmaceutical and cosmeceutical industries (Martins *et al.* 2011). Therefore, obtaining added-value functional compounds from natural sources, such as tomatoes, is highly desirable by the food industrial sector. Furthermore, the global nutraceutical market has grown in the last decade and a large percentage of the developed nutraceuticals and functional foods are driven by plant-based products (Lokesh *et al.* 2015).

Tomato is a key element of the Mediterranean diet (Altomare *et al.* 2013) and the second most important vegetable crop worldwide, being consumed either fresh or in the form of processed products. In Trás-os-Montes, North-eastern Portugal, native population's lifestyle has highlighted the importance of local tomato varieties, which are grown using extensive farming techniques and considered as very tasty and healthy foods (Carvalho and Morales 2010). Among them, the common variety of tomato, locally known as "tomate Redondo" (round tomato), was reported as a source of *p*-coumaric acid and quercetin derivatives, as well as of the non-phenolic compound benzyl alcohol dihexose (Barros, Dueñas, Pinela, *et al.* 2012). The *p*-coumaric acid has antioxidant, antilipidemic, antihypertrophic and cardioprotective properties (Roy and Stanely Mainzen Prince 2013, Stanely Mainzen Prince and Roy 2013). Quercetin shows a wide range of biological and pharmacological effects, including antioxidant, anti-inflammatory, antitumor and antibacterial activities, as well as neuroprotective, hepatoprotective, cardioprotective, anti-atherosclerotic, anti-thrombotic and antihypertensive effects (Russo *et al.* 2012, Song *et al.* 2014, Dajas *et al.* 2015, Nabavi *et al.* 2015). In tomato, quercetin is commonly found in the glycoside, *i.e.,* esterified with rutinose. Rutin, known as vitamin P, also display a remarkable array of health-

promoting effects and is widely used in the industry (Chua 2013). In turn, benzyl alcohol, an aromatic alcohol, is used in cosmetic formulations, as local anaesthetic, and as a flavouring substance in foods and beverages (European Commission 2002). Furthermore, epidemiological studies support the protective effect of tomatoes against certain degenerative diseases associated to oxidative stress, including cardiovascular diseases and various types of cancer (Canene-Adams *et al.* 2005). Meanwhile, there has been an increasing concern to develop and include phenolic-rich functional foods in the diet in order to improve the nutritional and health status.

Extraction is an important analytical step in the isolation of compounds from plant materials prior to chromatographic identification, or from a preparative point of view, to produce functional ingredients to use in new formulations (Dai and Mumper 2010, Lokesh *et al.* 2015). Today, microwave-assisted extraction (MAE) is gaining many merits due to the higher extraction rate and superior products quality at lower cost. In fact, this novel green technology is considered as a potential alternative to conventional solid-liquid extraction of bioactive compounds from plant matrices (Li *et al.* 2012). However, the MAE efficiency depends on several variables which may not be generalised for all plant materials due to the diverse nature of existing bioactive phytochemicals, being necessary to select and optimize the processing conditions as a function of the used matrix and taking into account the desired responses.

Apart from the large amounts of industrial by-products derived from tomato processing, sometimes a surplus production of this fruit occur, which can be sustainably used for functional ingredients recovery. In a previous study conducted by Li *et al.* (2012), optimal extraction conditions were determined based on the ferric reducing antioxidant power (FRAP) and oxygen radical absorption capacity (ORAC) assays. These optimized conditions were then used in the analysis of phenolic compounds. However, non-phenolic compounds can influence antioxidant responses. Therefore, an RSM optimization based on chromatographic analysis is more accurate and desired, once the optimal conditions obtained from antioxidant responses may not match the conditions for the extraction of individual compounds. In addition, the low range of extraction time (≤ 3.68 min) originated non significant results. Our study aimed at determining the optimal MAE conditions for maximizing the recovery of functional phenolic compounds and the antioxidant capacity of extracts from tomato. Different variables (processing time, temperature, ethanol concentration, microwave power, and solid/liquid ratio) were investigated and the extraction process optimized using a central composite design coupled with response surface methodology (RSM). The content of the major phenolic compounds (two phenolic acids: benzyl alcohol dihexose and a *cis p*-coumaric acid derivative; and two flavonoids: quercetin

pentosylrutinoside and quercetin-3-*O*-rutinoside) and the antioxidant activity (DPPH free-radical scavenging activity and reducing power) were evaluated as responses.

5.4.2. Material and methods

5.4.2.1. Standards and reagents

HPLC-grade acetonitrile was from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (*p*-coumaric acid, caffeic acid and rutin) were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Sigma (St. Louis, MO, USA). All the other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

5.4.2.2. Preparation of tomato extracts

Plant material

A common farmers' variety of tomato, known as "tomate redondo or batateiro" (round tomato), widely cultivated in rural communities from Miranda do Douro, North-eastern Portugal, was chosen for this study. Fruits at the ripen stage were hand-harvested randomly from the middle of six plants, in selected homegardens of two villages in the studied area. Ripeness was established according to local consumers' criteria based in morphological descriptors such as size, texture, and colour patterns of pericarp. According to local standards, the visual tonality of mature tomatoes was evaluated as corresponding to nº 42 in Red Group, using the colour chart of the Royal Horticultural Society. Six tomato fruits (pericarps without jointed pedicels and seeds) were lyophilized (Free Zone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) and kept at -20 °C until analysis.

Microwave-assisted extraction

The MAE process was performed using a Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) in closed vessels of high-precision glass. Ethanol:water mixtures were used since ethanol has low toxicity and efficiency for the extraction of phenolic compounds. The presence of a polar hydroxyl group and a non-polar end was also taken into account. The solvent volume was fixed at 20 mL. The powdered samples were extracted using different time (*t*), temperature (*T*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) conditions that ranged as defined by the RSM design (**Table 51**). During processing, samples were stirred at 600 rpm using a magnetic stirring bar and irradiated at 200 W (a

preliminary study presented in **Annexe 11** indicated that the microwave power has no effect on the extraction process). After that, the mixture in the extraction vessel was quickly cooled in the processing chamber. The mixture was centrifuged at 6000 rpm for 10 min, the pellet was discarded and the supernatant was carefully collected for further analysis. The residue obtained from each solution was evaluated to determine the extraction yield (g extract/g sample).

Table 51 Coded and natural values of the optimization parameters used in the RSM analysis. The four independent variables X_1 (time, min), X_2 (temperature, ºC), X_3 (ethanol concentration, %) and X_4 (solid/liquid ratio, g/L) were combined in a 5-level full factorial design of 25 combinations and 7 replicates at the centre of the experimental domain.

Coded values	Natural values			
	X_1: t (min)	X_2: T (ºC)	X_3: Et (%)	X_4: S/L (g/L)
-2	0	60	0	5
-1	5	90	25	15
0	10	120	50	25
+1	15	150	75	35
+2	20	180	100	45

5.4.2.3. Chromatographic analysis of phenolic compounds

After the MAE process, the extract solutions were purified using Sep-Pak® C-18 3 cc Vac Cartridges (Phenomenex, Torrance, CA, USA), wetted and activated with methanol followed by water; sugars and other polar substances were removed with 10 mL of water, and phenolic compounds were further eluted with 5 mL of methanol. The methanolic extracts were concentrated under vacuum, re-dissolved in 1 mL of water:methanol (80:20, v/v) and filtered through 0.22 µm disposable LC filter disks. The analysis of the main compounds in the tomato extracts was performed by high-performance liquid chromatography (HPLC) (Shimadzu 20A series UFLC, Shimadzu Corporation, Kyoto, Japan) as described previously by Barros, Dueñas, Pinela, *et al.* (2012). Double online detection was carried with a diode array detector (DAD) operating at 280 and 370 nm as preferred wavelengths. The target phenolic compounds were identified according to their UV spectra and retention time. For the quantitative analysis, a baseline to valley integration with baseline projection mode was used to calculate peak areas. External standards were used for quantification. The results were expressed in mg per g of extract.

5.4.2.4. Evaluation of the antioxidant activity

Two *in vitro* assays were applied to evaluate the antioxidant activity of the tomato extracts, which were successively diluted to different concentrations using the same extraction solvent.

DPPH free-radical scavenging activity

The solutions with different concentrations (30 µL) were mixed with a methanolic solution (270 µL) containing DPPH free-radicals (6×10^{-5} M) in a 96-well plate. The reaction mixture was left to stand for 60 min in the dark. After that, the reduction of DPPH free-radicals was determined by measuring the absorbance at 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc, Winooski, VT, USA) (Pinela *et al.* 2012). The nonlinear dose-response of the asymptotic end-point values of the solutions was calculated by the Weibull model as previously described by Prieto *et al.* (Prieto, Curran, *et al.* 2015) using the Eq. (1).

$$DPPH^{\bullet}(A) = K \exp\left[-(\ln 2)^{1-\alpha} \left(\frac{2 v_m A}{K \alpha} \right)^{\alpha} \right] \quad (1)$$

in which A is the dose of antioxidant. The parameter *K* is the starting value of DPPH free-radicals (30 µM). The α shape parameter is related with the maximum slope of the response. The parameter *vm* corresponds to the average number of DPPH molecules reduced per g of extract (µM DPPH/g extract), which is a value of maximal predictability and, therefore, was used as response.

Reducing power

The reducing power assay evaluates the capacity of the extracts to convert potassium ferricyanide (Fe^{3+}) into potassium ferrocyanide (Fe^{2+}), which reacts with ferric chloride to form a ferric-ferrous complex that can be monitored spectrophotometrically. The solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mM, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and then trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured into the 48-well plates, with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm using the microplate reader described above (Pinela *et al.* 2012). The dose-response results showed a linear dependency and the linear Eq. (2) with zero intercept was used to compute the average number of reduced molecules.

$$RP(A) = mA \quad (2)$$

in which A is the dose of antioxidant. The slope parameter *m* corresponds to the average number of molecules that are reduced per g of extract (µM Fe^{2+}/g extract) and was used to compute the potential antioxidant activity of the extracts.

5.4.2.5. Experimental design

Experimental design

The influence of different independent variables was investigated using a one-factor-at-a-time approach to select the significant ones and to determine a preliminary range for each variable. Based on these experimental results presented in **Annexe 11**, the variables X_1 (time, min), X_2 (temperature, ºC), X_3 (ethanol concentration, %) and X_4 (solid/liquid ratio, g/L) were selected for the RSM design. Then, the combined effects of these four variables on the extraction of phenolic acids and flavonoids and production of functional (antioxidant) ingredients from tomato were studied using a *central composite design* as proposed by Box and Hunter (1957). The responses were solved using 25 independent combinations and 7 replicates at the centre of the experimental domain, which implies 625 possible combinations. In this design, the points of experiments are generated on a sphere around the centre point. The centre point is supposed to be an optimum position for the response and is repeated to maximize the prediction (Box *et al.* 2005). This design also requires 5 levels of each factor. The number of repetitions n_0 of the centre point was calculated using the formulas presented in Eq. (3) for k factors based on uniform precision.

$$\gamma = \frac{(k+3)+\sqrt{9k^2+14k-7}}{4(k+2)} \quad ; \quad \text{where:} \quad n_0 = floor\left(\gamma\left(\sqrt{2^k}+2\right)^2 - 2^k - 2k\right) \tag{3}$$

where *floor* designates the highest integer value smaller than the argument. The number of experiments n for k factors is given as:

$$n = 2^k + 2k + 1 \tag{4}$$

Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. The independent variable coded values and the natural ones of the factorial design were coded and decoded by the expressions in Eq. (5).

$$v_c = (v_n - v_0)/\Delta v_n \quad \text{and} \quad v_n = v_0 + \Delta v_n \times v_c \tag{5}$$

where v_n and v_c are the natural (n) and coded (c) values in the centre of the experimental domain, v_0 is the initial value and Δv_n is the increment of v_n for unit of v_c.

Box-Behnken mathematical model

The response surface models were fitted by means of least-squares regressions using the following Box-Behnken design equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \tag{6}$$

where Y is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is

the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and *n* the number of variables. The responses of the parametric estimations of the antioxidant activity assays and of the chromatographic quantification of the main phenolic acids and flavonoids were used as dependent variables.

5.4.2.6. Fitting procedures and statistical analysis

The fitting procedures of equations to the responses were performed by means of a Microsoft Excel spreadsheet. Coefficients estimation and statistical calculations of the experimental results to the proposed equations were carried out in three phases:

1) Coefficients estimation was obtained by minimization of the sum of quadratic differences between the observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* (Kemmer and Keller 2010).

2) The significance of the coefficients of the parametric confidence intervals was calculated using the "*SolverAid"* (Prikler 2009). The model was simplified by dropping terms, which were not statistically significant (*p*-value > 0.05).

3) The uniformity of the model was checked by applying the following statistical assessment criteria: a) The Fisher *F*-test (α=0.05) was used to determine whether the constructed models were consistent to describe the observed data; b) The 'SolverStat' macro was used for the assessment of the parameter and model prediction uncertainties (Comuzzi *et al.* 2003); c) R^2 and R^2_{adj} were interpreted as the proportion of variability of the dependent variable explained by the model; d) The fitting to experimental data was evaluated by calculating the bias and accuracy factors of all equations, such as the Mean Squared Error (MSE), the Root Mean Square of the Errors (RMSE), the Mean Absolute Percentage Error (MAPE); and the Durbin-Watson coefficient (DW).

5.4.3. Results and discussion

5.4.3.1. Response criteria for the RSM analysis

Figure 48 shows the phenolic profile of the tomato extract obtained under the run nº 9 of the RSM design, whose processing conditions are presented in **Table 52**. Benzyl alcohol dihexose (P_1) and *cis p*-coumaric acid derivative (P_2) were the major phenolic acids, while quercetin pentosylrutinoside (F_1) and quercetin-3-*O*-rutinoside (F_2) were the main flavonoids, in agreement to Barros, Dueñas, Pinela, *et al.* (2012). These compounds were identified by comparison of their UV spectra and retention time with those of commercial standards. The quantification results are presented in **Table 52** for the different runs of the RSM design. The levels of phenolic acids ranged from 0.94 to 6.80 mg/g extract for P_1 and from 2.14 to 17.86 mg/g extract for P_2 and were achieved with the runs nº 14 and 21, respectively. For the flavonoids, the amounts of F_1 ranged from 0.38 to 2.94 mg/g extract and were achieved with

the runs nº 5 and 20, respectively; while the F_2 contents ranged from 0.61 to 4.83 mg/g extract and were achieved with the experimental runs nº 16 and 12, respectively. The results of this chromatographic quantification were used as response criteria to optimize the MAE conditions by RSM.

The MAE conditions were also optimized based on two antioxidant activity assays. **Figure 49** illustrates the responses of the DPPH free-radical scavenging activity (on the left-hand side) and reducing power (on the right-hand side) for the extracts obtained under the conditions designed by the RSM. Each half of **Figure 49** shows 25 subfigures that correspond to the dose-response antioxidant activity of the extracts obtained with the 25 genuine combinations of the RSM design. In each subfigure, dots (●) represent the values of standardized substrate (µM of DPPH free-radicals or reduced Fe^{2+}) and lines (—) represent the fitted responses to the model of Eq. (1) (for the DPPH free-radical scavenging activity responses) and Eq. (2) (for the reducing power responses). The obtained parametric fitting values, confidence intervals and statistical information are presented in **Annexe 12**. All coefficients showed significant parametric intervals at the 95% confidence level (α=0.05) and the correlation coefficients were always higher than 0.98. **Table 52** shows the parametric values of *vm* (µM DPPH/g extract) and *vm* (µM Fe^{2+}/g extract) achieved with the Eqs. (1) and (2), respectively, for the 32 runs of the experimental RSM design. The values of *vm* ranged from 0.27 to 5.96 µM DPPH/g extract and were achieved with the runs nº 23 and 16, respectively; while the values of *vm* ranged from 18.5 to 173.9 µM Fe^{2+}/g extract and were attributed to the experimental runs nº 2 and 8, respectively. These parametric values were used as response criteria in the RSM optimization.

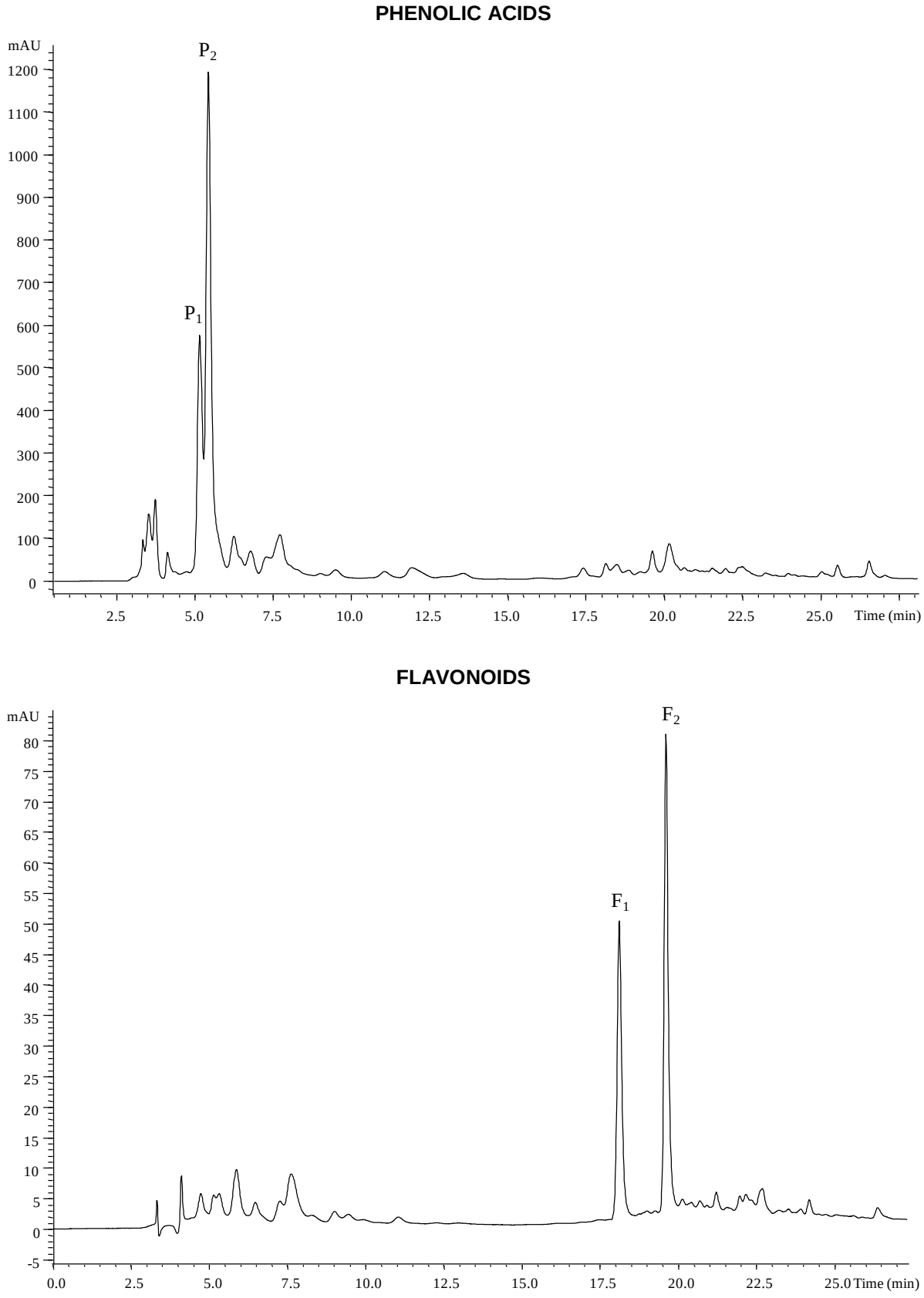

Figure 48 HPLC profiles of phenolic compounds in the tomato extracts (a representative case of the run nº 9 presented in **Table 52**). Phenolic acids (P_1, benzyl alcohol dihexose and P_2, *cis p*-coumaric acid derivative) were recorded at 370 nm and flavonoids (F_1, quercetin pentosylrutinoside and F_2, quercetin-3-*O*-rutinoside) were recorded at 280 nm.

Table 52 Numerical values of the responses obtained under the conditions designed in **Table 51**. The values of phenolic acids and flavonoids were obtained by HPLC quantification. The estimated numerical values of *vm* (μM DPPH/g extract) and *m* (μM Fe^{2+}/g extract) were achieved using the Eq. (1) and Eq. (2), respectively.

Run	Experimental domain				Experimental responses					
					Phenolic acids		***Flavonoids***		***Antioxidant activity***	
	X_1: *t*	X_2: *T*	X_3: *Et*	X_4: *S/L*	P_1	P_2	F_1	F_2	*DPPH*	*RP*
	min	*ºC*	*%*	*g/L*	*mg/g extract*	*mg/g extract*	*mg/g extract*	*mg/g extract*	*μM DPPH/g extract*	*μM Fe^{2+}/g extract*
1	-1(5)	-1(90)	-1 (25)	-1(15)	3.30	9.81	2.58	3.67	1.92	31.8
2	1(15)	-1(90)	-1 (25)	-1(15)	4.19	13.27	2.28	3.29	1.16	18.5
3	-1(5)	1(150)	-1 (25)	-1(15)	1.31	3.85	0.40	0.67	0.48	55.4
4	1(15)	1(150)	-1 (25)	-1(15)	2.49	8.25	0.87	1.49	1.30	85.9
5	-1(5)	-1(90)	1 (75)	-1(15)	1.61	3.96	2.94	4.51	2.42	96.6
6	1(15)	-1(90)	1 (75)	-1(15)	3.08	10.04	1.34	2.75	1.47	68.7
7	-1(5)	1(150)	1 (75)	-1(15)	1.14	3.75	1.02	1.22	2.74	158.5
8	1(15)	1(150)	1 (75)	-1(15)	2.01	7.32	0.59	0.68	3.47	173.9
9	-1(5)	-1(90)	-1 (25)	1(35)	5.00	14.13	2.42	3.35	2.21	50.8
10	1(15)	-1(90)	-1 (25)	1(35)	3.57	9.23	1.72	2.46	1.50	37.8
11	-1(5)	1(150)	-1 (25)	1(35)	5.76	16.29	1.82	2.88	1.83	70.6
12	1(15)	1(150)	-1 (25)	1(35)	5.45	12.88	2.73	4.83	2.66	99.1
13	-1(5)	-1(90)	1 (75)	1(35)	3.35	9.81	2.35	3.50	2.32	52.6
14	1(15)	-1(90)	1 (75)	1(35)	0.94	2.14	0.47	0.84	1.60	39.2
15	-1(5)	1(150)	1 (75)	1(35)	3.21	9.43	1.20	1.70	4.75	56.8
16	1(15)	1(150)	1 (75)	1(35)	1.73	4.30	0.42	0.61	5.96	71.3
17	-2(0)	0(120)	0 (50)	0(25)	2.20	6.14	2.48	3.69	2.26	50.3
18	2(20)	0(120)	0 (50)	0(25)	1.47	4.37	0.72	1.24	1.98	41.6
19	0(10)	-2(60)	0 (50)	0(25)	2.44	6.06	2.63	3.90	2.09	55.8
20	0(10)	2(180)	0 (50)	0(25)	2.00	5.48	0.38	0.79	3.51	119.7
21	0(10)	0(120)	-2 (0)	0(25)	6.80	17.86	2.14	3.47	0.96	34.0
22	0(10)	0(120)	2 (100)	0(25)	2.60	7.50	0.80	1.32	2.80	104.0
23	0(10)	0(120)	0 (50)	-2(5)	1.21	4.79	1.63	2.53	0.27	51.5
24	0(10)	0(120)	0 (50)	2(45)	4.50	11.72	1.68	2.62	1.72	33.1
25	0(10)	0(120)	0 (50)	0(25)	4.60	12.64	1.65	2.54	1.13	29.0
26	0(10)	0(120)	0 (50)	0(25)	4.73	12.87	1.77	2.71	1.14	28.6
27	0(10)	0(120)	0 (50)	0(25)	4.53	12.35	1.59	2.45	1.13	28.8
28	0(10)	0(120)	0 (50)	0(25)	4.71	12.75	1.61	2.47	1.06	28.8
29	0(10)	0(120)	0 (50)	0(25)	4.72	12.79	1.62	2.48	1.11	31.8
30	0(10)	0(120)	0 (50)	0(25)	4.70	12.74	1.60	2.47	1.09	29.0
31	0(10)	0(120)	0 (50)	0(25)	4.74	12.84	1.62	2.49	1.04	30.7
32	0(10)	0(120)	0 (50)	0(25)	4.73	12.80	1.62	2.48	1.08	28.8

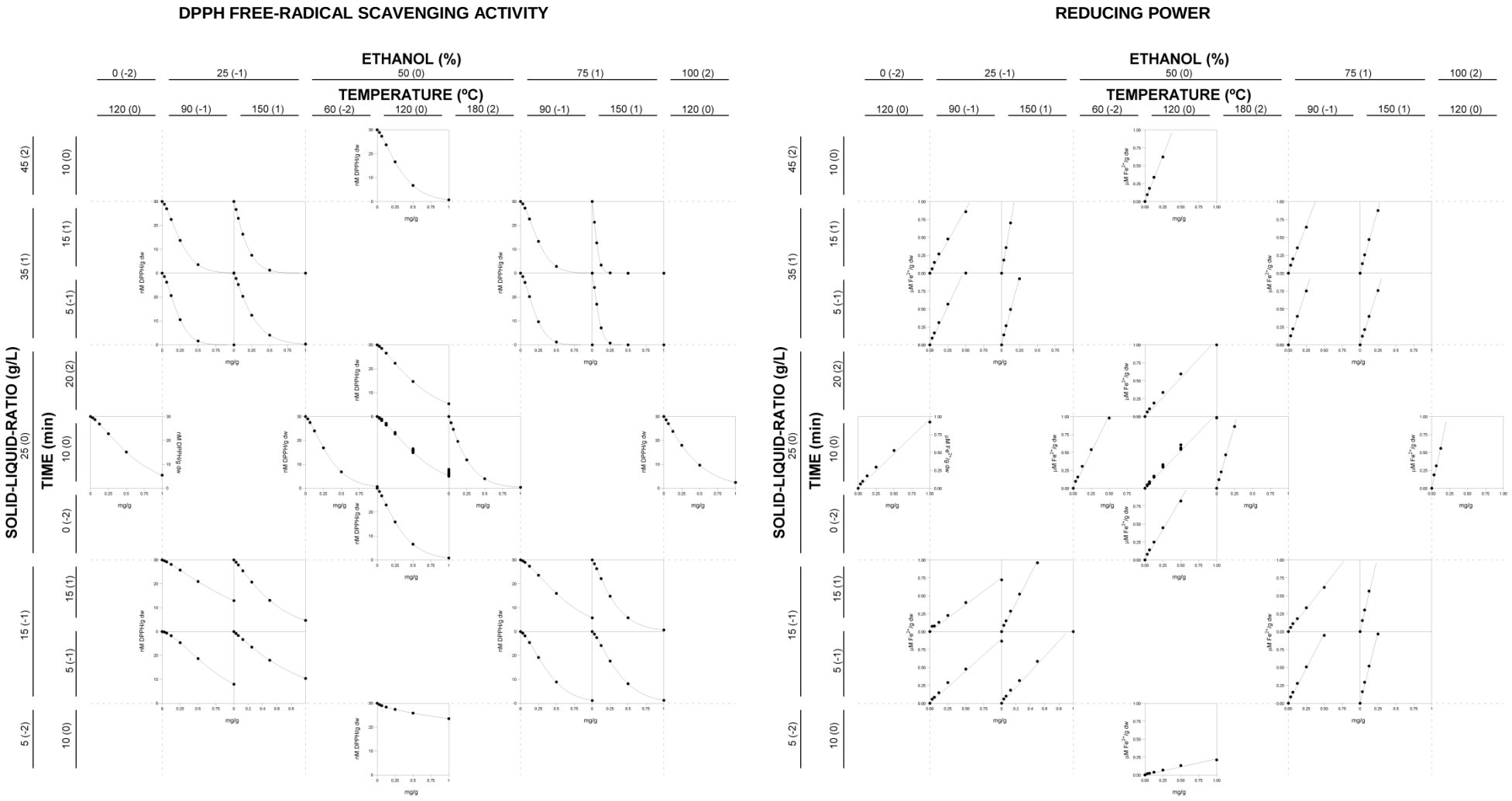

Figure 49 Illustration of the responses obtained for the DPPH free-radical scavenging activity (left-hand side) and reducing power (right-hand side) under the RSM experimental design presented in **Table** 1. Each graph illustrates one of the 25 independent variable combinations. In all cases, the response shows dots (●) representing the standardized substrate (μM of DPPH radicals or μM of reduced Fe^{2+}) values in a dose-response manner; and lines (—) representing the fitted responses to the mathematical models of Eq. (1) and Eq. (2). The parametric fitting values obtained by least-squares estimations are presented in **Annexe 12**.

5.4.3.2. Development of the theoretical response surface models and statistical verification

Fitting the models to the selected responses is crucial to elucidate how precisely the RSM mathematical model can predict ideal variances. The models for each response were built by fitting the Box-Behnken second-order polynomial model of Eq. (6) (independent variables in coded values) to the experimental values (**Table 52**) through nonlinear least-squares estimations. The resulting models are presented below.

When the peaks of the major phenolic acids were considered:

$$Y_{P1} = 4.7 - 0.7x_3 - 0.9x_4 - 0.7x_1^2 - 0.6x_2^2 + 0.4x_3^2 - 0.6x_1x_3 + 0.5x_2x_3 - 0.4x_3x_4 \quad (7)$$

$$Y_{P2} = 12.8 + 1.3x_3 - 2.4x_4 - 1.7x_1^2 - 1.6x_2^2 + 1.0x_3^2 - 2.4x_1x_3 + 1.3x_2x_3 - 1.1x_3x_4 \quad (8)$$

When the peaks of the major flavonoids were considered:

$$Y_{F1} = 1.6 - 0.3x_1 - 0.5x_2 - 0.3x_4 - 0.3x_1x_2 - 0.3x_1x_4 + 0.3x_2x_3 - 0.2x_3x_4 \quad (9)$$

$$Y_{F2} = 2.4 - 0.4x_1 - 0.7x_2 - 0.5x_4 + 0.4x_1x_2 - 0.5x_1x_4 + 0.6x_2x_3 - 0.3x_2x_4 - 0.4x_3x_4 \quad (10)$$

When the antioxidant activity assays were considered:

$$Y_{DPPH} = 1.1 + 0.5x_2 + 0.5x_3 + 0.6x_4 - 0.3x_1^2 - 0.5x_2^2 - 0.3x_4^2 - 0.4x_1x_2 + 0.4x_2x_3 + 0.6x_2x_4 \quad (11)$$

$$Y_{PR} = 29 + 21x_2 - 10x_3 + 17x_4 + 6x_1^2 + 16x_2^2 + 5x_3^2 + 12x_4^2 + 10x_1x_2 - 9x_2x_3 - 21x_3x_4 \quad (12)$$

where X_1 (processing time), X_2 (temperature), X_3 (ethanol concentration), X_4 (solid/liquid ratio), Y is the response, sub-indices indicate the analytical criterion used as response for RSM.

Not all the parameters of Eq. (6) were used for building the model, since some coefficients were statistically non-significant (**Table 53**). The significant ones were empirical and useful to predict the results of untested operating conditions (Ranic *et al.* 2014). The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level, and when a factor has a negative effect, the response is lower at high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable.

The complexity of the developed response models was very similar. Almost all of them present a combination of linear, quadratic and interactive coefficients and, in all of them, the four variables involved played a significant role. Nonetheless, based in the mathematical expressions, the responses of the antioxidant activity were more complex than those found for the individual phenolic acids and flavonoids. The antioxidant activity depends on the global contribution of different compounds, including interactions among them, and not only on a single molecule determined by HPLC. Indeed, as noted, the complexity of the mathematical equations can be related to the number of factors that affect the response.

Table 53 Parametric estimations of the 5-level full factorial design fitted to the second-order polynomial model of Eq. (6), confidence intervals of the estimated parameter values (α=0.05) and statistical information of the model proposed for each response.

		Phenolic acids		**Flavonoids**		**Antioxidant activity**	
		P_1	*P_2*	*F_1*	*F_2*	*DPPH*	*RP*
		mg/g extract	*mg/g extract*	*mg/g extract*	*mg/g extract*	*μM DPPH/g extract*	*μM Fe^{2+}/g extract*
Fitting coefficients obtained by Eq. (6) and showed in Eqs. (7)-(12)							
Intercept	b_0	4,70±0.25	12,82±0.70	1,58±0.07	2,44±0.12	1,14±0.20	29,44±8.29
Linear effect	b_1	ns	ns	-0,33±0.09	-0,39±0.14	ns	ns
	b_2	ns	ns	-0,48±0.09	-0,69±0.14	0,48±0.13	20,97±4.79
	b_3	-0,93±0.16	-2,40±0.46	-0,30±0.09	-0,46±0.14	0,64±0.13	16,99±4.79
	b_4	0,69±0.16	1,33±0.46	ns	ns	0,45±0.13	-10,33±4.79
Quadratic effect	b_{11}	-0,70±0.15	-1,76±0.41	ns	ns	0,32±0.12	6,07±4.32
	b_{22}	-0,60±0.15	-1,64±0.41	ns	ns	0,49±0.12	16,53±4.32
	b_{33}	ns	ns	ns	ns	0,26±0.12	11,85±4.32
	b_{44}	-0,44±0.15	-1,01±0.41	ns	ns	ns	5,17±4.32
Interactive effect	b_{12}	ns	ns	0,29±0.10	0,43±0.17	0,42±0.16	9,79±5.86
	b_{13}	ns	ns	-0,32±0.10	-0,47±0.17	ns	ns
	b_{14}	-0,63±0.20	-2,41±0.56	ns	ns	ns	ns
	b_{23}	ns	ns	ns	-0,28±0.17	0,60±0.16	ns
	b_{24}	0,53±0.20	1,34±0.56	0,34±0.10	0,63±0.17	0,41±0.16	-8,79±5.86
	b_{34}	-0,44±0.20	-1,05±0.56	-0,25±0.10	-0,43±0.17	ns	-21,54±5.86
Statistical information of the fitting analysis							
Obs		32	32	32	32	32	32
DF		22	22	23	22	21	20
R^2		0.9545	0.9482	0.9420	0.9348	0.9502	0.9407
R^2adj		0.9421	0.9284	0.9137	0.8964	0.9165	0.8996
MSE		4.52	30.99	1.00	2.34	2.61	2649.6
RMSE		2.12	5.56	1.00	1.53	1.61	51.47
MAPE		9.19	9.008	11.54	10.97	10.09	12.93
DW		1.36	1.15	1.99	2.12	1.43	1.50

ns: non-significant coefficient; *DF:* Degree of freedom; *R^2:* Correlation coefficient; *R^2adj:* The adjusted determination coefficient for the model; *MSE:* The mean squared error; *RMSE:* The root mean square of the errors; *MAPE:* The mean absolute percentage error; and *DW:* The Durbin-Watson statistic.

The lack of statistical fit, used to test the adequacy of the obtained models, demonstrated that no considerable improvement was achieved by the exclusion of the statistically non-significant effects (**Table 53**). This was also verified by the high values of R^2 and R^2_{adj} indicating the percentage of variability of each response that is explained by the model (**Table 53**). Additionally, the distribution of residuals was always randomly scattered around zero and grouped data and autocorrelations were not observed (data not shown).

This means that these models are workable and can be applied in the subsequent prediction and optimisation stages. It also indicates a good agreement between the experimental and predicted values. Finally, **Annexe 13** shows the results of the analysis of variance (ANOVA) for each of the nonlinear regression Eqs. (7) to (12). All coefficients were highly significant ($p < 0.01$). The lack of fit, used to verify the adequacy of the model, was not significant ($p > 0.05$), indicating that the model could adequately fit the experimental data.

The patterns of the extraction can be explained by means of the parametric values of the second-order polynomial models described in Eqs. (7) to (12), but can also be depicted by their graphical representation. **Figure 50**, **Figure 51** and **Annexe 14** show the 3D response surfaces of phenolic acids, flavonoids and antioxidant activity in function of the four studied variables. The individual 2D graphical responses of all the studied independent variables are presented in **Annexe 15**. The variables excluded in each 3D and 2D graphs were positioned at the centre of their experimental domain, *i.e., t*=10 min, *T*=120 ºC, *Et*=50 % and *S/L*=25 g/L.

5.4.3.3. Effect of extraction variables on the main phenolic compounds

In the phenolic profile (**Figure 48**) of the tomato extracts was possible to notice two main peaks corresponding to benzyl alcohol dihexose (P_1) and a *cis p*-coumaric acid derivative (P_2). Both compounds were affected in a similar way by the processing conditions, as can be observed comparing each 3D graph showed in the bottom diagonal part of **Figure 50** (for P_2) with those presented in the bottom diagonal part of **Annexe 14** (for P_1). The variables excluded in each 3D graph were positioned at the centre of their experimental domain, *i.e., t*=10 min, *T*=120 ºC, *Et*=50 % and *S/L*=25 g/L. Focussing on the particular case of P_2, it was verified an increase in the extraction yield with the increase in *T* until 144.64 ºC followed by a gradual decrease probably due to degradation phenomena. The *t* had a similar quadratic effect; the recovery of P_2 increased up to 3.15 min of processing and then decreased. The effect of the type of extraction solvent was linear and water (*Et*=0%) was the preferred extraction medium. In fact, phenolic compounds are polar molecules, so the extraction yield increases with increasing water content according to the "like dissolves like" principle (Zhang *et al.* 2008). Additionally, water may enhance swelling of cell material, increasing the contact surface area between plant matrix and solvent, resulting then in an increased extraction yield (Hayat *et al.* 2009). The *S/L* had linear and quadratic effects and the higher ratio (45.0 g/L) favoured the extraction yield. At an industrial scale, high ratios are desirable since it is important to maximize the extraction (thus productivity) with a minimal solvent consumption (more sustainable process). It was also found an interactive effect between *S/L* and the other 3 extraction variables (**Table 53**).

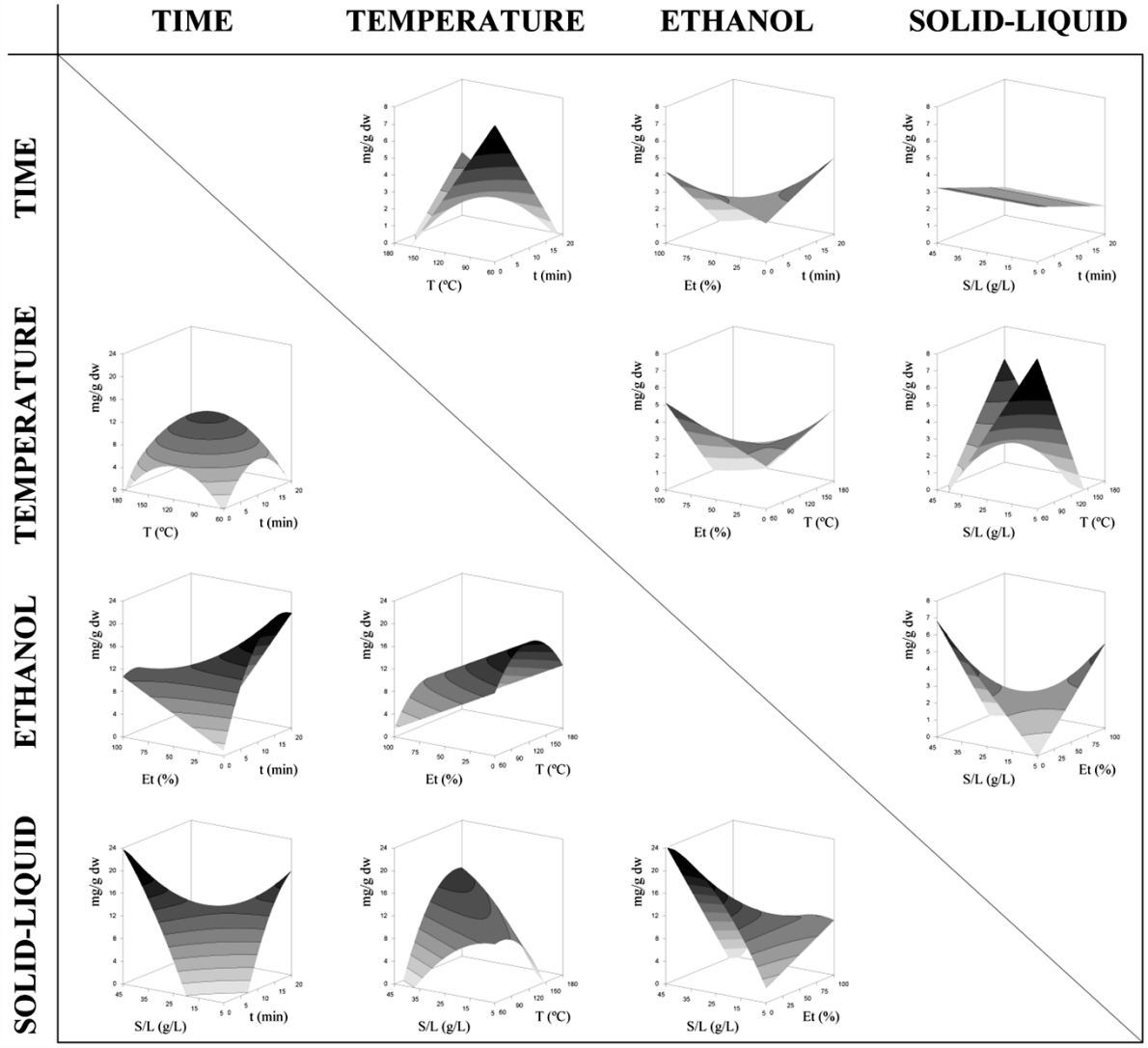

Figure 50 Matrix combination for the response surfaces of selected phenolic compounds (F_2 and P_2). In the top diagonal part is presented the response surface of F_2 (quercetin-3-*O*-rutinoside) and in the bottom diagonal part is presented the response surface of P_2 (*cis p*-coumaric acid derivative). For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The parametric fitting values are presented in **Table 53**.

In the HPLC profile (**Figure 48**), recorded at 280 nm, it was also possible to assign quercetin pentosylrutinoside (F_1) and quercetin-3-*O*-rutinoside (F_2) as being the two most abundant flavonoids in the tomato extracts. The interactive effects of the studied independent processing variables on the extraction of these functional compounds are presented in **Figure 50** and **Annexe 14**. The top diagonal part of **Annexe 14** shows the response surfaces of F_1 and the top diagonal part of **Figure 50** illustrates the response surfaces of F_2. The variables excluded in each 3D graph were positioned at the centre of their experimental domain, *i.e., t*=10 min, *T*=120 ºC, *Et*=50 % and *S/L*=25 g/L. As verified for phenolic acids, the processing conditions also affected the extractability of both flavonoids in a similar way. The increased in *t* and *T* probably led to a decomposition of the analytes, decreasing linearly the

recovery of these compounds. Similarly, the higher *Et* revealed a less affinity for the selected flavonoids. The *S/L* had a non-significant effect, but it interacted with *T* and *Et* (**Table 53**).

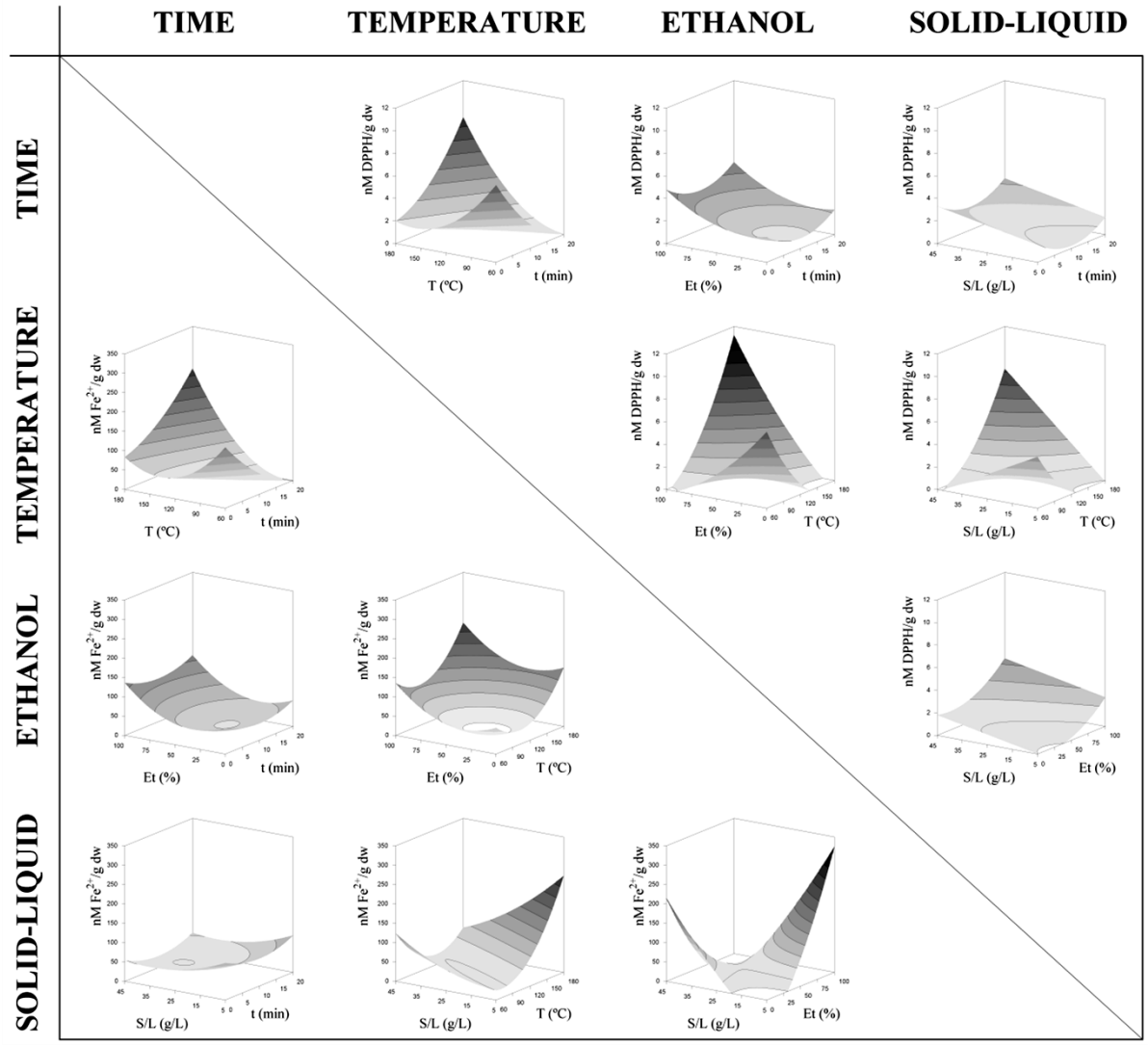

Figure 51 Matrix combination for the response surfaces of the antioxidant activity of the tomato extracts. In the top diagonal part is presented the response surface of the DPPH free-radical scavenging activity and in the bottom diagonal part is presented the response surface of the reducing power. For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The parametric fitting values are presented in **Table 53**.

5.4.3.4. Effect of extraction variables on the antioxidant activity

The effects of the studied independent variables on the antioxidant activity of tomato extracts are presented in **Figure 51**. The top diagonal part shows the response surfaces of the DPPH free-radical scavenging capacity and the bottom diagonal part represents the response surfaces of the reducing power. The variables excluded in each graph were positioned at the centre of their experimental domain, *i.e., t*=10 min, *T*=120 ºC, *Et*=50 % and *S/L*=25 g/L. The results are expressed in µM of protected substrate (DPPH free-radical or Fe^{2+}) per g of

extracted material. The response surfaces of both *in vitro* assays were somewhat similar, except for *S/L*, *i.e.,* high ratios favoured the DPPH free-radical scavenging activity in a positive linear manner, while low ratios led to a higher reducing power in linear and quadratic forms. It was found that higher processing *t* and *T* allowed obtaining tomato extracts with stronger antioxidant properties. Pure ethanol (100 %) was suitable to increase the antioxidant potential of the extracts, as observed for flavonoids, but in a manner contrary to that observed for phenolic acids. In fact, non-phenolic compounds can contribute to the antioxidant activity since increasing the *Et* also increases the solvent affinity towards less polar compounds. An interactive effect of *T* with *t* and *S/L* (**Table 53**) affected the response surfaces of both *in vitro* assays. Noteworthy consequences on the DPPH free-radical scavenging capacity were also induced by an interactive positive effect between *T* and *Et*. The response surfaces of the reducing power were also influenced by the interaction between *Et* and *S/L*.

5.4.3.5. Optimal extraction conditions

The operating conditions that maximize the extraction of the major phenolic acids and flavonoids and the antioxidant activity of the tomato extracts are presented in **Table 54**. The optimal processing conditions for each phenolic acid were as follows: *t*=5.51 min, *T*=146.69 ºC, *Et*=0.0 %, and *S/L*=45 g/L for benzyl alcohol dihexose (P_1); and *t*=3.15 min, *T*=144.64 ºC, *Et*=0 %, and *S/L*=45 g/L for the *cis p*-coumaric acid derivative (P_2); and allowed obtaining the following maximum recovery: 8.99±0.58 mg/g extract for P_1 and 24.8±0.9 mg/g extract for P_2. These optimal conditions were very similar, which can facilitate the obtainment of both compounds simultaneously, as reinforced by the following intermediate conditions optimised for both compounds: *t*=4.38 min, *T*=145.6 ºC, *Et*=0 %, and *S/L*=45 g/L. In fact, there were only slight differences in *t* and *T*. Regarding flavonoids, the optimal processing conditions for quercetin pentosylrutinoside (F_1) and quercetin-3-*O*-rutinoside (F_2) were exactly the same: *t*=20 min, *T*=60 ºC, *Et*=100 %, and *S/L*=45 g/L; and allowed obtaining a maximum recovery of 6.78±0.45 mg/g extract for F_1 and 11.7±0.6 mg/g extract for F_2. Compared with the optimal operating conditions for phenolic acids, the extraction of flavonoids demanded a lower *t*, *T* and *S/L* but a higher *Et*.

Table 54 Optimal processing conditions in natural values that lead to optimal response values.

Criteria	Optimal processing conditions				Response optimum	
	X_1: t (min)	*X_2: T (ºC)*	*X_3: Et (%)*	*X_4: S/L (g/L)*		
For each phenolic acid						
P1	5.51	146.69	0.0	45.0	8.99±0.58	*mg/g extract*
P2	3.15	144.64	0.0	45.0	24.8±0.9	*mg/g extract*
For each flavonoid						
F1	2.0	60.0	100.0	5.00	6.78±0.45	*mg/g extract*
F2	2.0	60.0	100.0	5.00	11.7±0.6	*mg/g extract*
For each antioxidant activity						
DPPH	20.0	180.0	100.0	45.0	14.28±0.6	*µM DPPH/g extract*
RP	20.0	180.0	100.0	5.0	445.0±3.1	*µM Fe^{2+}/g extract*
Intermediate conditions for phenolic acids, flavonoids and antioxidant activity						
P1	4.38	145.6	0.0	45.0	8.96±1.22	*mg/g extract*
P2					24.7±3.37	*mg/g extract*
F1	2.0	60.0	100.0	5.0	6.78±1.11	*mg/g extract*
F2					11.75±1.9	*mg/g extract*
DPPH	20.0	180.0	100.0	45.0	6.91±1.58	*µM DPPH/g extract*
RP					265.4±60.6	*µM Fe^{2+}/g extract*
Global processing conditions						
P1	20.0	180.0	0.0	45.0	7.57±0.77	*mg/g extract*
P2					18.8±1.9	*mg/g extract*
F1					3.67±0.37	*mg/g extract*
F2					7.47±0.76	*mg/g extract*
DPPH					4.02±0.41	*µM DPPH/g extract*
RP					203.7±20.6	*µM Fe^{2+}/g extract*

Curiously, the optimal processing conditions that allowed obtaining tomato extracts with maximal antioxidant activity differed in the *S/L* (45.0 g/L for the DPPH free-radical scavenging activity and 5.0 g/L for the reduction power), but the other processing conditions were exactly the same for both *in vitro* assays. As observed for flavonoids, a higher *Et* (100.0 %) favoured the antioxidant activity of the tomato extracts. Moreover, contrary to that observed for phenolic acids and flavonoids, a longer processing *t* (20.0 min) was required as well as a higher *T* (180.0 ºC). The MAE was conducted in closed vessels; so it was possible to increase the temperature above the boiling point of the solvent. The increase in *T* may improved the extraction efficiency by increasing desorption of antioxidants from active sites in the tomato matrix, and because the decrease in the surface tension and solvent viscosity may improved sample wetting and matrix penetration, respectively (Xie *et al.* 2015). These results diverge from those previously described by Li *et al.* (2012), achieved with the FRAP

and ORAC assays. The authors also verified that the independent variables of *T* and *Et* had significant effects on the response of both *in vitro* assays. However, the proposed optimization model was characterized by a shorter *t* (< 2.06 min) and a lower *T* (96.5 ºC) and *Et* (< 66.2 %). These divergent results can be justified by the different mechanisms of action of the performed *in vitro* assays and by variations in the antioxidants profile of the analysed tomato varieties. The FRAP assay is based on the reducing power of antioxidants, whereas the ORAC assay has been used to evaluate the antioxidant capacity of hydrophilic compounds against the peroxyl radical-induced oxidation initiated by thermal decomposition of AAPH (2,20-azobis-(2-methylpropionamidine) dihydrochloride).

In this study, global processing conditions were also computed in order to promote all the evaluated responses and thus originate tomato extracts with high amounts of phenolic acids and flavonoids and increased antioxidant properties. These MAE conditions were calculated using a simplex method tool to solve linear problems. Restrictions were made to the variable coded values to avoid the variable involved in the equations to consider unnatural conditions (*i.e.,* lower times than 0). As observed in **Table 54**, the global optimum processing conditions were based on high processing *t* (20 min), *T* (180 ºC) and *S/L* (45.0 g/L) and low *Et* (0.0 %), and allowed obtaining tomato extracts with the highest responses as possible. Thus, based on the different processing conditions shown in Table 4, the tomato samples could be processed differently according to the intended purpose, namely for recovery of phenolic acids, recovery of flavonoids, production of extracts with maximized antioxidant activity, or ingredients with increased levels of functional phenolic compounds and with high antioxidant capacity.

5.4.4. Conclusions

The combined effects of the independent variables of *t*, *T*, *Et* and *S/L* on the extraction of phenolic compounds and production of antioxidant extracts from tomato were investigated. A 5-level full factorial Box-Behnken design of 25 combinations and 7 replicates at the centre of the experimental domain was successfully implemented for MAE optimization by RSM. The MAE conditions were optimized for each response, as well as for the set of all responses. Under the global optimum conditions (*t*=20 min, *T*=180 ºC, *Et*=0 %, and *S/L*=45 g/L), the values for P_1, P_2, F_1 and F_2 were 7.57±0.77 mg/g extract, 18.8±1.9 mg/g extract, 3.67±0.37 mg/g extract and 7.47±0.76 mg/g extract, respectively; for DPPH free-radical scavenging activity and reducing power the values of 4.02±0.41 µM DPPH/g extract and 203.7±20.6 µM Fe^{2+}/g extract were obtained, respectively. The proposed optimization model was statistically validated by the high values of the adjusted coefficient of determination and by the observed non-significant differences between the experimental and predicted results. This study highlighted the analysed tomato variety as a source of added-value phenolic compounds.

Moreover, using the optimal processing conditions, it was possible to produce functional extracts with high potential as nutraceuticals or as active ingredients in the design of functional foods, which can be also extended to other industrial fields such as pharmaceutical and cosmeceutical industries.

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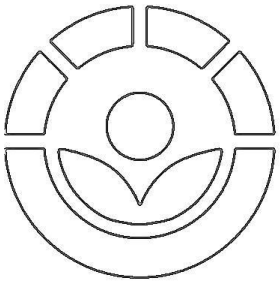
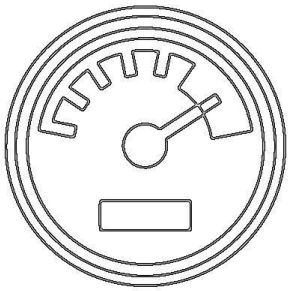
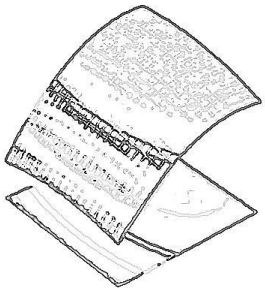
6. Concluding Remarks and Future Work

This **6th chapter** synthesizes the general conclusions of the work, highlights relevant features of the studied traditional foods and of the applied preservation and extraction technologies, and presents future perspectives.

6.1. Concluding remarks

6.1.1. Overall conclusion

This study was focused on valorisation and processing of traditional plant foods, including medicinal plants (prepared in herbal beverages), leafy vegetables, and tomato farmers' varieties, but also tomato wastes (source of biomolecules). The preservation of the dried medicinal plants *T. lignosa* and *M. neglecta* and the fresh vegetables *N. officinale* and *R. induratus* by using γ-ray irradiation treatments and/or inert gas-enriched modified atmospheres was investigated. The main goal was to extend the shelf-life of these perishable foods without negatively affect quality parameters (*i.e.,* visual, nutritional, chemical and bioactive attributes), and therefore reduce food waste. Argon was a suitable choice for preserving the overall postharvest quality of the selected vegetables during refrigerated storage. The adequacy of post-packaging irradiation treatments for shelf-life extension was also demonstrated. This study also highlights the bioactive compounds of tomatoes as health promoters and described for the first time the nutritional composition and antioxidant properties of four tomato farmers' varieties in North-eastern Portugal homegardens. The optimization of microwave-assisted extraction and high hydrostatic pressure processes for recovery of valuable compounds from tomato and watercress, respectively, was performed using RSM, and considering relevant independent variables and green solvents. In further studies it will be interesting to investigate the hurdle effect between MAP, γ-ray irradiation and natural bioactive coatings, which can be obtained using the MAE and HHP techniques.

6.1.2. Specific conclusions

6.1.2.1. The relevance of traditional plant foods for today's society

WEPs are alternative sources of nutrients and bioactive compounds, such as vitamins, minerals, fibre, unsaturated fatty acids, and phenolic compounds. Their interest as contemporary foods is not only due to their nutritional value but also to their peculiar organoleptic characteristics, which makes them differentiated foods to be included in new recipes and gourmet dishes. Given the high potential of wild foods and local farmer' varieties, different retrieval strategies were proposed in order to highlight their potential and promote their use in agro-food, pharmaceutical and environmental sectors. Some of the proposed retrieval strategies were adopted in this study and based on the obtained results it was possible to conclude:

- *For medicinal plants:* The obtained results give scientific support to the traditional uses of *T. lignosa* and *M. neglecta* in folk medicine, highlighting the prepared herbal beverages as having powerful antioxidant properties. In the case of *T. lignosa*, the decoction was the preferable preparation method to obtain a higher phenolic content

and antioxidant activity. This medicinal plant was also highlighted as a source of high added-value molecules (ellagitannin derivatives), which may be responsible for the therapeutic properties attributed to this plant.

- *For leafy vegetables:* The interesting nutritional and antioxidant properties of *N. officinale* and *R. induratus* were demonstrated. *p*-Coumaric acid was identified as the most abundant phenolic compound in *N. officinale*, although the group of flavonoids predominated over phenolic acids, and four kaempferol derivatives were identified for the first time in this vegetable. These findings highlight the potential of these leafy vegetables in the minimally processed food sector in order to be included in contemporary diets.
- *For tomato farmer' varieties:* The tomato farmer' varieties were identified as functional foods rich in antioxidant compounds such as ascorbic acid, carotenoids (in particular lycopene), and phenolic compounds (mainly phenolic acid derivatives). The so-called round tomato proved to be the most powerful in antioxidant activity, phenolic compounds and carotenoids, while the variety locally known as yellow tomato revealed an interesting nutritional composition, characterized by higher levels of fructose, glucose, α-linolenic acid and total tocopherols. A *cis p*-coumaric acid derivative was the most abundant phenolic compound in the yellow and round tomato varieties, while 4-*O*-caffeolyquinic acid predominated in the long and heart varieties. Quercetin pentosylrutinoside was the most abundant flavonoid in the four varieties. Additionally, the phenolic profile of these local farmer' varieties differed from varieties available in other countries, which is certainly related to genetic features, cultivation conditions, and handling and storage methods.

6.1.2.2. The suitability of the studied postharvest preservation treatments

The decontamination/preservation of minimally processed fruits and vegetables remains an important unsolved technological problem. However, the tested postharvest treatments were useful for shelf-life extension of leafy vegetables and, due to its various advantages, can be used as effective and safer alternatives to the chemical treatments conventionally used. Based on these preservation studies it was possible to conclude:

- *For dwarf mallow (Malva neglecta):* The levels of total phenolics and flavonoids were lower in hydromethanolic extracts prepared from samples irradiated at 1 kGy (dose that induced colour changes) and in decoctions prepared from those irradiated at 8 kGy, which also showed a lower antioxidant activity. In turn, irradiation at 5 kGy favoured the amounts of total phenolics and flavonoids. It was concluded that the irradiation-induced modifications on colour parameters depend not only on the applied dose but also on the plant material under investigation.

- *For perennial spotted rockrose (Tuberaria lignosa):* The preparation method had higher influence in the phenolic profile and antioxidant activity of this plant than the irradiation treatment; and colour was more sensitive to the drying method than to irradiation. Furthermore, the irradiation treatment (up to 10 kGy) did not significantly affect the antioxidant properties of the prepared herbal beverages. It was also demonstrated that the ellagitannin derivatives (~90% of the phenolic fraction) were not significantly affected by the 10 kGy dose; while the 5 kGy dose improved their extraction using water as extraction solvent.
- *For buckler sorrel (Rumex induratus):* This vegetable was more suitably stored under an Ar-enriched atmosphere, since the treatment allowed preserving relevant postharvest quality attributes; while the 6 kGy dose was a good option to preserve PUFA and the ratios of PUFA/SFA and n-6/n-3 fatty acids.
- *For watercress (Nasturtium officinale):* Air-packaged samples were those that presented the most dissimilar profiles from the non-stored control. In turn, Ar-enriched MAP was the most suitable choice to preserve the overall postharvest quality of fresh-cut watercress. Overall, samples irradiated at 2 kGy were those with the most similar quality profiles to the non-stored control samples.
- *Irradiation vs. modified atmosphere:* Overall, MAP proved to be a more suitable postharvest treatment than the post-packaging irradiation treatment for preserving quality parameters of *N. officinale* and *R. induratus* during storage at 4 ºC.

6.1.2.3. The efficacy of the tested non-conventional extraction methods

The recovery of valuable molecules from plant materials may help to tackle the societal challenges of the 21st century. Therefore, the extraction of phenolic compounds from watercress (a fast-growing plant) and tomato (a possible surplus crop) was optimized using two nonconventional methods:

- *High hydrostatic pressure:* The obtained results highlighted this promising emerging technology to cold extract phenolic compounds (phenolic acids and flavonoids) from *N. officinale* in a selective way using a green solvent and reduced extraction times.
- *Microwave-assisted extraction:* This technique was successfully used in the recovery of hydrophilic and lipophilic antioxidants, phenolic acids (benzyl alcohol dihexose and a *cis p*-coumaric acid derivative) and flavonoids (quercetin pentosylrutinoside and quercetin-3-*O*-rutinoside) from the round tomato framer' variety, and proved to be a powerful and efficient non-conventional extraction methodology.

Using the optimal processing conditions determined in these studies, it was possible to produce functional extracts with high potential to be used as functional ingredients in the development of functional foods and nutraceuticals, which can be also extended to other

industrial sectors such as pharmaceutical and cosmeceutical industries. In addition, it has also been found that the irradiation treatment at 5 kGy can promote the extraction of ellagitannins (mainly punicalagin derivatives) from *T. lignosa* aerial parts. Moreover, the applied full factorial designs coupled to RSM proved to be a time-saving and robust optimization tool for this type of studies.

6.2. Closing remarks and future prospects

Regarding the retrieval of traditional foods: Valorisation strategies were adopted in this study to promote their sustainable use of the selected WEPs and farmer' varieties of tomato. Given the high biodiversity and number of WEPs available in the North-eastern region of Portugal, it will be interesting to promote the sustainable use of other plant foods traditionally consumed in this region and also the valorisation of plant wastes and by-products of different crops. The revival, agricultural production, commercialization and conservation of WEPs can generate knowledge, new practices, and relevant goods and services, and contribute for an ecologically sustainable development. It will be interesting to adopt other retrieval strategies:

- The evaluation of other bioactivities (such as anti-inflammatory, antitumor and antimicrobial activity) and health promoting effects;
- Development of new based products (such as functional foods and functional beverages), recipes and dishes;
- Agricultural and *in vitro* production in order to minimize wild-harvesting practices, obtain greater availability and to overcome a possible lack of such species in the regions where they are harvested; at the same time as these species and the ecosystem are conserved;
- Publicize and promote the commercialization of these foods in restaurants and gourmet food stores, since consumers are looking for foods with different organoleptic properties from those daily eaten.

Regarding the application of preservation and extraction technologies: The following parameters and conditions should be tested and relevant aspects considered:

- Evaluate the effect of the studied preservation methods on other quality attributes (including sensorial and microbiological aspects) and physiological parameters (respiration rate of fresh vegetables under a modified atmosphere);
- Test different combinations between the studied technologies and other preservation factor (*e.g.,* the irradiation of plant foods under a modified atmosphere and the storage of coated food products under a modified atmosphere) in order to obtain possible synergistic or at least additive effects; in fact, the hurdle concept fits with the

actual consumer trend for minimally processed foods and is expected to gain more popularity and practical applications;

- Test other packaging gases, active packaging systems with natural bioactive compounds, and other sources of ionizing radiation (e.g., electron beam);
- Assess the suitability of the HHP treatment to preserve herbal beverages and fresh vegetables;
- Consumers should receive education about the risks and benefits of food irradiation so that more consumers can embrace it;
- Since lycopene is the major carotenoid in tomatoes, the cold extraction of this antioxidant pigment by high hydrostatic pressure is under investigation.
- Future trends in food preservation and extraction of high added-value compounds from plants cannot be considered independent of sustainability, eco-friendly, innovation, and advanced technologies, always intending to obtain safe and high quality foods or high yields of functional target compounds, respectively.

Annexes

Annexe 1 Percentage of contribution to Recommended Dietary Allowances (RDA) for adults (31 through 50 years) of proteins, carbohydrates, dietary fiber, mineral elements, folate, vitamin C and tocopherols of the edible parts (100-g portions) of the selected WEPs.

Plant species		**Proteins**	**Carbohydrates**	**Fiber**	**Na**	**K**	**Ca**	**Mg**	**Cu**	**Fe**	**Mn**	**Zn**	**Folate**	**Vitamin C**	**Vitamin E***
RDA (male/female)[1]		56/46 g/kg/day	130 g/day	38/25 g/day	1500 mg/day	4700 mg/day	1000 mg/day	420/320 mg/day	0.9 mg/day	8/18 mg/day	2.3/1.8 mg/day	11/8 mg/day	400 µg/day	90/75 mg/day	15 mg/day
A. ampeloprasum	Ep1	3.0/3.7	12.8	11.1/16.8	3.7	9.3	7.0	3.3/4.4	12.2	7.5/3.3	4.8/6.1	6.8/9.4	36.3	4.6/5.5	0.2
A. azurea	Ep1	3.4/4.1	1.0	10.3/15.6	1.9	12.0	15.8	6.0/7.8	14.4	23.8/10.6	10.4/13.3	3.9/5.4	69.6	0.7/0.9	2.4
A. nodiflorum	Ep1	2.9/3.5	0.9	7.1/10.8	16.3	3.5	15.2	6.7/8.8	8.9	22.5/10.0	12.6/16.1	4.5/6.3	31.3	9.9/11.9	1.5
A. acutifolius	Ep1	2.7/7.0	4.2	11.3/17.2	1.3	9.3	2.4	3.1/4.1	18.9	6.1/2.7	7.4/9.4	8.7/12.0	54.3	28.4/34.1	50.0
B. maritima	Ep1	4.6/5.7	2.8	15.5/23.6	13.8	26.0	9.6	17.4/22.8	23.3	28.0/12.4	35.7/45.6	8.0/11.0	75.5	11.3/13.5	3.4
B. officinalis	Ep1	2.1/2.6	7.3	-	5.9	4.6	23.2	4.8/6.3	5.6	34.9/15.5	70.4/90.0	3.2/4.4	-	1.4/1.7	7.7
B. dioica	Ep1	10.0/12.2	2.5	14.7/22.4	2.2	13.3	7.5	10.2/13.4	20.0	12.5/5.6	16.1/20.6	10.6/14.6	10.8	14.7/17.7	21.7
C. intybus	Ep1	5.2/6.3	2.7	16.1/24.4	5.1	10.2	12.1	5.7/7.5	12.2	12.9/5.7	9.1/11.7	3.3/4.5	63.4	4.9/5.9	6.6
C. ambrosioides	Ep1	-	-	-	-	-	-	-	-	-	-	-	-	6.1/7.3	364.1
C. juncea	Ep1	3.8/4.6	2.1	20.5/31.2	1.6	18.3	27.3	12.6/16.6	47.8	49.6/22.1	42.2/53.9	14.8/20.4	22.6	2.2/2.6	3.8
F. vulgare	Ep1	3.8/4.6	2.4	-	5.1	8.6	22.7	8.6/11.3	5.6	13.0/5.8	27.0/34.4	3.3/4.5	67.9	15.3/18.4	2.9
	Ep2	2.3/2.8	16.8	-	-	-	-	-	-	-	-	-	-	16.6/19.9	4.9
	Ep3	2.1/2.6	14.2	-	-	-	-	-	-	-	-	-	-	9.5/11.4	7.9
	Ep4	2.0/2.4	14.9	-	-	-	-	-	-	-	-	-	-	4.6/5.5	0.1
	Ep5	2.5/3.0	17.5	-	-	-	-	-	-	-	-	-	-	5.0/6.0	0.9
G. hederacea	Ep1	2.3/2.8	16.2	-	-	-	-	-	-	-	-	-	-	5.1/6.1	490.2
H. stoechas	Ep1	-	-	-	-	-	-	-	-	-	-	-	-	33.6/40.4	168.9
H. lupulus	Ep1	7.7/9.3	1.2	13.7/20.8	1.9	11.0	8.9	8.6/11.3	15.6	11.4/5.1	15.7/20.0	10.3/14.1	36.0	14.2/17.1	3.9
M. sylvestris	Ep1	5.2/6.3	13.0	-	6.2	13.9	24.0	67.4/88.4	23.3	45.1/20.1	21.3/27.2	14.4/19.8	-	4.5/5.4	132.3
	Ep2	4.1/5.0	16.5	-	-	-	-	-	-	-	-	-	-	29.2/35.1	25.7
	Ep3	3.2/3.9	31.4	-	-	-	-	-	-	-	-	-	-	16.3/19.6	7.5
	Ep4	5.7/7.0	12.6	-	-	-	-	-	-	-	-	-	-	9.7/11.7	43.1
M. pulegium	Ep1	5.2/6.3	26.5	-	-	-	-	-	-	-	-	-	-	3.6/4.3	187.9
M. fontana	Ep1	2.9/3.5	1.5	11.6/17.6	5.0	7.6	3.1	7.6/10.0	5.6	16.3/7.2	46.5/59.4	3.5/4.8	10.5	17.1/20.6	9.7

Plant species		**Proteins**	**Carbohydrates**	**Fiber**	**Na**	**K**	**Ca**	**Mg**	**Cu**	**Fe**	**Mn**	**Zn**	**Folate**	**Vitamin C**	**Vitamin E***
RDA (male/female)[1]		56/46 g/kg/day	130 g/day	38/25 g/day	1500 mg/day	4700 mg/day	1000 mg/day	420/320 mg/day	0.9 mg/day	8/18 mg/day	2.3/1.8 mg/day	11/8 mg/day	400 µg/day	90/75 mg/day	15 mg/day
N. officinale	Ep1	2.9/3.5	3.2	4.7/7.2	0.8	5.9	17.5	6.0/7.8	-	20.6/9.3	-	0.8/1.1	32.0	0.7/0.8	6.7
O. vulgare	Ep1	4.1/5.0	30.9	-	-	-	-	-	-	-	-	-	-	4.6/5.5	32.7
P. rhoeas	Ep1	6.6/8.0	2.7	14.4/22.0	1.4	15.1	20.0	7.9/10.3	37.8	69.0/30.7	29.1/37.2	13.0/17.9	38.1	15.2/18.3	7.5
P. oleracea	Ep1	4.5/5.4	2.1	2.4/3.6	1.4	11.5	16.0	34.0/44.7	30.0	27.0/12.0	21.7/27.8	4.2/5.8	-	29.6/35.5	-
P. tridentatum	Ep1	11.1/13.5	23.8	-	-	-	-	-	-	-	-	-	-	17.4/20.9	23.9
R. ulmifolius	Ep1	-	-	-	-	-	-	-	-	-	-	-	-	97.6/117.1	20.2
	Ep2	-	-	-	-	-	-	-	-	-	-	-	-	33.7/40.4	0.1
R. acetosella	Ep1	1.6/2.0	6.6	-	-	-	-	-	-	-	-	-	-	51.0/61.2	38.0
R. induratus	Ep1	3.8/4.6	4.8	-	-	-	-	-	-	-	-	-	-	57.4/68.9	32.9
R. papillaris	Ep1	4.3/5.2	1.5	11.6/17.6	1.7	7.5	6.0	10.7/14.1	8.9	12.5/5.6	32.6/41.7	3.3/4.5	46.8	43.2/51.9	8.6
R. pulcher	Ep1	5.7/7.0	2.5	12.4/18.8	5.3	14.1	5.0	7.4/9.7	13.3	23.1/10.3	14.3/18.3	7.0/9.6	126.6	22.6/27.2	2.9
S. hispanicus	Ep1	3.2/3.9	2.6	18.4/28.0	2.6	22.1	23.5	22.4/29.4	10.0	29.5/13.1	16.1/20.6	4.5/6.3	25.9	1.0/1.2	0.1
S. vulgaris	Ep1	5.5/6.7	2.2	9.5/14.4	1.9	21.0	9.3	16.2/21.3	7.8	10.0/4.4	32.6/41.7	5.2/7.1	66.9	24.5/29.4	9.9
S. marianum	Ep1	1.1/1.3	0.8	6.8/10.4	5.4	15.3	13.2	4.0/5.3	8.9	6.3/2.8	4.3/5.6	2.4/3.3	10.4	0.5/0.6	0.3
S. oleraceus	Ep1	4.1/5.0	1.9	10.3/15.6	8.3	10.8	15.6	7.4/9.7	5.6	7.9/3.5	20.0/25.6	5.2/7.1	21.5	3.1/3.7	11.3
T. communis	Ep1	5.7/7.0	3.2	12.4/18.8	1.4	9.3	5.2	6.2/8.1	13.3	10.4/4.6	9.6/12.2	7.7/10.6	9.5	47.0/56.4	17.5
T. obovatum	Ep1	2.9/3.5	2.5	18.4/28.0	2.3	12.0	11.7	4.3/5.6	16.7	44.6/19.8	14.3/18.3	4.5/6.3	27.7	2.0/2.3	3.4
T. mastichina	Ep1	3.9/4.8	28.2	-	-	-	-	-	-	-	-	-	-	3.2/3.9	1.1
T. pulegioides	Ep1	6.6/8.0	34.2	-	-	-	-	-	-	-	-	-	-	3.5/4.1	44.1

[1]Source: Otten et al. (2006). *As α-tocopherol.

Source of a certain nutrient (a 100-g portion provides 15% or more of the RDA for a certain nutrient)

High content of a certain nutrient (a 100-g portion provides 30% or more of the RDA for a certain nutrient)

Annexe 2 Composition in oxalic acid (mg/100 g) of the selected WEPs. It is presented the mean value and, in parentheses, the range of variability of the literature data. Oxalic acid/Ca ratios calculated based on the mean value of oxalic acid and Ca levels presented in **Table 5**.

Plant species	Ep	Oxalic acid	Oxalic acid/Ca ratio	Reference
A. ampeloprasum	Ep1	36 (6-92)	0.5	Sánchez-Mata *et al.* (2012), García-Herrera, Morales, *et al.* (2014)
A. azurea	Ep1	378 (111-644)	2.4	Morales *et al.* (2014)
A. nodiflorum	Ep1	618 (110-920)	4.1	Morales (2011)
A. acutifolius	Ep1	93 (88-99)	3.9	Pereira *et al.* (2013)
B. maritima	Ep1	544 (152-1009)	5.7	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
B. officinalis	Ep1	563.74 (559-568)	2.4	Pereira *et al.* (2013)
B. dioica	Ep1	69 (nd-124)	0.9	Sánchez-Mata *et al.* (2012, Pereira *et al.* (2013)
C. intybus	Ep1	5 (3-9)	0.0	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
C. ambrosioides	Ep1	1545 (1463-1627)	5.6 *	Barros *et al.* (2013)
C. juncea	Ep1	36 (7-93)	0.1	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
F. vulgare	Ep1	263 (124-403)	1.2	Sánchez-Mata *et al.* (2012)
	Ep5	669 (658-680)	-	Pereira *et al.* (2013)
H. stoechas	Ep1	130 (126-134)	-	Pereira *et al.* (2013)
H. lupulus	Ep1	59 (51-68)	0.7	Sánchez-Mata *et al.* (2012)
M. sylvestris	Ep4	6 (-)	0.0	Barros *et al.* (2013)
M. fontana	Ep1	367 (169-565)	11.8	Tardío *et al.* (2011), Pereira *et al.* (2013)
N. officinale	Ep1	503 (251-754)	2.9	Pereira *et al.* (2013), Pinela, Barreira, Barros, Antonio, *et al.* (2016)
O. vulgare	Ep1	164 (163-164)	-	Pereira *et al.* (2013)
P. rhoeas	Ep1	455 (124-768)	2.3	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
P. oleracea	Ep1	517 (371-753)	3.2	Petropoulos *et al.* (2015)
P. tridentatum	Ep1	56 (43-70)	-	Pereira *et al.* (2013)
R. ulmifolius	Ep2	80 (74-86)	-	Pereira *et al.* (2013)
R. acetosella	Ep1	137 (136-138)	-	Pereira *et al.* (2013)
R. induratus	Ep1	0.70 (0.66-0.74)	-	Pinela, Barreira, Barros, Cabo Verde, Antonio, Oliveira, *et al.* (2016)
R. papillaris	Ep1	158 (80-273)	2.6	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
R. pulcher	Ep1	256 (58-576)	5.1	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
S. hispanicus	Ep1	265 (15-955)	1.1	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
S. vulgaris	Ep1	210 (202-219)	2.3	Sánchez-Mata *et al.* (2012)
S. marianum	Ep1	719 (171-1889)	5.4	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
S. oleraceus	Ep1	539 (180-898)	3.5	Morales *et al.* (2014)
T. communis	Ep1	62 (12-186)	1.2	Sánchez-Mata *et al.* (2012), Pereira *et al.* (2013)
T. obovatum	Ep1	10 (3-37)	0.1	Sánchez-Mata *et al.* (2012), Morales *et al.* (2014)
T. mastichina	Ep1	257 (234-280)	-	Pereira *et al.* (2013)

* Oxalic acid/Ca ratio calculated based on the Ca value of USDA.

nd: not detected

Annexe 3 Parametric results of the three-level full factorial design presented in Eq. [5] combining the effects of extraction time (X_1) and irradiation dose (X_2) on the recovery of phytochemicals from *T. lignosa* as function of the extracted residue. The analysis of significance of the parameters (α=0.05) and the statistical information of the fitting procedure to the model are presented.

	Fitting coefficients obtained after applying the Box-Behnken model						**Statistical information of the fitting analysis**						
	Intercept	**Linear effect**		**Quadratic effect**		**Interactive effect**	Obs	R^2	R^2adj	MSE	RMSE	MAPE	DW
	b_0	b_1 (t)	b_2 (D)	b_{11} (t^2)	b_{22} (D^2)	b_{12} ($t \times D$)							
Residue	10.17±0.56	8.49±0.39	-0.89±0.39	-1.69±0.68	*ns*	-0.48±0.27	27	0.9902	0.988	64.1	8.2	5.6	2.3
DPPH• scavenging activity	32.65±1.17	17.12±0.64	*ns*	-16.58±1.11	1.57±1.11	*ns*	27	0.9947	0.994	332	18.2	3.0	2.5
Reducing power	25.50±1.51	12.47±0.83	*ns*	-14.22±1.43	1.78±1.43	-1.56±1.01	27	0.9859	0.984	196	14.0	4.9	1.9
β-Carotene bleaching inhibition	30.53±10.76	17.55±7.61	*ns*	-12.98±13.17	*ns*	*ns*	27	0.5438	0.560	574	24.0	33.5	2.4
TBARS formation inhibition	6.17±2.22	3.21±1.57	*ns*	-2.96±2.72	*ns*	*ns*	27	0.5039	0.468	22.5	4.7	83.4	2.6
Oxalic acid	21.70±6.27	8.76±4.43	*ns*	-12.95±7.68	*ns*	*ns*	27	0.5447	0.526	209	14.4	51.1	1.8
Quinic acid	17.57±1.27	6.50±0.90	3.45±0.90	-11.07±1.56	*ns*	3.92±1.10	27	0.9622	0.954	93.7	9.7	10.5	2.5
Shikimic acid	47.54±2.31	15.96±1.26	2.15±1.26	-30.10±2.19	-2.22±2.19	*ns*	27	0.9857	0.983	490	22.1	5.8	2.7
Succinic acid	2.01±0.01	18.95±2.38	-6.08±2.38	16.94±2.38	*ns*	-9.11±2.92	27	0.9508	0.948	514	22.7	8.5	1.6
TOTAL	85.33±9.28	50.16±6.56	*ns*	-35.17±11.36	*ns*	-7.93±8.03	27	0.9278	0.913	2744	52.4	13.2	1.5
Punicalin	5.47±0.77	4.35±0.55	1.05±0.55	-1.12±0.95	*ns*	1.64±0.67	27	0.9355	0.922	20.35	4.51	16.01	2.7
Punicalagin (isomer 1)	15.25±1.18	7.80±0.65	0.63±0.48	-6.14±1.12	-1.96±1.12	*ns*	27	0.9724	0.971	66.22	8.14	6.61	3.2
Punicalagin gallate (isomer 1)	1.24±0.27	0.79±0.19	*ns*	-0.45±0.33	*ns*	0.28±0.23	27	0.7975	0.792	0.81	0.90	32.89	1.4
Punicalagin (isomer 2)	31.51±1.88	17.22±1.03	*ns*	-13.32±1.78	-1.47±1.35	*ns*	27	0.9842	0.983	310.2	17.61	5.88	2.5
Punicalagin gallate (isomer 2)	1.75±0.35	1.65±0.43	0.55±0.43	*ns*	*ns*	1.18±0.53	27	0.8975	0.876	4.1	2.6	58.1	1.4
TOTAL	55.67±3.65	31.81±2.00	2.32±2.00	-21.34±3.46	-3.78±3.46	4.75±2.45	27	0.9839	0.980	1036	32.2	6.3	1.8
Luteolin-6-*C*-glucose-8-*C*-glucose	48.43±2.53	27.13±1.38	*ns*	-22.83±2.40	2.29±2.19	*ns*	27	0.9888	0.988	789.9	28.11	4.9	3.3
5-*O*-*p*-Coumaroylquinic acid	45.08±4.21	29.62±2.98	-7.29±2.98	-15.46±5.16	*ns*	-10.34±3.65	27	0.9597	0.950	966	31.08	11.2	2.2
Luteolin-8-*C*-glucoside	79.82±6.21	69.09±4.39	13.60±4.39	*ns*	-12.93±7.60	17.30±5.38	27	0.9814	0.980	4553	67.5	7.3	2.4
Apigenin-8-*C*-glucoside	134.2±0.87	72.90±0.62	1.56±0.62	-61.34±1.07	*ns*	2.34±0.76	27	0.9997	1.000	5637	75.1	0.8	1.7
Quercetin-3-*O*-rutinoside	22.94±1.41	19.21±1.00	3.06±1.00	-3.73±1.73	*ns*	4.23±1.22	27	0.9873	0.985	342.8	18.52	6.5	1.3

	Fitting coefficients obtained after applying the Box-Behnken model						Statistical information of the fitting analysis						
	Intercept	**Linear effect**		**Quadratic effect**		**Interactive effect**	Obs	R^2	R^2adj	MSE	RMSE	MAPE	DW
Apigenin-6-*C*-glucoside	121.1±1.56	70.29±1.10	2.42±1.10	-50.84±1.91	*ns*	2.95±1.35	27	0.9989	0.999	4988	70.6	1.7	3.1
Kaempferol-3-*O*-rutinoside	64.34±2.69	50.67±1.90	-6.19±1.90	-13.67±3.30	*ns*	-4.11±2.33	27	0.9931	0.992	2312	48.09	5.1	2.7
Luteolin-6-*C*-hexoside	1.34±0.37	1.99±0.26	-0.31±0.26	0.65±0.45	*ns*	*ns*	27	0.9210	0.903	3.90	1.98	41.0	1.6
TOTAL	520.7±7.85	340.9±4.30	7.87±4.30	-173.6±7.45	-9.36±7.45	13.13±5.27	27	0.9993	0.999	1084	329	1.2	1.9

ns: non significant coefficient; R^2: Correlation coefficient; R^2adj: Adjusted coefficient of determination; *MSE*: Mean squared error; *RMSE*: Root mean square error; *MAPE*: Mean absolute percentage error; and *DW*: Durbin-Watson statistic.

Annexe 4 ANOVA table for the models developed with Eq. [5] for the *in vitro* assays used to evaluate the antioxidant activity of the extracts.

Source	***df***	***SS***	***MS***	$F_{statistic}$	***Pr > F***
DPPH• scavenging activity					
Model	5	536282.7	107256.5	128.9	< 0.0001
Error	21	17478.9	832.329		
Lack of fitting	19	16497.3	868.2	1.8	0.4225
Pure error	2	981.7	490.8		
Total corrected	26	553761.6	0.000		
Reducing power					
Model	5	330499.6	66099.9	88.4	< 0.0001
Error	21	15699.5	747.594		
Lack of fitting	19	14744.8	776.0	1.6	0.4490
Pure error	2	954.7	477.358		
Total corrected	26	346199.1	0.000		
β-Carotene bleaching inhibition					
Model	5	520924.4	104184.9	4.6	0.0055
Error	21	477845.3	22754.5		
Lack of fitting	19	475661.7	25034.8	22.9	0.0426
Pure error	2	2183.6	1091.7		
Total corrected	26	998769.7	0.000		
TBARS formation inhibition					
Model	5	22401.0	4480.2	5.5	0.0022
Error	21	17193.0	818.7		
Lack of fitting	19	17172.2	903.8	87.0	0.0114
Pure error	2	20.8	10.		
Total corrected	26	39594.0	0.000		

df: degree of freedom; *SS*: Sum of squares; *MS*: Mean square.

Annexe 5 ANOVA table for the models developed with Eq. [5] to evaluate the effects of the ionizing radiation dose on the extraction kinetics of organic acids.

Source	***df***	***SS***	***MS***	$F_{statistic}$	***Pr > F***
Oxalic acid					
Model	5	48.3	9.757	11.3	< 0.0001
Error	21	18.0	0.858		
Lack of fitting	19	15.3	0.807	0.6	0.7827
Pure error	2	2.7	1.337		
Total corrected	26	66.3			
Quinic acid					
Model	5	30.5	6.145	43.7	< 0.0001
Error	21	2.9	0.139		
Lack of fitting	19	2.0	0.105	0.2	0.9748
Pure error	2	0.9	0.470		
Total corrected	26	33.4			
Shikimic acid					
Model	5	155.2	31.05	122.1	< 0.0001
Error	21	5.3	0.254		
Lack of fitting	19	5.0	0.263	1.5	0.4650
Pure error	2	0.3	0.170		
Total corrected	26	160.5			
Succinic acid					
Model	5	315.6	63.18	90.4	< 0.0001
Error	21	14.7	0.698		
Lack of fitting	19	14.4	0.760	7.0	0.1322
Pure error	2	0.2	0.109		
Total corrected	26	330.3			
TOTAL					
Model	5	1374.6	274.9	138.5	< 0.0001
Error	21	41.7	1.984		
Lack of fitting	19	35.1	1.846	0.6	0.8055
Pure error	2	6.6	3.299		
Total corrected	26	1416.2			

df: degree of freedom; *SS*: Sum of squares; *MS*: Mean square.

Annexe 6 ANOVA table for the models developed with Eq. [5] to evaluate the effects of the ionizing radiation dose on the extraction kinetics of ellagitannins.

Source	*df*	*SS*	*MS*	$F_{statistic}$	*Pr > F*
Punicalin					
Model	5	10.5	2.12	78.5	< 0.0001
Error	21	0.6	0.027		
Lack of fitting	19	0.5	0.025	0.5	0.8316
Pure error	2	0.1	0.048		
Total corrected	26	11.1			
Punicalagin (isomer 1)					
Model	5	32.3	6.54	69.9	< 0.0001
Error	21	1.9	0.092		
Lack of fitting	19	1.8	0.097	2.2	0.3584
Pure error	2	0.1	0.044		
Total corrected	26	34.2			
Punicalagin gallate (isomer 1)					
Model	5	0.4	0.1	21.1	< 0.0001
Error	21	0.1	0.003		
Lack of fitting	19	0.1	0.004	4.6	0.1931
Pure error	2	0.0	0.001		
Total corrected	26	0.4			
Punicalagin (isomer 2)					
Model	5	155.4	31.1	100.8	< 0.0001
Error	21	6.5	0.308		
Lack of fitting	19	6.4	0.338	11.0	0.0866
Pure error	2	0.1	0.031		
Total corrected	26	161.8			
Punicalagin gallate (isomer 2)					
Model	5	1.9	0.477	17.5	< 0.0001
Error	21	0.5	0.022		
Lack of fitting	19	0.5	0.024	13.1	0.0731
Pure error	2	0.0	0.002		
Total corrected	26	2.4			
TOTAL					
Model	5	526.1	105.2	94.0	< 0.0001
Error	21	23.5	1.119		
Lack of fitting	19	23.5	1.236	96.4	0.0103
Pure error	2	0.0	0.013		
Total corrected	26	549.6			

df: degree of freedom; *SS*: Sum of squares; *MS*: Mean square.

Annexe 7 ANOVA table for the models developed with Eq. [5] to evaluate the effects of the ionizing radiation dose on the extraction kinetics of flavonoids and phenolic acid.

Source	*df*	*SS*	*MS*	$F_{statistic}$	*Pr > F*
Luteolin-6-C-glucose-8-C-glucose					
Model	5	384.3	76.9	677.2	< 0.0001
Error	21	2.4	0.113		
Lack of fitting	19	2.0	0.107	0.6	0.7743
Pure error	2	0.3	0.173		
Total corrected	26	386.7			
5-O-p-Coumaroylquinic acid					
Model	5	564.1	112.8	254.1	< 0.0001
Error	21	9.3	0.444		
Lack of fitting	19	9.0	0.471	2.6	0.3164
Pure error	2	0.4	0.183		
Total corrected	26	573.4			
Luteolin-8-C-glucoside					
Model	5	2665.4	533.1	109.6	< 0.0001
Error	21	102.1	4.86		
Lack of fitting	19	100.9	5.30	8.4	0.1111
Pure error	2	1.3	0.62		
Total corrected	26	2767.5			
Apigenin-8-C-glucoside					
Model	5	2778.3	555.7	390.2	< 0.0001
Error	21	29.9	1.42		
Lack of fitting	19	29.8	1.56	26.6	0.0368
Pure error	2	0.1	0.059		
Total corrected	26	2808.2			
Quercetin-3-O-rutinoside					
Model	5	197.1	39.4	206.0	< 0.0001
Error	21	4.0	0.19		
Lack of fitting	19	3.9	0.20	3.7	0.2354
Pure error	2	0.1	0.056		
Total corrected	26	201.1			
Apigenin-6-C-glucoside					
Model	5	2568.0	513.6	320.5	< 0.0001
Error	21	33.7	1.60		
Lack of fitting	19	33.6	1.76	38.7	0.0255
Pure error	2	0.1	0.05		
Total corrected	26	2601.7			
Kaempferol-3-O-rutinoside					
Model	5	1419.2	283.8	1350.2	< 0.0001
Error	21	4.4	0.210		
Lack of fitting	19	4.1	0.215	1.3	0.5316
Pure error	2	0.3	0.169		
Total corrected	26	1423.6			
Luteolin-6-C-hexoside					
Model	5	2.3	0.5	76.1	< 0.0001
Error	21	0.1	0.006		
Lack of fitting	19	0.1	0.006	2.1	0.3678
Pure error	2	0.0	0.003		
Total corrected	26	2.5			
TOTAL					
Model	5	60556.1	12111.2	536.5	< 0.0001
Error	21	474.1	22.5		
Lack of fitting	19	473.4	24.9	77.8	0.0128
Pure error	2	0.6	0.32		
Total corrected	26	61030.2			

df: degree of freedom; *SS*: Sum of squares; *MS*: Mean square.

Annexe 8 Pearson's correlation coefficients (*R*) of phenolic compounds and antioxidant activity[1] in fresh-cut watercress submitted to different postharvest preservation treatments.

Compounds	DPPH free-radical scavenging activity		Reducing power		β-Carotene bleaching inhibition capacity		TBARS formation inhibition capacity	
	R	Sig.	*R*	Sig.	*R*	Sig.	*R*	Sig.
Quercetin-3-*O*-sophoroside	-0.547**	0.006	-0.369	0.076	-0.382	0.066	0.250	0.238
p-Coumaric acid hexoside	0.567**	0.004	0.023	0.916	0.599**	0.002	-0.028	0.897
Quercetin-3-*O*-manolylglucoside-7-*O*-glucoside	-0.593**	0.002	-0.083	0.698	-0.758**	0.000	-0.487*	0.016
Ferulic acid hexoside	0.508*	0.011	-0.500*	0.013	-0.357	0.087	-0.087	0.686
Roseoside	-0.527**	0.008	-0.424*	0.039	-0.405*	0.049	-0.100	0.642
Caffeic acid	-0.097	0.654	0.050	0.818	0.090	0.674	0.008	0.971
p-Coumaric acid	0.134	0.533	0.221	0.300	-0.103	0.633	0.267	0.207
Quercetin-3-*O*-rutinoside-7-*O*-glucoside	0.330	0.116	0.302	0.152	0.264	0.212	-0.251	0.236
Ferulic acid	0.408*	0.048	0.588*	0.003	0.418*	0.042	-0.033	0.878
Sinapoylmalic acid	-0.142	0.509	0.455*	0.026	-0.119	0.579	-0.110	0.610
Quercetin-3-*O*-rutinoside (rutin)	-0.706**	0.000	-0.118	0.582	-0.839**	0.000	-0.421*	0.041
Quercetin-*O*-sophoroside-*O*-rutinoside	0.000	1.000	0.161	0.453	0.057	0.793	-0.219	0.304
Quercetin-*O*-coumaroylsophoroside	-0.942**	0.000	-0.496*	0.014	-0.843**	0.000	0.115	0.593
Quercetin-*O*-sophoroside-*O*-malonylhexoside	-0.810**	0.000	-0.256	0.228	-0.942**	0.000	-0.355	0.089
Quercetin-O-dihexosyl-O-malonylhexoside	-0.880**	0.000	-0.473*	0.020	-0.906**	0.000	-0.101	0.639
Quercetin-*O*-sinapoylhexoside-*O*-rutinoside	-0.943**	0.000	-0.510*	0.011	-0.852**	0.000	0.085	0.692
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-hexoside	-0.799**	0.000	-0.400	0.053	-0.672**	0.000	0.106	0.622
Isorhamnetin-*O*-sophoroside-*O*-hexoside	-0.751**	0.000	-0.363	0.082	-0.616**	0.001	0.171	0.424
Isorhamnetin-*O*-hydroxyferuloylhexoside-*O*-malonylhexoside	-0.870**	0.000	-0.345	0.098	-0.950**	0.000	-0.270	0.201
Isorhamnetin-*O*-sophoroside-*O*-malonylhexoside	-0.694**	0.000	-0.037	0.862	-0.863**	0.000	-0.423*	0.039
Kaempferol-*O*-feruloylhexoside-*O*-rutinoside	-0.897**	0.000	-0.548**	0.006	-0.784**	0.000	0.082	0.704
Kaempferol-*O*-feruloylhexoside-*O*-hexoside	-0.891**	0.000	-0.555**	0.005	-0.769**	0.000	0.098	0.648
Kaempferol-*O*-hydroxyferuloylglucuronide-*O*-malonylhexoside	-0.881**	0.000	-0.394	0.057	-0.810**	0.000	0.079	0.713
Kaempferol-*O*-feruloylhexoside-*O*-malonylhexoside	-0.778**	0.000	-0.210	0.324	-0.884**	0.000	-0.265	0.210

Compounds	DPPH free-radical scavenging activity		Reducing power		β-Carotene bleaching inhibition capacity		TBARS formation inhibition capacity	
	R	Sig.	*R*	Sig.	*R*	Sig.	*R*	Sig.
Total phenolic acids	-0.105	0.625	-0.004	0.984	0.095	0.660	0.253	0.233
Total flavonoids	-0.908**	0.000	-0.463*	0.023	-0.889**	0.000	-0.063	0.771
Total phenolic compounds	-0.761**	0.000	-0.407*	0.048	-0.614**	0.001	0.142	0.509

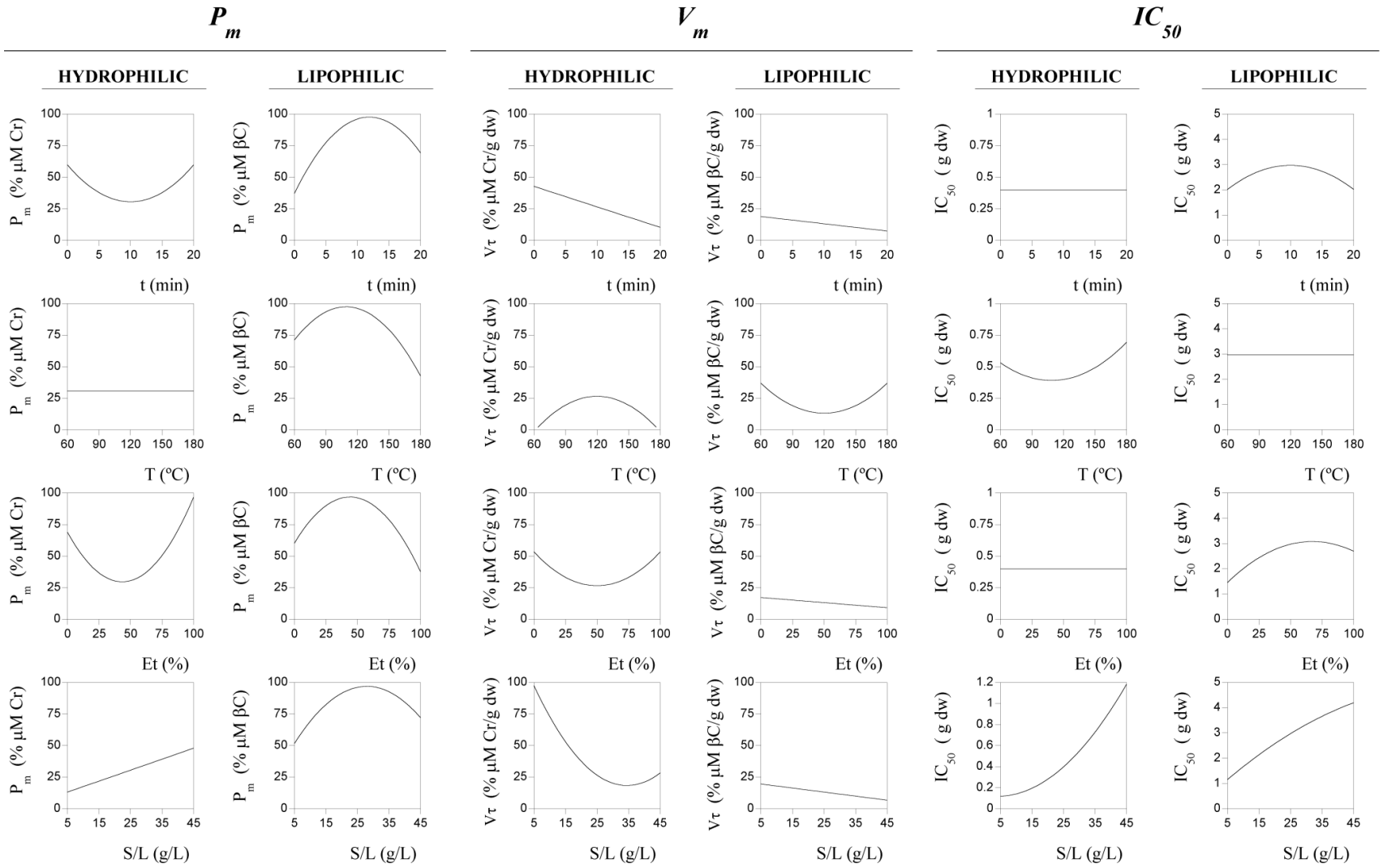
[1]Results of the antioxidant activity published in Pinela, Barreira, Barros, Antonio, et al. (2016) and Pinela, Barreira, Barros, Cabo Verde, et al. (2016).

*Significant at $P \leq 0.05$.

**Significant at $P \leq 0.01$.

≥ 0.9 Very strong correlation
0.7–0.9 Strong correlation
0.5–0.7 Moderate correlation

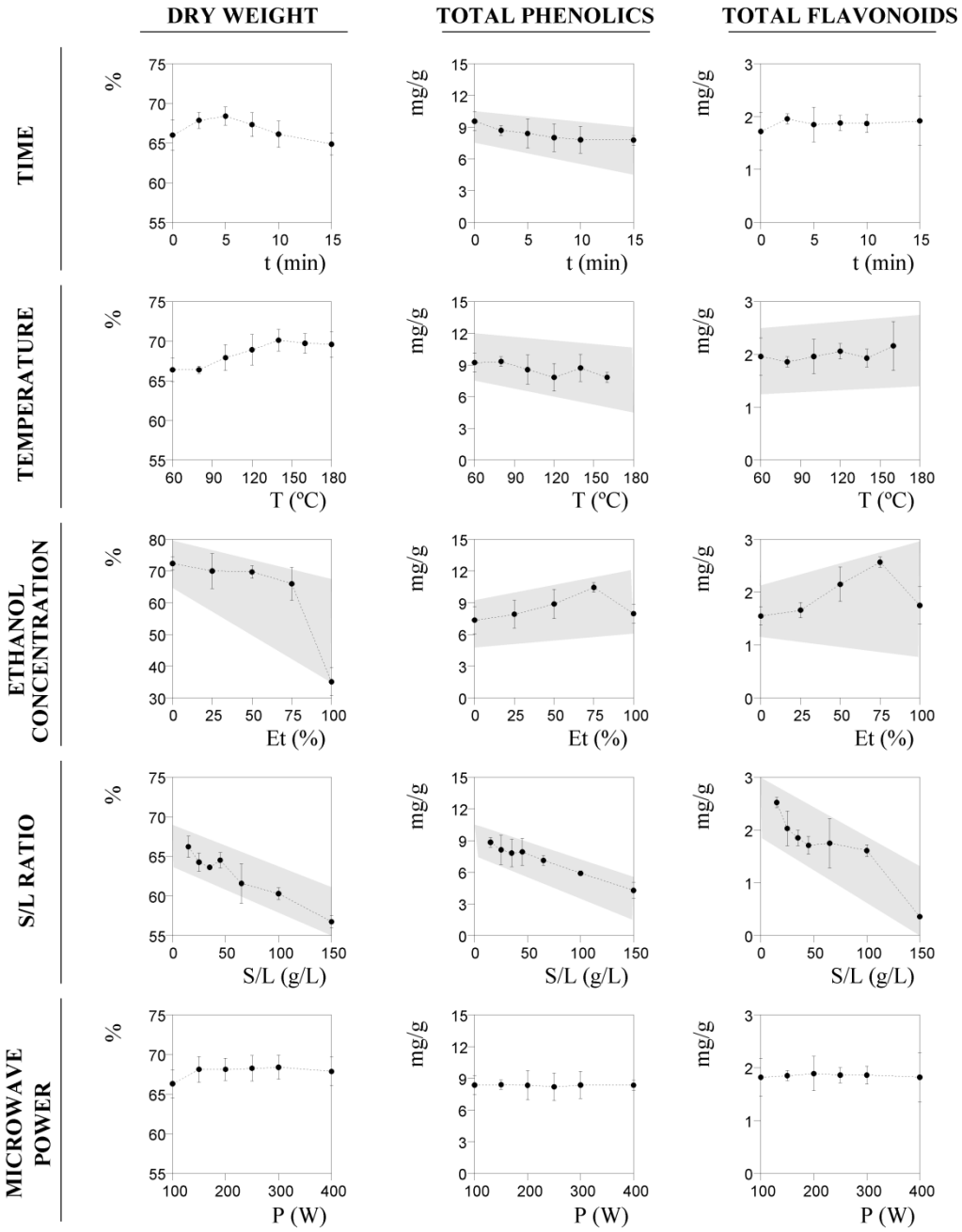
Annexe 9 Individual responses for the *H* and *L* antioxidant reactions obtained for the parametric coefficients P_m (% µM of Cr), V_m (% µM of Cr/g extract) and IC_{50} (g extract). The variables excluded in each graph were positioned at the center of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L).



Annexe 10 Partial derivatives of all the response criteria (P_m, V_m and IC_{50}) are presented in the multivariable characterization Box-Behnken second-order polynomial model of Eqs. (9) to (11) for the *H* reaction and in Eqs. (12) to (14) for the *L* reaction. Note that X_1 (extraction time), X_2 (temperature), X_3 (ethanol concentration) and X_4 (solid-liquid-ratio) are the independent variables and *Y* the response in the tested criteria.

	$\partial Y / \partial x_1$	$\partial Y / \partial x_2$	$\partial Y / \partial x_3$	$\partial Y / \partial x_4$
Hydrophilic reaction				
P_m	$\frac{\partial Y_{Pm}^{H}}{\partial x_1} = 14.6x_1 + 13.1x_2$	$\frac{\partial Y_{Pm}^{H}}{\partial x_2} = 13.1x_1 - 4.5x_3$	$\frac{\partial Y_{Pm}^{H}}{\partial x_3} = 8.7 - 4.5x_2$	$\frac{\partial Y_{Pm}^{H}}{\partial x_3} = 6.9 + 26.2x_4$
V_r	$\frac{\partial Y_{V\tau}^{H}}{\partial x_1} = -8.1$	$\frac{\partial Y_{V\tau}^{H}}{\partial x_2} = 13.8x_2$	$\frac{\partial Y_{V\tau}^{H}}{\partial x_3} = 18.2x_3$	$\frac{\partial Y_{V\tau}^{H}}{\partial x_3} = 14x_4$
IC_{50}	$\frac{\partial Y_{IC_{50}}^{H}}{\partial x_1} = 0.16x_2$	$\frac{\partial Y_{IC_{50}}^{H}}{\partial x_2} = 0.04 + 0.1x_2 + 0.16x_1 - 0.05x_3$	$\frac{\partial Y_{IC_{50}}^{H}}{\partial x_3} = 0.1x_3 - 0.05x_2$	$\frac{\partial Y_{IC_{50}}^{H}}{\partial x_3} = 0$
Lipophilic reaction				
P_m	$\frac{\partial Y_{Pm}^{L}}{\partial x_1} = 8.1 - 21.4x_1 - 7.7x_2$	$\frac{\partial Y_{Pm}^{L}}{\partial x_2} = -7.1 - 19.6x_2 - 7.7x_1 + 13.1x_3$	$\frac{\partial Y_{Pm}^{H}}{\partial x_3} = 5.1\text{-}17.2x_3 + 13.1x_2$	$\frac{\partial Y_{Pm}^{L}}{\partial x_3} = -5.6 - 23.6x_4$
V_r	$\frac{\partial Y_{V\tau}^{L}}{\partial x_1} = -2.9 + 2.3x_3 + 1.6x_4$	$\frac{\partial Y_{V\tau}^{L}}{\partial x_2} = 11.8x_2 - 1.7x_3 + 6.2x_4$	$\frac{\partial Y_{V\tau}^{L}}{\partial x_3} = -3.2 + 2.3x_1\text{-}1.7x_2$	$\frac{\partial Y_{V\tau}^{L}}{\partial x_3} = -2 + 1.6x_1 + 6.2x_2$
IC_{50}	$\frac{\partial Y_{IC_{50}}^{L}}{\partial x_1} = \text{-}0.48x_1 - 0.24x_4$	$\frac{\partial Y_{IC_{50}}^{L}}{\partial x_2} = 0.43x_3$	$\frac{\partial Y_{IC_{50}}^{L}}{\partial x_3} = 0.76 - 0.014x_3 + 0.43x_2 + 0.27x_4$	$\frac{\partial Y_{IC_{50}}^{L}}{\partial x_3} = 0.31 - 0.44x_4 - 0.24x_1 + 0.27x_3$

Annexe 11 Results of the preliminary study carried out to select significant variables and determine optimum ranges for an appropriate RSM design. The independent variables of extraction time (0-15 min), temperature (60-180 ºC), ethanol concentration (0-100 %), solid/liquid ratio (5-150 g/L), and microwave power (100-400 W) were investigated. The extraction yield (% of dry weight) and the amounts of total phenolic (mg of gallic acid equivalents per g of extract) and flavonoids (mg of catechin equivalents per g of extract) were evaluated as responses. The shaded results showed statistically significant differences and the corresponding independent variables were selected for the MAE optimization by RSM.



Annexe 12 Parametric estimations and statistical information of the mathematical models of the Eq. (1) for the DPPH free-radical scavenging activity and Eq. (2) for the reducing power. All coefficients showed effects with significant parametric intervals at the 95% confidence level.

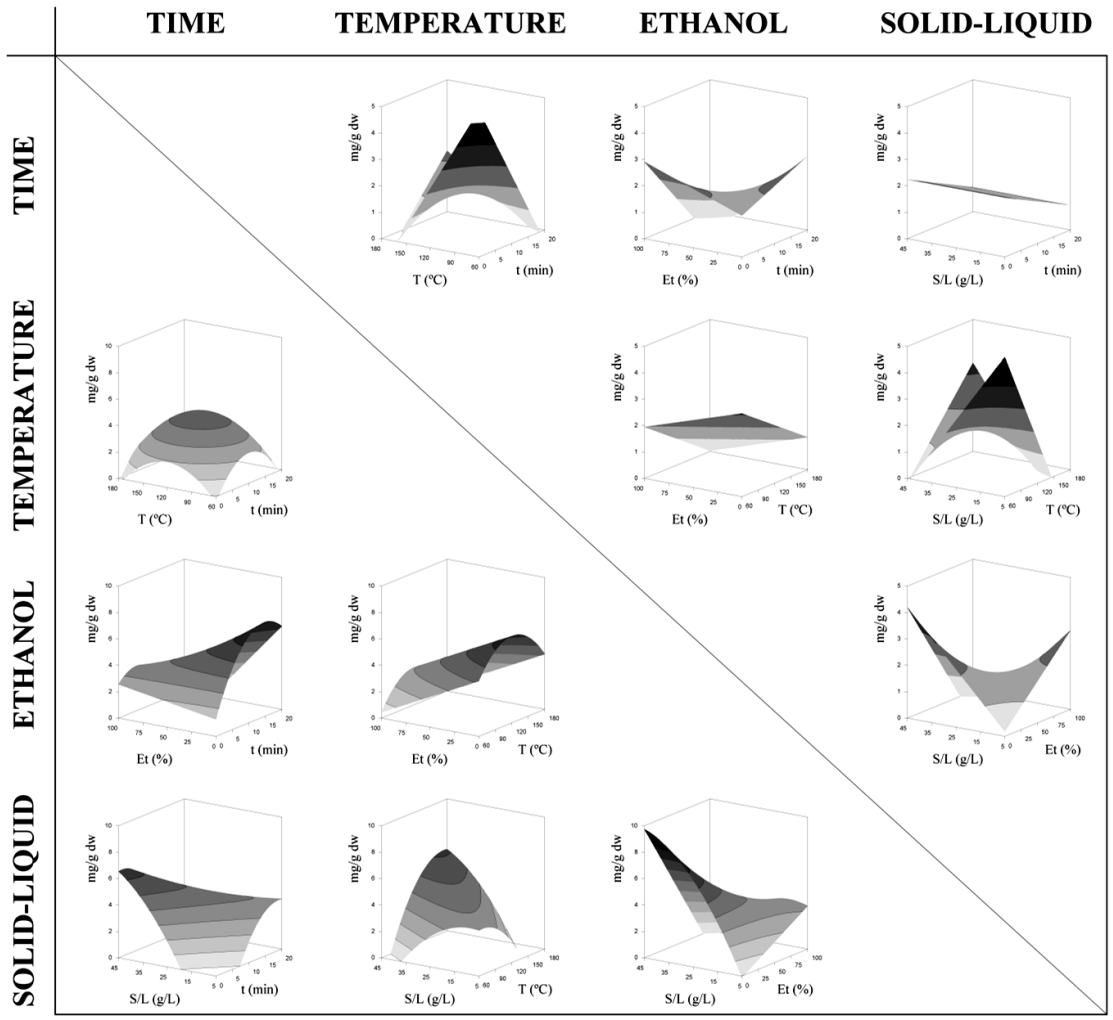
Run	Experimental domain				Parametric estimations						
					DPPH free-radical scavenging activity					Reducing power	
	X_1: t	X_2: T	X_3: Et	X_4: S/L	K	IC_{50}	a	vm	R^2	m	R^2
	min	ºC	%	g/L	µM DPPH	g extract	--	µM DPPH/g extract	--	µM Fe^{2+}/g extract	--
1	-1(5)	-1(90)	-1(25)	-1(15)	30.0	9.70	1.49	1.60	0.993	42.68	0.994
2	1(15)	-1(90)	-1(25)	-1(15)	30.0	12.82	1.23	1.00	0.999	35.75	0.994
3	-1(5)	1(150)	-1(25)	-1(15)	30.0	10.04	1.06	1.10	0.992	49.89	0.996
4	1(15)	1(150)	-1(25)	-1(15)	30.0	6.43	1.16	1.88	0.996	91.48	0.997
5	-1(5)	-1(90)	1(75)	-1(15)	30.0	5.08	1.44	2.94	0.996	95.21	0.998
6	1(15)	-1(90)	1(75)	-1(15)	30.0	8.03	1.39	1.80	0.997	59.10	0.997
7	-1(5)	1(150)	1(75)	-1(15)	30.0	4.62	1.29	2.92	0.997	179.16	0.996
8	1(15)	1(150)	1(75)	-1(15)	30.0	3.69	1.22	3.44	0.997	190.39	0.997
9	-1(5)	-1(90)	-1(25)	1(35)	30.0	6.62	1.48	2.32	0.995	40.79	0.996
10	1(15)	-1(90)	-1(25)	1(35)	30.0	8.05	1.44	1.86	0.998	34.23	0.994
11	-1(5)	1(150)	-1(25)	1(35)	30.0	7.13	1.18	1.72	0.994	75.54	0.998
12	1(15)	1(150)	-1(25)	1(35)	30.0	4.88	1.19	2.53	0.991	112.97	0.999
13	-1(5)	-1(90)	1(75)	1(35)	30.0	6.34	1.51	2.49	0.995	53.33	0.997
14	1(15)	-1(90)	1(75)	1(35)	30.0	7.89	1.54	2.02	0.997	46.10	0.995
15	-1(5)	1(150)	1(75)	1(35)	30.0	2.54	1.35	5.52	0.989	56.96	0.998
16	1(15)	1(150)	1(75)	1(35)	30.0	1.86	1.34	7.47	0.986	66.49	0.997
17	-2(0)	0(120)	0(50)	0(25)	30.0	6.69	1.25	1.95	0.994	46.77	0.996
18	2(20)	0(120)	0(50)	0(25)	30.0	12.20	1.27	1.08	0.988	29.43	0.991
19	0(10)	-2(60)	0(50)	0(25)	30.0	7.17	1.36	1.97	0.996	58.03	0.995
20	0(10)	2(180)	0(50)	0(25)	30.0	4.83	1.12	2.42	0.996	101.85	0.998
21	0(10)	0(120)	-2(0)	0(25)	30.0	12.63	1.32	1.09	0.992	25.12	0.993
22	0(10)	0(120)	2(100)	0(25)	30.0	8.13	1.15	1.47	0.991	97.27	0.990
23	0(10)	0(120)	0(50)	-2(5)	30.0	21.68	0.72	0.34	0.960	32.15	0.986
24	0(10)	0(120)	0(50)	2(45)	30.0	12.66	1.33	1.10	0.997	36.79	0.997
25	0(10)	0(120)	0(50)	0(25)	30.0	12.50	1.33	1.11	0.994	29.44	0.995
26	0(10)	0(120)	0(50)	0(25)	30.0	12.40	1.35	1.13	0.996	27.79	0.996
27	0(10)	0(120)	0(50)	0(25)	30.0	12.91	1.37	1.11	0.997	28.15	0.994
28	0(10)	0(120)	0(50)	0(25)	30.0	14.11	1.25	0.92	0.997	28.21	0.993
29	0(10)	0(120)	0(50)	0(25)	30.0	13.33	1.34	1.05	0.996	34.09	0.998
30	0(10)	0(120)	0(50)	0(25)	30.0	13.51	1.30	1.00	0.997	28.58	0.996
31	0(10)	0(120)	0(50)	0(25)	30.0	14.00	1.13	0.84	0.997	32.01	0.997
32	0(10)	0(120)	0(50)	0(25)	30.0	13.29	1.24	0.97	0.998	28.07	0.994

Annexe 13 ANOVA table for the five-level Box-Behnken central composite design for the combined effect of *t*, *T*, *Et* and *S/L* on the extraction yield of phenolic acids and flavonoids and on maximizing of the antioxidant activity of the extracts according to Eq. (6) and presented in Eqs. (7)-(12).

Source	***df***	***SS***	***MS***	$F_{statistic}$	***Pr > F***
a) Analysis of the MAE extraction of presented in Eq. (7)					
Model	9	74.42	5.3154	37.03	< 0.0001
Error	22	2.44	0.1435		
Lack of fitting	10	2.40	0.2402	43.71	< 0.0001
Pure error	7	0.04	0.0055		
Total corrected	31	76.86			
b) Analysis of the MAE extraction of presented in Eq. (8)					
Model	9	506.26	36.161	29.72	< 0.0001
Error	22	20.68	1.216		
Lack of fitting	10	20.49	2.048	73.62	< 0.0001
Pure error	7	0.19	0.027		
Total corrected	31	526.94			
c) Analysis of the MAE extraction of F1 presented in Eq. (9)					
Model	8	16.25	1.1611	24.46	< 0.0001
Error	23	0.81	0.0475		
Lack of fitting	10	0.78	0.0784	23.67	0.0002
Pure error	7	0.02	0.0033		
Total corrected	31	17.06			
d) Analysis of the MAE extraction of F2 presented in Eq. (10)					
Model	8	37.58	2.6844	20.17	< 0.0001
Error	23	2.26	0.1331		
Lack of fitting	10	2.21	0.2214	31.55	< 0.0001
Pure error	7	0.05	0.0070		
Total corrected	31	39.84			
e) Analysis of the DPPH free-radical scavenging activity presented in Eq. (11)					
Model	10	42.45	3.0323	25.32	< 0.0001
Error	21	2.04	0.1198		
Lack of fitting	10	2.03	0.2028	171.02	< 0.0001
Pure error	7	0.01	0.0012		
Total corrected	31	44.49			
f) Analysis of the reducing power presented in Eq. (12)					
Model	11	42563.8	3040.2	20.84	< 0.0001
Error	20	2479.9	145.8		
Lack of fitting	10	2470.5	247.1	185.1	< 0.0001
Pure error	7	9.34	1.31		
Total corrected	31	45043.7			

df: degree of freedom; *SS*: Sum of squares; *MS*: Mean square.

Annexe 14 Matrix combination for the response surfaces of selected phenolic compounds. The top diagonal part shows the response surface of F_1 (benzyl alcohol dihexose) and the bottom diagonal part shows the response surface of P_1 (quercetin pentosylrutinoside). For representation purposes, the variables excluded in each 3D graph were positioned at the centre of the experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L).



Annexe 15 Individual responses of all studied parameters. The variables excluded in each of the 2D graphs were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L).