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ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

OHMIC-HEATING TREATMENT AS A NEW PROCESS FOR DEVELOPMENT OF FUNCTIONAL INGREDIENTS THROUGH VALORISATION OF TOMATO AND GRAPE BY-PRODUCTS

Thesis submitted to *Universidade Católica Portuguesa, Universidade do Minho and Universidade de Aveiro* to attain the degree of PhD in

Ciência Tecnologia Alimentar e Nutrição

Marta Isabel Correia Coelho

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Marta Isabel Correia Coelho

Supervisor: ***Professor Maria Manuela Estevez Pintado***
Co-supervisors: ***Professor José António Teixeira***
Professor António Sebastião Rodrigues

October 2021

To my family and friends:

"Some men see things as they are and say why, I dream things that never were and say, why not?"

George Bernard Shaw

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Resumo

A produção de tomate e vinho são os principais sectores agroindustriais em Portugal. Estas atividades produzem e acumulam grandes quantidades de resíduos, sobretudo, sementes, cascas e restos de polpa, com elevada preocupação económica e ambiental em todo o mundo. Estes resíduos são tipicamente ricos em compostos bioactivos (CB), como por exemplo, proteínas, açúcares e lipídos, bem como compostos fenólicos (CF) e carotenoides. Assim sendo, podem constituir uma fonte económica, de elevado valor agregado, com potenciais aplicações na área alimentar, cosmética ou farmacêutica. No entanto, os métodos de extração atuais (por exemplo, extração com solventes químicos) além de serem um perigo ambiental, podem também degradar os CB e promover a sua toxicidade, reduzindo propriedades biológicas e benefícios à saúde, dificultando o seu uso como produtos de valor agregado. O principal objetivo desta tese foi explorar o valor de bagaço de uva e de tomate para promover o princípio do desenvolvimento sustentável, com a obtenção de ingredientes de valor acrescentado e resíduo zero. Assim, foi realizado um estudo de otimização da extração de CB a partir destes resíduos, utilizando o aquecimento óhmico (OH) e solventes utilizados na indústria alimentar, para garantir a manutenção da integridade composicional, funcionalidade e segurança dos extratos bioativos obtidos e comparar com o método tradicional o qual utiliza solventes orgânicos (CONV).

Inicialmente foi utilizado um grupo alargado de subprodutos da uva, nomeadamente amostras de engaço e bagaço utilizadas na produção de vinho branco e tinto, assim como o bagaço de tomate, obtido da indústria de processamento. Procedeu-se à caracterização composicional e propriedades fitoquímicas dos subprodutos para verificar o potencial da matriz a ser utilizado e definir a estratégia de valorização. O bagaço de uva apresentou maior teor de proteína e de carboidratos e de CB do que o engaço, que se caracterizou principalmente por um alto teor de fibra. Relativamente ao bagaço do tomate, utilizaram-se amostras da mesma cultivar de tomate Heinz, de duas indústrias diferentes e compararam-se os resultados. Ambas as amostras apresentam teores de proteínas, entre 16,3 e 19,4 g / 100 g PS; teor de fibra entre 57,8 e 59,0 g / 100 g PS, além disso, são ricos em ácidos gordos 17,0 g / 100 g, principalmente, polinsaturados, ácido linoleico, oleico e o ácido palmítico. Após caraterização completa, selecionaram-se como mais promissores o bagaço de uva tinta e de tomate e aplicou-se OH para extrair os CB e reduzir os impactos ambientais com base numa estrategia de na economia circular. Como solventes foram usados a água isoladamente ou combinada com etanol, para promover a extração de compostos mais lipofílicos, como por exemplo os carotenos. Em paralelo procedeu-se a comparação dos resultados com o método CONV. Após a extração obtiveram-se duas frações diferentes, o extrato líquido (FL) rico em CB solubilizados e ainda, o remanescente, a fração sólida (FS) que também possui relevante potencial nutricional e funcional. No caso do bagaço, a FL, não apresentou diferenças significativas ($p>0.05$) entre o CONV e o OH para os compostos fenólicos totais (CFT), 2,84 ± 0,037 e 3,28 ± 0,46 mg / g PS equivalente de ácido gálico, respetivamente. A mesma tendência foi encontrada para a actividade antioxidante (AA), onde CONV e OH apresentaram valores de 2,02 ± 0,007 g / 100 g e 2,34 ± 0,066 g / 100 g equivalente de ácido ascórbico, respetivamente. As principais antocianinas identificadas foram malvidina-3-*O*-acetilglucosídeo, delfinidina-3-*O*-glucosídeo, petunidina-3-*O*-glucosídeo. Esses extratos exibiram potencial antimicrobiano contra os microrganismos *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Salmonella* Enteritidis, *Staphylococcus aureus* sensível à meticilina, *S. aureus* resistente à meticilina (MRSA) e *Bacillus cereus*. A FS obtida por OH mostrou uma

composição com potencial ingrediente funcional, tendo evidenciado maiores quantidades de proteínas e CF ligados à fibra do que o CONV. Relativamente ao bagaço de tomate, obteve-se uma FL rica em carotenoides, polifenóis, e açúcares e uma FS rica em fibras ligada a polifenóis e carotenoides. Também, novas moléculas foram identificadas pela análise LC-ESI-UHR-OqTOF-MS, como feno-di-hexano e N-acetil-D-triptofano. Após uma primeira avaliação da exequibilidade da aplicação do OH, fez-se um desenho experimental para obter melhores rendimentos. Foram utilizados também diferentes campos elétricos moderados (MEF) de diferentes intensidades (ou seja, 4, 6 e 11 V.cm-1) para identificar a presença de efeitos não térmicos no processo de extração e a sua influência nas propriedades bioativas dos compostos extraídos. A extração de CF usando OH foi otimizada com sucesso com as melhores condições de extração correspondendo a 70 ºC por 15 min usando etanol 70% como solvente, que permitiu uma maior recuperação de rutina em 77% do que as amostras controlo. Permitiu recuperar até 4,93 µg / g de licopeno dos subprodutos do tomate sem recorrer a solventes orgânicos, mostrando-se como método de extração seletivo, dependendo dos compostos de interesse. Como não existem estudos sobre o impacto das metodologias de extração e da ação do trato gastrointestinal (TGI) na bioacessibildiade de CB obtidos a partir de subprodutos do tomate, simulou-se o TGI para cada uma das FL e FS obtidas pelo métodos OH e CONV. Na FL os resultados mostraram que a extração influencia significativamente a bioacessibilidade dos CB presentes, com OH demonstrando um impacto positivo na preservação dos CF e consequentemente nas propriedades biológicas associadas, como propriedades antioxidante, anti-hipertensiva, prebiótica e anti-inflamatória. Os principais CB identificados por UPLC-qTOF-MS foram o ácido *p*-cumárico (163 m / z), naringenina (271 m / z) e luteolina (285 m / z). Além disso, o extrato obtido por OH após simulação do TGI exibiu efeito pré-biótico sobre diferentes estirpes de *Bifidobacterium* e *Lactobacillus.* Relativamente à FS os resultados mostraram que o tratamento OH originou uma farinha (FSOH) com maior fibra total do que as farinhas obtidas com o CONV (FSCONV), 62,47 ± 1,24 e 59,06 ± 0,67 g / 100 g PS, respetivamente. Ambas as farinhas apresentaram alto teor de proteína resistente, representando entre 11 a 16% da fibra alimentar insolúvel. Os principais carotenoides identificados foram o licopeno, fitoflueno e luteína, todos conhecidos como promotores de saúde. Apesar dos valores iniciais maiores de polifenóis e carotenóides na FSCONV, os CB da FSOH foram mais bioacessíveis e apresentaram maior capacidade antioxidante do que os presentes no FSCONV, em todo o TGI.
Finalmente, e porque existe um suporte científico sobre os efeitos positivos sobre a saúde funcional de prebióticos na microbiota intestinal avaliou-se a fermentabilidade das FSOH e FSCONV de tomate. Os resultados mostraram um maior crescimento de Bacteroidetes com FSOH e os maiores valores de Bacteroides para FSCONV. Uma correlação entre o crescimento de microrganismos e ácidos gordos de cadeia curta também foi encontrada. Assim sendo, o tratamento OH permitiu rendimentos de recuperação semelhantes com tempos de tratamento menores, e sem necessidade de solventes orgânicos (rotas de extração verdes).
Em suma, este trabalho irá contribuir para a sustentabilidade das indústrias do vinho e do processamento do tomate num contexto de economia circular, na medida em que apresenta um processo amigo do ambiente, rápido e económico capaz de recuperar CB com elevado potencial de aplicação, gerando ingredientes funcionais para aplicação alimentar, nutracêutica ou cosmética.

Palavras-chave: residuos; bagaço de uva, bagaço de tomate; aquecimento ohmico; economia circular; bioacessibilidade, benefícios para a saúde

Abstract

Tomato and wine production are the main agro-industrial sectors in Portugal. These activities produce and accumulate large amounts of waste, especially seeds, peels and pulp residues, with high economic and environmental concerns worldwide. These residues are typically rich in bioactive compounds (BC), such as proteins, sugars and lipids, and phenolic compounds (PC) and carotenoids. Therefore, they can be an economical source of high added value, with potential food, cosmetic, or pharmaceutical industry applications. However, current extraction methods (e.g. extraction with chemical solvents) and environmental hazards can also degrade BC and promote toxicity, reducing biological properties and health benefits, making it difficult to use as value-added products. The main objective of this thesis was to explore the value of grape and tomato bagasse to promote the principle of sustainable development, with the achievement of ingredients of added value and zero waste. Thus, an optimisation study of BC extraction from these by-products was carried out, using ohmic heating (OH) method and solvents used in the food industry, to ensure the maintenance of compositional integrity, functionality and safety of bioactive extracts obtained and compared with the traditional method in which it is used organic solvents (CONV).

Initially, a group of grape by-products was used, namely stalk and bagasse samples from white and red wine and tomato bagasse, obtained from the processing industry. The compositional characterisation and phytochemical properties of the byproducts were carried out to verify the potential of the matrix to be used and to define the recovery strategy.

Grape bagasse presented higher protein, carbohydrate, and BC content than stalks, mainly characterised by high fibre content. Concerning tomato bagasse, samples of the same Heinz tomato cultivar from two different industries were used, and the results were compared. Both samples presented protein contents between 16.3 and 19.4 g / 100 g DW; fibre content between 57.8 and 59.0 g / 100 g DW, mainly polyunsaturated, linoleic acid, oleic and palmitic acid. After complete characterisation, the grape and tomato bagasses were selected as the most promising byproducts and OH was applied to extract the BC and reduce the environmental impacts based on a circular economy strategy. As solvents, water was used alone or combined with ethanol to promote the extraction of more lipophilic compounds, such as carotenoids. In parallel, a CONV method was used to compare the results. After extraction, two different fractions were obtained: the liquid fraction (LF) rich in BC solubilised and the remnant, the solid fraction (SF) with relevant nutritional and functional potential. In the case of bagasse, LF did not present significant differences ($p>0.05$) between CONV and OH for total phenolic compounds (TPC), 2.84 ± 0.037 and 3.28 ± 0.46 mg / g DW equivalent of gallic acid, respectively. The same trend was found for antioxidant activity (AA), where CONV and OH presented values of 2.02 ± 0.007 g / 100 g and 2.34 ± 0.066 g / 100 g ascorbic acid equivalent, respectively. The main anthocyanins identified were malvidin-3-*O*-acetylglucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside. These extracts exhibited antimicrobial potential against the microorganisms *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Salmonella* Enteritidis, Methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA) and *Bacillus cereus*. SFOH showed a composition with potential functional ingredients, showing higher proteins

and BC bound to fibre than CONV. Regarding tomato bagasse, a LF rich in carotenoids, polyphenols, and sugars and a SF rich in fibres linked to polyphenols and carotenoids were obtained. Also, new molecules were identified by UPLC-qTOF-MS analysis, such as phene-di-hexane and N-acetyl-D-tryptophan. After first evaluating the feasibility of applying OH, an experimental design was made to obtain better yields. Different moderate electric fields (MEF) of different intensities (i.e., 4, 6 and 11 V.cm-1) were also used to identify non-thermal effects in the extraction process and their influence on the bioactive properties of the extracted compounds. BC extraction using OH was successfully optimised with the best extraction conditions corresponding to 70 ºC for 15 min using 70% ethanol as a solvent, which allowed a more significant recovery of rutin in 77% than the control samples. It allowed to recover up to 4.93 µg / g lycopene of tomato by-products without resorting to organic solvents, showing itself as a selective extraction method, depending on the compounds of interest. Since there are no studies on the impact of the extraction methodologies and the action of the gastrointestinal tract (GIT) on the bioaccessibility of BC obtained from tomato by-products, the GIT was simulated for each of the LF and SF obtained by the OH and CONV methods. In LF, the results showed that extraction significantly influences the bioaccessibility of the BC present, with OH demonstrating a positive impact on the preservation of BC and consequently on the associated biological properties such as antioxidant, antihypertensive, prebiotic and anti-inflammatory. The main BC identified by UPLC-qTOF-MS were p-cumáric acid (163 m/z), naringenin (271 m/z) and luteolin (285 m/z). In addition, extract obtained by OH after GIT simulation exhibited a prebiotic effect on different strains of *Bifidobacterium* and *Lactobacillus*. Regarding FS, the results showed that the OH treatment originated a flour (SFOH) with higher total fibre than the flours obtained with CONV (SFCONV), 62.47 ± 1.24 and 59.06 ± 0.67 g / 100 g DW, respectively.

Both flours presented high resistant protein content, representing between 11 and 16% of the insoluble dietary fibre. The main carotenoids identified were lycopene, phytofluene and lutein, all known as health promoters. Despite the higher initial polyphenols and carotenoids in SFOH, BC were more bioaccessible and presented higher antioxidant capacity than those present in SFCONV, throughout the simulated GIT.

Finally, and because there is scientific support on the positive effects of prebiotics on the intestinal microbiota, the fermentability of SFOH and SFCONV of tomato were evaluated. The results showed a higher growth of Bacteroidetes with SFOH and the highest values of Bacteroides with SFCONV. A correlation between the growth of microorganisms and short-chain fatty acids was also found. Therefore, the OH treatment allowed similar recovery yields with reduced treatment times and without the need for organic solvents (green extraction routes).

In a sense, this work will contribute to the sustainability of the wine and tomato processing industries in the context of circular economy, as it presents an environmentally friendly, fast and economical process capable of recovering BC with high application potentials, such as functional ingredients for food, nutraceutical or cosmetic application.

Keywords: wastes; grape bagasse, tomato bagasse; Ohmic heating; circular economy; bioaccessibility, health benefits

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Abbreviations

AA – Antioxidant activity

AAPH – 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

ADF – Acid detergent fibre

AOAC – Association of Official Analysis Chemists International

BC – Bioactive compounds

BCFA – branched-chain fatty acids

BI – Bioaccessibility index

BPC – Bound phenolic compounds

BPH – Benign prostatic hyperplasia

C1 – Company 1

C2 – Company 2

CBE – Circular bio-based Europe

CE – Circular economy

CFU – Colony-forming unit

CN – Negative control

CONV – Conventional method

CP – Positive control

CSP – Chelator soluble pectin

DAD – Diode Array Detector

DF – Dietary fibre

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DW – Dry weight

EC – European Commission

EDTA – ethylenediaminetetraacetic acid (EDTA)

EU – European Union

FI – Faecal inocula

FOS – Fructooligosaccharides

FPC – Free phenolic compounds

GAE – Gallic acid equivalent

GID– Gastrointestinal digestion

GIT – Gastrointestinal tract

GP – Grape pomace

GUAE – Galacturonic acid equivalent

HPLC – High-Performance Liquid Chromatography

HSP – Hydroxide soluble pectins

HTST – High-temperature short-time HTST

HVDE – High voltage electric discharge

iACE – Angiotensin-converting enzyme

IDF – Insoluble dietary fibre

IL6 – Interleukine 6

LF – Liquid fraction

LFCONV – Liquid fraction conventional

LF-GP – Liquid fraction from grape pomace

LFOH – Liquid fraction ohmic heating

LF-T – Liquid fraction from tomato bagasse

MAE – Microwave-assisted extraction

MH – Mueller-Hinton

MRSA – Methicillin-resistant *Staphylococcus aureus*

MSSA – Methicillin sensitive *Staphylococcus aureus*

MUFA – Monounsaturated fatty acid

MW – Molecular weight

n.d. – Non detected

n.i. – Non identified

n.q. – Non quantified

NDF – Neutral detergent fibre

OD – Optical density

OH – Ohmic heating

ORAC – Oxygen radical absorbance capacity

PBMCs – Peripheral blood mononuclear cells

PBS – Phosphate-buffered solution

PCA – Principal component analysis

PEF – Pulsed electric field

PHWE – Pressurized Hot Water Extraction

PLE – Pressurised liquid extraction

PSA – Prostate antigen

PTFE – Polytetrafluoroethylene

PUFA – Polyunsaturated fatty acid

R-CONV – red pomace with CONV application

R-GB – Raw grape by-products

RGP – Red grape pomace

RGS – Red grape stems

RI – Recovery index

RID – Refractive index detector

R-OH – Red pomace with OH application

R-TB – Raw tomato by-products

RT – Retention time

RTB – Raw tomato bagasse

SCFAs – Short-chain fatty acids

SD – Standard deviation
SDF – Soluble dietary fibre
SF – Solid fraction
SFA – Saturated fatty acid
SFCONV – Solid fraction conventional
SFE – Supercritical fluid extraction
SF-GP – Solid fraction from grape pomace
SFOH – Solid fraction ohmic heating
SFPC – Soluble free polyphenols
SF-T – Solid fraction from tomato bagasse
SLE – Solid-liquid extraction
STE –Stem grape extract
TA – Total anthocyanins
TE – Trolox equivalent
TNF-α – Tumour necrosis factor-alpha
TPC – Total phenolic compounds
TSP – Total soluble pectins
UAE – Ultrasound-assisted extraction
UFA – Unsaturated fatty acid
W-CONV – White pomace with CONV technique applicatio;
WGP – White grape pomace
WGS – White grape stems
WSP – Water-soluble pectin

Scope and Outline

This thesis is organized into five major parts, which are subdivided into 12 chapters. Figure 1 represents the schematic layout of the thesis structure.

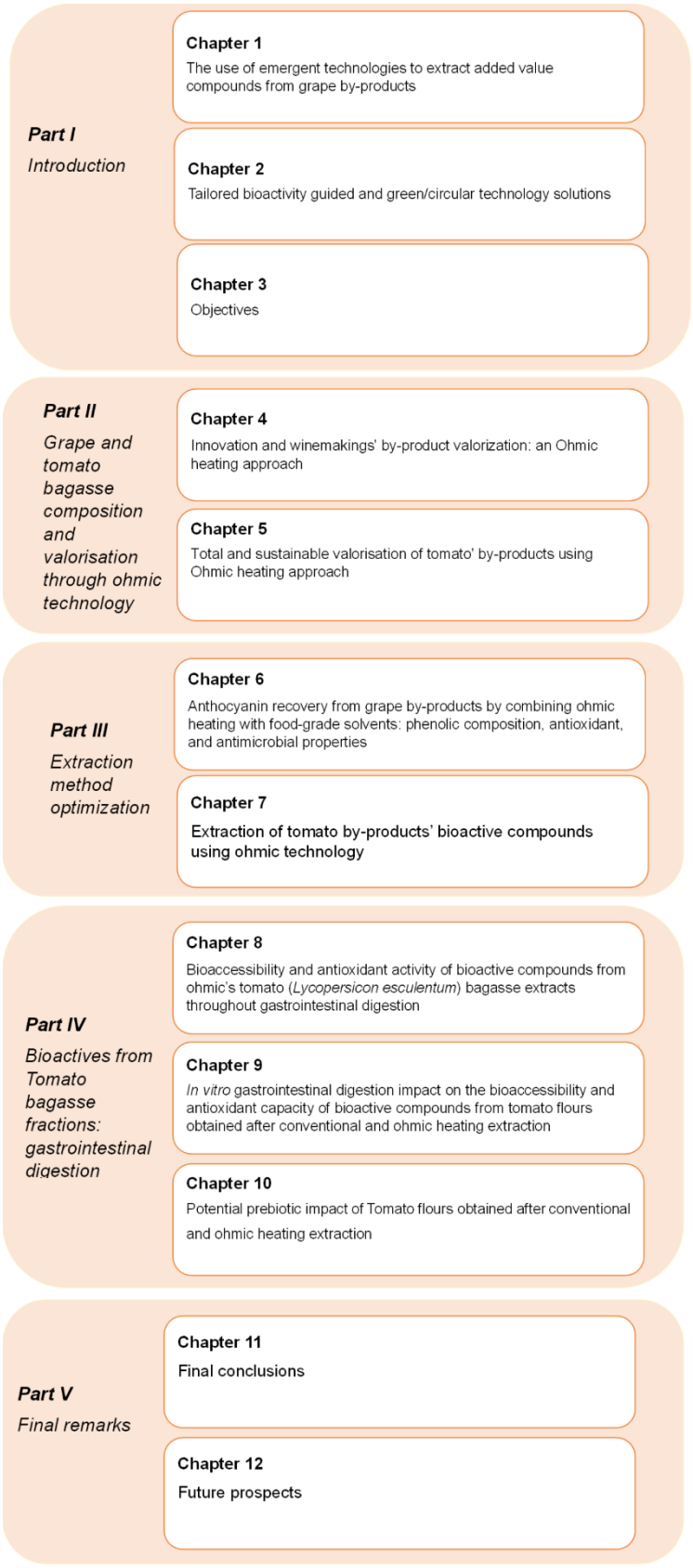

Figure 1. Thesis outline.

The first part is composed of three chapters, and the Chapter 1 corresponds to state of the art of the evaluation of Bioactive Ccompounds (BC) generated during the winemaking process and the use of emergent technologies to valorise these by-products, Chapter 2 corresponds to a literature review on the valorisation of the tomato process by-products and health benefits associated and Chapter 3 comprises the objectives of this thesis. Parts II, III and IV encompass the experimental work. Part II includes Chapters 4 and 5, which focus on the valorisation of grape and tomato by-products by the characterization of raw material and the application of a green methodology, ohmic heating (OH) technology using food grade solvents to valorise both grape (Chapter 3) and tomato by-products (Chapters 4). Part III comprises Chapters 6 and 7, focusing on the strategies to optimize the extraction of BC using the OH technology from grape (Chapter 6) and tomato (Chapter 7) by-products. In Chapter 6 we have used OH technology with food-grade solvents, composed essentially by water, and compared with conventional chemical solvents. Also, the safety of grape bagasse extracts and bioactive properties, such as antioxidant and antimicrobial properties, were evaluated. Regarding Chapter 7, an experimental design was performed to obtain higher recovery yields of phenolics and carotenoids extraction from tomato bagasse.

Then, to study the impact of gastrointestinal digestion on each key bioactive compound and some relevant properties of by-products fractions, due to the high number of fractions, we have focused on one of the by-products, and due to the existent higher number of studies on grape by-products, we have selected the tomato by-products fractions to proceed, both the liquid and solid fractions.

So, part IV includes Chapter 8, Chapter 9 and Chapter 10 and comprises the BC characterization of liquid (Chapter 8) and solid fractions (chapter 9 and 10) obtained from tomato bagasse through OH and conventional methods. Chapter 8 and chapter 9 include the tomato liquid and solid fractions' bioactivity characterisation throughout gastrointestinal digestion simulation. Explicitly, these chapters encompass the study of the liquid- and solid fractions obtained after ohmic and conventional extractions from tomato bagasse throughout the simulated gastrointestinal digestion, and understand how it affects their chemical composition as multifunctional powders and their different biological properties, including the antioxidant capacity, antihypertensive and anti-inflammatory activity. Finally, Chapter 10 describes the impact of tomato solid fractions upon gut microbiota and some related potential gut health benefits using an *in vitro* faecal model. Finally, Part V comprises Chapters 9 and 10, which correspond to the general conclusions (Chapter 11) and future work (Chapter 12).

Figure 2 represents the scheme of the grape and tomato samples obtained and evaluated throughout this thesis.

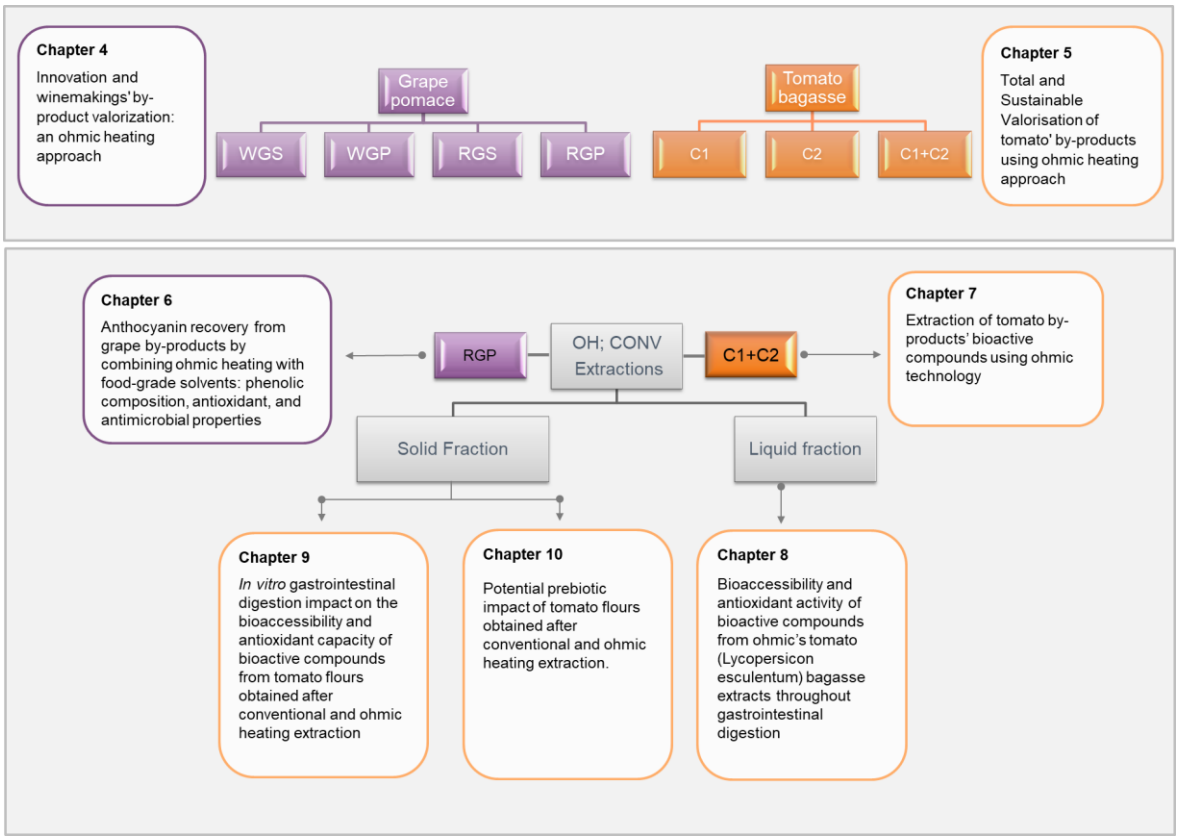

Figure 2. Scheme of the grape and tomato functional ingredients evaluated throughout this thesis. WGS – white grape stems; WGP – white grape pomace; RGS – red grape stems; RGP – red grape pomace; C1 tomato by-products provided from company 1; and C2 tomato by-products provided from company 2; OH – ohmic heating; CONV – conventional method

The core of this thesis is composed of nine articles, five already published (Chapter 1; Chapter 6; Chapter 7; Chapter 8 and Chapter 9) and 4 under submission (Chapter 2; Chapter 4; Chapter 5 and Chapter 10) in international peer-reviewed journals, according to the following list:

Chapter 1

Coelho, M. C., Pereira, R. N., Rodrigues, A. S., Teixeira, J. A., & Pintado, M. E. (2020). The use of emergent technologies to extract added value compounds from grape by-products. In *Trends in Food Science and Technology* (Vol. 106, pp. 182–197). https://doi.org/10.1016/j.tifs.2020.09.028

Chapter 2

Coelho, M. C., Rodrigues, A. S., Teixeira, J. A., & Pintado, M. E. (2021). Tailored bioactivity guided and green/circular technology solutions. (submitted to Food Chemistry).

Chapter 4

Coelho, M. C., Galhamara, S., Pereira, R. N., Rodrigues, A. S., Teixeira, J. A., & Pintado, M. E. (2021). Innovation and winemakings' by-product valorization: an Ohmic heating approach (submitted to Food and Bioproducts Processing).

Chapter 5

Coelho, M. C., Campos, Débora, Galhamara, S., Ribeiro, Tânia, Pereira, R. N., Rodrigues, A. S., Teixeira, J. A., & Pintado, M. E. (2021). Total and sustainable valorisation of tomato' by-products using ohmic heating approach (submitted to Food Research International jounal).

Chapter 6

Coelho, Marta, Silva, Sara, Costa, Eduardo, Pereira, Ricardo N., Rodrigues, António S., Teixeira, José A., Pintado, Manuela. (2021). Anthocyanin recovery from grape by-products by combining ohmic heating with food-grade solvents: phenolic composition, antioxidant, and antimicrobial properties. Molecules 26,13: 3838. https://doi.org/10.3390/molecules26133838

Chapter 7

Coelho, M., Pereira, R., Rodrigues, A. S., Teixeira, J. A., & Pintado, M. E. (2019). Extraction of tomato by-products' BC using ohmic technology. *Food and Bioproducts Processing*, *117*, 329–339. https://doi.org/10.1016/j.fbp.2019.08.005

Chapter 8

Coelho, M. C., Ribeiro, T. B., Oliveira, C., Rodrigues, A. S., Teixeira, J., & Pintado, M. (2021). Bioaccessibility and antioxidant activity of bioactive compounds from ohmic's tomato (Lycopersicon esculentum) bagasse extracts throughout gastrointestinal digestion (Food and Function, under reviewing).

Chapter 9

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Chapter 10

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PART I

Introduction

Chapter 1.
The use of emergent technologies to extract added value compounds from grape by-products

Abstract

Background: The current circular economy system-based sustainability and social lifestyle trends have led to a developed structure that is restorative or regenerative by purpose and innovation. It substitutes the end-of-life idea of a by-product adding value to it, shifts towards the usage of environmentally friendly solutions, eliminating harmful chemicals, which impair reuse.

Scope and Approach: Wine production is one of the most critical agro-industrial sectors worldwide, generating large amounts of by-products with environmental impact, but also with high economic and nutritional potential. This review aims to evaluate the effects of alternative green technologies on the functionality and recovery of Bioactive compounds (BC) from wine by-products.

Key Findings and Conclusions: These agro-industrial by-products, e.g., skins and pulp remnants, are rich in BC with health benefits such as supporting the immune system, anti-tumoral, and preventing cardiovascular diseases. Besides, the consumer has increased interest in diet and health, demanding suppliers to consider the reuse of agro-food by-products. Thus, the application of green recovery technologies eliminates harmful effects compared to conventional technologies, can be recycled into the food chain as functional additives for different products and applications, guaranteeing the sustainability and reducing the amount of winemaking by-products.

1.1. Introduction

According to the United Nations (2017), the global population is about to reach 9.8 billion in 2050, which could not only lead to a decrease in food security but also new food crises. Urbanization, people's lifestyles, such as travel, intensive exploitation of natural resources, and land-use modification, may increase the likelihood of pandemics, such as the currently experienced COVID-19 pandemic. Moreover, the current food systems are not sustainable (Galanakis, 2020). Specifically, approximately 1.3 billion ton per year of food produced worldwide is wasted (the equivalent to 3300 Mtn of CO_2 emissions per year), with food losses amounting to almost 14% in stages before the retail level, such as agriculture, harvest, catch, and slaughter (FAO, 2019; Galanakis, 2020).

Therefore, it is crucial to think about alternatives that can feed, and at the same time, promote health (Galanakis, 2020). Currently, the industrial and agricultural sectors produce large amounts of post-harvest losses and processing by-products and wastes, representing a significant disposal problem for the industry (Gómez-García et al., 2020). Those losses and processed materials are, in general, susceptible to the growth of pathogens requiring biological stability and fast treatment (Galanakis, 2012). These by-products usually constitute a promising source of Bioactive compounds (BC) that can be used due to their nutritional properties and biological potential. This can lead to benefits from by-products for health promotion and together with the high value-added of generated ingredients, also endorses business (Coman et al., 2019; Galanakis, 2020). Therefore, the collection of by-products at the source, as well as reducing transportation time, is essential. Following the considerations above and the “Universal Recovery Strategy,” it is possible to implement affordable, feasible and safe recuperation of high value from food losses and by-products through an all-encompassing methodology (Figure 1.1.) (Galanakis, 2012).

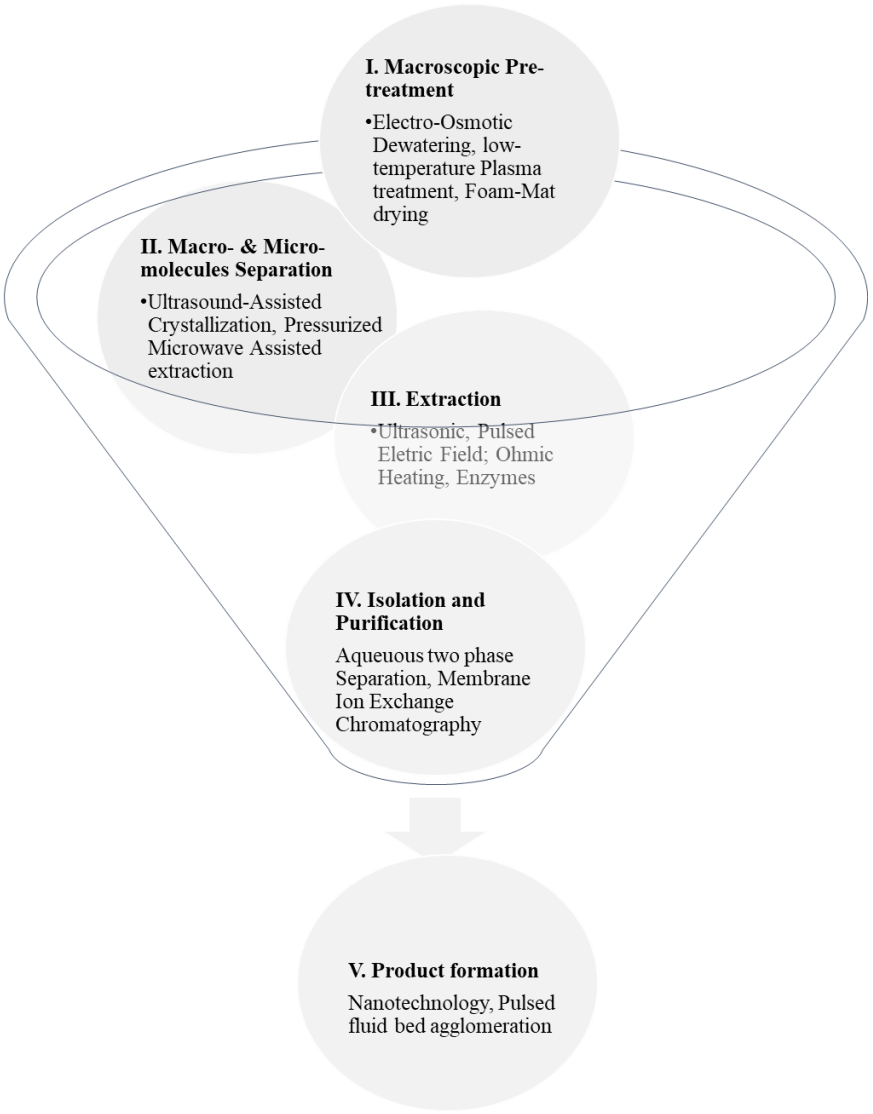

Figure 1.1. Recovery stages of high-added-value components with new technologies, adapted from Galanakis 2012.

However, many of the current technologies applied to the re-use of by-products have limitations, namely the use of toxic organic solvents that impact on human health, but also on the environment (Ananey-Obiri et al., 2018; Galanakis, 2012; Gullón et al., 2020; Zinoviadou et al., 2015). Besides, most of them comprise low efficiency, long extraction times, high energy consumption, and sometimes thermal and hydrolytic compound degradation (Deng et al., 2015; Sarfarazi et al., 2020). Accordingly, it is imperative to find cleaner and safer alternative techniques and with reduced environmental impact. Currently, the “green extraction” alternatives have been increasingly studied and implemented without the need for “clean-up” processes to recover the compounds that may be safely and immediately consumed or further used in the manufacture of foods (Bursać Kovačević et al., 2018; Deng et al., 2015). Numerous authors have suggested alternative extraction methods, such as ultrasonic, microwaves, electrical technologies (pulsed electric fields, high electric discharges, and ohmic heating) and mechanical treatment (pressurized hot water extraction and subcritical fluid extraction) (Coelho et al., 2019; Deng et al., 2015; Galanakis, 2012, 2015a; Galanakis et al., 2018; Kalli et al., 2018). These are processes suitable for the improved recovery of valuable compounds

since they use green solvents and reduce solvent consumption, and at the same time, facilitate plant cell disruption and compounds extraction, minimizing the impact on the BC (Barba et al., 2016). Furthermore, these technologies present selectivity for target compounds that influence the extractions yields.

Thus, this may lead to zero waste and with maximum value, thus taking advantage of the potential of by-products. Moreover, as the use of these techniques for extraction is still very little used, in many of them, their effects are not known, not only at the organoleptic level but also their impact on phytochemicals that allow their food, cosmetic, pharmaceutical use, among others (Gómez-García et al., 2020; Zinoviadou et al., 2015).

Among the natural products recovered from agro-industrial by-products, the polysaccharides are of significant interest, due to their physical-chemical and biological properties, namely prebiotic, anti-inflammatory, anticancer, and antioxidant activities, and applications for example in the formulation of vaccines (Beres et al., 2016; P. Gullón et al., 2020). Also, antioxidant compounds (e.g., vitamins, polyphenols) are other interesting BC due to their ability to delay or to prevent the production of free radicals or to protect cells from their harmful effects (Afonso et al., 2014; Barba et al., 2016).

Grapes are one of the most widely produced fruit in the world, with approximately 75 million tonnes produced each year. Their production is aimed towards fresh consumption as table fruit, juice, and raisins, but the principal use is in winemaking, a relevant traditional activity in several European countries. The wine agro-industrial sector is a significant player in the Portuguese economy as the country is the 11st largest producer of wine and the 10th exporter worldwide (OIV, 2018). As a consequence, high amounts of by-products are generated with associated economic and environmental costs (Galanakis, 2015a; Karovičová et al., 2015). These by-products are often underexploited, and their potential value is frequently lost. Nevertheless, these by-products can be used as a source of high-value materials, thus avoiding thermal destruction, or sending them to disposal. These by-products should be adding value to the industry using environmentally friendly methods and in line with the new philosophies on sustainable industrial development (Chemat et al., 2020; Kalli et al., 2018).

Various benefits are associated with the application of a recovery strategy: help in the reduction of by-products that accumulate in the vinification process (causing pollution problems); producers would be getting advantages from decreasing disposal costs of waste and the opportunity of extra-income; consumers and industries would profiting from the application of some BC (Barba et al., 2016; Maroun et al., 2017a).

The standard vinification procedure follows a multiple-step process, including destemming, crushing, storage, screening, fermentation, maturation, stabilization, and bottling (see Figure 1.2).

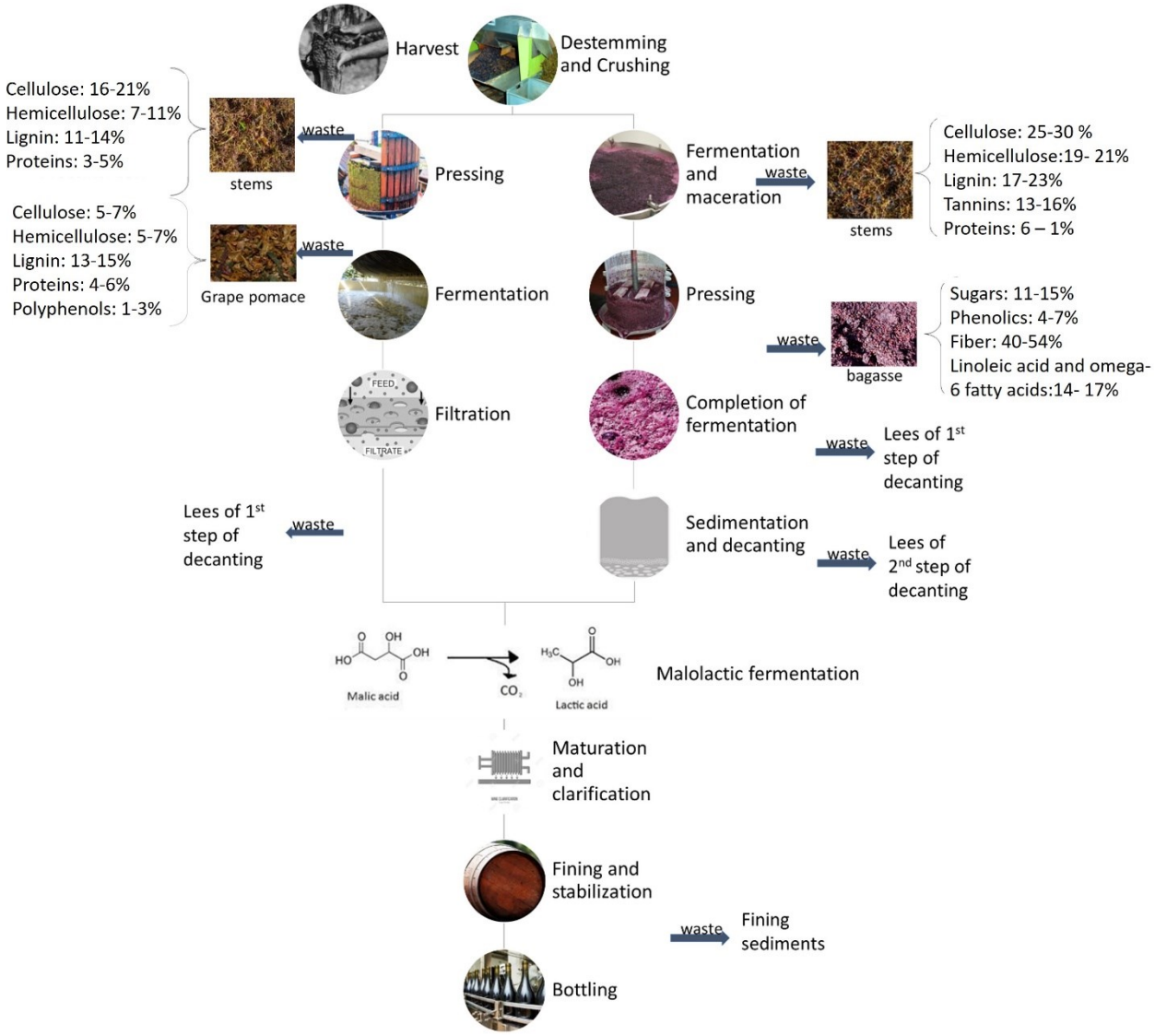

Figure 1.2. By-products generation during winemaking process based on Hogervorst et al., 2017 and Prozil et al., 2012**.**

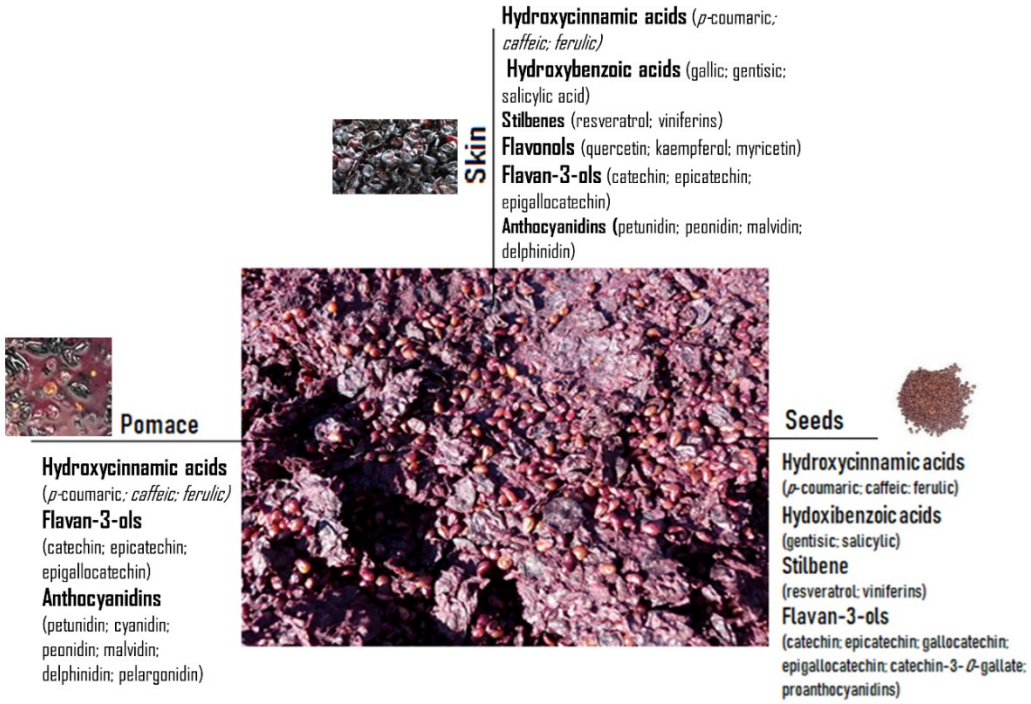

Figure 1.3. Pomace of red grapes (seeds, pulp and skins).

Grape (*Vitis vinifera L.*) pomaces are the major by-products generated in the winemaking process, with 23 million tons produced in Europe (García-Lomillo & González-SanJosé, 2014). They consist of skins, pulp, seeds, in some cases, stems being is used to extract grape seed oil (see Figure 1.3). A distinct winery by-product may be grape marc that only contains skins and pulp (O'Shea et al., 2012).

The by-products generated in this process are also rich in BC, namely fibre, phenolic compounds (tannins, phenolic acids, anthocyanins, resveratrol), proteins, lipids, carbohydrates, vitamins, and minerals (O'Shea et al., 2012; Sousa et al., 2014). The characteristics of BC present in grape pomace depend on the grape cultivar, grape pre- and post-harvest conditions, and processing conditions. They may exert beneficial effects as antioxidants, antimutagens, and anti-tumoral agents and anti-inflammatory modulators (Karovičová et al., 2015).

Grape pomace has been reported as a rich source of dietary fibre (mainly cellulose, small proportions of pectins, and hemicelluloses) (O'Shea et al., 2012). Nevertheless, concerning grape skin there is a lack of knowledge about the composition and structure of its chemical components.

Therefore, grape by-products constitute a promising source of BC (Table 1.1) that can be used for their nutritional properties and biological potential with nutritional and pharmaceutical applications (e.g., anticancer, anti-inflammatory, cardiovascular prevention) (Hogervorst et al., 2017; Ianni & Martino, 2020; Karnopp et al., 2017). Also, given the current situation of COVID, they also can strengthen the immune system, which is fundamental to the response to the virus, although new studies must be performed to specifically demonstrate this action (Galanakis, 2020).

1.1.1. Dietary Fibre

According to CODEX, dietary fibre is a group of food components, which is resistant to hydrolysis by human digestive enzymes and may have one or more physiological and health benefits. It consists of carbohydrate polymers with ten or more monomeric units, polysaccharides, oligosaccharides, and lignin (see Figure 1.4) (Karovičová et al., 2015).

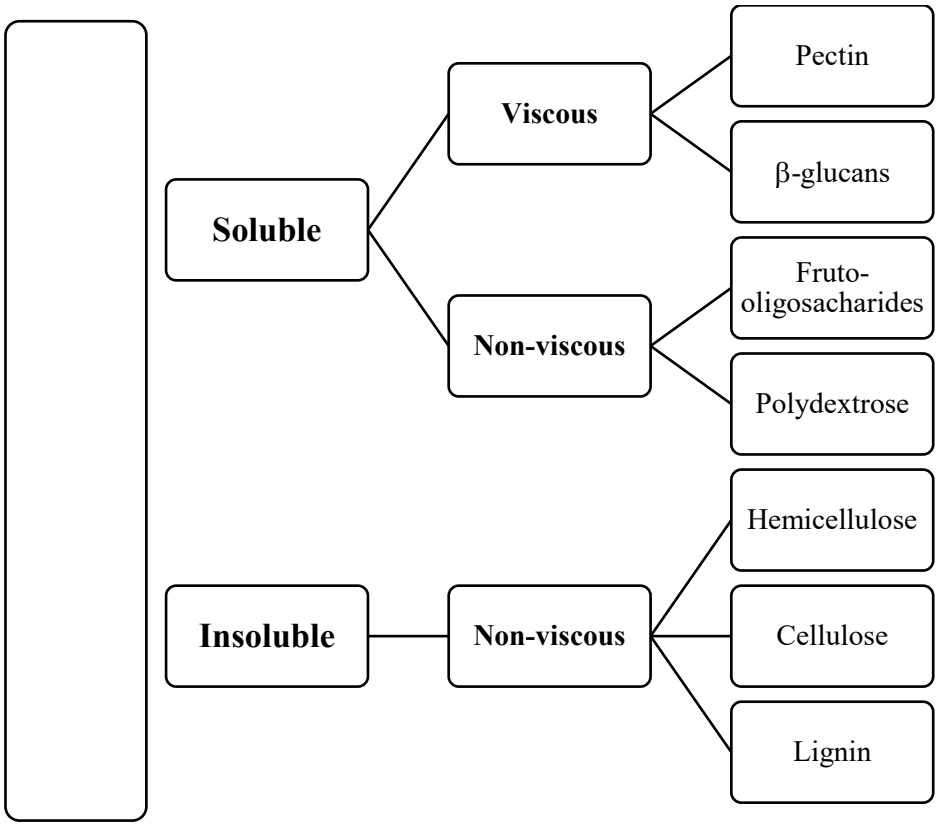

Figure 1.4. Scheme of grape dietary fibre based on its chemical properties adapted from Arranz et al., 2012.

Table 1.1. Chemical Composition of grape by-products concerning Fibre, Proteins, Minerals, Vitamins, and Polyphenols.

Classes	Compounds	Grape by-products								Biological activity	References
		Red				White					
		pomace	skin	seeds	stems	pomace	skin	seeds	stem		
Total Fibres		51.4- 77.9	73.5		50.33 - 77.2	56.3 - 71.6	59	56	34.8-77.2	Fibres have potential effects on regulation of bowel functions and water retention	González-Centeno et al. 2010; Sousa et al. 2014; Beres et. al. 2016; Hanušovský et al., 2020
Soluble		3.8 -9.8				10.3					
Insoluble		50.1-73.5				61.3					
Proteins		9.0-13.8	9.8 - 12	12	6.2	10.5 - 11.0	12	12	8.59	Proteins are required for the growth, development, regeneration, and reconstruction of the body and are responsible for the production of antibodies, blood cells, hormones, and enzymes.	Sousa et al. 2014; Beres et. al. 2016; Hanušovský et al., 2020
Vitamins	Vitamin E			20						Vitamins are needed for normal cell function, growth, and development.	Karnopp, 2017; Choi and Lee 2009; Sousa et al. 2014
	Vitamin C	2.6	0.5-0.01								
Minerals	Total minerals	1.0 - 6.0			0.9 - 6.8	0.9 - 5.7	1.3	3.8	0.9 - 7.4	Minerals are required for growth, reproduction, and a good health state.	Sousa et al. 2014; Abdrabba and Hussein 2015; Hanušovský et al., 2020
	Calcium	0.04 -1.6									
	Magnesium	0.01 – 0.8	5.8	2.5							
	Potassium		25.4	0.83		0.14-19.5					
	Iron	18.1									
	Manganese	0.8									
	Phosphorus	0.02 – 2.4	8.6	0.65							
	Sulfur	0.09									
	Zinc	0.1									
	Calcium	1.6	31.6	7.5							
	Iron	0.07	0.16	1.2							
Polyphenols (mg.kg)										Polyphenols are generally involved in the protective responses to different stresses *e.*	(Cádiz-Gurrea et al.,

Flavonols	Quercetin	5.6	0.48-1.04		*g.* ultraviolet radiation; DNA damage; pathogens	2017; Maroun et al., 2017a; Yammine et al., 2014)
Phenolic acids	Gallic acid	1.9-19.6	0.17	12.6		
	(-)-Epicatechin		15.05			
Anthocyanidins	Cyanidin					
	Peonidin					
	Delphinidin					
	Petunidin					
	Malvidin					

Several studies described in Table 1.1., report the presence of dietary fibre in grapes and its by-products, ranging from 51.4 and 83.6% dry matter (DM). Moreover, there is no indication of significant differences in the amount of total fibre between red and white grape cultivars (Afonso et al., 2014; Apolinar-Valiente et al., 2015; Beres et al., 2016; González-Centeno et al., 2013; Karovičová et al., 2015; O'Shea et al., 2012). Nonetheless, differences were found among insoluble and soluble fractions content from white or red grape pomace. The insoluble fibre is constituted mainly by cellulose, hemicellulose, and lignin, representing 61.3 and 73.5 of total fibre in white and red grape pomace, respectively, while the soluble fraction is mainly constituted by uronic acids and contains 3.7 and 10.3% of the fibre in red or white, respectively (Sousa et al., 2014). Indeed, the grape cultivar, growth climate, and processing conditions influence the dietary fibre amount.

As it turns out, winemaking by-products are a rich source of dietary fibre with demonstrated beneficial effects, including the regulation of glucose absorption, obesity prevention, reduction of blood cholesterol, and cardiovascular risk. Besides, grape pomace is a good source of fibre ingredient for the industry with higher potential on the regulation of bowel functions and water retention (Ianni & Martino, 2020; O'Shea et al., 2012).

1.1.2. Proteins, peptides and amino acids

Food proteins are highly complex biochemical macromolecules with a significant function in many biological processes. Proteins are comprised of ca. 20 amino acids and nine of them (essential amino acids) cannot be synthesized by adult humans and must be obtained through the diet (Atanacković Krstonošić et al., 2017).

The properties that govern protein functionality include size, shape, net charge and distribution of charges, polarity, hydrophobicity/hydrophilicity ratio, structure (secondary, tertiary and quaternary), composition, sequence, interaction with other food components, and physical parameters such as pH and chemical environments (Tahergorabi & Hosseini, 2017).

The predominant proteins found in must and wine are thaumatin-like proteins and chitinases. A bottle deposit is caused by protein aggregation during storage. Regarding white wine, proteins play a significant role in colloidal stability and clarity (Tahergorabi & Hosseini, 2017).

The amounts of proteins reported in grape by-products ranged from 6.2 to 13.8% of DM (Beres et al., 2016; Hanušovský et al., 2020; Karnopp et al., 2017; Karovičová et al., 2015; T. P. Ribeiro et al., 2018; Tseng & Zhao, 2013). This difference depends on both grape cultivar and type of winemaking by-products. While for grape pomace, the red cultivars present superior values of proteins than the white grapes pomace, for stems higher significant values of proteins than the red grapes have been found (Hanušovský et al., 2020).

1.1.3. Vitamins

Vitamins are defined as a heterogeneous group of micronutrients with different structures and functions, which are present in food in small amounts (traces), and that an organism needs in small amounts for normal functioning because they are involved in metabolic reactions; vitamin deficiency causes several pathologies or even death. However, as the organism cannot synthesize some vitamins (or in enough quantities), they must be obtained through the diet.

The amount of Vitamin C in the skin of grapes ranges from 0.5 to 1.2% of DM, while in grape pomace 2.6 % DM (Sousa et al., 2014).

Vitamin E, also known as tocopherol, is a constituent of grape seeds, varying from 10 to 20% of DM, and it depends on the grape variety, origin, and environmental growing conditions (de Souza et al., 2020). It acts as a lipid-soluble chain-breaking antioxidant, being involved in the protection of polyunsaturated fatty acids and lipoproteins from lipid peroxidation, preserving the integrity of biological membranes, lipoproteins, and lipid stores against oxidation (Barba et al., 2016; de Souza et al., 2020; Maroun et al., 2017a). These mechanisms relate to the prevention of many diseases, such as atherosclerosis, cancer, aging, cataracts. Additionally, animal studies have shown significant anti-inflammatory effects with dietary supplementation containing grape pomace (Kafantaris et al., 2018; Urquiaga et al., 2015).

1.1.4. Minerals

Minerals are micronutrients; some of these considered essential because they are necessary for growth, reproduction, and good health state. Several minerals have been used as components of functional foods due to their demonstrated preventive or therapeutic effects on chronic diseases (Phan-Thien et al., 2012).

According to García-Lomillo et al. 2014, the grape by-products, essentially skin and pomace, are rich in minerals. Skin presented 11% DM of ash content and grape pomace 14% DM, while seeds presented the lowest ash content (3% DM). Potassium (K) has been reported as the major mineral present in the skin of grape by-products (ranging from 25.4 to 43.0% DM). Other minerals are also found in grape skins, namely calcium (Ca) (with ranges from 20.0 to 31.6% DM), phosphorous (P) (with ranges from 8.6 to 30.0% DM), and sodium (Na) (with ranges from 0.1 to 10.0% DM). Relatively to grape pomace, the mineral content includes iron (Fe), K, zinc (ZN), Ca, manganese (Mn) and phosphorus (1.8; 0.1; 0.1; 0.04; 0.013 and 2.4%, respectively). Grape seeds present higher amounts of K, Na, Ca, Mg and P (ranging from 59.0 to 95.2; 1.9 to 3.4; 52.1 to 60.0; 13.8 to 17.1 and 6.5 to 42.1% DM, respectively) (Abdrabba & Hussein, 2015; García-Lomillo et al., 2014).

As verified before, there are differences in the mineral content of grape by-products associated with the edaphoclimatic conditions, viticultural practices and winemaking process. Besides, the duration and type of maceration process (in the winemaking process) have a strong influence on the minerals extraction and reabsorption, conditioning the minerals content of grape by-products (García-Lomillo et al., 2014; Hanušovský et al., 2020).

Essential minerals are also found in vine-shoots, including Ca, Fe, K, Mg, P, and Zn. Other minerals such as N, S, Al, K, and Ca are also found and could be used as fertilizers (Mendívil et al., 2013). Sánchez-Gómez et al. (2014) have studied mineral extraction from Airén vine-shoots suggesting the mineral content are dependents of the extraction technique used, namely conventional solid-liquid extraction (CSLE), solid-liquid dynamic extraction (SLDE-Naviglio), microwave extraction (ME), or pressurized solvent extraction (PSE).

1.1.5. Polyphenols

Polyphenols (mainly flavonoids and anthocyanins) are specific plant secondary metabolites with a phenolic hydroxyl group attached to the aromatic ring. Polyphenols classification is based on their chemical structural similarity (Figure 1.4). In this way, four major polyphenols classes can be found: phenolic acids, flavonoids, lignans, and stilbenes (Ianni & Martino, 2020; Namir et al., 2015;).

Polyphenols exist in structures, such as vacuoles of plant cells and lipoproteins bilayers, which are not accessible to solvents (Boussetta et al., 2015; Parniakov et al., 2016). Thus, their recovery is complicated, and usually, the conventional method to extract polyphenols is based on a solid-liquid solvent extraction.

Several epidemiological studies have demonstrated that polyphenols have anti-inflammatory, anti-viral, anti-tumoral, furthermore prevent heart diseases and cancers (Boussetta et al., 2015).

The potential health benefits of these BC and the increasing interest in the biopreservation of food components represent a challenge towards improving traditional heat-based methods (which may also lead to loss of BC) (Deng et al., 2015; Kalli et al., 2018). Nevertheless, these traditional technologies may lead to the loss of BC; thus it is imperative to develop new, less aggressive and cleaner processing technologies to allow the BC application in the formulation of different food (e.g. colorants and antioxidants) (Galanakis, 2018) and pharmaceutical products (e.g. nutraceuticals) (Barba et al., 2015).

Among fruits, grapes are one of the significant sources of polyphenols, and they are mainly present in their skins, seeds, and short stems. Grape pomace is still rich in secondary metabolites, including phenolic acids and flavonols, and they differ due to the grape variety (Ianni & Martino, 2020). The main polyphenols present in red grape pomace are anthocyanins, flavonols, and phenolic acids. The main monomeric anthocyanins include 3-*O*-monoglucosides of the five free anthocyanidins, namely, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside, representing 46.5 to 50.0% of the anthocyanins available. Quercetin is the main flavonol (0.92 to 5.66% DW) and gallic acid (1.9 to 19.7% DW) the main phenolic acid present (O'Shea et al., 2012). As a supplement to the effect of the grape cultivar, the winemaking process also influences the individual compounds, including the polyphenols content. In contrast to red pomace, the white grape by-products are exposed to short-time maceration and leaving little time for the extraction of soluble polyphenols. The polyphenolic compounds that are mainly present are (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate (González-Centeno et al., 2013).

Relatively to grape seeds, the principal flavonoids that could be found are (-)-epicatechin, catechin, epicatechin gallate, and dimeric procyanidins B1 and B2 (Cádiz-Gurrea et al., 2017; de Souza et al., 2020).

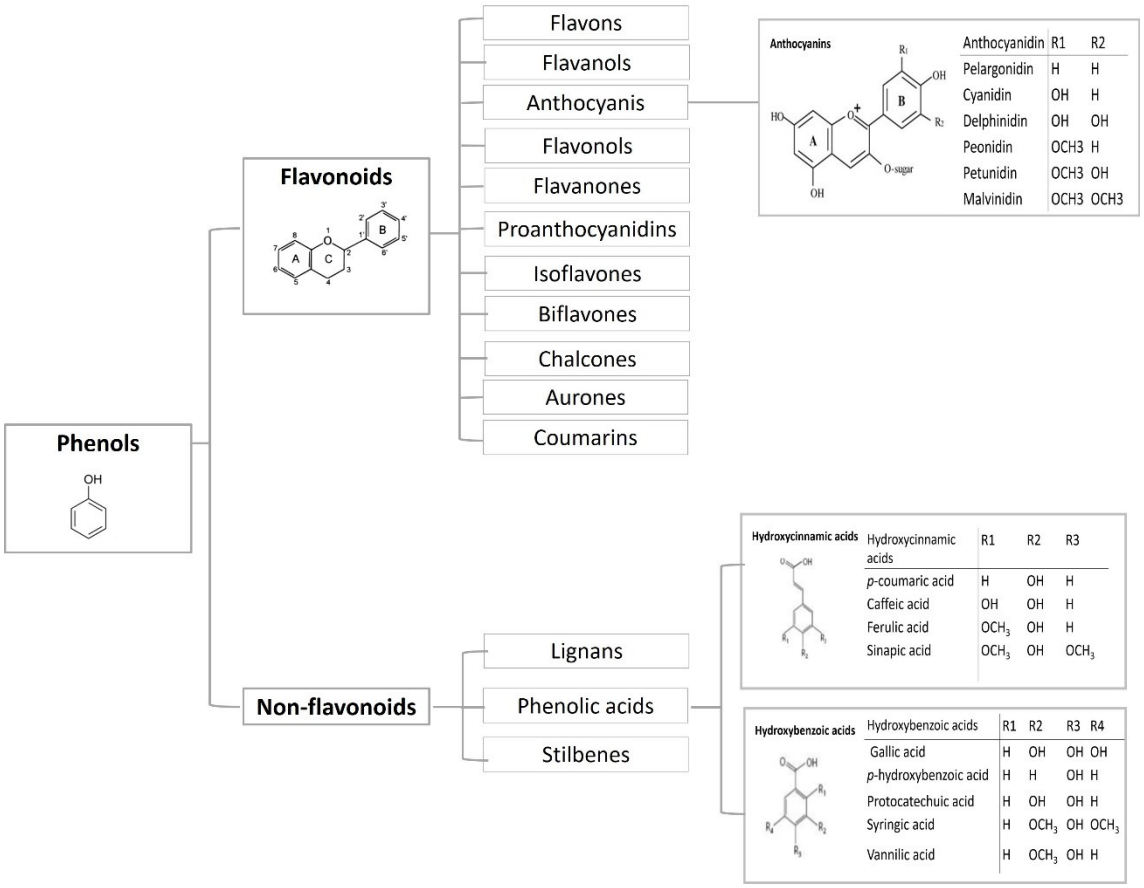

Figure 1.5. Classification of polyphenols of grape by-products according to the solubility.

1.1.6. Lignans

The lignans are monolignol dimers from hydroxycinnamic acids (*p*-coumaric, ferulic and sinapic acids) bonded at carbon 8 (C_8-C_8). They are considered as bioactive, non-nutrient, non-caloric phenolic plant compounds(Peterson et al., 2010).

The lignan content in grapes is approximately 130 µg per 100 g, and the predominant lignan compounds presents are lariciresinol, matairesinol, pinoresinol, secoisolariciresinol, syringaresino and medioresinol (Peterson et al., 2010).

1.1.7. Stilbenes

Stilbene are organic compounds with aromatic groups bond to each end of a carbon-carbon double bond. They derived from the phenylpropanoid pathway (present in many plant families). These compounds can be found in the wine by-products such as stems, leaves, and berries. They derive from *trans*-resveratrol (3,5,4'-trihydroxystilbene) and occur as glucosylated derivatives, or oligomeric forms denominated viniferins (Ianni & Martino, 2020; Piñeiro et al., 2017).

1.2. Recovery of BC from by-products

As mentioned above, grape by-products have beneficial properties conferred by their BC content. Thus, it is imperative to minimize the adverse effects of the extraction and recover as much as possible without loosing those BC and consequently, its beneficial properties (Galanakis, 2012). The recovery strategy must aim a final purpose:

- maximizing yield extraction of target compounds
- adaptation of the extraction process to the demands of industrial processing
- purification of high added-value BC from co-extracted compounds, impurities, and toxics
- avoid deterioration, autoxidation, and reduction in BC functional properties
- ensure the food grade characteristics of the final product and the sustainability of the process within the food industry.

Different steps are needed to extract BC from food by-products, and these follow the rules of advanced analytical chemistry (Deng et al., 2015; Galanakis, 2012), with progress from the macroscopic to the macromolecular level and afterwards to the recovery (extraction, removal) of specific BC (Deng et al., 2015; Galanakis, 2013; Martillanes et al., 2018). In case of fruits (solid by-products) it includes a pre-treatment of by-product (e.g., milling the by-product) to allow better recovery followed by extraction of homogeneous grape by-products; purification of extracts and finally drying of purified extracts, see Figure 1.5. (Galanakis, 2015a; Galanakis, 2012; Galanakis, 2013).

Extraction and separation constitute a critical issue for the rational valorization of agro-food by-products (Galanakis, 2015b). The extraction process depends not only on the qualitative and quantitative composition of the extracts from grape by-products, the source of the plant material and the tissue considered (leaves, stems, seeds, peels) but also on the physical and chemical properties of the desired compounds, i.e., solubility, polarity, hydrophobicity and thermal stability (Hogervorst et al., 2017; Manna et al., 2015; Yammine et al., 2014).

The principal advantage of this recovery strategy is the capacity of simultaneous extraction of several ingredients in various streams, which means that micromolecules, such as polyphenols, can be recovered with an ethanolic extraction. At the same time, the macromolecules, like pectin, can be a recovered in the insoluble ethanol residue. On the other hand, if the target compound is just a macromolecule, such as protein, the second stage could be omitted (Galanakis, 2015a, 2015b). These methods typically comprise water or organic solvent extraction (percolation, infusion, steam distillation, soxhlet extraction), supercritical CO_2 extraction, pressurized liquid extraction, and microwave-assisted extraction (Table 1.2.).

Table 1.2. Effects of extraction methods on compounds extracted from grape-by-products.

Extraction Method	Application matrix	Detected Compounds	Important issues	Effects	References
SLE	milled grape	trans-resveratrol; ferulic acid;			Karacabey and Mazza 2008
	grape seed extract and grape pomace	Gallic acid; galocatechin, catechin, procyanidin	The enzymes utilization as a solvent hydrolyze some or all components of by-products matrixes, and increase the yields of extraction		Chamorro et al. 2012
	Cannes	Hydroxybenzoi acid (ellagic; vanillic; syringic); Hydroxycinnamic acid (ferulic; *p*-coumaric; caffeic; trans-caftaric); Flavanol ((+)-catechin); Flavonol (Quercetin; myricetin; kaempferol); stilbenes (trans-resveratrol)		To cause reactions and/or strong interactions between phenolic compounds and the solid materials	Burin et. al. 2014;
	grape seeds and skins	Proanthocyanidins	Authors utilize enzymes to increase the yield extraction, cellulase, tanase, pectinases mixture	The links between polyphenols and polysaccharides are broken	Fernández et al. 2015
	winegrapes skin	Anthocyans, flavonols reactive to vanillin, proanthocyanidins			Benucci et al. 2017
MAE	grape seeds	Polyphenols		tissue disruption and compounds extraction into solvent	Li et al. 2011
	grape skins	Anthocyanins	higher significant influence of solvent extrcation; MAE allows recover acyl derivates, while with conventional methods application lowest levels of these compounds may be extracted		Liazid et al. 2011
	grape juice wastes	Anthocyanins	the antioxidant capacity increases progressively with the application up to 400 w and decays when this voltage is exceeded	osmotic effect; cell rupture, compounds diffusion into solvents and na effectiveness extraction	Varadharajan et al. 2017
SFE	grape pomace				Campos et. Al, 2008
	grape skins	polyphenols	use co-solvents increase the SFE efficiency		Ghaffor et al. 2010
	grape pomace				Oliveora et al. 2013
	grape seeds	Total polyphenols		High pressure and temperature increase the CO_2 solvating power	Manna et. al. 2015
				Temperatures higher than 50 ºC may cause the polyphenolic compounds denaturation	

	grape skins	Total Anthocyanins		Improve both solute solubility and the diffusion coefficient	
	grape seeds	Flavonol (quercetin-3-β-d-glucoside); phenolic acids (caffeic acid; trans-Ferulic acid; *p*-coumaric acid; Trans-Cinnamic acid*);* *flavanol* ((-)-epicatechin; (+)-catechin; naringenin); stilbene (resveratrol); isoflavone (formononetin)			de Souza et. al., 2020
PEF	grape by-products	Total phenolic content			
	grape by-products	Anthocyanins monoglucosides		PEF application allows the substitution pattern of B ring in the flavylium structure, as well as the different glycosilation of A and C rings	Corrales et al. 2008
			effect of electroporation by cellular membranes breakage		Vorobiev and Lebovka 2011
					el darra 2013
	grape skins			skin tannins depolymerization	Barba et al. 2016
				vacuolar tannins modification	

1.2.1. Solid-liquid extraction

Solid-liquid extraction (SLE) implies the contact of the solid material matrix with a liquid solvent and allows soluble components to be separated from solids (Baiano, 2014; Barba et al., 2016).

It is one of the most used techniques in the industry to obtain anthocyanins, usually with hydroalcoholic mixtures, being ethanol the preferred alcohol when the product is for human consumption (Galanakis, 2015a; Galanakis, 2012).

The selection of the solvents must be carefully made considering the chemical and physical properties of the target substances to eliminate or minimize matrix interferences. Experimental parameters (temperature, time, pH value, solid-to-liquid ratio, particle size, stirring, solvent polarity) must be chosen accurately to facilitate the extraction of the target molecules. This technique is frequently used to extract oils and is not suitable for thermolabile substances (Baiano, 2014).

Disadvantages of solvent extraction are associated with their toxic, inflammable, explosive and hazardous properties, costs, and the long times needed (Table 1.3). Moreover, this process could require a purification stage after extraction, as filtration or centrifugation (Baiano, 2014; Barba et al., 2016). The purification step can be avoided if clean solvents such as water are being used combined with new extraction techniques to extract hydrophobic compounds, such as lycopene (Nagarajan et al., 2019). Nagarajan and co-authors were able to extract lycopene and pectin throughout the formation of the lycopene-pectin complex from pink guava decanter under the hydrated condition (Nagarajan et al., 2019). The extraction involved two steps, in the first step generated the lycopene-pectin colloidal complex, by the mixture of plant sample (2.5 g) with water (50 mL) into an extraction vessel. This step encompasses an environmental-friendly method, affordable with high recovery yields than others reported in the literature. The second step involved the separation of lycopene and pectin from the colloidal complex, applying organic solvent extraction (e.g., tetrahydrofuran, THF). Although this method uses only 2 mL to extract, it does not eliminate its use, being necessary to evaporate the organic solvent that may be present in the pectin (fibre). However, it represents a breakthrough for extractions involving hydrophobic compounds, and it uses lesser volumes than other methods. This extraction is believed possible due to the pectin properties, which exist as an emulsifying agent and envelops the lycopene bodies, which may disperse it as emulsions in a water medium (Nagarajan et al., 2019).

Moreover, the development of the lycopene-pectin complex may be associated with the high-methyl esterified pectin, which forms a three-dimensional system through hydrophobic interaction; the non-polar methyl ester groups in pectin minimize their contact with water by coalescing with each other (Nagarajan et al., 2019). Intrinsically, the pectin-stabilized emulsions containing the hydrophobic compounds anchored themselves by its non-polar methyl ester groups and established the cross-linked network structure. Eventually, the lycopene-pectin complex is flocculated and recovered as the sediment (Nagarajan et al., 2019). SLE is referred to in some studies to extract BC from grape by-products. Karacabey and Mazza (2008) optimized the solid-liquid extraction conditions for resveratrol and other phenolic compounds extraction from milled grape canes. To flavonoid's extraction, both ethanol and the temperature are the dominant process variables. Also, the yield

extraction of ferulic acid is better at lower temperatures 16.4 ºC and ethanol concentration of 35%. Nevertheless, for trans-resveratrol and other phenolics, some studies refer that the yields increase with ethanol concentration ranging from 50 and 70% and the highest temperatures (83.6 ºC) (Karacabey & Mazza, 2008).

Burin et al. (2014) studied the effects of different methods of extraction, including, solid-phase extraction on BC extraction from grape by-products, observing SPE did not recover those hydroxybenzoic acids (gallic acid, protocatechuic acid), flavanols ((-)-epicatechin) and stilbenes (tyrosol). It might be explained by reactions or interactions occurring between compounds and solid materials. Furthermore, the different yields obtained during SLE extraction by several researchers have demonstrated that both phenolic acids and flavonoid extraction are dependent on the type and the concentration of solvent.

Notwithstanding, the interest for improving the extraction yield of target BC from agro-industrial by-products and the relevance of the environmentally friendly procedures highlight the need for the introduction of a pre-extraction step combining the application of enzymes, with some studies reporting benefits for winemaking by-products. Fernández et al. (2015) compared the extraction yield effectiveness of enzymes, like pectinases, cellulases, and tannases, in the proanthocyanidins extraction from grape seeds and skins. They observed an increase of compounds compared to controls. Other authors use β-glucanase, xylanase, polygalacturonase to also extract polyphenols from winegrapes skins and pomace (Benucci et al., 2017). Despite the high extraction yield and being an environmentally friendly nature, the high costs of the process and the low popularity in the food application has caused disinterest from the industry (Fernández et al., 2015; Gligor et al., 2019).

1.2.2. Microwave-assisted extraction

Microwave-assisted extraction (MAE) is similar to solid-liquid extraction combined with microwave heating (Gligor et al., 2019; Sarfarazi et al., 2020). Nevertheless, it allows increased yields, better process monitoring, and reduces the volume of solvent, extraction time, energy consumption, and cost, which constitute the significant advantages of MAE comparatively to conventional methods (Kwiatkowski et al., 2020; Maroun et al., 2017a; Sarfarazi et al., 2020).

It is electromagnetic waves, i.e., “an electric and a magnetic field which oscillate perpendicularly to each other at frequencies ranging from 0.3 to 300 GHz” (Baiano, 2014; Chemat et al., 2020). Microwaves interact directly with molecules by ionic conduction and dipole rotation, particularly with polar molecules, to generate heat. The use of microwave energy in combination with temperature and controlled pressure allows high diffusion of fluids and may be applied to different matrices to extract BC (Galanakis, 2014).

MAE efficiency on compounds extraction depends on factors as power, particle size, irradiation time, and liquid-to-solid ratio. This method can be applied to extract polar molecules, but it is not suitable for dry materials or very wet matrices using non-polar solvents (Ameer et al., 2017; Rocha & Noreña, 2020).

Grape by-products contain a high amount of water, which efficiently absorbs microwaves' energy and raises internal temperature causing cell disruption and improvement of the extraction compounds. Also, shifting of dissolved ions occurs, allowing the solvent penetration into the internal matrix of samples and consequently allowing higher compounds extraction (Khan et al., 2018; Krishnaswamy et al., 2013).

Some studies refer to both advantages and disadvantages of MAE use in the recovery of BC from grape by-products. MAE conditions applied (power, time, and temperature) can improve the polyphenols' recovery or their degradation (Chemat et al., 2020; Gligor et al., 2019). Varadharajan et al. (2017) extracted anthocyanins from grape juice wastes with the application of MAE power up to 400 W. Authors observed a gradual increase of Trolox equivalent antioxidant capacity, but when the power exceeded the 400 W, the Trolox equivalent antioxidant capacity value dropped gradually. Other authors reported an improved polyphenols extraction with power increases from 100 to 200 W and a polyphenols degradation combining temperature increases and power of 200 W. These results may be due by the MAE acceleration of solvent's movement, cell rupture and diffusion of compounds able to be extracted into solvents and consequently an increase in the effectiveness of the extraction of BC. On the other hand, the increase of MAE power and irradiation time lead to a heating effect and can cause compounds degradation and, consequently, a decrease of phenolic compounds, in this case, glycosidic-bound fractions.

Furthermore, this technique could also cause a reduction of bound phenolic compounds (mainly esterified and glycoside bound phenolic acids), suggesting that MAE could have the capacity to break down -ester and glycoside-bound phenolic acids (Khan et al., 2018). In addition, at low power, higher anthocyanin concentrations were found by other authors. They refer to an immediate diffusion of anthocyanins present in the fresh pulp, but when they were present in the skin, other methods were required to make possible their extraction (Chemat et al., 2020). Li et al. (2011) reported that it is possible a recovery of 90% of polyphenols from seed grapes in 4.6 min using MAE, while with conventional techniques are needed much more time (200 min).

The same method was used to extract anthocyanins from grape skins. A fractional experimental design taking into account six variables (solvents, stirring, temperature, time, microwave power, and volume), was performed by Liazid and colleagues (2011). They determined that the best extraction conditions were 25 mL of methanol/water (40%), 100 ºC at 500 W as system power for 5 min., being able to recover acyl derivates (which were not extracted at significant levels with the conventional method). They got these results by a combination of methanol/water as a solvent for anthocyanins extraction, short time extraction, and the power required to recover the anthocyanins.

Piñeiro and their colleagues (2017) used MAE to extract stilbenes from woody vine material. They used 80% of ethanol in water as solvent at 125 ºC and a time of extraction of 5 min and yields extraction similar to conventional methods (longer time of extraction).

These enhanced extraction yields of BC can be attributed to the dipolar molecule's rotation and the solvent heating due to this electromagnetic radiation (Rocha & Noreña, 2020)

Table 1.3. Green technologies aspects, comparison between methods, advantages and disadvantages of emerging technologies extraction adapted from Khan 2018.

Methods	Major Applications	Temperature (°C)	Pressure (atm)	Energy imput (KJ / Kg)	Solvent	No. of Stage	Extraction Time (min)	"green" characteristics	Advantages	Disadvantages	References
Conventional	Plant samples, essential oils	25-200	1	20 to 2000	organic	01/05		not applied	Commonly used; easiest method	Chances of impurities; Introduction of Analytical errors	(Deng et al., 2015)
PHWE	Plant samples, medicinal compounds	100-374	10-200		Water	1	3-150	Shorter extraction time with higher extraction yields	Less solvent; Less time of extraction	No suitable for thermolabile compounds	(Bursać Kovačević et al., 2018)
MAE	Essential oils, thermolabile compounds	25-70	1-100		Organic		15- 120	Minimized solvent amounts, thus reducing waste production or the emission of CO_2,	Shorter extraction times; lower volumes of solvent; Speed uniformity of heating (in some cases this uniformity may be reduced); Selective heating (microwaves couple selectively into materials that are more absorptive of the energy although greater efficiency can be achieved, temperature profiles can develop in multi-component food systems); Can be turned on or off instantly; Colors, flavors, and nutrients are preserved;	Lack of experimental data needed to model MW heating; The need for engineering intelligence to understand and minimize uneven heating or thermal runaway; High capital cost	Li et al. 2011; Liazid et al. 2011; Varadharajan et al. 2019
SFE	Thermolabile compounds	31.1<	73	32 - 254	CO_2	1 (recycled)	10-60	Higher temperature and pressure allow recover polar and non-polar molecules with water	Solvent has a low critical point of pressure and temperature (31°C and 7.3 MPa); It is non-toxic, non-flammable, nonexplosive, and it is considered as a food-grade solvent (GRAS); CO_2 is non-reactive, non-toxic, easily available, and less costly; Contain exceptional mass transfer properties; environmentally friendly and energy-efficient process; sustainable solvent easy to obtain, cheap, and it allows to obtain a solvent-free extract	High pressures increase the process costs; Polar extracts are not soluble in the CO2 mobile phase	Campos et al. 2008; Ghaffor et. Al. 2010; Oliveira et al. 2013; Manna et al. 2015; Herrero et al. 2015
PEF	Thermolabile compounds	25-150	1	1 to 800	organic/ water	1	2 - 60	Shorter extraction time with higher extraction yields; low energy consumption	No toxicity; short treatment time;	Difficult to use with conductive materials	Baiano 2014; Barba et al. 2016
OH	Thermolabile compounds	25-150	1	30 and 180	water	1	1 - 60	Shorter extraction time; low energy consumption with higher extraction yields and at the same time microbial inactivation;	Reduced maintenance costs because the lack of moving parts; no residual heat transfer after shutting off of the current; low maintenance costs and high energy conversion efficiencies; instant shut down of the system; environmentally friendly system; reduce the risk of fouling on heat transfer surface;	Lack of generalized information; Requested adjustment according to conductivity of the dairy liquid; Narrow frequency band; Difficult to monitor and control; Complex coupling between temperature and electrical field distribution	Sakr & Liu, 2014; Pereira et al. 2015, 2020

Based on recent studies (Dimić et al., 2020), MAE has been referred for oil extraction from grape seeds. The authors refer to yields superior to the conventional methods (soxhlet) and advantages such as the extraction time and low temperatures that reduce the deterioration of oily compounds. However, more studies are needed to verify the method's ability to isolate purity oils. Overall, the advantage of this method is that the volume of solvent needed and the extraction time are reduced,despite the purification stage after extraction being required as in any other conventional extraction process (Ameer et al., 2017; Baiano, 2014).

Furthermore, it is imperative to establish the process conditions to promote simple tissue disruption and the migration of compounds to the surrounding solvent and consequently obtain higher yields extractions.

Based on studies, the BC extraction efficiency by MAE can be explained by a fast increase in both temperature and pressure inside the cells. Which, it breaks the cell walls and releases the polyphenols.

1.2.3. Supercritical Fluid Extraction (SFE) SC-CO_2 extraction

Supercritical fluid extraction has been used over the years in different matrices.

Supercritical fluids have characteristics of both gases and liquids and present higher diffusion coefficients, lower viscosity, and surface tension than conventional solvents (Baiano, 2014; Manna et al., 2015). Extraction with supercritical fluids can be done for matrices both in the liquid and in the solid phase, and the extraction selectivity can be controlled by adjusting the temperature, and the pressure of extraction as supercritical fluid dissolving capacity depends on its density. Based on the increase or decrease of temperature, the solubility of extract change, and the extracting agent can be separated from the solvent (Baiano, 2014; Barba et al., 2016). These facts were shown by several authors when applying the SFE to grape by-products (de Souza et al., 2020; Ghafoor et al., 2010; Manna et al., 2015).

The most used solvent in SFE is carbon dioxide (CO_2) as it is cheap, safe, non-toxic, easy to recycle, and its supercritical conditions may be easily reached. Supercritical extraction CO_2 also generates cleaner extracts than other conventional extraction techniques. Nevertheless, in some situations, this technique presented disadvantages due to reduced efficiency extraction compared with conventional techniques, *e.g.,* soxhlet extraction. Moreover, although feasible, longer extraction times were needed leading to a higher CO_2 consumption to obtain higher yields of polyphenols from grape skin (Manna et al., 2015). SFE could be modified by co-solvents (ethanol) to change its polarity and increase extraction's yields (Baiano, 2014). Several researchers have used this method to improve BC extraction. Ghafoor et al. (2010) reported that the use of co-solvents is essential to get polyphenols from grape skins and established the need to use ethanol concentrations higher than 6% to improve the SFE efficiency. In addition, Oliveira et al. (2013) applied this technique on grape pomace showing an enhanced extraction yield with an increase of ethanol concentration up to 15%. However, the increase of ethanol concentration did not necessarily result in higher extraction efficiency, for instance, a decrease of extraction yield by 30% at higher concentrations was observed

in the same study (Manna et al., 2015). These contradictory results reported by researchers in the literature could be related to CO_2 saturation -with ethanol increased at precise system conditions of temperature and pressure-; the ethanol effect - the hydrogen from ethanol molecule (C_2H_6O) forms hydrogen bonds with the oxygen of other molecules. Thus, when the solution has higher ethanol concentration, the energy to separate ethanol molecules is not enough, and it causes a decrease of extraction and, consequently, a decrease in process yield.

In the case of total polyphenols and anthocyanin's extraction, both CO_2 temperature and pressure cause significant effects (p<0.05) (Ghafoor et al., 2010). The possible explanation is the increase in the solvating power of CO_2 with higher pressures and temperatures. Also, the increase of temperature could improve the polyphenols extraction by enhancing both solute solubility and the diffusion coefficient. However, temperature cannot be increased indefinitely since the polyphenols degradation may occur for temperatures above 50 ºC.

This technique also has the high potential for oil recovery from grape seeds. Dimić et al. (2020), examined how SFE operating parameters affect fatty acid profiling, comparing with conventional technique soxhlet extraction and green methodologies, such as UAE and MAE, accounting for yields and lipophilic antioxidant potential. Regarding the oil seeds profile, they showed that the increase in pressure decreases the total tocopherol, while the increase in temperature increases the content of this compound. This highest tocopherol content could be explained by the promotion of higher solubility of the solute associated to temperature increase, which enhances the mass transfer of a solute from the matrix to the SFE solvent.

1.2.4. Pressurize hot water extraction

The Pressurized Hot Water Extraction (PHWE) has gained popularity in the last decade due to its eco-friendly nature. This process uses pressurized hot water or even steam too, a relatively cheap solvent, under controlled pressure, temperature conditions (100 ºC to critical temperature Tc), flow rate and additives to recover compounds from plants, food matrix, and some chemical mixtures. Compared to conventional extraction methods used in the industry, this method is faster. Nevertheless, more studies are necessary for industry scale-up (Bursać Kovačević et al., 2018; Teo et al., 2010).

The PHWE method comprises four steps: 1) desorption of solutes from different active sites in the sample matrix under the pressurized and highest temperature conditions; 2) diffusion of recovery fluid into the matrix; 3) breaking of bonds between the solute and the matrix and release to the extraction solvent; 4) solutes removing from system throughout chromatographic elution (Teo et al., 2010).

Several authors refer to the highest total phenolic extraction with a temperature increase with the PHWE process (Bursać Kovačević et al., 2018; Teo et al., 2010). The application of higher temperatures can promote complete cell disruption (Teo et al., 2010) by the hydrolytic reactions promoted by the increase in ionization constant of water at subcritical conditions. Furthermore, it also

conduces to plant cell wall components degradation, such as lignin's into phenols; the water polarity decrease (low dielectric constants), promoting the total phenolic compounds solubilization (Bursać Kovačević et al., 2018).

It allows better diffusion rates, and consequently, reaction and extraction rates. Also, it presents lower surface tension and a better solubility to less polar compounds (Bursać Kovačević et al., 2018; Teo et al., 2010).

The method also can be applied to hydrophobic compounds extraction, like carotenoids, chlorophylls. Bursać Kovačević et al. (2018) refers to the possibility to extract chlorophyll with a maximum at 160 ºC for 10 min. Although the authors have used a higher temperature, it was not sufficient to degrade chlorophylls, and it may be related to the duration time of PHWE process (10 min). Moreover, as already mentioned, this process at high temperatures changes the water properties, which are closer to the properties of the compound of interest (Bursać Kovačević et al., 2018; Teo et al., 2010). Generally, the water viscosity reduces with an increase of temperature enhancing the solute solubility, and it can also assist with breaking of analyte-matrix bonds facilitating the diffusion of these analytes (Bursać Kovačević et al., 2018).

Some researchers applied the PHWE to extract BC from grape by-products. Vergara-Salinas et al. (2013) applied this technique to grape pomace, and they also achieved high recovery rates of BC such as proanthocyanidin, when high temperatures were applied during this process. The authors concluded that both temperature and time are critical to anthocyanins stability. Maximum temperatures at 100 ºC during short times exposure (5 min) are enough to obtain higher amounts of anthocyanins. Nevertheless, temperatures higher than 100 ºC and long-time exposure could degrade this compound. The anthocyanins degradation can be caused by the pyrilium ring of anthocyanins opening by the increase of temperature (greater than 100 ºC), or by its sugar moiety cleavage to form a more labile anthocyanin aglycon (Vergara-Salinas et al., 2013).

The same authors (Vergara-Salinas et al., 2015) in other study detected the presence of phenolic compounds, such as (+)-catechin, (−)-epicatechin, kaempferol and myricetin, and hydroxymethylfurfural (5-HMF) from grape pomace. Furthermore, they found that the increase of temperature changes the extraction of the phenolic compounds. At 100 ºC favors the (+)-catechin, kaempferol and myricetin extraction, while the (−)-epicatechin content decrease. The differences could be explained by the impact of temperature on phenols stability (as explained earlier). Besides, the best results to (−)-epicatechin extraction at higher temperatures (200 ºC) could be explained by the breakdown of tannin bonds, which release the subunits.

The disadvantage of the method is the water corrosion at higher temperatures and pressures. Thus, the material should be appropriate to prevent it.

The PHWE is very similar to the SFE, only introduces some alterations. The PHWE needs more time to extract than SFE due to the different resistances' usages, which water needs throughout (Ameer et al., 2017). This method can be applied to other than food matrices (e.g chemical mixtures) and obtain a wide variety of compounds (Plaza & Marina, 2019), while SFE could be limited to the food

matrix due to the higher pressures used and its unimpressive efficiency to use in high-capacity industrial chemical processes.

Comparing this method with one previously described, MAE, the PHWE does not require a secondary "clean-up" methodology; this can be intrinsic in the process (Ameer et al., 2017; Plaza & Marina, 2019). Besides that, to recover compounds, the PHWE requires less time of extraction than the conventional method, as the Soxhlet. Nevertheless, it spends more extraction time compared with MAE, but is less expensive.

1.2.5. Pulsed Electric Field

The application of emerging, novel processing techniques such as pulsed electric energy can be used to quality improvements and consumer benefits (Barba et al., 2016; Cholet et al., 2014; Comuzzo et al., 2020).

Although pulsed electric energy was discovered in 1791 by Luigi Galvani, only two centuries later, the effect of electroporation was established, and its ability to potentiate the extraction of anthocyanins from grape by electrical breakage of cellular membranes was observed. Nowadays, this assisted processing includes a pulsed electric field (PEF), pulsed ohmic heating (OH), and high voltage electrical discharge techniques (Maroun et al., 2017; Rocha et al., 2018).

PEF technology is a non-thermal method of food preservation that involves the discharge of high voltage electric pulses to liquid or semi-solid foods placed between two electrodes for a few to several hundred microseconds. This technique allows the extraction of intracellular compounds by the formation of pores in the membrane (electroporation) without affecting the structure of cell walls noticeably. The extraction efficiency depends on parameters such as pulse duration (pulse width), number of pulses, and pauses between pulses (Baiano, 2014). In skins and pulp, the energy input is lower in PEF when compared with conventional methods (mechanical or enzymatic), representing 1 to 15 and 20 to 100 KJ / Kg, respectively, which demonstrate to be an environmentally friendly technology (Rocha et al., 2018).

PEF technology is advantageous when compared with traditional thermal treatments because it causes microbial inactivation with minimal detrimental effect on food quality attributes (maintaining original color, flavor, texture, and nutritional value of the unprocessed food). Also, it avoids or significantly reduces detrimental changes in the sensory and physical properties of foods (Baiano, 2014; Barba et al., 2016; Comuzzo et al., 2020).This technology has been used by several food industries to scale-up the extraction process and the pre-treatment of food matrices (Barba et al., 2016).

Corrales *et al.* (Corrales et al., 2008), used PEF to extract anthocyanins from grape by-products. They reported that after 1 h extraction (performed in a water bath at 70 ºC during 1 h), the total phenolic content of samples was 50% higher than the control samples. The application of novel technologies also increased the antioxidant activity of the extracts being the extractions carried out with PEF fourfold higher than the control extraction. Besides, they also observed that anthocyanin mono-glucosides extraction was higher when PEF was applied, while acylated anthocyanin was

better extracted by high hydrostatic pressure. The PEF application induces the acylated glucoside anthocyanins trapping inside the matrix or establish hydrogen bonds with cell wall polysaccharides. The solid/liquid ratio (1:20) and the holding times used is very important to obtain the highest yields of anthocyanins extraction with PEF. Their stability and chemical structure depend on the ratio anthocyanins: solution, pH value, the OH- and OCH_2- groups at position R_1 in $C_{3'}$and R_2 in $C_{5'}$ of B ring and the sugar moieties or phenolic acyl groups of the C ring (Figure 1.**Erro! A origem da r eferência não foi encontrada.**5). Also, PEF changes pH values to pH < 4 allowed for better extraction of acylated anthocyanins due to their stability at this pH value.

This technique was also used to extract total tannins and polysaccharides by Cholet et al. (2014). They applied two PEF treatment parameters, and firstly, they use a voltage of 4 kV/cm with a duration of 1 ms and, secondly, 0.7 kV/cm with a duration of 200 ms. The authors verified that the first treatment has a lower impact on phytochemicals than the second treatment. These results obtained by authors indicate that the duration of PEF treatment has a high impact on the organization of the cell wall of the skin and causes skin tannins depolymerization. It has also been observed that the highest applied electric field modified the vacuolar tannins. Also, while the highest PEF energy can induce damage on parietal and cell walls tannins of grape skins, the highest PEF strength modified the vacuolar tannins leading to the increase of the degree of permeabilization of the cellular membrane and allowing the release of intracellular compounds. Higher yields of extraction were obtained by Comuzzo and colleagues (2020) when compared the effects of PEF as pre-treatment (applying energy of 2, 10, and 20 kJ. Kg) extraction enzymes on color and polyphenols during skin maceration from red grapes. The 10 and 20 kJ.Kg energies exhibited higher phenolic compounds than 2 kJ.Kg and the enzymatic extraction. The results suggest that the increase in PEF intensity create large pores in the cell membrane, which may allow the release of more complex phenolic compounds and protecting others (e.g., anthocyanins) towards oxidation.

1.2.6. Ohmic Heating (OH)

OH is an environmentally friendlier and more efficient thermal technology than traditional methods currently used in food processing; in OH, an electric current is passed directly through materials having electrical resistance, generating heat and increasing the instantaneous and homogeneous temperature inside the product (Coelho et al., 2019; Pereira, Rodrigues, et al., 2016). This technology has an energy efficiency of over 90% and, when compared to the conventional thermal method, which is less efficient, can achieve energy savings of up to 70% (Rocha et al., 2018).

By applying OH, some BC are not degraded as in traditional heat treatments. This method is efficient in inactivating enzymes and microorganisms. Nevertheless, few studies are reporting its impact on structural changes in food or BC present in by-products, which may compromise safety and bioavailability. Moreover, it allows more efficient extraction of BC from organic by-products minimizing extraction time, reducing organic solvent consumption, and maintaining recovery of compounds of interest (Maroun et al., 2017).

The OH method advantages compared to conventional heating include the more uniform and faster heating, higher yield, and higher retention of nutritional value of food (Rodrigues et al., 2015a). It is mainly due to its ability to heat materials rapidly and uniformly, leading to a less aggressive thermal treatment. Nevertheless, their effects on plant tissues are scarcely known. In grape skins, tissue damage was observed, maybe induced by electrical breakdown, or an electroporation mechanism. Furthermore, at low frequencies, cell walls build up charges and form pores altering membrane permeability (Kaur et al., 2016; Pereira et al., 2011, 2020).

An example is the extraction of polyphenols that was increased in red grape pomace by the application of OH. It is due to an alteration in cell membranes and, consequently, an acceleration of the polyphenols diffusion kinetics. El Darra et al. (2013) obtained a 36% higher extraction yield in pretreated extracts of grape pomace with OH (400 V/cm) compared to the conventional method. On the other hand, literature has shown degradation of heat-sensitive compounds, as anthocyanins compounds. As previously mentioned, the anthocyanins are very unstable, and their degradation is also dependent on the voltage applied (Maroun et al., 2017).

OH applied at moderate electric fields have shown the potential to enhance extraction of anthocyanins and other BC (phenolic compounds, including anthocyanins) from winemaking residues such as grape skins and seeds (results not published), suggesting that OH is a promising industrial method for BC extraction from grape by-products.

1.2.7. High voltage electric discharge (HVED)

HVED method is based on the treatment of liquid food placed in a chamber between a needle electrode and a plated grounded electrode using short electric pulses (typically, 40–60 kV/cm, 2 – 5 μs). The most promising application is for enhancing the extraction of oil from oilseeds (Barba et al., 2016).

The method is optimized by controlling the number of pulses, pH, water/press-cake ratio, and temperature.

Boussetta et al. (2009) showed that the HVED method is a promising technique for the extraction of polyphenols from grape pomace (composed of stems seeds and skins). This method was applied to the pomace samples mixed with distilled water and at temperatures ranging from 20 to 60 ºC using 80 successive discharges (40 kV, pulse repetition rate 0.5 Hz). The HVED treatment allowed an increase of the final yields of solutes with a decrease of time of diffusion. Nevertheless, the pilot-scale of HVED-assisted extraction spent a high volume of solvent and required higher treatment energies. Afterward, Boussetta et al. (2013) compared this technology (HVED 40 kV) with PEF (8 to 20 kV/cm, 0 to 20 μs) and grinding (180 W, 40 s) on polyphenols extraction from grape seeds. The authors showed an extraction efficiency by HVED and grinding, with subsequent ethanol extraction, higher than PEF. Rajha et al. (2015) also compare the effects of alternative physical treatments (HVED, PEF, and ultrasound) on dead-end ultrafiltration of vine shoot. They analyzed the cellular damage that was higher for grinding and followed by HVED (360 kJ/kg and Z = 1 (242 kJ/Kg and Z=

0.71, respectively) showing proteins concentrations above 0.097 mg/mL to grinding and 0.082 mg/mL to HVED. Total phenolic concentrations were 0.4 and 0.3 mg/mL for grinding and HVED, respectively). These results were obtained because authors with both treatments caused cell disintegration, improving the extraction of the intracellular compounds. In addition, higher yields of extraction could be obtained during HVED application due to the electrical breakdown followed by different phenomena, namely high-amplitude pressure shock waves, bubbles cavitation, creation of liquid turbulence, and production of active species leading to particle fragmentation and cell rupture of tissues and consequently.

1.3. Applications

In this review, we focus on emerging extraction processes and their effects on the recovery of compounds. It is noteworthy that the period between by-product production and its valorisation, in conjunction with recover procedures, have a direct impact on phytochemicals and, therefore, on the potential of BC. This compound recovery strategy is crucial for the valorisation of this by-product, to ensure a safe and high-quality compounds recovery, in a circular economy context. The BC from grape by-products have been reported in the literature applied to a large number of foods, cosmetic, and pharmaceutical applications since they act as multipurpose ingredients or additives.

1.3.1. Food applications

As food applications, these BC, due to their multifunctional characteristics, could be used to develop novel and functional foods. The dietary antioxidants recovered from grape by-products, such as polyphenols (e.g., phenolic acids, flavonoids), and vitamin E could be used as fortification of meat and bakery products, oxidative protection in olive and vegetable oils (Galanakis et al., 2018b) and enhancement of color stability in meat products (Galanakis, 2018; Kalli et al., 2018), in particular of cooked beef and pork patties (Rojas & Brewer, 2008). Rojas & Brewer (2008), reported increased stability in oxidative and color of raw beef and pork patties during 4 months stored frozen in a vacuum packaging. Also, extracts from grape pomace and grape seeds rich in antioxidants, polyphenols (phenolic acids, namely *p*-coumaric, cinnamic, caffeic, ferulic, gallic, sinapic, chlorogenic, protocatechuic, syringic and vanillic acids as well as flavonoids), vitamin E, were able to extend the lamb meat shelf-life after a week of storage (Guerra-Rivas et al., 2016). Phenols reduced lipid peroxidation and meat discoloration (Guerra-Rivas et al., 2016) by hydrogen donating and sequentially quenching of radicals (Galanakis, 2018). Both phenols and vitamin E are also used as antimicrobials (e.g., against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*), retarding rancidity and enhancing the shelf life of bakery products, as pastry and sugar-snap cookies and crackers (Galanakis et al., 2018a, 2018b). Also, phenols recovered from Merlot grape seed flour were used to enriched noodles, cereal bars and pancakes, with high antioxidant activity and consumer acceptance. The dietary fibre was used as a supplement and with a possible prebiotic function (Kalli et al., 2018).

1.3.2. Cosmetics and sunscreen industry

The term cosmeceutical is resulting from the combination of “cosmetic” and “pharmaceutical,” suggesting that the products have active components. These products take components with medical or drug-like benefits and are developed to change positive physical outcomes at the cellular level in addition to enhancing the surface’s appearance. The fundamental premise is that new BC from natural sources are safer and equally or more efficacious than synthetic ones. These compounds should have extremely efficient and stable attributes for therapeutic usage with reduced possible toxicity. Recently, agro-food by-products have been demonstrated as rich sources of structurally diverse, biologically active compounds with considerable cosmeceutical potential (Dzah et al., 2020; Galanakis, 2018b).

Presently, there are considerable research activities in progress regarding developing and characterization of extract full formulation to concurrently accomplish several goals, e.g., anti-inflammatory and anti-aging effects. The main BC used are polyphenols, flavonoids and carotenoids. These compounds maintain both antioxidant and UV protection. For example, quercetin improves the photostability of two conventional UVA (320–400 nm) and UVB (290–320 nm) filters (Galanakis, Tsatalas, & Galanakis, 2018a, 2018b).

It should be noted that antioxidants not only prevent against reactive species as increase UV absorption of sunscreen agents (Galanakis, Tsatalas, & Galanakis 2018a).

Other compounds, such as vitamin C and vitamin E and ubiquinone, coenzyme Q10, showed photostability of avobenzone, reducing the skin damage induced by UV (Afonso et al., 2014). Galanakis, Tsatalas, & Galanakis (2018b) obtained more active UV filters in a broader region of UVB and UVA to olive phenols from mill wastewater than to ascorbic acid and α-tocopherol. The authors showed that these by-products are effective against UV radiation, with great potential to be used in sunscreens and cosmetics. Nevertheless, the polyphenols used in cosmetic is accomplishing with some problems, such as solubility in water which compromises its diffusion through the skin (Galanakis, Tsatalas, & Galanakis 2018a), chemical instability, and low bioavailability (Kalli et al., 2018a). Thus, they can be removed with seawater immersion and compromise its effectiveness. In this sense, studies have been carried out to overcome the phenols’ water solubility (Galanakis, Tsatalas, & Galanakis, 2018a, 2018b). They have shown that encapsulation can increase the bioavailability and protect the degradation of the BC (Galanakis, Tsatalas, & Galanakis 2018a; Kalli et al., 2018). Galanakis, Tsatalas, & Galanakis (2018a) showed that the silica particles could increase the water-resistance of olive phenols’ cosmetic formulations. If, during the extraction processes of agro-industrial by-products silica is also recovered, we are facing a sustainable alternative that fits into the current circular economy context. Thus, the utilization of the by-products allows the cosmetic industry (a) to formulate more economical and sustainable skin products, (b) to obtain purer compounds to formulate clean-beauty, and finally (c) to adopt the REACH directives regarding the advisable solvents (Galanakis, Tsatalas, & Galanakis, 2018b).

1.4. Industry scale-up

The technologies presented before, are considered environmentally friendly compared to conventional methods, indicating a current alternative towards sustainable industrial production (Del Borghi et al., 2020). On the other hand, most of these processes have only been tested at the laboratory scale, and additional studies are needed for scaling up these procedures. Depending on the technology involved, it may make more sense to scale-up the recovery processes for BC at the industrial level. For example, concerning solid-liquid extraction, this scale-up process is minimal, not only because it uses an enormous amount of solvents, but also has high energy costs (Del Borghi et al., 2020). As alternatives, PHWE, MAE, PEF, OH extractions, and the use of supercritical fluids have also been investigated for the recovery at scale-up level from grape by-products (Chizoba Ekezie et al., 2017; Cravotto et al., 2018; Elliott et al., 2017; Guo et al., 2017). Regarding the MAE, although increasing the extraction time increases the yield, it is minimal. Besides, when looking at an industrial scale, a higher number of samples are instilled as well as the fact that it is a stepwise process, other requirements such as increasing solvents and a higher number of microwave systems are necessary (Ekezie et al., 2017). Therefore, MAE is not an advisable method for industrial purposes. The PHWE, PEF and OH technologies, in general, needs lower energy consumption compared with conventional pretreatment methods and extraction technologies. However, its industrialization implies working with much higher processing volumes when compared to the laboratory scale. This industrialization presents some challenges, such as the need to work with larger treatment chambers or fluxes, with larger electrode surface areas and electrode gap (Rocha et al., 2018).

Consequently, high energy capacity increased output voltage, but higher pulse frequency is required. But these processes could use a renewable source of energy (e.g., hydroelectric power) to produce electricity. Which, together with the current output with defined shape and pulse/wave frequency, still represents a challenge (Rocha et al., 2018). However, the increase in the application of MEF and PEF systems in the food industry contributes to its reliability increase and cost reduction during the process. Also, the investment in the development and innovation by partnerships with universities and companies has contributed to the optimization, not only in the materials used in the projects of electrical treatment chambers but also in the continuous operation mode, which has contributed to the investigation of industrial viability (Rocha et al., 2018).

It should be noted that high energy efficiency results in an overall reduction in energy consumption. Associated with the increase in the BC recovery rate and some cases the solvents reduction, or even its elimination, these technologies allow to reduce the use of non-renewable energies, increasing the value-added of the by-products and contributing to a more sustainable world within the scope of a circular economy.

1.5. Conclusion and Future Remarks

Grape pomace represents the main fraction of solid grape by-products and are a good source of BC, such as total dietary fibre (almost 60%), polyphenols (6 to 15%) (mainly flavonols: quercetin,

kaempferol, and myricetin; flavan-3-ols: catechin and epicatechin; tannins and anthocyanins: and proteins (5 to 12%). Polyphenols are generally responsible for sensorial properties but also for health benefits, namely, antimicrobial, anti-inflammatory, anti-cancer, and preventing cardiovascular diseases. In this way, the application of emerging food technologies has been useful to minimize the adverse effects of conventional techniques, facilitate the production of valuable natural products, which guarantee food sustainability and meet consumer demands and to reduce grape by-products. Also, both OH and SFE have higher potential due to their time-efficiency, quickly to scale up to the industrial level, and both technologies could directly use ethanol of a winemaking industry, heat, electricity already available, and CO_2, avoiding logistics costs. Furthermore, both techniques allow a selective extraction, and they have been successfully applied for different BC, including anthocyanins by OH and fibres by SFE.

Some technologies comparisons, such as OH, microwave, and solvent extraction, for anthocyanins extraction from grape by-products, have not been studied before. Furthermore, the combination of SLE with enzymes and new technologies can significantly enhance the BC recovery. For instance, they can break the bound polyphenols to proteins and polysaccharides, e.g., hemicellulose, cellulose, and pectin, in the food matrix. Nevertheless, there are a lack of information about the recovery and reuse of products obtained after the application of enzymes, ionic liquids together with green technologies presented before. For this reason, feasibility for BC recovery must be deeply studied in the future to understand the benefits of this type of solvents and technologies and their toxicity, stability and bioactivity This study is of high relevance since an optimal recovery of fibres, and other BC from grape by-products brings the high potential for product development according to current industry and consumer demands based on a circular economy system.

Chapter 2.
Tailored bioactivity guided and green/ circular technology solutions

Abstract

The tomato processing industry is one of the world's most important markets. This industry aims to optimize production, minimize energy costs and waste streams while ensuring high-quality products. This sector produces substantial amounts of by-products frequently disposed of as waste rather than reintroducing them with a new intent into the supply chain. However, these by-products are rich in bioactive compounds (BC), including carotenoids, phenolics, fibre, which exhibit antioxidant, anti-inflammatory and chemopreventive properties, and cardiovascular protection. Reusing these compounds is favourable to reduce the environmental impact and enables the development of added-value products with various possible uses such as food and feed additives, nutraceuticals, cosmeceuticals, etc. This review summarizes relevant issues towards the recovery and valorisation of BC from industrial tomato by-products within a circular economy context.

2.1. Introduction

Per year, around 1.3 billion tons of food are wasted or destroyed worldwide (FAO 2019), being fruit and vegetables one of the most generators of industrial by-products (Gustavsson et al. 2011). The losses and waste of fruit and vegetables (the final products not reused or used for other purposes) constitute up to 50% of production in the processing and post-harvest periods (Sogi and Siddiq, 2011, de Brito Nogueira et al., 2020). The preparation of fruit and vegetables (canning, drying, freezing, juices, jams and jellies) prolong the shelf-life of the products. According to the International Fresh-Cut Produce Association (IFPA), the fruit and vegetable industry consists of products that undergo cleaning, cutting, mixing and packing. They are the non-product flows of crude materials, which have a trade value lower than collection and restoration costs and are consequently disposed of as waste.

According to Eurostat, almost 2.2 million hectares of vegetables were consumed and processed in the European Union (EU) in 2017. The most valuable was the tomato crop, representing 11.7% of the total EU planted area (EC 2017). In the 2018 global tomato market, 184 million tons were predicted to rise more in the Compound Annual Growth Rate (CAGR) of 3.1% to attached 221 million tons over the projected period of 2019 to 2024. The growth of the tomato market and the improvement of tomatoes also fuel the progress of the industry. It has a heavy focus on the tomato industry, and the ten biggest developing countries account for 83% of the world's annual production.

Nevertheless, the indicators processed outside of those ten countries have slowly risen in recent years. Over the 2017-2018 trading year, seven primary manufacturing and trade countries shipped nearly 6.34 million tons of finished goods in Europe, China, the US, Turkey, Iran, Chile and Ukraine. Almost a third (40 million tons of valued tomatoes out of 130 million tons) of tomatoes turned out to be processed worldwide. About 10% of overall tomato production fails to meet the customers' requirements, leading to essential losses during harvest and minimal processing (Coelho et al. 2019). On a technical basis, most tomatoes are produced for processing into food products, namely sauce, paste, juice and canned tomatoes, generating a large volume of by-products (Figure 2.1).

Tomato and derived by-products contain various biologically active substances that mostly are lost as waste (or may be used in animal feeding) despite being a promising source of dietary fibres, proteins, carotenoids, tocopherols, polyphenols, vitamins and other compounds (Chaudhary et al., 2018; Viuda-Martos et al., 2014). These bioactive molecules contain various biochemical properties, including anti-inflammatory, anti-allergenic, antimicrobial, vasodilatory, coronary and antioxidant effects (Szabo et al., 2018; Viuda-Martos et al., 2014). However, the knowledge regarding how specific tomato BC's concentration, structure, and ratios affect their activity and uptake in the human body is still limited. Thus, due to their high BC content, the growing interest in using tomato waste is explored through modern extraction methods to obtain new value-added compounds.

The chronic consumption of functional foods to reduce the risk of non-communicable s is an emerging subject in food and nutritional science and an opportunity to respond to the changing market preferences and the food industry's competitiveness.

This review aims to present an overview of the functional and biological properties of the principal BC present in tomato by-products, their bioavailability and their integral valorisation.

2.2. Industrial Tomato by-products

The question is, “how much tomato is wasted along the food supply chain to produce a tomato sauce?”. When 344 g of tomato sauce are obtained, about 80.5 g of losses and waste are generated (Secondi et al. 2019). Tomato pomace is the most critical tomato by-product (Figure 3). Dried tomato pomace consists of 33% seed, 27% skin and 40% pulp (Viuda-Martos et al. 2014; Allison and Simmons 2017).

Some steps are associated with processing tomatoes. After harvest, tomatoes enter a sorting station, where extraneous materials are removed and discoloured, green and damaged tomatoes. The ideal tomatoes are chopped, and then their pulp can undergo a cold (pre-heated from 65 to 75 ºC) or hot (pre-heated from 85 to 95ºC) break, depending on the type of paste desired. The pulp obtained, comprising fibre, juice, pores and skin and seeds, passes through different sieves, allowing the pulp to end up either coarser or smoother. Usually, 95% of pulp pass through sieves and only 5% corresponds to bagasse by-product (fibre, seeds and skin), used as feed for livestock or others applications. The evaporation step is the most energy-intensive of the tomato paste process. In this step, water is removed, and the juice, which is still 5% solid, becomes 28 to 36% concentrated tomato paste. Finally, the pulp is aseptically packaged. Tomato bagasse's average composition (in dry weight) is 59.03% fibre, 25.73% sugars,19.27% protein, 7.55% pectin, 5.85% fat, and 3.92% minerals chemical characterization in various stages of the industrial production process. Regarding these results, tomato bagasse could be used as a potential source of fibre and protein. The tomato fibre can provide up to 80% of all dietary fibre, which means that it is much better than other vegetable by-products on a dry weight basis.

Thus, by-products with high potential are generated during tomato processing (Figure 2.1), with potential added value and health benefits.

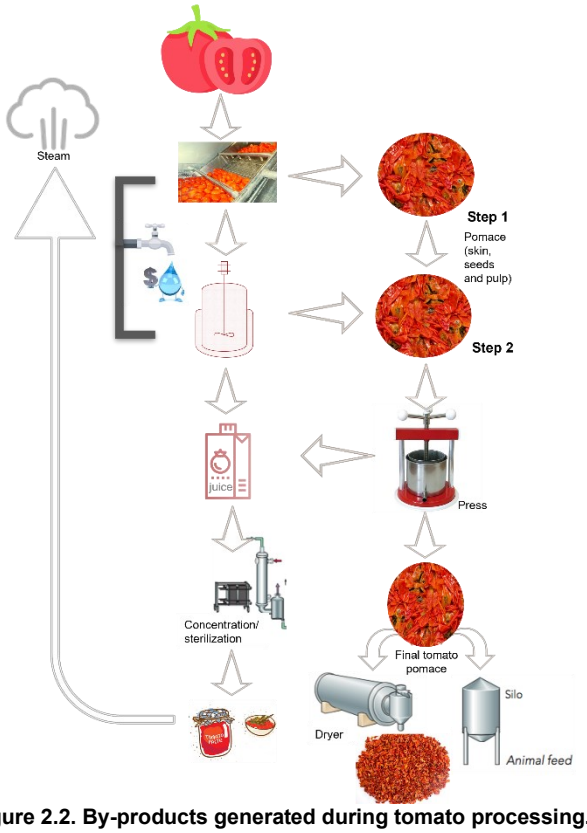

Figure 2.2. By-products generated during tomato processing.

2.3. Bioactive compounds

The central tomato BC are carotenoids (e.g., lycopene), tomato fibre, tomato seed oil and enzymes (Table 2.1). They can be recycled into the food chain as functional ingredients/additives for different products and applications (Shi and Le Maguer 2000; Del Valle et al. 2006; Taveira et al. 2010; Coelho et al. 2020).

Both polyphenols and carotenoids represent a set of essential metabolites abundantly present in fruits and vegetables (Coman et al., 2019). Their antioxidant properties play a crucial role in preventing chronic illnesses, mainly cancers or cardiovascular diseases (Kumar & Goel, 2019).

Table 2.1. Main BC present in tomato processing by-products and associated bioactivities.

BC		Bioactivities	References
Classes	*Subclasses*		
Carotenoids	Lycopene	Antioxidant activity	Singh et al 2015
		Prevention of heart diseases	Szabo et al. 2018
		Anti-cancer	Young et al 2014
		Immune defences	Zhao et al 2020
	β-carotene	Antioxidant capacity	Marti et al. 2016
		Improve immune defences	Domínguez et al. 2020
		Prevention of cardiovascular diseases	Domínguez et al. 2020
Dietary fibre		Control glucose absorption	Jones 2014
		Decrease the risk of cardiovascular diseases	Szabo et al. 2018
		Decrease the probability of colon cancer	Coelho et al. 2020; Ribeiro et al 2020
Fatty acids	linoleic acid	Emollient	Giannnelos et al., 2005
	oleic acid	UV protection	Persia et al., 2003
	palmitic acid		
Peptides		Antimicrobial activity against Gram-positive bacteria	Taveira et al., 2010

2.3.1. Carotenoids

Carotenoids are a class of fat-soluble plant- and microorganism-based pigments that produce various colours such as purple, orange and red. To date, more than 750 natural carotenoids have been reported, but only 20 have been identified in the blood and tissue of humans. Carotenoids are phytochemicals with antioxidant properties, and they serve as important dietary sources of vitamin A. They interact synergistically with other antioxidants to protect cells and tissues from oxidative damage (Szabo et al., 2018).

Carotenoids are grouped according to their chemical constituents into two categories, namely xanthophylls and carotenes. Oxygenated derivatives are known as xanthophylls; additionally, aldehyde groups (β-citraurin), epoxide groups (neoxanthin, antheraxanthin, and violaxanthin), oxo/keto groups (canthaxanthin and echinenone), and oxygen substituents (zeaxanthin and lutein)

are categorized as complex xanthophylls. At the same time, hydrocarbon derivates only carotenoids (lycopene, α-carotene, and β-carotene) are named carotenes (Tan & Norhaizan, 2019).

Thanks to the association of carotenoids to human wellbeing, numerous recent experiments have been conducted to revaluate tomato industrial by-products by extracting carotenoids. The method of extraction applied can strongly affect the amount and quality of this BC. Tomato pomace drying conditions, light exposure and tomato cultivar may also be of significant value in the recovery of carotenoids. Due to these reasons, to reduce losses, carotenoids should be extracted/used directly after treatment or with a limited period after drying (Szabo 2018).

2.3.1.1 Lycopene

Lycopene is the most common tomato carotenoids (lycopene, phytoene, phytofluene, β-carotene, γ-carotene; δ-carotene; lutein; neuroporene, and α-carotene). It is a photosynthesis and photoprotection red carotenoid (Martí et al. 2016; Caseiro et al. 2020). The acyclic carotenoid $C_{40}H_{56}$ is unsaturated, and its optimum pH stability is 3.5-4.5. Several studies have demonstrated the possible health benefits of lycopene (Riso et al. 2006; Nagao 2011; Bergougnoux 2014; Szabo et al. 2018; Caseiro et al. 2020). This antioxidant compound (Singh et al., 2015) lowers the likelihood of coronary disorders (Szabo et al., 2018), cancer (primarily the prostate) (Young et al., 2014) and immunological illnesses (Zhao et al. 2020). Since 1997 lycopene may be used as a colouring in food and drinks and is classified as E160d. It can be used as a dietary supplement in the food industry (Faustino et al. 2019). However, this compound is highly hydrophobic and can be deficient in the human body's bioavailability. It is insoluble in water and methanol, typically solvent-solubilized in organic solvents such as chloroform, hexane, carbon disulphide, acetone, petroleum ether and gasoline (Caseiro et al. 2020). Thus, it is necessary to apply different technologies to increase its solubility and bioavailability (Caseiro et al. 2020).

Tomato by-products may contain lycopene in a range of 80 to 150 mg/kg, while the tomato pulp contains 110 mg/kg and the tomato peel contains 540 mg/kg. Tomato cooking and processing improve the bioavailability of lycopene (Navarro-González et al. 2011; Kalogeropoulos et al. 2012; Nour et al. 2018).

2.3.1.2 β-carotene

β-carotene is a regularly used plant pigment and is one of the most studied in the carotenoid family. Often, Vitamin A has been the main nutritional precursor. Linear lycopene is turned into β-carotene by adding beta-ionone rings at both ends of the molecule, under the enzyme lycopene-cyclase (β-Lcy) (Rosati et al., 2000). β-carotene dietary origins are black and light green vegetables found in carrots, orange, kale, spinach, turnip greens, apricot, and tomatoes. β-carotene is the second most widely detected carotenoid in industrial tomato waste after lycopene, as indicated by the latest biological research (Domínguez et al., 2020; Urbonavičienė et al., 2018). A study reported that the tomato by-products, constituted mainly by skins and seeds, contain higher amounts of β-carotene 149.8 ± 6.4 mg dry weight) than the whole Heinz hybrid tomatoes (86.1 ± 4.4 mg dry weight) ((Domínguez et al., 2020; Kalogeropoulos et al., 2012). Various epidemiological studies have

revealed that β-carotene may improve immune function and have antioxidant capacity. β-carotene blood levels in humans are reversely associated with the risk of Type 2 diabetes and obesity, which are significant cardiovascular risk factors. However, these benefits seem to disappear when β-carotene is administered as a pharmacological supplement in high doses (Domínguez et al., 2020; Martí et al., 2016).

The most clearly shown feature of β-carotene is provitamin A development in humans. Because of its chemical composition, β-carotene can be hypothesized to create two retinol molecules through the enzyme β-carotene-15,15′–oxygenase, whereas the other provitamin A carotenoids can produce only one molecule (Domínguez et al., 2020; Marcelino et al., 2020). In a study by Weber and Grune (2012), the importance of β-carotene to human vitamin A generation has been addressed in depth. Because of different factors influencing bioconversion and bioavailability of provitamin A carotenoids, it is difficult to evaluate the recommended dietary dose to achieve maximum vitamin A absorption. In terms of nutrients, one of the main measures to avoid vitamin A deficiency is to improve the food supply containing vitamin A in the least developed areas (Mapelli-Brahm et al., 2018; Maurya et al., 2020; Weber & Grune, 2012). Industrial tomato waste is a cheap source of BC, meaning that the issues associated with vitamin A deficiency can be minimised.

2.3.2. Dietary fibre

Dietary fibres (DF) consist of polymers of 10 or more monomeric carbohydrates such as polysaccharides, oligosaccharides and lignin. These food components are resistant to digestive enzymes (Coelho et al., 2020). Insoluble and soluble fibres can control glucose absorption and reduce plasma cholesterol, thereby avoiding obesity, decreasing the risk of cardiovascular disorders, colon cancer and enhancing the digestive process (Jones 2014; Szabo et al. 2018; Coelho et al. 2020; Ribeiro et al. 2020).

The content of tomato DF reported in the literature ranges between 50.74 and 59.03 g/100 g (Szabo et al. 2018; Silva et al. 2019), from which 40.5 g/100 g were insoluble fibre (Alvarado et al., 2001). Therefore, this by-product could be applied in the food industry as an excellent source of fibre. In food labelling it can be referred "source of fibre," when it has at least 3 g/100 g or 1.5 g/100 g dietary fibre, under Regulation 1169/2011/EU (The European Parliament and the Council of the European Union, 2011).

2.3.3. Lipids

Tomato by-products contain a lipid content in the range of 32 to 60.0 g/kg for peel and between 40.4 and 63.7 g / kg for seed (Giannelos et al., 2005; Porretta et al., 2009; Rossini et al., 2013; Del Valle et al., 2007). Authoritative researchers reported that such by-products are rich in unsaturated fatty acids (77.04%), with only 22.72% of saturated fatty acids. The only main fatty acids present in this by-product is linoleic acid, followed by oleic and palmitic acid (Giannelos et al., 2005; Persia et al., 2003). The tomato seed oil is used in cosmetic formulations as emollient and UV protection.

2.3.4. Protein

The mean protein level reported in tomato pomace was 21.9% for dried weight and 38.7% for defatted tomato seeds. Defatted tomato seeds have been ascribed with hypocholesterolemic properties and can be used as a food product (Knoblich et al., 2005; Szabo et al., 2018, 2019). After hexane extraction, comparable protein content in tomato seeds was reported more than double the protein content of tomato seeds found in most wheat varieties (Sarkar et al., 2016).

Several studies were centred on tomatoes and pomace recycling ability, but few assessed the potential for tomato seeds reuse. The truth is that during industrial production, the additional step in the method of recovery of the seeds and the results of the protein content in tomato seeds shows their nutritional value and their functional applicability (Szabo et al., 2018).

2.4. Extraction of BC from tomato by-products

Tomato by-products are good sources of BC. The efficacy of the extraction of BC can depend on the implementation of crucial steps: I) pre-treatment; II) extraction; III) purification; and IV) drying (Galanakis 2012b).

2.4.1. Pre-treatment

Tomato by-products contain high moisture, making them perishable due to contaminations with microorganisms, aerobic rot and nutrient losses (Méndez-Llorente et al. 2014). The drying process may be appropriate depending on the extraction technique; also, dehydration allows the transport and storage of waste. Tomato dryer, drum dryer and fluid bed dryer types are among the big dryers used in the tomato industry. The extraction method is enhanced by homogenisation and the reduction of the particle size of these wastes. On the other hand, OH as pre-treatment may improve the extraction since the current passes through the food matrix, decreasing the microbial biomass and improving the extractions process by electroporation effects (Chapter 1).

2.4.2. Extraction

BC from tomato by-products, including mainly carotenoids and fibre, can be commonly recovered by chemical extraction. However, this process involves using toxic chemicals solvents, such as benzene, chloroform, and methylene chloride. Thus, the interest in environmentally friendly processes for industrial BC production has grown. This extraction includes dissolving BC in a suitable solution, followed by a separation process that aims to isolate soluble materials from insoluble or less soluble constituents. This step is critical because BC must be extracted without destroying its beneficial properties (Chapter 1). The principal techniques used in the recovery of BC are traditional solid-liquid extraction, sonicated extraction and microwave-assisted extraction (Coelho et al. 2019; Maroun et al. 2017; Sarfarazi et al. 2020). Many conditions used for the extraction of BC from food by-products have been thoroughly evaluated. Some studies suggest that the most efficient carotenoid extraction method from tomato seeds and peels is organic solvents, including hexane and ethyl acetate. Indeed, the yields of cis isomers are increased as the solid-liquid ratio declines and

the proportion of ethyl acetate increases. The conventional method of carotenoids extraction generally consumes large quantities of costly, toxic and dangerous organic solvents (Ascenso et al., 2013; Caseiro et al., 2020).

In Chapter 7 it is used OH extraction to recover different compounds from tomato by-products is studied. It showed not only that the new technology enables recovery of phenolic compounds such as rutin, kaempferol and naringenin at superior yield, but also that these same extractions can be selective for other compounds, like carotenoids (Chapter 7). Therefore, applying the same technology with varying temperatures, water-ethanol ratios, and extraction times can extract polyphenols and carotenes or increase the recovery rate of polyphenols, relatively to carotenes vice-versa, thus, allowing to improve the richness of BC in the final extracts. The low-temperature application helps to prevent thermal losses, and with an earlier rehydration stage, a recovery yield may be improved. Furthermore, different phases of industrial tomato processing are carried out with the pulsed electric field (PEF). PEF was used to maximize further juicing yield, presenting a 90.2% average yield to residues from the first tomato juicing stage (seeds, peels and a fraction of tomato). This technique improved the lycopene extraction from 9.84 mg lycopene/100 g to 14.31 mg/100 g tomato residue at 1.0 kV/ cm for 7.5 ms. Total phenolic compounds isolated doubled their concentration (56,16 mg gallic acid/kg) with the treatment of 2 kV/cm (700 pulses). Targeted PEF pre-treatment in tomatoes' industrial production generally leads to lower energy requirements (Andreou et al., 2020). The impact on the recovery rate of lycopene in either acetone or ethyl lactate from industrial tomato peels was investigated by Pataro and colleagues by the effect of PEF pre-treatment with different field intensity (E = 1–5 kV/cm) and the energy input (WT=5–10 kJ/ kg). The authors have demonstrated that with PEF treatment (5 kV/cm, 5 kJ/kg) both acetone and ethyl lactate extracts improved considerably, with lycopene (12–18%) and antioxidant strength (18.0–18.2%), respectively. Yet acetone yielded the most significant amount of lycopene. HPLC analyses found that lycopene's significant carotenoid derived and degradation was not evident (Pataro et al., 2020).

Super-critical fluid extraction (SFE) is suitable for recovering carotenoids and polyphenols from tomato by-products, as it reduces the use of toxic solvents since it generates solvent-free extract at moderately high selectivity and yield temperatures. SFE is a non-toxic and non-inflammable method, but because of its non-polar nature, it requires a stabiliser and a cosolvent. Besides, during any extraction phase, carotenoid degradation and/or isomerisation can occur. As an alternative to supercritical carbon dioxide, the use of ethane may also result in a less costly process, faster extraction, higher recuperation of compounds due to higher polarizing and low critical temperature and pressure (305.4 kg and 48.2 kg, respectively) (Arab et al., 2019; Ascenso et al., 2013; Caseiro et al., 2020).

High hydrostatic pressure (HHP) is an emerging non-thermal, non-conventional technology first studied as a technique for food preservation. It has been used to extract BC from fruit and vegetable products and contributes to the improvements in the bioaccessibility of BC (Coelho et al., 2020; Domínguez et al., 2020). It is simpler and more efficient than traditional extractions. In an effective solvent-free procedure, phylloquinone was obtained from tomato leaf waste (29.17 ± 0.96 μg^{-1}) using

high-pressure CO_2 and a room temperature of 180 bar (Arab et al., 2019). The same authors also reported higher vitamin K1 recovery rates for subcritical CO_2 than conventional extraction methods. Phenolics (240 mg (GAE). g^{-1}) and flavonoids (184 mg QE g^{-1}) were also present in tomato leaf. High protein content (24.47 ± 0.38%) are also present in tomato leaf, and the dominant free amino acids are aspartic acid, glutamic acid, and leucine (13 ± 0.1, 15:1 ± 0.2; 12.8 ± 0.1 mg g−1 dry weight, respectively). Other authors reported that the extraction of lycopene from the tomato paste waste by HHP was promising. They improved the recovery process with different laboratory conditions for the HHP process, such as solvents (chloroform, ethanol 95% and purified water), and ethanol levels (45–95% v/v), pressures (100–700 MPa), durations (1–10 min) and solid/liquid ratios (1:1 to 1:8 g/mL). HHP allowed lycopene extraction from tomato waste without heating in just 1 minute at room temperature. The fastest recuperation (92%) was achieved by extracting at 500 MPa, 1 minute, 75% ethanol and 1:6 (g/ml) solid/liquid (Jun, 2006).

Microwave-assisted extraction (MAE) is a new method of extraction that combines microwave and conventional solvent extraction (Domínguez et al., 2020). MAE is a quick technique to recover all-trans and total lycopene, whereas conventional extraction provides a higher percentage of cis isomers. MAE causes fast heating of the polar components, increasing the migration of carotenoids into the extraction solvent and limiting the heat exposure of non-polar components with limited treatment time (Caseiro et al., 2020).

Ultrasound-assisted extraction (UAE) has improved solvent penetration into plant cells and cell wall destruction, facilitating BC release and interaction between solvent and analyte (Caseiro et al., 2020; Domínguez et al., 2020). UAE was applied to tomato by-products to recover lycopene. The authors used three independent variables: ultrasonic pressure (30–70 W/m2), solid/oil (3.18–36.82% w/v), time to remove (1.59–18.41 min) and lycopene quality samples. They verified that the UAE allowed 87.25% of the lycopene extraction yield instead of conventional solvent recovery (63.66 mg/100 g with hexane: acetone: methanol at 2:1:1 v/v and 91.49 mg/100 g with UAE method) (Rahimi & Mikani, 2019).

2.4.3. Extracts' purification

The purification of the extract is essential for interferences and impurities to be eliminated, and a stable product is obtained after the extraction process. This purification involves the isolation of such compounds or fractions with high precision from complex mixtures. It encompasses techniques such as chromatography (partition, modification, adsorption, ion exchange, removal from scale or gel filtration), membrane filtration and crystallization (Galanakis 2015).

2.4.4. Extracts Drying

The drying technique is good for reducing the chance of oxidation of their BC. The widely used methods include freeze-drying, spray drier, sprinkling, and rotary drying vacuum.

2.5. Bioaccessibility, Bioavailability and Metabolism

The bioavailability of BC is the most significant element when designing functional foods. This is defined as the measure of a BC that will enter the bloodstream. When BC are administrated orally, these compounds pass through the mouth, stomach and gut to become available for bloodstream absorption (bioaccessibility) (Ribeiro et al., 2020). Protection against this gastrointestinal tract (GIT) conditions involves protection against digestive enzymes, pH values, and temperature. It is essential to change the constancy of BC and change their concentration by epithelium cells to change bioavailability. Thus, the term bioavailability includes the bioaccessibility of compounds. The organism's absorption, bioavailability, disintegration, distribution and storage are affected by many modulating factors (Caseiro et al., 2020; Desmarchelier & Borel, 2017; Szabo et al., 2018; Tan & Norhaizan, 2019).

Regarding carotenoid compounds, they are probably digested and absorbed as hydrophobic molecules such as lipids (Figure 2.2). Thereafter, through bile salts and pancreatic lipases, they may be integrated into micelles that are assimilated into the mucosal cells by passive absorption (Nagarajan et al., 2017). After that, carotenoids are carried from the intestine mucosa by chylomicrons. In some studies, lycopene, like other carotenoids, has been present in wet protein-carotenoid complexes and crystalline aggregates in most nutrients, resulting in some critical barriers to absorption (Hackett et al., 2006; Shi & Maguer, 2000; Uenojo & Pastore, 2010). Some studies evaluated tomato fruit bioaccessibility (Bot et al., 2018; Dewanto et al., 2002; Martínez-Huélamo et al., 2016). Nevertheless, there is no straightforward correlation between process-induced matrix delay and BC extractability and bioavailability.

The absorption, bioavailability, degradation, distribution and preservation of carotenoids are affected by many factors. There are not enough studies to explain all the complexity involved in the bioavailability of carotenes. The same is confirmed by the difference mentioned in the studies related to the proportion of β-carotene bioavailability, which ranges between 3.5% and 90% (Desmarchelier & Borel, 2017).

Carotenoid bioavailability can be reduced by the competition between carotenoids during absorption when eaten within the same meal. Dietary fibre, for example, was discovered to minimize carotenoid absorption from tomato plant sources, and bioavailability could be reduced in the location of carotenoids with chromoplasts and plant chloroplasts (Mapelli-Brahm et al., 2018; Tan & Norhaizan, 2019). The release of carotenoids from the food matrix depends heavily on their condition and interactions with other food components, including protein. In contrast with those immersed in lipid droplets, the microcrystalline type of carotenoids, for example, lycopene form in the tomatoes, have lower bioavailability. Earlier data have shown that almost 5% of carotenoids are absorbed by the gut, whereas up to 50% are absorbed from the micellar solutions. Also, at the same meal of carotenoid ingestion (cooked vegetables or raw vegan salads), dietary intakes of fat (for example, extra virgin olive oil or whole egg) have been shown to improve the absorption of some carotenoids effectively (Tan & Norhaizan, 2019).

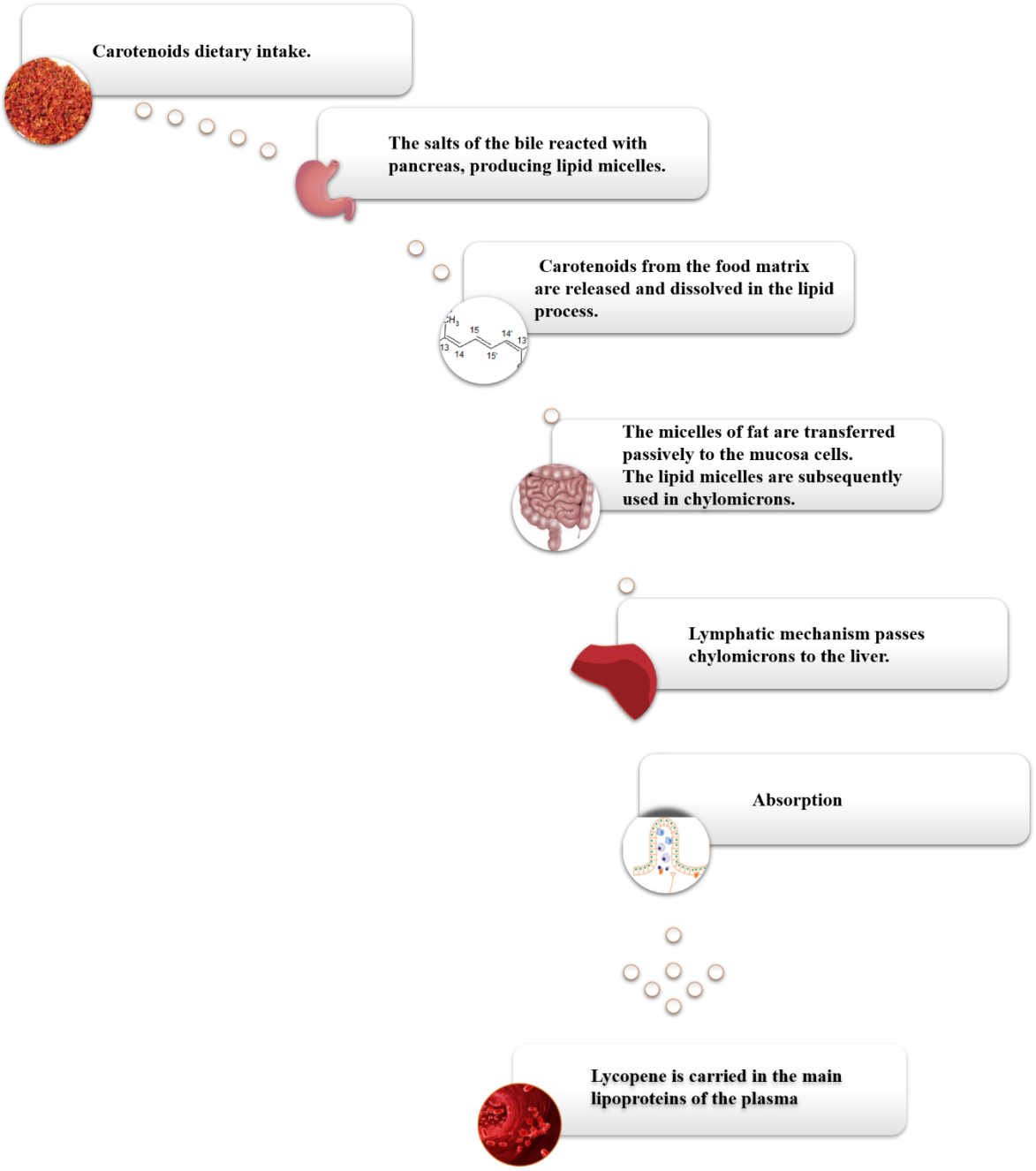

Figure 2.2. The absorption process of carotenoids from dietary intake (Adapted from Caseiro et al. 2020).

This result suggests the crucial importance of the physical form of carotenoids in intestinal mucous cells. Several experiments have shown that thermal mechanisms enhance carotenoid bioaccessibility and promote absorption by loosening the bond and disrupting cell walls.

Relatively to specific carotenoids, such as lycopene, little is understood considering dietary biological impact, particularly its behaviour in the human gastrointestinal tract. However, it is presumed to undergo a similar pathway as β-carotene.

2.6. Human wellbeing benefits from the consumption of tomato by-products

Tomato and its by-products intake are inversely proportional to coronary diseases and different kinds of cancer. The carotenoids and phenolic compounds with high antioxidant activity are responsible for these favourable properties (see Figure 2.3) (Szabo et al., 2018; Viuda-Martos et al., 2014).

2.6.1. Antioxidant properties

In the finishing of oxidative chain reactions, antioxidants play a critical role in scavenging free radical intermediates. Antioxidants regulate autoxidation, either by disrupting the development of free radicals or by suppressing the spread of free radicals (Başaran et al., 2017; Nabi et al., 2020; Paiva & Russell, 2013).

The BC in tomato by-products related to the antioxidant activity are essentially carotenoids, polyphenols, and specific vitamins. Carotenoids and other phytochemicals are recommended to shield the body from various reactive oxygen species (ROS)-mediated conditions, such as neurodegenerative diseases, cancer, eye-related and photosensitive disorders (Nabi et al., 2020; Viuda-Martos et al., 2014).

Recent studies have shown that daily tomato intake significantly decreases DNA damage arising from Fe^{2+} and increases the defence against UV and transition metal ions. Tomato peel extract has been tested by Elbadrawy and Sello (2016) for its antioxidant and nutritional activity. The authors used petroleum ether and chloroform extracts, concluding that the peel can be used as a functional component in the foodstuff to increase the diet's antioxidant level (Elbadrawy & Sello, 2016).

It has been experimentally demonstrated that lycopene effectively scavenges free radicals, extinguishing singlet oxygen, thiol and sulfonyl radicals. Its chain structure is also significant because of its biological properties, such as its resistance to oxidative degradation.

Lycopene is known amongst common carotenoids as the best antioxidant, shown by experimental *in-vitro* systems (Maoka, 2020; Pérez-Gálvez et al., 2020; Szabo et al., 2018; Tan & Norhaizan, 2019).

Lycopene is stated to be the single most effective quencher in the carotenoids. The ability to quench depends primarily on the number of double conjugate bonds and, to a lesser extent, on the presence of cyclical or acyclic terminals.

A blood antioxidant status study in rats showed that lycopene and GSH, and phase II GST enzymes could induce certain antioxidant enzymes such as superoxide dismutase (SOD), glutathione reductase and glutathione peroxidase (GSHPx) (Bhatia et al., 2018).

The antioxidant effects of lycopene can be enhanced by other BC, such as carotenoids and vitamins, due to synergistic antioxidant activity. This hypothesis has been the subject of numerous studies confirming the positive correlation between the antioxidant properties of lycopene and interactions with other studied BC (Viuda-Martos et al., 2014). Combined double bonds allow electrons from reactive species to be accepted and free radicals to be neutralized. A mixture of two lipophilic

antioxidants (e.g., vitamins E, C, and ß-carotene) has synergetic results, which can be considerably more significant than the one-effect combination of scavenging reactive nitrogen and lipid peroxidation (Milani et al., 2017).

2.6.2. Cardiovascular diseases

The most prevalent causes of mortality and incapacity in the world are cardiovascular diseases (CVDs). According to the World Health Organization, almost 17.9 million people die from CVD, representing 31% of deaths globally (World Health Organization, 2019). Obesity, blood pressure, elevated cholesterol in the blood, physical inactivity and smoking are the major causes of CVDs. Coronary artery disease and stroke that gradually progresses to endothelial dysfunction, inflammation, vascular over-rehabilitation and atherosclerosis are among the most prevalent forms of these conditions (Chaudhary et al., 2018; Nagarajan et al., 2017; Szabo et al., 2018; Tan & Norhaizan, 2019). The CVDs include modifiable and unmodifiable risk factors, as detailed in an analysis performed by Kulczinski and the co-authors (Kulczyński et al., 2017).

Metabolism and lifestyle affect modifiable risk factors. The main risk factor for CVD is an increased level of low-density lipoprotein plasma (LDL). The elevated risk of atherosclerosis, a lifelong condition with a strong health impact, is expected to be linked with increased LDL oxidation causes.

Fortunately, a series of epidemiological research suggests that the consumption of bioactive nutrients, including food containing lycopene, is inversely linked to the occurrence of CVD (Arab and Steck, 2000; Sesso et al., 2004). Studies have shown that a modest amount of dietary fats, such as olive oil or avocados, ingested as a whole food supplement of tomato soup, tomato-puree, tomato-paste or other tomato-drink lead to plasma carotenoids increase, especially lycopene.

The recommended daily intake of lycopene has been set at 35 mg, obtained by consuming two glasses of tomato juice or combining tomato products.

Although the precise mechanism(s) are still uneven, lycopene is likely to defend against atherosclerosis. It has been shown that lycopene inhibits in vitro ROS development and prevents LDL oxidation (Visioli et al., 2003). The development of foam cells, atherosclerotic lesions and CVD may also include the oxidative alteration of the LDL particles (Szabo et al., 2018).

Balestrieri et al. (2004) found that the endothelial cell acyl platelet activated factor biosynthesis in oxidative stress seems to be enhanced by combination with α-tocopherol or lipophilic tomato extract. The lipophilic compounds of tomatoes can prevent cardiovascular disease by modulating the atherogenic processes by oxidized lipoprotein regulated in the vascular endothelium (LDLs). Alshatwi et al. (2010) recorded a reduction of one-fifth of the overall serum cholesterol, tomato powder, lycopene-rich in tomato, and serum cholesterol low-density lipoprotein cholesterol by more than a third of their respective control amounts.

Further evoked mechanisms include endothelial injury prevention, lipid metabolism modulation by (i) cholesterol synthesis control and oxysterol toxic activities, (ii) reduction of inflammatory reactions by changes in cytokine production, (iii) inhibition of smooth-based muscular cell proliferation through regulation cell-proliferation molecular pathways (Palozza et al., 2010b).

Carotenoids can also stop coronary problems and suppress hyperlipidaemia, reactive protein and homocysteine in addition to their antioxidant properties. A study with 2856 adults (men and woman) showed a critical inverse relationship between LDL cholesterol and β-carotene Intake ($p<0.05$) and lutein+zeaxanthin ($p< 0.001$) and a decrease of total homocysteine with intakes of β- carotene ($p<0.05$), lycopene ($p<0.05$) and total carotene ($p<0.05$). Lutein consumption was also correlated positively with HDL cholesterol ($p<0.01$) and β-carotene serum inverting hsCRP (P-interaction <0.05) levels along with zeaxanthin (Wang et al., 2014).

Several in vitro and in vivo studies have been included in a previous study on lycopene and cardiac protection. The general finding was that the leading cause for the inflammatory mechanism could be tomatoes and tomato products' antioxidant and anti-inflammatory mechanisms. However, more studies are required to validate these results of CVD prevention.

If we understand the health implications of a dietary ingredient, the influence of one compound is difficult to distinguish from that of many compounds in whole foods and whole diets. If tomato lycopene affects wellbeing, it is an essential active ingredient or acts with other bioactive agents in tomatoes in conjunction with it (provitamin A, flavonoids, vitamin C, fibre, each other.). It has been documented that tomato flavonoids, including rutin, quercetin, naringenin, have possible health effects. The neutrophil-induced LDL oxidation of quercetin is reduced (Liu, 2003). These findings show that tomatoes and tomato derivatives can play a role in their health effects. Other experiments have demonstrated that glycoalkaloids in tomatoes have many biological effects as well. Also, recent research has shown that water-soluble tomato’s extracts can minimize platelet aggregation, which is a risk factor for cardiovascular diseases (Bhatia et al., 2018; Chaudhary et al., 2018; O’Kennedy & Duttaroy, 2021). The body of evidence shows that whole food is more effective in reducing the disease risk than individual compounds.

2.6.3. Eye disorders

The second most frequent cause of blindness has been visual impairment. The most prevalent vision loss in the elderly is diabetic retinopathy, glaucoma, cataract and age-related macular degeneration (AMD). AMD is not only triggered by age but may also raise the risk of other causes, such as dietary habits, oxidative stress and smoking (Desmarchelier & Borel, 2017). A high degree of phototoxic and oxidative stress comes from constant skin sensitivity to light and oxygen. Therefore, many studies have assessed the role of antioxidants in preventing light and oxygen damage and age-related cell and tissue damage.

β-carotene, lycopene, lutein and zeaxanthin have been the most studied carotenoids. β-carotene also offers additional advantages besides its antioxidant properties since it can be transformed into vitamin A, while lutein and blue light penetrate the eye (Tan & Norhaizan, 2019). Such a carotenoid (β-carotene, α-carotene, β-cryptoxanthin) can be classified as pro-vitamin A, which consists of a β-ionone ring that can be converted into retinal. The macular pigments present in human retinas are two dietary carotenoids, namely zeaxanthin and lutein. Macular pigments have antioxidants that absorb short and high wavelengths (Tuj Johra et al., 2020; Wu et al., 2015).

Vitamin A deficiency influences immunity and could destroy receptors that are sensitive to light (Awasthi & Awasthi, 2020). Furthermore, vitamin A deficit may also cause xerophthalmia and progress to irreversible blindness (Maurya et al., 2020). The retina, particularly in the macula lutea and the lens, possesses a possibly particular role in these two essential ocular tissues and has a unique lutein and zeaxanthin concentration (Tuj Johra et al., 2020). Many clinical trials and epidemiological research endorse lutein and zeaxanthine's possible role in preventing and treating a wide range of eye disorders, including age-related macular degradation, cataracts and retinitis pigmentosa (Tan & Norhaizan, 2019).

Zeaxanthan/Lutein (2 mg/10 mg) reduced the risk of cataract surgery. Further, compared to persons who seldom or never eat carotenoids, AMD is inversely associated with dietary supplementation intakes in carotenoid-rich diets (5–10 mg/day). Oxidative stress also appears to be an essential element in the growth of prematurity and diabetic retinopathy.

Indeed, a randomized controlled clinical trial has demonstrated that lutein is neonatally anti-inflammatory to preterm children. In contrast, forward trials have shown that in patients with non-proliferative diabetic retinopathy, lutein and zeaxanthin serum levels are slightly lower than standard subjects. In a previous study, carotenoid supplementation, including zeaxanthin (2 mg/day per year) and lutein (10-20 mg/day per year), could increase the optical density level of the macular pigment [104,105]. Several studies have also shown that zeaxanthin/lutein can improve its visual output (2 mg/10 mg/day/year), including regeneration of photo stress, glare resistance, and contrast. Collectively, the consumption of carotenoids may be a possible solution to oxidative stress improvement and potentially have benefits to eye protection and work.

On the other hand, more lung cancers in the β-carotene vs no β-carotene group (23 [2.0%] vs 11 [0.9%], minimal P=0.04) were found in the broad Age-Related Eye Disease Analysis (AREDS) (Chew et al., 2013). The expectation of lutein/zeaxanthin was not a risk.

2.6.4. Neurodegenerative Diseases

The gradual degradation of neural structure or activity, including neuronal killing, is neurodegeneration. As a consequence of neurodegenerative surgery, several neurodegenerative disorders — including lateral amyotrophic sclerosis, Parkinson, Alzheimer's and Huntington's — arise. Many similarities exist between neurodegenerative diseases and cell death caused, including atypical protein assemblies (Tan & Norhaizan, 2019).

The greatest risk factor for neurodegenerative diseases is ageing. Mitochondrial DNA mutations as well as oxidative aerobic stress both contribute to ageing. Many of these diseases are late-onset, meaning some element changes as a person ages for each illness. One constant factor is that neurons gradually lose function in each disease as the disease progresses with age. It has been proposed that DNA damage accumulation provides the underlying causative link between ageing and neurodegenerative illness.

Previous research shows that the accumulation of carotenoids in cognitively intact and cognitively disabled people is passively correlated with cognitive success (Tan & Norhaizan, 2019). Several

reports indicate that carotenoids can reduce the neuronal harm caused by free radicals, an alterable risk factor for cognitive decline. National Health and Nutrition Survey from 2011 to 2014, including 2796 participants over age 60, showed that lutein and zeaxanthin supplementation (2.02 mg/day) would help reduce cognitive loss (Christensen et al., 2020). Carotenoids delay the development of neurodegenerative diseases by many means, such as suppressing proinflammatory cytokines (Hadad & Levy, 2017), triggering the synthesis of peptides (Lin et al., 2017), and reducing oxidative stress (Wang et al., 2018). Since it has high binding energy from receptors (histone-deacetylase and P53 kinase receptors) associated with Alzheimer's, carotene is likely to become an Alzheimer's disease antagonist. The inflammatory cytokines (for example, TNF—, NF-B, IL-1) and transforming growth factor-beta (TGF-) in the brain also decrease with lycopene (1–4 mg/kg body weight/14 days) (Sachdeva & Chopra, 2015). Alzheimer's disease is decreasing in high levels of serum carotenoids such as lycopene, zeaxanthin and lutein (Crowe-White et al., 2019). Carotenoids collectively play an essential role in delays in neurodegenerative disease development as an antioxidant.

2.6.5. Chemoprevention

With almost 9.6 million deaths and 18.1 million new cases in 2018, cancer constitutes the second most frequent cause of death worldwide. Emerging literature indicates that 30-50% of deaths can be avoided by changes in primary risk factors such as physical exercise, bodyweight conservation, alcohol reduction and tobacco avoidance (Szabo et al., 2018; Viuda-Martos et al., 2014).

In addition, oxidative stress was implicated in cancer development through increased DNA mutations or disruption to DNA, genome instability, and cell proliferation (Szabo et al., 2018). These disorders can lead to various cancers, particularly prostate, lung, and stomach, which has decreased with consuming tomato and tomato products.

The reverse relationship between this kind of diet and colorectal cancer was reported in a recent study on the key components of the Mediterranean diet (olive oil, red wine and tomatoes). Colorectal carcinoma can be prevented by tomatoes, which are considered key to the Mediterranean diet and a principal source of lycopene (Farinetti et al., 2017). The tomato and carrot juice effects on the gastrointestinal lumen have been studied to assess whether carotenoid-rich foods can change the related processes during colon-carcinogenesis. After two weeks, increased levels of carotenoids were found in faeces following ingestion of juices with elevated β-carotene and lycopene content (Dixon et al., 2007). However, these two carotenoids provided alone are not associated with anticarcinogenic effects (Szabo et al., 2018). Any of the most critical epidemiological data support a connection between the intake of tomatoes and a decreased occurrence of prostate cancer and benign prostatic hyperplasia (BPH). A prospective retrospective analysis of 47.365 men in 1986, 1990 and 1994 among the population obtained by the Food Frequency Question (FFQ) has been done. The study showed a relation between the consumption of tomato sauce servings each week with the reduced risk for prostate cancer (Grainger et al., 2008).

At least 12 clinical trials have been investigated with the association of tomato products or lycopene with prostate cancer since 1999. Any of these experiments have measured prostate antigen (PSA)

in particular. Consumption of tomatoes and tomato products daily (target intake level 25 mg/day lycopene) for eight weeks reduced serum PSA levels in 34% of the subjects.

Various carotenoids such as lutein, zeaxanthin and lycopene have been shown to reduce the development of the inflammatory mediator, such as TNF-α, IL-1β, and IL-6, by blocking the NF- B pathway (Cha et al., 2017; Tan & Norhaizan, 2019).

The activation of intercellular gap interconnection communications is another important biochemical mechanism of action. β-carotene, canthaxanthin and lutein are effective inducers of intercellular gap interconnection, whereas α-carotene and lycopene are less involved. The ingestion of cis-lycopene has a significant cancer prevention activity (Caseiro et al., 2020; Jimenez-Lopez et al., 2020; Tan & Norhaizan, 2019). Lycopene tends to have the most significant impact in delaying the growth of the disease (Caseiro et al., 2020; Jimenez-Lopez et al., 2020; Tan & Norhaizan, 2019).

A dose-response systematic study of the dietary consumption or blood concentrations of carotenoids concerning prostate cancer risk revealed the inverse association of α-carotene and lycopene, but not of β-carotene with prostate cancer risk; thus, α-carotene and lycopene did not decrease advanced prostate cancer risk (Saint-John, 2017). Also, *in vitro* IGF-1-induced prostate cell proliferation has been decreased by quercetin and rutin.

Research by Cui and others showed that the use of lycopene is inversely linked to a positive risk of breast cancer in postmenopausal women (n = 84.805) for estrogenic and progesterone-receptors and was followed up over a 7.6-year average (Cui et al., 2008).

A randomized, placebo-controlled, double-blind crossover study performed by Voskuil et al. (2008) found a two-month reduction in free insulin-like growth factor I (IGF-I) over two months in premenopausal women at high risk of breast cancer (n = 36). There was also a substantial decline in the chances of people who ate the highest compared to the lowest amount of nutrient lycopene from two case-control trials comparing the nutritional habit of women with and without breast cancer. The high hydrophobicity of lycopene, an obstacle to cell culture studies, is a weakness of cell culture studies. Because lycopene is water-insoluble, steps must be taken before *in vitro* studies to improve its solubility inside cell culture media/buffers (Voskuil et al., 2008).

Tomato powder and distilled lycopene supplements have demonstrated antineoplastic effects in animal studies. Boileau et al. (2003) reported significant inhibition of N-methyl-N-nitrosourea – testosterone-induced carcinogenesis in male Wistar-Unilever rats following consumption of tomato powder (13 mg lycopene/kg diet). In contrast, no effects were observed with lycopene supplementation per se (161 mg lycopene/kg diet). This study suggests the synergistic effects of lycopene with other antioxidants in tomatoes in exerting an antineoplastic effect (Boileau et al., 2003).

Autocrine/paracrine loops in the model of Dunning prostatic cancer interact with lycopene and vitamin. Also, lycopene can only suppress prostate cancer development at higher concentrations than tomato antioxidant supplementation, as seen by *in vitro* and animal studies. Karas et al. (2000) further documented lycopene inhibitory effects on MCF7 human mammalian cancer cell growth due to the involvement in the signalling of the IGF-1 receptor and progression of the cell cycle (Karas et

al., 2000). Thus, the main pathologies in which lycopene prevents prostate and breast cancer development are seen as the involvement of androgen metabolism and inhibition of growth factors and cytokines. Additionally, also shown to avoid modification to the p53, p53 phosphorylation, and p53 target genes due to tobacco smoke exposure in the gastric mucosal of ferrets were the addition of tomato lycopene (1.1 mg/kg/day, equivalent to 15 mg lycopene intake of 70 kg individual) was tested. This result indicates more protection against gastric cancer by lycopene (Liu et al., 2006).

The antioxidant function may also be due to the protective effect of carotenoids against cancer. Indeed, carotenoids such as Lycopene's anti-cancer abilities are modulated by various pathways, including apoptosis, arresting cell cycles, detoxifying the phase II enzymes, and signalling for the growth factor (Jimenez-Lopez et al., 2020).

2.6.6. Immune system regulators

Lycopene's ability to stimulate the expression of antioxidant genes and control signalling pathways likely to induce inflammatory mediators has beneficial effects on inflammation and redox imbalances. Several anti-inflammatory properties of lycopene can be summarized as follows: a) regulation of inducible nitric oxide synthase; b) modulation of cyclooxygenase and lipoxygenase expression; c) interference NFkB NFkB as well as with activator protein-1 (AP-1) and with the signalling of MAPK (Caseiro et al., 2020).

A study demonstrated that tomato ketchup's hydrophilic and hydrophobic extracts also presented anti-inflammatory activity (Hazewind et al., 2014).

Lycopene also can activate the adaptive immune response, maintaining an adequate defence against microorganisms (Caseiro et al., 2020). Thus, it protects against bacterial infection and radiation (Ascenso et al., 2013).

An investigation with male mice found that lycopene would decrease plasma interleukin (IL)-6 and TNF-α considerably after intraperitoneal lipopolysaccharide injection, preventing inflammations of the brain tissue at six hours.

Serum lycopene is reversely correlated with plasma glucose concentrations and fasting insulin (Caseiro et al., 2020). Cross-cutting research observed that a rise of 1 mg in lycopene intake lowers the likelihood of gestational diabetes mellitus to 0.005 mmol/L, fasting blood glucose. This study promoted a decrease in blood glucose intake in dietary lycopene, which may decrease the risk of gestational diabetes mellitus (Gao et al., 2019).

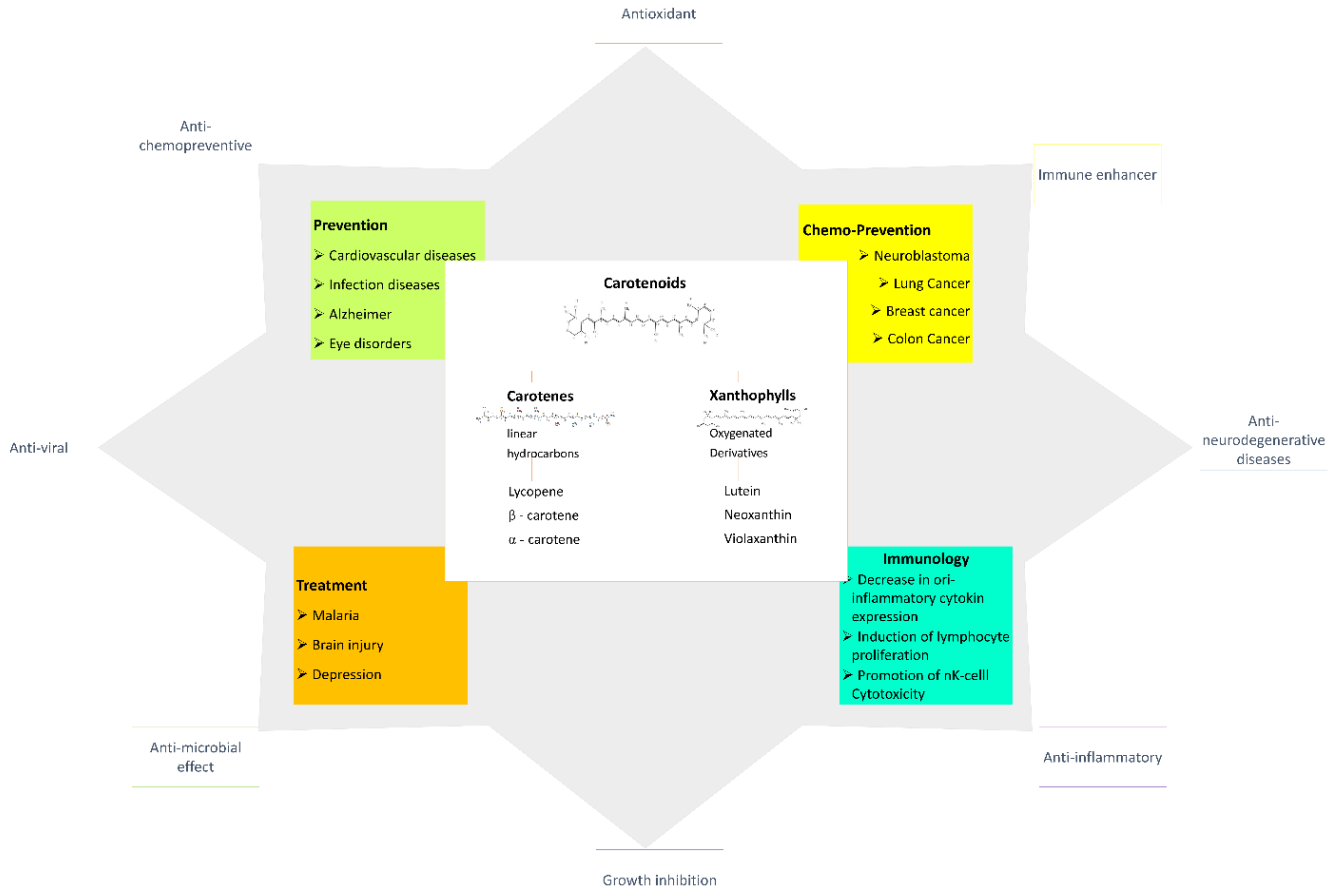

Figure 2.3. Chemical scheme of carotenoids division and its biological properties used to treat various diseases (adapted from Nabi et al. 2020).

2.7. Valorisation approaches to tomato by-products

The diverse composition and components of fruit and vegetable wastes imply a highly multidisciplinary task when the beneficial recovery of those molecules is expected and suited to the circular bio-based Europe (CBE) and public policies (FAO, EFSA, WHO). As stated earlier, this waste is rich in BC and can be used to obtain new valuable products instead of disposing of them with environmental impact (Coelho et al., 2020; de Britoi Nogueira et al., 2020). The utilization of fruit and vegetable wastes has included extraction of BC for food, nutraceutical and cosmetic applications, animal feed production, fertilizers, and bioenergy production. Extracts of tomato seeds have demonstrated Gram-positive bacteria antimicrobial activity (Taveira et al., 2010).

There are few studies based on its valorisation under the CBE method on tomato by-products. Generally, the tomato-bagasse is used as cattle feed or spilt into controlled sites, causing significant shipping costs, environmental impact, consisting of around 60% seed and 40% peel. Secondi et al. (2019) described the leading factors for tomato losses and waste in the supply chain for tomato sauce and the reuse of these by-products according to the CBE method. In this report, 85.9% of overall losses and surplus are priced in alternate industries. All the destinations mentioned have

been treated as low to medium value uses according to the CBE approach: feed and treatment of livestock (14.5%); recovered for energy (12.5%); not harvested (57.9%), and for human consumption (0.7%) of overall tomato losses and waste are used.

Many researchers investigated a potential high-quality, total recovery solution by applying tomato bagasse and its fractions as powder as a whole in food. Tomato bagasse, which is a rich food in lycopene, peroxides, dietary fibre, unsaturated fatty acids and essential amino acids with no harmful effects on the sensory and textural properties, has been integrated as ingredients into meat products, wheat flour-based foods and into the tomato paste (Lu et al. 2019). For example, it is possible to add tomato bagasse powder (2 g/100 g) or crude lycopene (50 and 100 mg/100g) to whole wheat flour cookies). Another excellent example of this is the use of fresh tomato bagasse in the manufacture of tomato ketchup, with an improved amount of food fibre (3.8 g/100 g) (Torbica et al. 2016).

To avoid the harmful influence of food on the sensory characteristics is essential to use lower tomato bagasse powder content, reduce its powder particle size, and non-use of warming or lyophilization techniques (Torbica et al. 2016; Belović et al. 2018; Bhat et al. 2020).

Besides using tomato bagasse in food as a whole, its bioactive fractions can be obtained, such as lycopene, dietary fibre, pectin, protein and oil. Usage of conventional and novel approaches for extraction lycopene has been the subject of tomato by-products' recovery (Løvdal et al., 2019). In the last years, the importance of tomato pomace valorization has also been reported. This valorisation includes, for example, the lycopene sequential recovery and anaerobic digestion (Allison & Simmons, 2017). Allison and Simmons (2017), valorised tomato bagasse with sequential lycopene recovery and anaerobic digestion. The authors used mixed and organic solvent processes constituted by hexane, acetone, and ethanol to obtain lycopene. After lycopene recovery, the authors also bioconvert methane via anaerobic digestion, using pre-treatments with ionic liquids. They verified extraction yields to lycopene above 293 to 476 mg g^{-1} DW. In another study, in 2018, the researchers have highlighted the use of tomato pomace as a raw material for the manufacture of biomaterials. The authors produced a mixture of unsaturated and polyhydroxylated fatty acids through hydrolysis of tomato pomace by-products; and a non-hydrolysable secondary residue. The authors showed a yield of approximate 31% w/w, which is compatible with the lycopene and proteins recovery by standard process (alkaline hydrolysis at medium temperatures followed by neutralization) (Benítez et al., 2018).

2.8. Conclusions

Biotechnology has brought a modern view of agriculture and health, which brings creativity and provides productive and economical ways of producing different products and resources. The tomato processing industry produces large quantities of waste, especially seeds and skins, which are wasted and can exacerbate the degradation of the environment. The use of green technologies, like the PEF and OH, have been applied to tomato waste with better results to recover BC than with other extraction methods. Thus, these green technologies and their combinations appear to be the best

methodology for obtaining target BC with market potential, namely carotenoids, polyphenols, fibres, proteins, among others. Also, this chapter showed that incorporating whole tomato by-product (bagasse) can be more beneficial for preventing the risk of some chronic diseases than individual compounds. As the tomato peel is rich in BC, such as lycopene, it would be possible to apply this by-product as ingredients to the food directly and obtain lycopene enriched products.

Similarly, more studies are necessary to overview the individual compounds' behaviour, absorption rate, metabolism, and bioavailability. It is also essential to determine the dose levels and enhance its cost-benefit efficiency for various applications, such as additives/ingredients for food and feed, cosmetics, and natural pesticides. These applications result in the acquisition of value-added products based on the circular economy, which could mitigate the adverse effects caused by tomato by-products accumulated in the landfills. Thus, the subproducts obtained were essential and must therefore be priced in line with the CBE principles.

Chapter 3.
Objectives

The general objective of this thesis was to valorise tomato and grape industrial processing by-products and/or wastes towards the recovery of BC by green technologies such as OH. Furthermore, the OH optimization was performed to efficiently extract BC and maximise bioactive properties, namely antioxidant activity. Bioavailability and toxicity of resulting tomato by-products fractions were also evaluated to assess their performance throughout the gastrointestinal tract and to guarantee their safety. Conventional extraction methods, such as solvent extraction, was used for comparison.

The specific objectives included:

I. Characterization of the composition of tomato and grape by-products in order to define an extraction and valorisation strategy;
II. Application of the green OH technology to tomato and grape by-products (and compare with classical extraction method) to obtain optimized fractions with antimicrobial and antioxidant BC;
III. determination of the compositional analysis (qualitative and quantitative) of the principle BC present in the extracts with higher activity, mainly focused on phenolic compounds and carotenoids, establishing the relationship between extracts composition and bioactivity;
IV. Assessment of genotoxicity and cytotoxicity by *in vitro* studies of resulting bioactive fractions to demonstrate their safety;
V. Determination of main biological activities of bioactive fractions focused on the antimicrobial, antioxidant activity, antimutagenic and prebiotic activity.
VI. *In vitro* study of bioaccessibility and bioavailability of primary BC from tomato by-product fractions throughout the gastrointestinal tract, and
VII. Establish the potential of digested tomato by-products fractions on the modulation of gut microbiota using an *in vitro* model.

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PART II

Grape and tomato bagasse composition and valorisation through ohmic technology

Chapter 4.
Innovation and winemakings' by-product valorization: an ohmic heating approach

Abstract

The winemaking processing by-products can represent chances for the development of new products. This study focused on the “zero waste” strategy development for by-products generated within winemaking from white and red grape varieties cultivated in the North of Portugal. The phytochemical properties of by-products were identified and characterized. The ohmic heating (OH) as a green extraction method was also applied to grape pomace due to their unknown effects on centesimal and phytochemical composition. Both protein and carbohydrates were shown to be higher in grape bagasse than in stems. Also, red bagasse is richer in bioactive compounds (BC) than white bagasse. The sugars content was 21.91 and 11.01 g/100 g DW in red and white grape bagasse, respectively. The amount of protein was 12.46 g/100 g DW for red grape bagasse and 13.18 g/100 g DW for white.

Regarding extraction methods, two fractions were obtained, a liquid fraction and solid (the remained after methodology application). The OH presented a higher antioxidant capacity than a conventional (CONV) method. Besides, both extracts presented similar content of anthocyanins, e.g., delphinidin-glucoside, petunidin-glucoside and peonidin-glucoside. The solid fraction presented higher amounts of protein and phenols bound to fibre than CONV, which allows its use as a functional ingredient. In conclusion, the OH can be an alternative extraction method compared with CONV methods, avoiding non-food grade solvents, thus contributing to circular economy implementation.

Keywords: grape pomace; valorisation; fractionation; food ingredients.

4.1. Introduction

Vitis vinifera l. (grape) are one of the most harvested fruits in the world. Approximately 78 million tons (mt) of grapes are produced annually, 37% produced in Europe, 34% in Asia, and 19% in America, representing a global vineyard surface area of 7.4 million hectares (OIV, 2018). Global grape production is approximately 78 mt. It is additionally one of the most fruits crops worldwide: while practically half of the grapes are utilized to make wine, 33% is expended as new foods, while the remaining part is dried, consumed as grape or put away as grape musts (regardless of whether concentrated or not) (FAO, 2017; Fereres & FAO-OIV FOCUS, 2012). For each 1.32 Kg of grape, one hectoliter of wine is produced. In 2018, world wine production reached a record volume of 293 million hectoliters (OIV, 2018). Nine million tons of grape by-products are produced annually globally, representing 20% (w/w) on average of the overall grapes used for wine production. The main by-product is bagasse, which is the remaining solid after pressing that usually contains the skins, pulp, seeds, and stems of the fruit (Teixeira et al. 2014; Coelho et al. 2020). Grape skins represent, on average, 65% of the overall material of grape bagasse, and they are rich in BC, depending on the vinification process and extraction method employed (solvent temperature, time, and other factors) (Coelho et al., 2020). Although the phytochemical profile of this by-product showed high potential, the lack of proper valorization operations makes its primary use as compost or discarded in public fields, possibly causing environmental issues. Also, most thermal treatments, such as pasteurization, extraction or blanching, are related to a decrease in nutritional properties and BC losses because of oxidation, filtering and different actions that lessen these antioxidant properties (Guida et al., 2013; Minatel et al., 2017).

Several studies explore the use of grape bagasse, a source of healthy and technological compounds that could be applied in animal feed, pharmaceutical, cosmetic or food industry to improve stability and nutritional characteristics, and in the cosmetic industry, where grape seeds oil is widely used (Ianni & Martino, 2020; Kalli et al., 2018).

However, many of the by-products generated by the winemaking industry remain without a sustainable recovery solution implemented. Such waste streams are partly valorized in various added-value levels (spread on land, animal feed, composting), whereas the first volumes are overseen as natural misuse, with applicable negative impacts on the sustainability of agro-industry (Jimenez-Lopez et al., 2020; Truong et al., 2019). Furthermore, by-products usually comprise high amounts of proteins, sugars, and fibres, making them a source of cheap nutrients and BC (Hanušovský et al., 2020).

Grape bagasse is a fibrous and tannin-rich material used in the oil and gas industry as a lost circulation material in oil-based drilling muds. Grape skin by-products have also been used as compost to regenerate vineyards (Kurosawa, 2006).

The European Union’s (EU) goal is to achieve zero food by-products by 2030, and obviously, this includes food loss reduction along food supply chains. Thus, it is essential to find alternatives to reuse or reintroduce the by-products in the supply chain.

The actual modus operandi to extract or make synthetic BC uses a large number of organic solvents, which are very toxic, prejudicial for both environment and health, and with higher costs when associated with cleaning solvents to use the materials (Coelho et al., 2020; Galanakis, 2014; Vidović et al., 2021). Thus, to make this a more sustainable process, it is necessary to break the linear economy. Thus, it is vital to use greener alternatives with lower impacts and allow direct by-products to develop new products further. Therefore, several technologies have been studied to reach these objectives. Ohmic heating (OH) has been highlighted as a good alternative due to its environmentally friendly character, faster and homogenous processing properties (El Darra et al. 2013; Pereira et al. 2016; Maroun et al. 2017). OH, also known as joule heating or resistive heating, consists of the passage of an alternating electric current through a food sample that acts as a semi-conductor, thus producing internal heat within a given material. OH can be used in various unit operations, such as pre-treatment or thermal processing, such as blanching, evaporation, dehydration, fermentation, recovery, sterilization, and pasteurisation. A disadvantage of this technique is that it depends on the product's electrical conductivity – for example, it does not occur in non-conducting materials, as these materials do not allow the passage of electrical current.

Regarding winemaking by-products, OH is used as a pre-treatment, and only in a few cases, its phytochemical impacts are evaluated. El Darra et al. (2013) showed that POH (Pulsed Ohmic heating) causes cell wall disruption on red grape bagasse. The study has shown the permeabilization increases with increasing temperature and electrical field power's intensity. Polyphenols extraction yields were 36% higher than in untreated samples, for a current of 400 V/cm, during 60 min, at 50 ºC in 30% of aqueous ethanol solution. Nevertheless, few studies about the OH impact on the nutritional composition of the residual winemaking by-products flour obtained after polyphenol extraction.

This study aimed to valorize the winemaking by-products. Firstly we evaluate the by-products phytochemicals potential from two Portuguese cultivars, Vinhão and Loureiro, red and white grape cultivars, respectively. Secondly, an alternative technology, the OH, was used to improve the extraction yields of BC compared to conventional (CONV) methods that use chemical solvents to recover the same compounds.

Overall, this work aims to create a new winemaking strategy that considers the benefits of a waste management policy.

4.2. Materials and methods

4.2.1. Chemicals

The 2, 20-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS diammonium salt), potassium sorbate, sodium carbonate, and ethylenediaminetetraacetic acid (EDTA were purchased from Sigma-Aldrich (Sintra, Portugal). Hexane, ethanol, Folin–Ciocalteu's reagent, and potassium persulfate were

purchased from Merck (Algés, Portugal). Standards of ascorbic acid, trolox, gallic acid, rutin, *p*-coumaric, and 4-hydroxybenzoic acid, were purchased from Sigma-Aldrich (Sintra, Portugal), while kaempferol, β-carotene, lycopene, zeaxanthin, and lutein (Extrasynthese, France) were purchased from Extrasynthese (Lyon, France).

4.2.2. Samples preparation

Grape bagasse (seeds, skins, pulp) and stems from two grape cultivars, “Vinhão” and “Loureiro” grape cultivars were kindly provided by two farms of North Portugal that produce white and red wine, respectively. These by-products were collected three times after production and were transported under refrigeration until the laboratory. After collecting these three batches, the samples were homogenized, packed in polyethene flasks and stored at -80 ºC until analysis.

4.2.3. Fractionation of grape by-products

White (WB) and red bagasse (RB), and white (WS) and red stems (RS) were dried in an oven at 55 ºC until levels of moisture ca. 5%, and then milled in a kitchen robot Thermomix TM5, obtaining a flour with particle size < 1mm to bagasse and >1mm to stems. All batches were mixed, each 100 g of the mixture was stored in sampling bags in a dark and dry place at room temperature until the analysis.

4.2.4. Characterization of the raw material

The production of solid by-products worldwide is dramatically increasing every year, and most of them are composed of food by-products rich in BC with high potential. So, a complete and preliminary characterization of each grape by-product was performed to envisage the value of new functional ingredients. Also, after by-products evaluation, a green recovery process, Ohmic heating technology (OH), was used and compared with conventional methods, which uses organic solvents.

4.2.4.1. Proximate composition

All by-products from cultivars were submitted to nutritional and phytochemical characterization with referenced methods. The moisture content of grape by-products bagasse (composed of seeds, peels, pulp) and stems (fibrous parts) was determined according to the oven method (Chemists, 2000) in fresh samples. The calculation was present as follows:

$$Moisture\ (dry\ matter)(\%) = \frac{W1 - W2}{W1} X\ 100\%$$

Where W1 is the weight (g) of the sample before drying and W2 is the weight (g) of the sample after drying.

The ashes were determined by resulting inorganic residue weight after ignition in a Muffle furnace at 550 ± 25 °C for 3 h.

Total nitrogen was obtained according to the Kjeldahl method, and protein content was then calculated using a conversion factor of 6.25 in all fresh samples. The fat content was determined gravimetrically by the Soxhlet method using petroleum ether (boiling point 60 - 80 ºC), according

to the method described in AOAC 2000. The crude fibre content was determined with an acid/ alkaline hydrolysis of insoluble by-products, as described in AOAC 2000. The total carbohydrates were measured by the phenol-sulfuric acid method, according to AOAC 2000.

All methodologies followed the recommendations of the Official Methods of Analysis (AOAC, 2000). All measurements were done in triplicate. The content of each parameter was expressed as g/ 100 g dry weight (DW).

4.2.4.2. Total Pectin

Total pectin content of stems and bagasse from red and white grapes were calculated based on the method described by Deng, Penner, and Zhao (2011), consisting of the sum of three fractions: water-soluble pectin (WSP), chelator soluble pectin (CSP), and hydroxide soluble pectin (HSP).

4.2.4.3. Cellulose, hemicellulose, and lignin

For the determination of the crude grape by-products, the WS, RS, WB, RB fraction tests, the technique of Sluiter et al. was followed for cellulose (as glucose), hemicellulose (as arabinose, mannose, galactose and xylose) and lignin (soluble and insoluble). The extractors were already extracted with ultra-pure water and absolute ethanol (SER 148, Velp, Usmate Velate MB, Italy) as a solvent in two stages. Then two steps of acid sequence hydrolysis were submitted for free-extractive samples, further cellulose determination/quantification, and high-performance liquid chromatography achieved hemicellulose quality (HPLC). Structural carbohydrates were calculated by HPLC (micro guard column: Amenex Carbo-P, Bio-Rad) and were used to measure the contents of the cellulose (as glucose) and hemicellulose (as arabinose, mannose, galactose and xylose) and the column for carbohydrates: Aminex HPX-87P, heavy metal, 300 ko-7.8 mm, bio-Wheel. Following hydrolysis residue filtration, the insoluble amount of lignin was gravimetrically measured, and the soluble lignin was determined by spectrophotometry for UV (ultraviolet) at 340 nm [23]. The findings have been seen in g/100 g DW.

4.2.4.4. Soluble sugars

In ultrapure water extracts, soluble sugars were measured using the total carbohydrate phenol-sulphuric acid technique (Ribeiro et al., 2020). The sugar content was determined by combining 80 µL of the soluble sugar solution with 2 mL, 98% H_2SO_4 and 320 µL of 5% phenol. The reaction mixture occurs at 100 ºC for 15 min and is measured at the absorption of 490 nm, using a D-(+)-glucose-grading curve and the results expressed as g glucose equivalents/100 g DW. Free sugar profiles were defined at 55 °C, and 35 mM H_2SO_4 as a mobile phase (flow rates: 0.5 ml/min) by HPLC combined to a refractive index detector using an Aminex 87-H column (Bio-Rad, Berkeley, CA, US) (Nielsen, 2010). Sugars have been identified by comparing the retention time of glucose and mannitol peaks and the results expressed in g/100 g DW.

4.2.5. Extraction methodologies to phenolic compounds recovery

4.2.5.1. CONV method

In the case of white bagasse and stems, an aqueous methanolic solution at 80% (25 mL) was added to 2.5 g of grape by-products and homogenized with an ultra-turrax (IKA T18, Wilmington, USA) operated at 12879 x g for 2 min (Oliveira et al., 2015). In the case of red bagasse and stems, the same method was used to obtain the anthocyanins extraction, with a slight modification, the solvent used was 80% of acidified aqueous methanol solution (methanol:water: hydrochloric acid, 12 N: 800 mL: 150 mL: 50 mL)(Oliveira et al., 2015). After the extracts were centrifuged at 4000 x g, 4 ºC for 10 min, the supernatants were filtered through a 0.45-mm cellulose acetate filter (Orange Scientific, Braine-l'Alleud, Belgium) and used for total activities measurement. The remaining solid fractions (red and white) were also characterized for fibres, bound protein and bound and free polyphenols.

4.2.5.2. OH technology

Based on the results of previous proximal characterization, both bagasse were chosen for further extraction procedures since they presented the highest polyphenol content – i.e., the level of free polyphenols in the stems was significantly reduced. Besides, electrical conductivity (0.03 ms/s), whereas bagasse presented a conductivity between 1.0 ms/s and 8 ms/s.

OH was carried out, according to Pereira et al. (2016). Briefly, 2.5 g of grape by-products and 12 mL of water were added to the reactor (composed of a double-walled water-jacketed cylindrical glass tube vessel, 30 cm total length, and a 2.3 cm inner diameter; the electrodes have a gap of 5 cm). A function generator to supply voltage (1 Hz-25 MHz and 1 to 10 V, Agilent 33220A, Penang, Malaysia) connected to an amplifier system (Peavey CS3000, Meridian, MS, USA) controlled the sample heating with electrical frequency at 25 kHz.

After 10 min of reaction, the extracts were centrifuged as mentioned before, and the liquid fraction (LF) and pellets corresponding to the solid fraction (SF) were also kept.

The total antioxidant activity and the quantitative phenolic compounds profile were analyzed on the liquid fractions obtained from the grape bagasse's extractions. The solid fractions were analyzed as previously described, and results were compared against a CONV method of extracting phenolic compounds (Silva et al., 2017).

4.2.6. Bioactive characterization

All samples were characterized concerning the content and profile of main polyphenols and the related antioxidant.

4.2.6.1. Total Antioxidant Capacity and Total Phenolic Content

After CONV extraction and OH extraction of bagasse from red and white grapes, LF and SF were obtained. In the LF the total antioxidant activity (AA) was measured through ABTS method, according to Gião et al. (2007) with slight modifications. Sample (10 µl) was added to a coloured solution of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation) ($ABTS^{\bullet+}$), with an

optical density (OD) measured at 734 nm and adjusted to 700 ± 0.020 in a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). After 6 min of reaction, the final OD was read, and the results were given in mg ascorbic acid equivalent per 100g DW.

As described elsewhere (Coelho et al., 2019), the total polyphenolic content of LF was evaluated through the Folin – Ciocalteu (TPC) spectrophotometric method. A mixture of the sample (5 µL*), Folin – Ciocalteu* reagent (15 µL), sodium carbonate at 75 g/L (60 µl of), Sigma-Aldrich and distilled water (200 µl) were performed, and the solutions were mixed. After samples were heated at 60 ºC for 5 min, the OD was read at 700 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). TPC was expressed as a milligram of gallic acid equivalent per dry weight material (mg GAE/g). The analyses were performed in triplicate, and a standard deviation was calculated.

4.2.6.2. Bounds polyphenols

The red grape bagasse (1 g) and the extractions derived solid fractions were washed with ethanol 3 times to eliminate the free polyphenols. An extraction residue was obtained after 4 h of reaction in 20 mL of NaOH (4 M). The solution obtained before was acidified with HCl (6 M) at pH 1.5 to 2.0 and centrifuged for 30 minutes at 12879 x g. An extraction was then performed with ethyl-acetate, for 15 min, 5 times. The supernatant was concentrated in a vacuum evaporator, resuspended with 10 mL of ethanol, and the polyphenols obtained were analyzed by HPLC-DAD.

4.2.6.3. Phenolic compounds quantification by HPLC

Polyphenols characterization (quantitative and qualitative) was carried out, according to Coelho et al. (2019). Analysis was conducted on HPLC-DAD (Waters Series 600. Mildford MA. USA). A Symmetry® C18 column, 250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size with a guard column (waters), was used and solvents elution consisted of solvent A – Acetonitrile (100%) with 0.2 % TFA; Solvent B: acetonitrile/water (5:95 v/v) (Merck pure grade and pure water) with 0.2% TFA (Sigma-Aldrich, Germany). Samples were analyzed in triplicate. Calibration curves were obtained at a detection wavelength of 280 nm to flavan-3-ols and 320 nm to flavonols. Standards solutions over the concentration ranging from 0.10 to 100.00 mg/L were prepared to identify and quantify the following compounds. In essence, rutin, naringenin, kaempferol gallic acid, protocatechuic acid, catechin, vanillic acid, syringic acid, *p*-coumaric acid and phloretin (Sigma, Sintra, Portugal) expressed as µg per mL of dry weight (DW) biomass of grape. All calibration curves were linear over the concentration ranges tested, with correlations coefficients of 0.999.

4.2.7. Statistical analysis

A SPSS v. 19 (Chicago IL, USA) evaluated the statistical differences using the non-parametric test Mann-Whitney. All experiments were performed in triplicated, and the results were expressed as the mean of triplicated analysis and respective standard deviation. Differences were considered significant at a 5% confidence level (p<0.05).

4.3. Results and Discussion

4.3.1. Fractionation approach

A higher water loss occurred during the drying of stems and bagasse fraction from white cultivar, due to their higher moisture (WGS: 76.42 ± 0.09, WGB: 72.71 ± 0.35) than the stems and bagasse obtained from red grape by-products (RGS: 69.37 ± 0.22, RGB: 68.99 ± 0.47).

Two separate flour fractions were produced after the drying phase and the dry fractionation (milling and sieving) of the stem and bagasse. For raw grape by-products (0.21–0.23 kg of 1 kg of wet grape), the bagasse percentage of both samples was around 69% and 73% DW (WGB and RGB samples) for both samples.

Mendes et al. (2013) developed a strategy for the integrated use of white grape skin pomaces. This strategy comprises a consistent or simultaneous removal of grapes with neutral organic solvent and reflux water. Bio-extract is a good raw source for oleanolic acid recovery. The aqueous extract (about 50% w/w) is primarily hexoses constituted and is adapted to produce high yields (up to 51%) of bioethanol at a peak cell growth rate (max) of 0.29 h^{-1}. The grape skin remaining is the structural polysaccharide complex. Ruiz-Moreno et al. (2015) tested an extract from grape stems from identifying its potential for replacing and/or reducing SO_2 in vinification with antioxidant and antibacterial activity in model wines. The authors showed significant antioxidant activity, suggesting a strong antioxidant extracts from grape stem.

The authors claim to valorize grape pomace by ultrasound phenol extraction (Goula et al., 2016). They examine the drying behaviour and the kinetics of total phenolic degradation in the drying process of that winery by-product. The effectiveness of diffusive properties was assessed by the slopes technique of the curve of dryness (working at 60 – 85 ºC). It examined the influence on phenol recovery yields from solvent type, extract temperature, solvent/solid ratio, amplitude level and pulse duration/pulse interval ratio. They demonstrated that the drying rate was enhanced when the temperature was raised, but drying was primarily done during the decreasing rate phase.

Neither study provided the output of each of the fractions produced by the fractionation of the grape pomace. In addition, the previous techniques have not explored the full potential of the grape pomace fractions, affecting the quality of the value-added products and the "zero waste" objective. In contrast, the fractionation approach proposed in this paper seems to provide a promising, sustainable alternative for the production, without any consumption of water or chemicals, of various added-value products from grape pomace biomass and, first, for higher value usages and secondly the energy utilization according to cyclical principles for bioeconomic efficiency.

4.3.2. Proximate composition

Regarding the grape's minerals content, the stem is higher than the one found in the grape bagasse, representing a difference between samples of approximately 4 g/100 g DW. These

results follow the reported literature (Hanušovský et al., 2020; Ribeiro et al., 2018). Also, the white grape stems presented more minerals than the red grape stems. Relatively to grape bagasse, significant differences (p<0.05) were found between cultivars. The red grape bagasse presents a mineral content higher than the white grape bagasse, 2.50 g / 100g to 1.23 g / 100g, respectively.

Regarding protein content in stems, no differences were found between cultivars. Nevertheless, protein content in the bagasse samples ranged from 8.31 to 8.52 g / 100 g, which is well compared with literature (Bravo and Saura-Calixto 1998; Llobera and Cañellas 2008; Ferreira 2010, Sousa et al. 2014; Valiente et al. 1995; Karnopp et al. 2017; Tahergorabi and Hosseini 2017). The literature also refers to these higher amounts of protein in peels than in the seeds content, which depends on the cultivar (Coelho et al., 2020; Hanušovský et al., 2020).

Carbohydrates and dietary fibres, as predicted in material rich in plant cells, have greater levels in bagasse samples than stems. Also, the red grape bagasse presented more carbohydrates than the white bagasse (p<0.05).

Since 1995, grape by-products have been shown as dietary fibre sources (predominantly cellulose, small proportions of pectin and hemicellulose) (Valiente et al. 1995; Costa et al. 2019; Coelho et al. 2020). Dietary fibre is composed of two fractions: soluble fibres, e.g. fructooligosaccharides, and pectin (soluble in water), which are readily fermented in the colon; and insoluble fibres (non-soluble in water), e.g. cellulose, hemicellulose, and lignin, which it is inert to digestive enzymes providing bulking, and may be poorly or non-fermented in the colon (Karnopp et al. 2017; Coelho et al. 2020).

The results showed that the white cultivar presented higher values of dietary fibre than the red cultivar. Concerning white stems had a significantly highest-total fibre value than red stems (Table 4.1) – i.e., 55.91 to 50.28 g/100 g - containing more cellulose than lignin (p<0.05). Relatively to both bagasse, it was possible to observe that they were mainly constituted by lignin. In general, bagasse contained more insoluble fibre than stems. The fibre content is in agreement with reported in the literature, and cultivars also present different fibre composition (González-Centeno et al., 2010; Hanušovský et al., 2020; Karovičová et al., 2015; Llobera & Cañellas, 2007; Sousa et al., 2014; Valiente et al., 1995). Valiente and colleagues studied the dietary fibre composition present in red grape bagasse, and they reported that total dietary fibre represents 77.89 % of dry matter. Also, they found that 90% of insoluble dietary fibre consists mainly of cellulose. The authors also reported the importance of seedless grape bagasse as a good source of fibre ingredient for industry and their potential beneficial effects on the regulation of bowel functions and water retention (Valiente et al., 1995).

Karovičová et al. (2015) found an amount of grape fibre ranging from 56.8 g / 100 g to 83.6 g/100 g. Both grape cultivar, climacteric, and processing conditions influence the dietary fibre amount (Karovičová et al., 2015). The results obtained for grape bagasse showed that it could be a good substitute due to its soluble dietary fibre content and low caloric content, with a better insoluble/soluble ratio and better functional properties than cereal, which represents the leading fibre supplier.

Besides, bagasse use contributes to a high economic value and supports the circular economy (Mendes, Prozil, et al., 2013)

Table 4.1. Chemical composition of raw grape pomace (C-GP) and grape fractions (LF-GP and SF-GP) obtained after OH and CONV extractions (g/100 g DW).

Chemical Components		R-GB				LF-GP				SF-GP			
		WGS	RGS	WGP	RGP	W_OH	W_CONV	R_OH	R_CONV	W_OH	W_CONV	R_OH	R_CONV
Proximate composition (g /100g)	Moisture	0.98± 0.12	0.97±0.19	0.97±0.10	0.92±0.09	2.97±0.32	3.03±0.21	2.70±0.17	2.96±0.15	0.58±0.04	0.72± 0.08	0.63±0.05	0.76±0.08
	Ash	6.40 ± 0.02	6.30 ± 0.01	1.23 ± 0.01	2.50 ± 0.01*	13.50 ± 0.09	12.71 ± 0.12	6.21 ±0.08	10 ± 0.07	1.29 ± 0.09	1.20 ± 0.01	1.93 ± 0.01	2.44 ± 0.01
	Protein	7.31 ± 0.01	7.30 ± 0.02	8.34 ± 0.02	8.51 ± 0.02	5.02 ± 0.32	2.18 ± 0.12	5.05 ± 0.15	4.26 ±0.05	8.51 ± 0.02	11.3 ± 0.5	8.34 ± 0.02	9.95 ± 0.25
	Fat	2.15 ± 0.12	2.96 ±0.15	14.14 ±0.17	12.58 ± 0.23	1.25 ±0.14	2.49 ± 0.08	3.08 ±0.12	4.87± 0.03	15.02 ± 0.45	12.18±0.2	11.86 ± 0.31	9.46 ± 0.21
	Crude Fibre	76.91 ± 0.80**	72.28 ± 0.73	57.82 ± 0.76*	55.98 ± 0.96	21.02 ±0.54	16.98 ± 0.25	20.98 ± 0.23	15 ± 0.76	68.98 ±0.69	70.56 ±0.45	70 ±0.44	71.02 ±0.53
	Carbohydrates	6.33 ± 0.04	7.80 ± 0.13**	11.03 ± 0.29	14.28 ± 0.05*	56.26 ±0.35	62.58 ± 0.34	62.03 ± 0.12	62.55 ±0.23	5.76 ±0.09	4.06 ± 0.12	6.98 ±0.13	8 ± 0.15
Structural Carbohydrates	Cellulose (as glucose)	16.33 ± 0.04	17.33 ± 0.69**	5.42 ± 0.68	6.77 ± 0.04*	0.50244	ND	ND	ND	6.03 ± 0.96	5.41±0.68	8.12 ±0.68	7.69 ± 0.40
	Hemicellulose	6.70 ± 0.44	6.85 ± 0.28	6.74 ± 0.16	8.38 ± 0.09*	ND	ND	ND	ND	7.83 ± 0.65*	6.73±0.16	11.23 ± 0.51*	9.52 ± 0.11
	Lignin	30.54± 0.10	30.24±0.09	40.46 ± 0.09	40.84 ± 0.40	ND	ND	ND	ND	21.53 ± 1.81*	18.44 ± 0.72	21.97 ±1.8	21.37 ± 4.00
	Insoluble	21.24 ± 0.39	21.07 ± 0.04	22.31 ±0.08	22.41 ±0.22	ND	ND	ND	ND	20.45 ± 0.10	16.91 ±0.09	17.53 ± 0.07	17.59 ± 0.07
	Soluble	9.33 ± 0.15	9.17±0.10	18.15 ± 0.12	18.42 ± 0.16	ND	ND	ND	ND	1.57 ± 0.08	1.52 ± 0.04	4.44 ± 0.17	3.78 ± 0.15
Pectins	TSP	13.72 ± 0.50	14.21 ± 0.60	7.75 ± 0.15	8.77 ± 0.19	4.09 ± 0.21	2.12 ±0.23	5.49 ± 0.12	6.08± 0.41	3.75± 0.22	5.75 ±0.49	3.77 ± 0.36	2.77 ± 0.24
	WSP	7.84 ± 0.75	6.53±0.73	3.04±0.18	3.12±0.06	1.98 ± 0.41	1.24 ± 0.17	2.12 ±0.31	1.41± 0.12	4.80 ± 0.32*	3.00±0.18	5.03 ± 0.14*	3.51 ± 0.18
	CSP	4.42±0.19	6.47±0.61	1.51±0.06	2.88±0.08	ND	ND	ND	ND	3.20 ± 0.69*	1.50±0.06	4.76 ± 0.12*	3.25 ± 0.24
	HSP	1.46±0.06	1.02± 0.06	3.20±0.15	2.80±0.28	ND	ND	ND	ND	4.20 ± 0.23*	3.18±0.15	4.53 ± 0.56	3.14 ± 0.81
Soluble sugars	total soluble sugars	6.74 ± 0.3	1.04 ± 0.98	10.57±1.76	17.24±1.25	12.36 ± 0.85	11.01 ± 1.3	21.91 ± 0.56	23.5 ± 0.31	1.79±0.03	0.44±0.01	4.67±0.08	6.26±0.06

R-GB – raw grape by-products; LF-GP – liquid fraction from grape pomace; SF-GP – solid fraction from grape pomace; WGS- grape stems from white grape by-products; RGS – grape stems from red cultivars; WGP – grape pomace from white cultivars; RGP – grape pomace from red cultivars; W-OH white pomace with OH application; W-CONV- white pomace with CONV technoque application; R-OH- Red pomace with OH application: R-CONV- red pomace with CONV application. NDF – Neutral detergent fibre; ADF – Acid detergent fibre. TSP – Total soluble pectins; WSP – Water-soluble pectins; CSP – Chelator soluble pectins; HSP – Hydroxide soluble pectins. ND – Not detected. [*1] g Glucose equivalent /100 g sample dry weight. Data were expressed as mean ± SD (n = 3). The different superscripts in the same row represent significant differences between samples ($p < 0.05$)

Relatively to the pectin present in grape by-products, higher values of Water Soluble Pectin (WSP) were found in stems from both cultivars compared with bagasse. Also, significant differences (p<0.05) were found in the red grape stems with higher CSP values than white grape stems. Relatively to grape bagasse, no differences (p>0.05) were found between cultivars; nevertheless, the tendency is the same as in the stems. The difference of pectin fractions is related to their structure, which influences functionality and application. The lowest CSP and HSP in white grape bagasse and stems indicates the small capacity to recover high-esterified pectin.

In contrast, a high-esterified pectin structure for red grape stems indicates a high potential for food applications. This pectin could be used on confection jellies, make a friendly gel system with a clean taste, and confer a great flavour. It may also be used to strengthen acidic protein beverages, e.g., drinking food, improving the mouthfeel and the flesh stability in juice beverages, and as a fat substitute in baked goods (Deng et al., 2011c; Güzel & Akpınar, 2019; Mendes et al., 2013; Van Buggenhout et al., 2009). Following the previous studies, winemaking procedures did not significantly impact the pectin distributions, except for the WSP that might be reduced due to the compression step (Deng et al., 2011b; Sauvignon et al., 1990).

4.3.3. Bioactivity Characterization

4.3.3.1. Antioxidant capacity and total polyphenolic content after CONV extraction

Significant differences (p<0.05) in antioxidant capacity were found between the different by-products. Red grape stems showed the highest antioxidant capacity values than white grape stems; the same was observed relative to red and white grape bagasse (Table 4.2).

Polyphenolic compounds have been related to beneficial health effects, namely antioxidant, anti-inflammatory, anti-tumoral, anti-obesogenic, and prevention of therapeutic neurodegenerative and cardiovascular diseases (Cory et al., 2018; Koch, 2019). These properties depend upon the number of polyphenols available in the lower parts of the digestive tract (bioaccessibility) or on the quantity effectively absorbed.

Table 4.2. Total phenolic compounds and antioxidant activity of bagasse and stems from red and white grapes by-products in LF (mg /100 g DW).

Samples	Bagasse		Stems	
	White grape	Red grape	White grape	Red grape
Total phenolic compound	4.03±0.01	6.62±0.05	3.02±0.04	9.75±0.03
Total antioxidant activity	5.18±0.66	22.65±0.84	8.30±0.69	34.56±0.94

*p < 0.05 between white and red cultivars.

Results showed a significant difference between the grape bagasse's total polyphenolics content from red and white grapes (Table 4.2).

The red grape by-products presented higher values than the white ones, with significant differences (p < 0.05). The results are justified by the grape composition and the vinification process described in the literature (Comuzzo et al., 2020; Deng et al., 2011b; Thimothe et al., 2007). In red wine

production, bagasse is produced after free juice is poured, leaving behind dark blackish-red debris consisting of grape skins and stems. The colour of red wine is derived from skin contact during the maceration period, which sometimes includes partial fermentation. The resulting bagasse is more alcoholic and tannic than bagasse produced from white wine production. In white wine production, the grapes are crushed and quickly pressed to avoid skin contact with the juice, resulting in a pale greenish-brown pressed by-product.

Furthermore, it is also possible to observe in Figure 4.1 a higher total phenolic content for red grape stem than in red bagasse, which is in line with previous studies about total phenolic content of grape by-products (Llobera and Cañellas 2007; Alonso et al., 2002; Spigno and De Faveri 2007; Doshi et al. 2015; Anastasiadi et al. 2012). The cultivar and the weather conditions determine the total phenolic compounds obtained for stems since they were isolated from grapes before the winemaking and did not endure any procedure (Coelho et al. 2020; Llobera and Cañellas 2008).

The results observed shown a directed correlation obtained between polyphenols content and antioxidant capacity (r 0.93). Other authors also reported these correlations (Abreu et al., 2019; Arina & Harisun, 2019).

Therefore, grape bagasse may be used in the food industry as a food preservative, changing or preventing the decay of nutrients by aerobic mechanisms and as an antimicrobial agent restricting the development of spoilage and pathogenic microorganisms.

4.3.4. Impact of OH *vs.* CONV method on phytochemical composition from grape bagasse

In this study, the use of OH was evaluated and compared against chemical extraction to evaluate if it can be an efficient alternative for the reuse/use of red grape bagasse with quality and safety without damaging inherent BC (Table 4.3).

4.3.4.1. Protein content

Regarding protein content on the SF after extractions, the values showed a protein content range from 8.6 to 14.65 g / 100 g, with the application of CONV methods, which are in agreement with the literature (Bravo & Saura-Calixto, 1998; Ferreira et al., 2001; Karnopp et al., 2017; Llobera & Cañellas, 2008; Sousa et al., 2014; Tahergorabi & Hosseini, 2017; Ueno et al., 2004; Valiente et al., 1995). In SF of white grape bagasse, a significant increase ($p < 0.05$) of protein content was observed to OH treatment application compared with the CONV method, 13.18 to 15.02 g / 100 g, respectively. A similar result was obtained for red grape bagasse when OH was compared to the CONV method, 13.86 and 12.46 g /100 g, respectively. Solvents and extraction techniques used during the extraction process could explain the protein content differences obtained from extraction methods (Burin et al., 2014; Coelho et al., 2020; Ferreira et al., 2001; Karnopp et al., 2017).

Furthermore, both methodologies used in this study have different approaches. The CONV extraction method used 80% of MeOH solution, while water was used in OH extraction. It is known that MeOH is a non-food grade solvent, and alternative methods, based on solvents-free and new technologies, such as pulsed electric field, high pressure, of extraction, have been studied. The OH extraction method causes cell wall electroporation but does not cause protein denaturation. Furthermore, at

concentrations higher than 40% of this solvent, denaturation of the proteins can occur (Kayser et al., 2020). Besides that, MeOH molecules adjacent to the protein side-chain can originate van der Waals interactions, which reduce intra-protein nonpolar interactions and lead to the full extension of protein tertiary structure (Shao, 2014). Also, the water molecules can establish hydrogen links with proteins and impact the strength of intra-protein hydrogen bonds, which increases the protein stability and decrease the extracted capacity in SF comparing with the CONV method (Pereira, Teixeira, and Vicente 2011; Pereira et al. 2010; Pereira and Vicente 2010; Rodrigues et al. 2015).

4.3.4.2. Dietary Fibre

SF found no significant dietary fibre content differences between methods applied to grape by-products and cultivars studied. To our best knowledge, there are no studies about the OH extraction method's effects on dietary fibre regarding grape by-products. This study shows a significant increase in the insoluble dietary fibre in SF after OH treatment than in the SF obtained from the CONV method (p<0.05). The increase of temperature during OH (100 ºC) intensifies the Maillard reaction products, quantified as insoluble dietary fibre (Huda et al. 2006).

The soluble fibre in SF is higher in OH application than CONV; oppositely, there was less soluble fibre in the LF treated by OH than in the CONV method. The CONV method may increase the extraction of arabinoxylans (soluble in water) from the cell wall included in the soluble fibre quantification (Ramírez-Jiménez et al., 2019a; Yadav et al., 2009, 2016). The CONV method has water with an organic solvent, allowing break wall cell the arabinoxylans release from pericarp under alkaline conditions.

Only, few researchers refer to the thermal effects on dietary fibre, and these also depend on the type fibre source and the processing method (Bader Ul Ain et al., 2019; Chang & Morris, 1990; Elleuch et al., 2011).

4.3.5. Bioactivity characterization

4.3.5.1. Total phenolic content and antioxidant activity of free and bound phenolics

As previously mentioned, the application extraction methods resulted in an LF rich in phenolic compounds. SF of red grape bagasse's presented a higher content in total phenolic compounds (Figure 4.1) with antioxidant capacity (Figure 4.2) than SF from white grape bagasse. Nevertheless, the remaining SF also contain polyphenols linked to fibres.

The total phenolic compounds in LF from white and red grape bagasse observed in Figure 4.1 were significantly higher (p < 0.05) with OH than the CONV method. OH allows increased polyphenols extraction due to its improved diffusion kinetics, resulting from electro-heating effects and membrane's alteration. Furthermore, this method is fast, and depending on the voltage applied, it may be used to extract heat-sensitive and unstable compounds, *e.g.,* anthocyanins (Coelho et al. 2019; Maroun et al. 2017; El Darra et al. 2013; Jesus et al. 2020). El Darra and his colleagues applied the OH to grape bagasse and obtained a higher extraction yield of 36 % than the CONV methods, with a hydroalcoholic solution used to extract polyphenols.

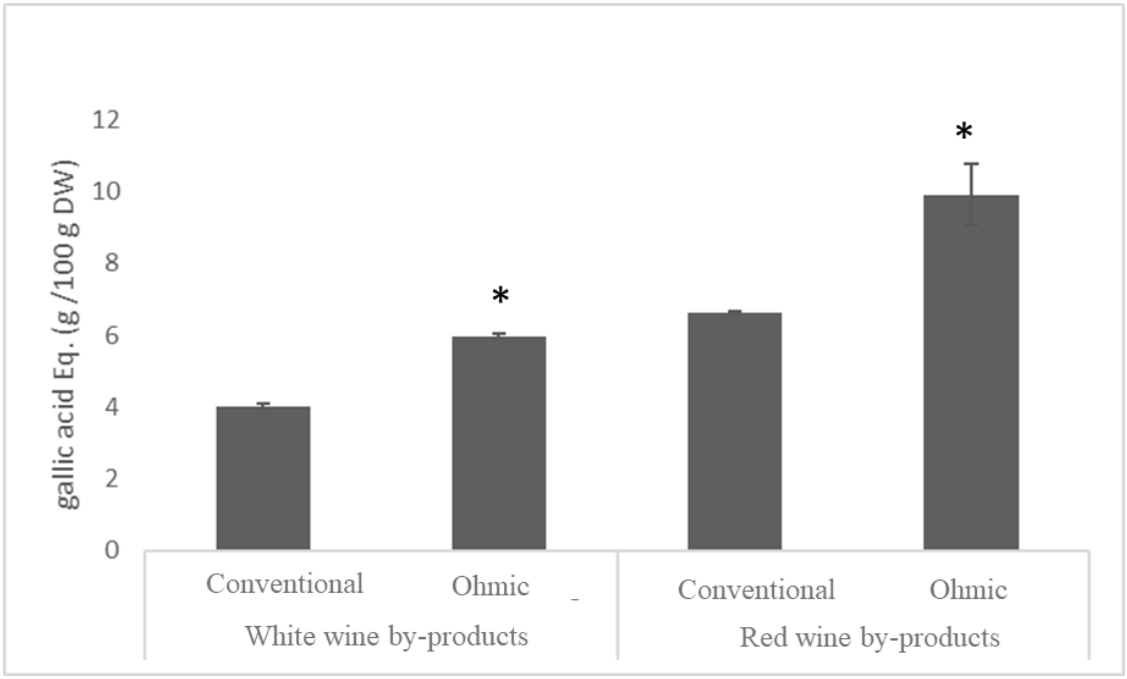


Figure 4.1. Total phenolic compounds content of LF obtained from red and white grape bagasse by CONV and OH treatments. *p < 0.05 compared with the CONV method

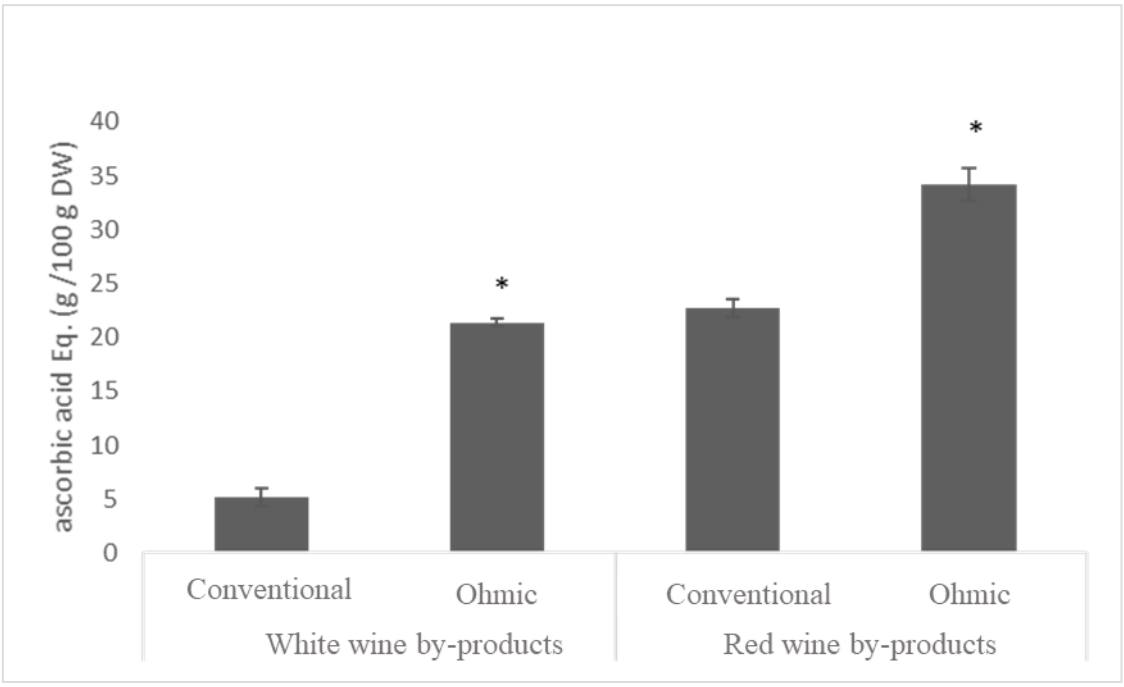


Figure 4.2. Total antioxidant activity of white and red by-products of LF submitted to CONV and Oh treatments. *p < 0.05 compared with the CONV method. *p < 0.05 compared with the CONV method.

4.3.5.2. Identification of phenolic compounds

It is relevant to evaluate OH's impact on the extraction of heat-sensitivity polyphenols compared to the CONV extraction method. The individual compounds found for red grape are shown in table 4.5. HPLC identified and quantified the main phenolic compounds identified and quantified by HPLC in

LF of both cultivars were: - (-) epicatechin, gallic acid, p-coumaric acid, syringic acid, ferulic acid, and caffeic acid. Significant differences were found between the methods applied ($p < 0.05$). In both methods, the majority of free compounds present were phenolic acids. Regarding bound phenolic in SF, gallic acid, catechin, and vanillic acid were the most predominant ones (32.7-54.2 μg/g DW).

In white grape bagasse, more individual compounds were identified to the CONV method than to the OH. Mainly epicatechin, syringic, ferulic, p-coumaric, caffeic and gallic acid; while for OH identified epicatechin, syringic and gallic acid. Regarding red LF, the content of the individual compound from OH is similar to CONV samples. Also, higher values were found for bound polyphenols in SF obtained by OH when compared with the CONV method, indicating that the OH preserves these compounds in the final product (Ramírez-Jiménez et al., 2019a). The red grape bagasse is richer in individual polyphenols than white samples.

The results showed grape bagasse extracted with OH rich in dietary fibre bound to polyphenols compounds, allowing their utilization as an effective enhancer in the food industry, improving drinks, or even as an element dried out organic product increased in phenolic content. Besides, the OH allowed obtaining a LF rich in polyphenols, including anthocyanins. Regarding the anthocyanins are considered sensitivity compounds, no differences were obtained between methods of extraction. The recovery yields of anthocyanins (e.g. delphinidin, petunidin, and peonidin) were similar in LF from the two extraction methods. The results obtained can also be explained by the difference in the solvents used.

Regarding the OH extraction, acidified water was used, a more polar solvent than methanol promoting higher solubilization of the anthocyanins following the “like dissolves like” principle (Roda-Serrat et al., 2021; Sabino et al., 2021). Thus, OH can be an excellent solvent-free alternative to extract these compounds. Other authors also corroborate using OH as an alternative to organic solvents (Coelho et al. 2020; Loypimai et al. 2015; Salari and Jafari 2020).

Free and bound phenolic compounds differences in OH extracts could be explained by the deliverance of bound phenolic acids just as the breakdown or mellowing of cell constituents of plant cells caused by the thermal application, prompting increased BC accessibility (Khan et al., 2018).

The results also showed that OH could be a selective method of extraction of phenolic compounds. The remaining bagasse is also rich in phenolic compounds bound to fibre, conferring a functional ingredient.

Table 4.3. Polyphenols quantitative profile identified by HPLC-DAD in the red and white grape bagasse (µg / g DW). Free polyphenols were measured in LF and bound phenols in SF.

Compound (µg / g)c	White grape bagasse		Red grape bagasse	
free phenolic compound (LF)	*CONV*	*OH*	*CONV*	*OH*
(-) -Epicatechin	145.3 ± 18.10*	38.95 ± 2.01	n.d.	n.d.
Syringic acid	1.14 ± 0.33	0.98 ± 0.03	n.d.	n.d.
Ferulic acid	0.39 ± 0.066*	n.d.		
p-coumaric acid	0.80 ± 0.021*	n.d.	n.d.	n.d.
Caffeic acid	0.79 ± 0.095*	n.d.	n.d.	n.d.
Gallic acid	7.46 ± 2.37	8.63 ± 1.81	10.83 ± 1.85	28.64 ± 0.96*
Esculin	n.d.	n.d	n.d.	1.77 ± 0.06*
Catechin hydrate			0.98 ± 0.07	1.63 ± 0.35*
Vanillic acid			0.23 ± 0.3	1.36 ± 0.05
Delphinidin-3-glucoside	n.d.	n.d	0.020 ± 0.001	0.020 ± 0.0003
Petunidin-glucoside	n.d.	*n.d.*	132.64 ± 1.45	133.80 ± 0.16
Peonidin-3-glucoside	n.d	n.d	0.021 ± 0.001	0.022 ± 0.001
bound phenolic compound (SF)				
Gallic Acid	15.30 ± 0.98	21.57 ± 1.84	26.7 ± 1. 15	54.2 ± 3.24*
Protocatechuic acid	n.d.	n.d.	5.35 ± 0.21*	n.d.
Catechin	n.d.	n.d.	13.4 ± 0.52	32.7 ± 2.50*
Vanillic acid	n.d.	n.d	4.67 ± 0.30	6.94 ± 0.61*
Caffeic acid	n.d.	n.d.	n.d.	0.03 ± 0.001
Syringic acid	n.d.	n.d.	0.88 ± 0.001	1.76 ± 0.0004
p-coumaric acid	n.d.	n.d.	2.62 ± 0.03	3.06 ± 0.01
Rutin	n.d.	n.d.	0.04 ± 0.0001	0.01 ± 0.001
Phloretin	n.d.	n.d.	1.24 ± 0.0002	n.d.

*n.d. non determined. The different superscripts in the same row were significantly different ($p < 0.05$).

4.4. Conclusion

The results show that bagasse presents higher protein, phenolic compounds (such as anthocyanins) and carbohydrates contents than stems, which could be helpful for food applications aiming health impact. The OH technology could allow integral valorization obtaining two valuable ingredients: one liquid fraction resulting from the extraction from the by-product and the correspondent solid fraction resulting from the leftover after extraction. With OH application, it is also possible to obtain higher amounts of phenolic compounds, including anthocyanins, compared with the CONV method. In this way, this method may be applied during the separation process and applied directly in bagasse as a continuous process improving compounds extraction and reusing the remaining solid fraction of a new product. Besides, this study unveils that the resulting solid by-products are a rich source of fibre linked to polyphenols, making them an ingredient with health benefits, and may be used as a potential ingredient.

Grape by-products and their extracted BC have the higher commercial potential as an ingredient or an integral product. Furthermore, the OH could be applied in the winemaking process, allowing valuable extract compounds, and contributing to the circular economy. New studies are necessary to optimize the extraction processes to eliminate organic solvents and to decrease possible contaminants.

Chapter 5.
Total and sustainable valorisation of tomato' by-products using ohmic heating approach

Abstract

Increased industrial demands for new sources of food ingredients at a competitive cost have led to research efforts to valorize agro-industrial by-products. An excellent example is tomato by-products from processing industries, which have a high potential to be reused as a source of bioactive compounds (BC). Reliable national data on tomato by-products and physicochemical characterization could inform and foster effective planning on tomato waste management in Portugal that is absent. Thus, selected Portuguese companies were contacted to obtain representative samples of by-products generation and physicochemical composition. Furthermore, an environmental-friendly method, applying OH, was also compared with conventional methods to explore new safe value-added ingredients. Tomato processing by-products revealed a high valorization potential since both collected samples from companies were rich in protein, between 16.3 to 19.4 g / 100 g DW; fibre content ranging from 57.8 to 59.0 g /100 g DW. In addition, these samples contain 17.0 g /100 g of fatty acids, including polyunsaturated, monounsaturated and saturated fatty acids, such as linoleic acid, oleic and palmitic acid, respectively. After understanding its compositional potential, OH was applied to identify added-value solutions to tomato by-products. With extractions, two types of fractions were obtained. The liquid fraction (LF) is rich in polyphenols, free sugars, and carotenoids and a solid fraction rich in fibre bound to polyphenols and carotenoids. This treatment has been shown to preserve carotenoids, such as lycopene, relatively to conventional methods. Nevertheless, new molecules were identified by LC-ESI-UHR-OqTOF-MS analysis, such as phene-di-hexane ana N-acethyl-D-tryptophan. According to the results, the OH boosts the potential of tomato by-products and can be directly introduced into the process, contributing to the circular economy and zero by-products.

Keywords: Tomato by-products; Ohmic heating; whole valorization, BC, phenolic compounds, carotenoids

5.1. Introduction

An expanding global human population, rapid urban development and economic growth increase waste production at an alarming rate (Ellen MacArthur Foundation, 2015; Esposito et al., 2020). Decoupling economic development from resource demand is essential, given the ever-increasing demands on our planet's limited resources. However, the shift to an eco-friendlier economy is a cause for concern and involves joint action by multiple stakeholders across borders. Stemming from an in-depth assessment of sustainable development goals on responsible expenditure and industry in recent years, a wide variety of stakeholders (political, scientists, financials, industry) has discussed and progressed on this shift to the circular economy to accelerate the implementation of multiple sustainable development goals. With the world population expecting to increase to about 10 billion in 2050, it is vital that companies progressively adapt the strategies for more resource-efficient use. Traditionally the system has been linear, where life, foods and other resources are utilized and then wasted. In a circular system, the idea is that materials will be recycled repeatedly in some weather than ending at the landfill (Campos et al., 2020; Foundation, 2015).

Lycopersicon esculentum L. (tomato) is one of the essential fruits consumed worldwide. According to the Food and Agriculture Organization of the United Nations (FAOSTAT, 2018), in 2016, the global tomato area harvested was about 5 million hectares, producing approximately 173 million tons (fresh and processed) with a total gross production value of 85 billion US dollars. This fruit is consumed in processed products such as tomato juice, paste, puree, ketchup, and salsa. Interestingly, this agro-industry produces significant amounts of solid by-products, namely seeds, peels, pulp and fibrous parts that account for 7.0–7.5% of raw materials (Nour et al., 2018). The by-products generated are mainly destined for animal feed without processing or dumped in landfills, thus representing costs and environmental concern for the tomato processing industry, which must be carefully considered(Silva et al., 2019). Fortunately, food industries and researchers have started to pay special attention to this by-product to recover relevant BC, *e.g.* polyphenols, carotenoids, vitamin E, sterols, for main applications in food, pharmaceutical and or/ cosmetic industries.

To accomplish the objectives implied in the concept of a circular economy and biorefinery, new approaches with green technologies to obtain different fractions with zero-waste must be evaluated.

Traditionally, liquid-liquid extraction has been employed to isolate and recover bioactive compounds (BC), such as polyphenols and carotenoids from tomato by-products. This method uses a large amount of hazardous organic solvents that adversely affect both health and the environment. Furthermore, it may jeopardize its reuse, such as incorporating into foods and, therefore, maintaining the food value chain (Akanbi et al., 2006).

Ohmic heating (OH) has become a promising technology for food products or by-products with some advantages, including preserving products' nutritional, functional and structural properties (Coelho et al., 2020). Furthermore, it is also fast, homogeneous with efficient energy transfer technology providing an environmentally clean methodology, reducing processing costs, and improving the products added value. (Hosainpour et al., 2014) used this technology to analyze the effects of voltage

gradients on tomato paste, obtaining electrical conductivity data and verifying the possibility of OH application in tomato industries. These authors observed significant differences in heating time and pH changes of samples caused by voltage gradient application, opening the possibility of using this technology at industrial levels to obtain processed tomatoes with preserved properties. Reduced process times in OH maintains the nutritional and sensory properties of nutrients. OH inactivates positively antinutritional elements like lipoxygenase (LOX), polyphenol oxidase (PPO), and pectinase, removing their dynamic metal radicals by the electric field application (Castro et al., 2006; Niakousari et al., 2019). Furthermore, this technique has been applied for several purposes, such as pasteurisation and processing juices, but few studies have reported the application of OH on food by-products. Additionally, the effects of OH on BC and, in particular, their recovery from by-products are still scarce.

Thus, the purpose of this work was to assess the phytochemicals profile of tomato by-products (polyphenols and carotenoids) and test for the first time the application of OH as an alternative to traditional liquid-liquid extraction to valorize this byproduct through the extraction of BC.

5.2. Materials and methods

5.2.1. Materials

The 2, 20-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS diammonium salt), potassium sorbate, sodium carbonate, and ethylenediaminetetraacetic acid (EDTA were purchased from Sigma-Aldrich (Sintra, Portugal). Hexane, ethanol, Folin–Ciocalteu's reagent, and potassium persulfate were purchased from Merck (Algés, Portugal). Standards of ascorbic acid, Trolox, gallic acid, rutin, *p*-coumaric, and 4-hydroxybenzoic acid, were purchased from Sigma-Aldrich (Sintra, Portugal), while kaempferol, β-carotene, lycopene, zeaxanthin, and lutein (Extrasynthese, France) were purchased from Extrasynthese (Lyon, France).

5.2.2. Samples preparation

Tomato processing by-products were obtained from two industrial processing companies in Portugal, Italagro from Vila Franca de Xira and Sugal Group from Benavente. Both organizations used tomatoes to produce different processed tomato products. The tomato from the first company was obtained fresh (C1), right after the stage in which the skins and seeds of the tomato were removed while processing the pulp; for the other sample, it was delivered to us after being dried in the sun (C2), to remove excess of humidity.

5.2.2.1. Samples processing and fractions production

The by-products formed during the tomato processing were collected, stored and transported at 4 ºC in a portable refrigerator. Once at the laboratory, the samples were maintained fresh for each company to perform phytochemical composition, dry matter, ashes, proteins, fibre, sugars, and fatty acids. After, the samples from both companies were mixed, forming a blend (Figure 5.1). The blended

sample was fractionated in four types of processing: i) fresh sample; ii) dried at 55 ºC in a convection oven for 24 h; iii) frozen at -20 ºC for 24 h, and iv) frozen at -80 ºC in an ultra-low temperature freezer for 24 h. Afterwards, for the different processed conditions, the samples were all dried (50.0 ºC ± 3.0, during 24 h), milled with a food processor (Bimby, Vorwerk, TM5) and sieved with a mechanical sifter using sieves of pore sizes 150 µm (particle size 150 µm) in the same conditions (Mphahlele et al., 2016; Oliveira et al., 2016).

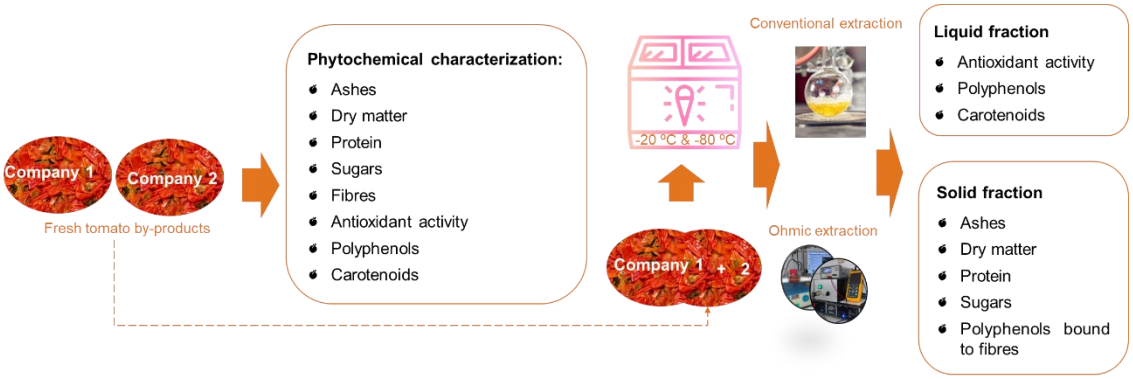


Figure 5.1. The schematic methodology applied to tomato bagasse.

Finally, the samples were kept at -20 ºC and were submitted to conventional and ohmic heating extraction methods, applying ethanol as a food-grade solvent.

5.2.3. Extraction methodologies employed

5.2.3.1. Polyphenols conventional extraction

Polyphenol extraction was performed according to Oliveira et al. (2016), with slight modifications. Tomato bagasse (2.5 g) was homogenized using an ultra-turrax (IKA, T18, Wilmington, USA) for 2 min with 25 mL of methanol (80%, v/v) at room temperature., followed by 30 min (300 rpm) stirred and centrifuged at 8000 x g, at 4 ºC, during 5 min. The resulting supernatant (liquid fraction) was analysed in terms of antioxidant activity, total phenolics content and phenolics quantitative profile. The solid fraction was also analysed for bound polyphenolics, those bound to the fibre. Both extracts, liquid and solid fractions, were stored at -80 ºC.

5.2.3.2. Carotenoid conventional extraction

A conventional carotenoids extraction method was conducted to compare and characterize the carotenoids present in the by-products. Briefly, 2.5 g of tomato by-products were suspended in 5 mL of cold ethanol and homogenized at 14000 x g for 3 min applying an ultra-turrax. Hexane (4 mL) was added to the homogenate, and the resulting mixture was homogenized for an additional 2 min and then centrifuged for 10 min at 8000 x *g*. The hexane layer containing the carotenoids was transferred to a polypropylene tube. A solution of saturated sodium chloride (2.5 mL) and an additional 4 mL of hexane were added to the slurry, and the resulting mixture was homogenized for 1 min. The mixture was centrifuged as described in the previous step, and the second hexane layer was recovered and mixed with the first hexane layer (Oliveira et al., 2015). The extracts (liquid fraction) and retentates

(solid fraction) were stored at -80 ºC. The liquid extract was analyzed for total carotenoids content and a carotenoid profile by spectrophotometric and HPLC UV/VIS DAD. All extracts and the analyses were performed in triplicate.

5.2.3.3. Ohmic heating extraction

An OH system was used to extract BC from tomato by-products to compare with a conventional method. Briefly, the OH method consists of a cylindrical glass reactor (30 cm total length with 207 cm diameter) with two stainless steel electrodes placed at each edge isolated by polytetrafluoroethylene (PTFE) caps. The bioactive extraction was performed based on previous studies (data not showed). A hydroethanolic solution (ethanol 30%, v/v) was used for 15 min at 55 ºC, 25 kHz of frequency and a supplied voltage of 60v. The extracts were centrifuged, and both solid and liquid fractions were stored at -80 ºC. The liquid extracts were analyzed in terms of total polyphenols content, total carotenoids and total antioxidant capacity. Also, the carotenoids and polyphenols profiles were evaluated by HPLC. On the other hand, the solid by-products were analyzed in terms of fibre and bound polyphenols profile. All analyses were performed in triplicate.

5.2.4. Compositional analysis

5.2.4.1. Proximate composition

The moisture content of fresh tomato bagasse (including seeds, peels, rest of pulp and fibrous parts) was determined according to the oven method (Chemists, 2000). The calculation was as follows:

$$Moisture\ (dry\ matter)(\%) = \frac{W1 - W2}{W1} X\ 100\%$$

Where W1 is the weight (g) of the sample before drying and W2 is the weight (g) after drying.

The ashes (inorganic deposit remaining after the water and organic matter were discarded by warming with oxidizing agents, which gives a proportion of the aggregate sum of minerals inside nourishment) were determined according to the AOAC official method 942.05. Briefly, one gram of tomato bagasse was weighed (w_1) into a previously weighed crucible (W_0) and placed in the muffle furnace at 600 ° C for 2 h. After cooling, it was placed in a desiccator, and the crucible was weighed at room temperature (w_2). The ashes were determined according to the equation:

$$Ash\ (dry\ basis)(\%) = \frac{(w_2 - w_0)}{(w_1 - w_0)} \times 100$$

Total nitrogen was analysed by the Kjeldahl method, and protein content was then calculated using a conversion factor of 6.25.

Fat extraction from tomato by-products was performed by hydrolysis with ether. The fat content was determined gravimetrically by the Soxhlet method using petroleum ether (boiling point 60-80 ºC), according to the method described in Association of Official Analysis Chemists International (AOAC),

2000). Subsequently, it was methylated to obtain fatty acid methyl esters. The characterization and quantification of individual fatty acids were analysed by gas chromatography (GC-FID). All analyses were performed in triplicate.

5.2.4.2. Fibre

The crude fibre content was determined with an acid/alkaline hydrolysis of insoluble by-products as described in (Association of Official Analysis Chemists International (AOAC), 2011). All measurements were done in triplicate. The chemical compounds were expressed as g/ 100 g dry weight (DW), and all analyses were performed in triplicate.

5.2.4.3. Cellulose, hemicellulose and lignin

Both carbohydrates and lignin were accurately measured in all flours (tomato bagasse, solid residues obtained after ohmic and conventional extractions) according to Sluiter et al. (2012). The method employed two-step sequential acid hydrolysis, 72% sulfuric compound at the beginning followed by 4% (v/v) sulfuric compound at the second measure to hydrolyze both cellulose and hemicellulose to sugars quantified via HPLC. The carbohydrates (sugars) issued were assessed to determine the hemicellulose sugar content. The results were expressed in g /100 g DW, and all analyses were performed in triplicate.

5.2.4.4. Total Pectin

The total pectin content of fresh samples was calculated based on the method described by Deng et al., (2011), comprising the three fractionated pectin water-soluble pectin (WSP), chelator soluble pectin (CSP) and hydroxide soluble pectin (HSP). All analyses were performed in triplicate. The results were expressed in galacturonic acid equivalents (GUAE) / 100 g

5.2.5. Bioactivity Characterization

5.2.5.1. Total Antioxidant Capacity

The antioxidant activity was performed using ABTS and ORAC methods. The ABTS was performed according to Gião et al. (2007) with slight modifications. The sample was added to a coloured solution of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation) ($ABTS^{\bullet+}$), with an optical density (OD) measured at 734 nm and adjusted to 700 ± 0.020 in a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). After 6 min of reaction, the final OD was read and the results were given in ascorbic acid equivalent.

The ORAC measurement of the different extracts of tomato by-products was assessed according to Dávalos et al. (2004). The extracts were dissolved with phosphate buffer (pH 7.4), and the Trolox standard curve (0-90 mg/ L) was performed. At the analysis time, 70 nM fluorescein and 14 mM AAPH results were made at ORAC buffer. The 96 wells coloured microplate was prepared to contain25 µL of blank control (ORAC buffer); standardized, control, or sample and 200 µL of fluorescein solution were added. Then, 50 µL of newly prepared AAPH solution was added. The microplate was incubated for 10 minutes at 37 ºC. The fluorescence readings were carried every 2 min within 104 cycles using the FLUO star OPTIMA plate reader (BMG Labtech, Offenburg,

Germany). The wavelength excitation was 485 nm, and the emission was 530 nm. Results were expressed in µmol Trolox equivalent / g DW, and each sample's measurements were performed in triplicate.

5.2.5.2. Total Polyphenols Content and bound phenolic compounds

The Total Polyphenols Content (TPC) were evaluated through Folin–Ciocalteu spectrophotometric method. A mixture of a sample (5 µL), Folin–Ciocalteu reagent (15 µL), sodium carbonate at 75 g/L (60 µl), and distilled water (200 µl) was performed. Next, samples were heated at 60 ºC for 5 min, and the OD was read at 700 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). TPC was expressed as a milligram of gallic acid equivalent per dry weight material (mg GAE/g). The analyses were performed in triplicate, and a standard deviation was calculated.

The bound phenolic analyses were performed on the tomato bagasse samples and the solid residues obtained after conventional and ohmic extractions. The tomato bagasse was washed three times with ethanol absolute to eliminate the free polyphenols. Concerning the other samples obtained after extraction, they were already washed during the extraction process. Then, for all the samples, a reaction of 4 h was carried out with 20 mL of NaOH (4 M) and 1 g of washed residue. The solution obtained was acidified with HCl (6 M) at pH 1.5 to 2.0 and then centrifuged for 30 min at 12000 x g. The extraction was then performed with ethyl-acetate, for 15 min, five times (Xie et al., 2015). The supernatant was concentrated in a vacuum evaporator, resuspended with 10 mL of ethanol absolute. The extracts obtained of the free total content of polyphenols and the bound polyphenols were analyzed by HPLC.

5.2.5.3. Determination of total carotenoids, lycopene, *β*-carotene and chlorophylls content

Total Carotenoids (TC) content of tomato bagasse were assayed using a spectrophotometric analysis, as described by (Kimura et al., 1990). The results were expressed in equivalent *β*-carotene, compared to a range of *β*-carotene standards prepared, starting from a stock ethanolic solution.

Essentially, 16 mL of acetone: hexane (4:6) solution was added to 1 g of fresh tomato bagasse of the two companies (C1 and C2) and shaken for 15 min. Two different phases were formed, and the hexane layer was measured at 453, 505, 645, 663 nm wavelength using a UV-vis spectrophotometer. The following equations were used to determine lycopene, β-carotene and chlorophylls a, b: Relatively to the lycopene, β-carotene, and chlorophylls, a, b determination was performed according to NAGATA and YAMASHITA (1992).

Chlorophyll *a* (mg / 100 mL) = $0.999 \times A_{663} - 0.0989 \times A_{645}$

Chlorophyll *b* (mg / 100 mL) = $-0.328 \times A_{663} + 1.77 \times A_{645}$

Lycopene (mg / 100 mL) = $-0.0458 \times A_{663} + 0.204 \times A_{645} + 0.372 \times A_{505} - 0.0806 \times A_{453}$

β-carotene (mg / 100 mL) = $0.216 \times A_{663} - 1.22 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$

Where A_{663}, A_{645}, A_{505}, and A_{453} represent the absorbance at 663, 645, 505, and 453 nm each other. The results were expressed in mg / Kg DW and the determinations were performed in triplicate.

5.2.5.4. High-Performance Liquid Chromatography - diode array detector (HPLC-DAD) analysis

Polyphenols profiles (quantitative and qualitative) of liquid fractions obtained from conventional and ohmic extraction were studied. Analysis was conducted on HPLC-DAD (Waters Series 600. Milford MA. USA). A Symmetry® C18 column, 250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size with a guard column (waters), was used and solvents elution consisted of solvent A – Acetonitrile (100%) with 0.2 % TFA; Solvent B: acetonitrile/water (5:95 v/v) (Merck pure grade and pure water) with 0.2% TFA (Sigma-Aldrich, Germany); Samples were analyzed in triplicate. Calibration curves were obtained at a detection wavelength of 280 nm to flavan-3-ols and 320 nm to flavonols. Standards solutions over the concentration range from 0.10 to 100.00 mg/ L were prepared for the identification and quantification of the following compounds: rutin, naringenin, kaempferol, gallic acid, protocatechuic acid, catechin, vanillic acid, syringic acid, *p*-coumaric acid and phloretin (Sigma, Sintra, Portugal) expressed as µg per mL of dry weight (DW) tomato biomass. All calibration curves were linear over the concentration ranges tested, with correlations coefficients of 0.999.

HPLC-DAD also analyzed carotenoid content (Vydac 201TP54 C-18 column, 250 mm - 4.6 mm), equipped with a C-18 pre-column. Chromatographic separation was performed as described by (Oliveira et al., 2004). Solvent A with ethyl acetate (Merck pure grade) and solvent B 90:10 acetonitrile: water (Merck pure grade and pure water, 1.0 ml/min flow rate, at room temperature. The UV–vis detector was set between 270 and 550 nm. Individual carotenoids were quantified based on a calibration curve built with pure standards: *β*-Carotene, lycopene, and lutein (Extrasynthese, Genay Cedex, France) and expressed as milligrams per kilogram of DW.

5.2.5.5. LC-ESI-UHR-OqTOF-MS analysis

An ESI source in negative mode was used to obtain the polyphenols profile (non-identified by HPLC) of fresh extracts according to Monforte et al.(Monforte et al., 2018). Furthermore, UltiMate 300 Dionex UHPLC (Thermo Scientific), coupled to an Ultra-High Resolution Qq-Time-Of-Flight (UHR-QqTOF) mass spectrometer with 50, 000 Full-Sensitivity Resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany). A volume of 5 µl was injected and an Acclaim RSLC 120 C18 column (100mm x 2.1 mm, 2.2µm) (Dionex) was used to identify BC with an increased gradient elution composed of two solutions: solvent A water and 0.1% of formic acid and solvent B acetonitrile and 0.1% formic acid at a flow elution of 0.4 ml per minute. The gradient started at 5% B and increased to 95% in 7 min, which was maintained constant for 2 min and returned to 5% B in 1 min and maintained at 5% B for an additional 5 min at a flow rate of 0.25 mL/min. Parameters for MS analysis were set using negative ionization mode with spectra acquired over a range from m/z 20 to 1000. The parameters were as follow: capillary voltage (4.5kV), drying gas temperature (200 °C), drying gas flow (8.0 L/min), nebulizing gas pressure (2 bar), collision RF (300 Vpp), the transfer time (120 µs) and prepulse storage (4 µs). Post-acquisition internal mass calibration used sodium formate clusters with the sodium methanoate delivered by a syringe pump at the start of each chromatographic analysis. High-resolution mass spectrometry was used to identify the phenolic compounds present in the fractions. The elemental composition for the compounds was confirmed

according to accurate masse and isotope rate calculations designated as mSigma (Bruker Daltonics). The accurate mass measurement was within 5 mDa of the assigned elemental composition, and mSigma values of < 20 were confirmed.

5.2.6. Statistical analysis

All experiments were performed in triplicated, and the results were expressed as the mean of triplicated analysis ± standard deviation. SPSS v. 19 (Chicago IL, USA), was used to evaluate the statistical differences using a non-parametric Mann-Whitney test. Furthermore, Statistica package software v. 10 (StatSoft Inc.) was also applied for principal component analysis (PCA). Differences were considered significant at a 5% confidence level (p<0.05).

5.3. Results and Discussion

5.3.1. Tomato bagasse nutritional analysis

5.3.1.1. Proximal composition

Given the aim of this study, the valorisation of tomato by-products (tomato bagasse) to obtain a suitable process with zero wastes, our work was divided into two main steps.

Firstly, the proximal composition of tomato bagasse obtained from two processing companies was performed, as shown in Table 5.1. The two samples were characterized immediately after arrival from each producer (C1 and C2).

The sample provided by the companies consisted of an average of 28% seeds and 72% peels and pulp by-products. The samples were characterized in proximal composition (proteins, fat, fibre, and ash), and the results are shown in Table 1.

A significant difference was found for moisture content present in tomato bagasse from the two companies, 12.67 ± 0.24 to 67.40 ± 0.91 g/100 g DW, which is explained by the differences in the processing conditions and methodologies employed by each company. As previously mentioned, the C1 was provided fresh as usually supplied for animal feeding to local farmers, and the C2 was pressed and dried under the sun to reduce the water content to considerably minimize transport costs associated with the final disposal (Valle et al., 2006; Lavelli & Torresani, 2011; Silva et al., 2019). Nevertheless, different authors found quite variable results for moisture content within fresh tomato samples, some of them in the same range of the fresh sample (C1) evaluated in this study and other authors have reported a moisture content ranging from 64.3 to 92.6 g/100 g DW and 62.3 to 70.1 g/100 g DW, as reported by Del Valle et al. in 2006 and by Silva et al. (2019), respectively. Also, Lavelli and Torresani (2011) have reported even high moisture content ranging from 90.0 to 92.7 g / 100 g. The variability of the reported values demonstrates the high variability associated with the moisture content found in tomato by-products, which might vary on the specific type of tomato, maturity degree, processing applied and other factors that directly affect the moisture content. This parameter may also differ if there is a previous pressure treatment before exiting the industry to reduce transport costs.

The minerals contents were similar for C1 and C2, ranging from 2.90 to 3.35 g/ 100g, respectively. These values are comparable with those reported by Del Valle et al. (2006) (3.92 g / 100 g DW), Rossini et al. (2013) (3.3 g / 100 g DW) and Silva et al. (2016) (3.33 ± 0.02 to 4.02 ± 0.05 g / 100 g dry basis) in dry tomato pomace, which one more time shows the impact of the processing applied to the samples at the final moisture content.

Table 5.1. Proximal composition of tomato by-products.

		RT	
Chemical Components		C1	C2
Proximate composition (g /100g)	Moisture	0.13 ± 0.24	0.67 ± 0.91
	Ash	2.90 ± 0.13	3.35 ± 0.01
	Protein	16.34 ± 0.62	19.41 ± 0.36
	Fat	16.52 ± 0.35	13.20 ± 0.50
	Crude Fibre	58.75 ± 0.42	57.80 ± 0.26
	Total Sugars	5.76 ± 0.28	5.18 ± 0.16
Fibre	NDF	30.01 ± 0.03	16.93 ± 0.04
	ADF	12.98 ± 0.64	8.60 ± 0.23
Total Lignin		9.80 ± 0.23	12.73 ± 0.49
Cellulose (as glucose)		11.27 ± 0.66	7.23 ± 0.25
Hemicellulose		19.58 ± 0.10	9.08 ± 0.13
Pectins	Water soluble Pectin (WSP)	6.10 ± 0.81	11.68 ± 0.73
	Chelator soluble pectin (CSP)	5.04 ± 0.39	2.87 ± 0.40
	Hydroxide Soluble Pectin (HSP)	0.97 ± 0.04	1.01 ± 0.28

The values are expressed at g/ 100 g DW (dry weight); C1 and C2 represent the two companies studied.

Considerable amounts of protein were found in C1 compared to C2, 16.34 ± 0.62 to 19.4 ± 0.36 g / 100 g DW, respectively, but the content was significantly different in samples from different companies. Other researchers also observed protein content with significant variability ranging from 10.0 to 20.9 g/ 100 g DW (Valle et al., 2006; Knoblich et al., 2005; Nour et al., 2018; Persia et al., 2003; Silva et al., 2019). The divergences may explain the differences in companies' processing and tomato varieties since seeds contain higher protein content than peels, an each company's peel to seed ratio differs.

Regarding the nutritional composition, the macronutrient present at the highest content was fibre 58.75 ± 0.42 and 57.80 ± 0.26 (g/ 100 g DW), reduced differences were found among producers, and the results were comparable to the results reported by Nour et al. (2018) 52.4 g/ 100 g DW. The differences in total fibre could occur for different reasons: tomato cultivars, growing conditions and

the processing method (amount of seeds, pulp, and skins in the by-product). The high fibre content in this by-product makes it a good supplement for new food formulation combined with the relatively high protein content (Nour et al., 2018).

Cell walls of tomato by-products contain pectin, a structural branched heteropolysaccharide. The total pectin present in tomato by-products was 2.11 ± 1.24 for C1 and 15.56 ± 1.41 g GUAE / 100 g DW for C2. Furthermore, there are significant differences between the amount of Water-soluble Pectin (WSP) from C2 and C1 ($p<0.05$). Other authors reported similar values of WSP. Chou and Kokini (1987) reported 7.0 g / 100 DW of WSP extracted from tomato seeds (Alancay et al., 2017) extract 8.70 ± 0.26 g/ 100 g of tomato paste waste. Higher values of Chelator soluble pectin (CSP) were found in C2 ($p<0.05$) compared to C1. (CHOU & KOKINI, 1987) found higher values of CSP (7.0 ± 1.0 g/100 g GUAE). The results could be explained by the methods used to extract pectin and the tomato by-products' conditions (C1 and C2). The WSP pectin recovered is a biopolymer weakly attached or linked by hydrogen bonds to the cell wall, which decreases the extracted yield of C1, a sample with more moisture than C2; while the pectin extracted by acid conditions is a polymer strongly attached to the cell wall through a covalent bond increasing the recovered yield compared to C2 than C1 (Alancay et al., 2017). Also, the identified differences are correlated to the tomato variety and the advanced ripening stage. Relatively to HSP, both companies present small amounts. Müller-Maatsch et al. (2016) reported values of 6.0 ± 3.0 g / 100 g GUAE. The pectins were halfway esterified and partially acetylated. The differences between samples can be explained through the advanced ripening stage of tomatoes used in the processing industry and the treatment of the starting material.

5.3.1.2. Total fat and individual fatty acids content

GC characterized the fatty acids composition. Both tomato by-products presented more than 10% of fatty acids, a good source of such compounds. C1 yield on the fatty acids content was 17.7%, while C2 content was 13.2 %, with the most representative fraction being polyunsaturated fatty acids, with 10.1 and 8.0%, respectively (Table 5.2). Our values are higher than those reported in the literature. Elbadrawy and Sello found 4.0 g /100 g of the total fatty acids, while Nour et al. (2018) report 2.19 g /100 g (Elbadrawy and Sello, 2016; Nour et al., 2018). The most predominant saturated fatty acid was C16:0 (palmitic acid), and the major monounsaturated fatty acid was oleic acid, and linoleic acid was the primary polyunsaturated fatty acid. Our results following the described by Elbadrawy and Sello (2016). The authors also reported linoleic acid as the main fatty acid (52.41 g/100 g) followed by oleic acid (19.14 g/100 g) and palmitic acid (15.19 g /100 g) from tomato peel. Similar results were found with tomato wastes by Nour et al. (2018), 51.91 g/100 g of linoleic acid, followed by 18.50 g / 100 g of oleic acid and 16.32 g/100 g of palmitic acid. The latter fatty acids have gained attention during the past decades due to their beneficial health effects. Besides, this by-product may be an excellent alternative to other vegetable oils, such as sunflower or soybean, due to their similar physicochemical characteristics (Nour et al., 2018; Silva et al., 2019).

Table 5.2. Fatty acids profile (identification and quantification) of tomato by-products.

Fatty Acid Name	C1	C2
Lauric acid (C12:0)	0.01	n.d.
Myristic acid (C14:0)	0.04	n.d.
Myristoleic acid (C14:1)	n.d.	n.d.
Pentadecanoic acid (C15:0)	0.01	n.d.
Cis-10-Pentadecanoic acid (C15:1)	0.01	n.d.
Palmitic acid (C16:0)	2.50	1.95
Palmitoleic acid (C16:1)	0.05	0.03
Heptadecanoic acid (C17:0)	0.02	0.01
Cis-10-Heptedecenoic acid (C17:1)	0.01	0.06
Stearic acid (C18:0)	1.03^{x}	0.67
Elaidic acid (C18:1n9t)	n.d.	2.41^{x}
Oleic acid (C18:1n9c)	3.82^{x}	n.d.
Linoleic acid (C18:2n6c)	9.64^{x}	7.62
Arachidic acid (C20:0)	0.09	0.05
y-Linoleic acid (C18:3n6)	0.43	0.35
Cis-11Eicosenoic acid (C20:1)	0.02	0.01
Heneicosanoic acid (C21:0)	0.01	n.d.
cis-11,14-Eicosadienoic acid (C20:2)	0.02	n.d.
Behenic acid (C22:0)	n.d.	0.01
Lignoceric acid (C24:0)	0.03	0.02
Total (mg FA/ 100 g DW)	17.71	13.20
Total saturated (mg FA/ 100 g DW)	3.78	2.72
Total monounsaturated (mg FA/ 100 g DW)	3.89	2.51
Total polyunsaturated (mg FA/ 100 g DW)	10.07	7.97

c/t, *cis/trans* double bond; DHA, docosahexaenoic acid; n.d: not detected.
<LOQ, concentration under quantitation limit.
^{x} Superscript letter in a value for significant differences in a same lipid fraction among raw C1 and C2 composition (p<0.05).
The values are expressed at g FA/ 100 g DW (dry weight); C1 and C2 represent the two companies studied.

5.3.2. Antioxidant capacity, total polyphenols content, total carotenoids content and individual compounds

Differences in the antioxidant activity of both tomato bagasse were found; C1 had higher antioxidant activity than C2, probably due to the initial state of the samples when receiving it at the laboratory. As described previously, one of the samples received an extra pressing and drying step, which might directly influence the antioxidant activity. The same behaviour was found when analyzing the data collected from ORAC analysis, supporting the previous conclusions. The authors Valdez-Morales et al. and Perea-Domínguez et al. (2018) found values ranging from 7.05 and 35.64 µmol TE / g DW. Also, the higher tomato bagasse effect on scavenging free radicals is attributed to its higher lycopene and phenolic compounds content (Capanoglu, 2010; Elbadrawy and Sello, 2016; Nour et al., 2018; Savatović et al., 2010). On the other hand, significant differences were found between total phenolic content from the two companies, 0.141 and 0.0924 g/100 g to C1 and C2, respectively (table 5.3),

which correlates well with antioxidant activity, and once again demonstrating that pressing and drying process may preserve some phenolics and consequently its relative antioxidant activity. Studies reported a positive correlation between antioxidant capacity and phenolic compounds (Catană et al., 2017). The difference found between samples could be explained by the tomato processing steps, peeling, and dehulling, which causes degradation and, consequently, reduction of phenolic compounds. The drying process may also reduce the antioxidant compounds of tomato by-products (Toor & Savage, 2005). Another critical factor is the tomato cultivar and all the maturation processes. Similar results were found by (Cherif et al., 2010), who reported an average of 0.150 g/100 g DW in two tomato by-products samples, while Nour et al. (2018) found 0.123 g GAE /100 g of DW.

Table 5.3. Antioxidant capacity and total polyphenolic content.

Sample	**C1**	**C2**
Total Phenolic compounds (GAE)*	0.14± 0.06	0.092 ± 0.0145
Total carotenoids content (*β*-carotene Equivalent)*	0.780 ± 0.12	0.67 ± 0.007.3
ABTS (AAE)*	0.115 ± 0.016	0.858 ± 0.024
ORAC (trolox eq.)**	2.31 ± 0.020	2.09 ± 0.011

*The values are expressed as g/ 100 g DW (dry weight); ** expressed at µmol / TE 100 g C1 and C2 represent the two companies studied.

No significant differences were observed between C1 and C2 samples regarding carotenoid content, which means that residual carotenoids present on bagasse are relatively resistant to the drying process. Many authors have reported the high carotenoid content in tomato processing by-products (Cherif et al., 2010; Knoblich et al., 2005; Navarro-González et al., 2011a; Nour et al., 2018).

The high content of polyphenols was discriminated by HPLC, showing that the by-product samples contain differences in the phenolic compounds identified (Table 5.4). The flavonoids were predominant in tomato by-products from both companies.

The common phenolic compounds identified in both by-products were chlorogenic, naringenin and trans-cinnamic acids, luteolin-7-glucoside and kaempferol, the main chlorogenic acid phenolic compounds present in both extracts. Also, rutin and quercetin were found in C1 and hydroxymethylfurfural and 4-hydroxybenzoic acid in C2. The scarcity of studies with tomato by-products precludes any comparison of individual polyphenol compounds. Nevertheless, there is information on the effect of tomato cultivar and ripening stage on individual compounds and the compounds present during tomato processing, which helps to understand that the differences obtained in our study are also due to the cultivars used and the tomato processing (García-Valverde et al., 2013; Navarro-González et al., 2011a).

Table 5.4. individual compounds identified by HPLC from the two companies.

Samples	Compounds identified	Concentration (mg /100 g DW)
C1	Rutin	1.54 ± 0.0027
	Quercetin	0.14 ± 0.000018
	Naringenin acid	0.0086 ± 0.00001
	Transcinnamic acid	0.12 ± 0.00030
	Chlorogenic acid	4.12 ± 0.023
	Luteolin-7-glucoside	n.q
	Kaempferol	n.q.
C2	4-hydroxybenzoic acid	0.11 ± 0.021
	Hydroxymethylfurfural	0.0050 ± 0.000010
	Naringenin acid	0.00178 ± 0.00001
	Trans-cinnamic acid	0.12 ± 0.00030
	Chlorogenic acid	2.72 ± 0.017
	Luteolin-7-glucoside	n.q.
	Kaempferol	n.q.

n.q. non-quantified

Nour et al. (2018) reported ellagic acid and chlorogenic acid as the most abundant polyphenols present in tomato by-products, 14.34 and 7.63 mg/100 g DW, respectively. The differences found in both samples depends not only on the cultivar used in the processed tomato industry but also the condition of by-products obtained (e.g. C1 and C2 samples). The results obtained are under described in the literature (Kumar & Goel, 2019; Szabo et al., 2018; Urbonavičienė et al., 2018).

5.3.3. Ohmic heating extraction vs Conventional extraction

After ohmic extraction was applied to the tomato bagasse, the samples were centrifuged to separate the liquid extract from the residue, which was later dried at 55 ºC for 48 h and was grounded to obtain a valuable flour.

5.3.3.1. Proximate composition

Table 5.5. presents the approximate composition of raw tomato samples, LF and SF. The solid proportion (0.80 to 0.95 g/100 g DW) presented less moisture than RTB. SFOH was greater in dried samples than SFCONV, with considerable differences for protein fat and fibre ($p<0.05$).

Table 5.5. Chemical composition of raw tomato bagasse (RTB) and tomato fractions (LF-T and SF-T) obtained after OH and CONV extractions (g/100 g DW).

Chemical Components		RTB	LF-T		SF-T	
			LFOH	**LFCONV**	**SFOH**	**SFCONV**
Proximate composition (g /100 g)	Moisture	0.95 ± 0.07	3.08 ± 0.03	3.14 ± 0.07	0.80 ± 0.09	0.90 ± 0.08
	Ash	3.16 ± 0.10	10.56 ± 1.22	12.45 ± 0.22	3.02 ± 0.12	2.98 ± 0.16
	Protein	17.65 ± 0.30	4.01 ± 0.15	4.32 ± 0.23	17.44 ± 0.25*	16.29 ± 0.59
	Fat	15.49 ± 0.90	4.22 ± 1.25	6.46 ± 0.87	19.02 ± 0.43*	17.82 ± 0.30
	Crude Fibre	57.60 ± 1.54	0.15 ± 0.08	0.10 ± 0.06	60.47 ±0.44*	59.06 ± 0.67
	Carbohydrates	5.70 ± 1.23	74.99 ± 2.23	73.53 ± 2.19	1.22 ± 0.34	2.91 ± .98
Fibre	IDF	24.42 ± 0.96	n.d	n.d	48.06 ± 0.11	46.01 ± 0.13
	SDF	11.13 ± 0.75^{b}	10.86 ± 0.85^{b}	12.98 ± 0.64^{a}	n.d	n.d
Klason Lignin		11.34 ± 1.49	*	*	13.06 ± 0.52 ^{b}	14.09 ± 0.27^{a}
Structural Carbohydrates	Cellulose (as glucose)	9.14 ± 1.66^{b}	*	*	13.74 ± 1.12^{a}	12.82 ± 0.87^{a}
	Hemicellulose	14.29 ± 0.12^{b}	*	*	25.2 ± 0.10^{a}	24.72 ± 0.30^{a}
	Xylose	14.69 ±	*	*	13.2 ± 0.10 ^{a}	8.89
	Galactose	0.75 ± 0.08	*	*	0.12± 0.02	0.31 ± 0.021
	Mannose	6.64± 0.05	*	4.10 ± 0.12 ^{a}	6.5 ± 0.18	*
	Fructose	3.25 ± 1.23	2.24 ± 0.87	0.99 ± 0.12	0.61 ± 0.05	2.74± 0.22
Resistant Protein		17.04 ± 0.09	n.d	n.d	16.03 ± 0.05 ^{a}	11.69 ± 0.03 ^{b}

R-T – raw tomato by-products; LF-T – liquid fraction from tomato bagasse; SF-T – solid fraction from tomato bagasse; LFOH- liquid fraction obtained in the OH extraction; LFCONV- liquid fraction obtained after CONV method; SFOH- solid fraction obtained after OH; SFCONV- solid fraction obtained after CONV method; IDF – insoluble dietary fibre; SDF- soluble dietary fibre. ND – Not detected. *1 g Glucose equivalent /100 g sample dry weight. Data were expressed as mean ± SD (n = 3). The different superscripts in the same row represent significant differences between samples ($p < 0.05$).

Compared to the solid fractions, the liquid fraction had more carbohydrates (73.53 to 74.99 g/100 g DW) and ashes (10.56 to 12.45 g/100 g DW).

The increased nutritional value of SFOHs than RTB and SFCONV is due mainly to the heating process during OH treatment, which leads to the development of pores (electro-permeabilization) and intracellular diffusion of compounds which probably remained in the SF following extraction of LF.

The significant differences observed in fat and ash content of LFCONV compared to SFOH probably is due to the higher affinity of compounds with chemical solvents used to their recovery.

The significantly higher protein content of SFOH than RTB and SFCONV might be associated with electric field interference, frequency, and consequently the temperature of the protein aggregation, denaturation, and soluble protein content of the matrix-induced by OH (Pereira et al., 2018; Rodrigues et al., 2015; Xue & Farid, 2015). In addition, OH causes ions and other charged molecules (e.g. proteins) to move in the opposite direction of the charging electrode (Rodrigues et al., 2015b). The impact of OH on whey protein was assessed by Pereira et al. (2016) and demonstrated that rapid joule-heating contributes to lower proteins aggregation and greater solubility of protein content in conjunction with a low electric field. While, with the CONV technique, the temperature is not used, and protein denaturation does not occur. This technique also utilizes methanol with ethanol-like protein extraction results (the latter is used in OH).

SFOH also had more fatty acids than samples of RT and SFCONV ($p<0.05$). The use of water in OH technique as a solvent is probably the principal cause for SFOH retention of higher fatty acids than SFCONV.

An excellent predictor of the nutritional value of dietary fibre is neutral detergent fibre (Ribeiro et al., 2020). Due to the restriction of the neutral detergent fibre technique in water-soluble fibre analysis, the fluid part was classified as "not detected". Although this approach is restricted, the dietary fibre was another nutritionally influenced component of SF favourable for the extraction of OH. The total fibre and insoluble fibres exhibited by SFOH were greater than the SFCONV samples ($p<0.05$).

When comparing the extraction effects of OH and CONV techniques, Ramírez-Jiménez et al. (2019) showed comparable data on corn flours. The results show that OH preserves substances (e.g. insoluble fibres), not removing the pericarp and aleurone layer during the process. In contrast, the removal of the pericarp, losses in the CONV technique, which utilizes organic solvent, may be ascribed to insoluble fibre (Ramírez-Jiménez et al., 2019a). The greater insoluble fibre content of SFOH makes OH an essential option to produce tomato flour with an enhanced bioactive profile, often linked with insoluble dietary fibre, retaining phenolic bonding fractions (Goñi et al., 2009; Ramírez-Jiménez et al., 2019a).

5.3.3.2. Soluble Sugars

The total soluble sugar content of the samples of RTB and their corresponding fractions is considerably different, except for the liquid fraction ($p<0.05$) (Table 5.1).

The HPLC study for soluble sugars revealed the greater content of mannose and fructose as determined by the results for tomato by-products previously shown.

The liquid fraction found that the quantity of mannose between LF and SF was considerably different. The higher concentration of mannose in LFCONV (4.10 g/100 g DW) than LFOH and SFOH (6.5 g/100 g) can be explained by their higher fat content and a possibly higher degree of maturation of its tomatoes. At the same time, mannose rises as tomato mesocarp absorbs oil throughout the tomato ripening process.

The intended separation by fractions technique often leads to a concentration of soluble sugars, mostly mannose, in the liquid fraction. Therefore, the LF was mainly a source of minerals and carbohydrates in nutrient composition (fructose and mannose). This composition may serve as an advantage in the formulation of sports food or health-benefited food items, particularly reducing carbohydrate intake that increases blood glucose levels (Ribeiro et al., 2020).

3.3.3. Total Phenolic Content and Antioxidant Activity of Free and Bound Phenolics

Regarding total phenolic compounds, differences were found between solid fractions obtained from OH and conventional extraction methods ($p<0.05$), see figure 5.1. Nevertheless, the flour fraction presents higher total phenolic compounds than fractions submitted to an extraction process.

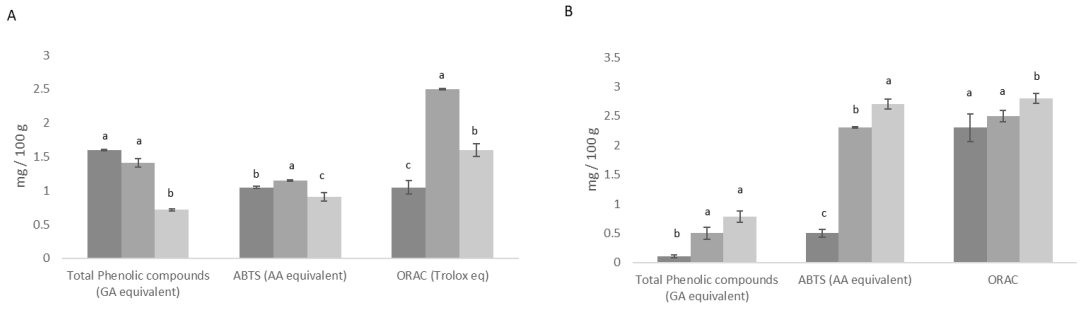


Figure 5.2. Total phenolics content, antioxidant capacity by ABTS, and ORAC method of free (A) and bound phenolics (B) from flour, conventional and OH extraction methods. The different superscripts in the same row represent significant differences between samples ($p < 0.05$).

The antioxidant activity was evaluated using different methods (ABTS and ORAC), and the samples showed different behaviours. While in tomato flour, the free phenolics had higher antioxidant capacity, in solid factions from extracts with ohmic and conventional heating, bound phenolics showed significantly higher antioxidant activity ($p<0.05$). It may be because of the higher overall phenolic content in these bound phenolic extracts than those at these free phenolic extracts ($p<0.05$). Higher values of ORAC were found for different fractions; nevertheless, the same trend was found for all samples.

The significant polyphenols identified as bound to fibres (table 5.6) from OH and conventional extracts ($p<0.05$) were *p*-coumaric acid, gallic acid, syringic acid, vanillic acid, and catechin (Palafox-Carlos et al., 2011). These phenolics acids were not extractable by aqueous methanol but issued upon alkaline hydrolysis. These results reinforce the previously presented results, whereas the bound phenolics showed significantly higher antioxidant capacity than free phenolics.

Table 5.6 Bound polyphenols complexed with tomato bagasse fibre, comparative analysis between different extraction techniques. Qualitative and quantitative analysis by HPLC.

Polyphenolic compounds	CONV	OH
4-hydroxybenzoic acid	2.14 ± 0.01	2.41 ± 0.08
Caffeic acid	3.36 ± 0.51	n.d.
Vanillin	2.64 ± 0.02	1.14 ± 0.01
p-coumaric acid	4.04 ± 0.02	2.14 ± 0.32
Rutin	8.04 ± 0.71	0.58 ± 0.001

The values are expressed at g/ 100 g DW (dry weight); CONV – Conventional extraction; OH – Ohmic heating extraction; n.d. – non-detected

The composition of bound polyphenols showed an antioxidant fibre-rich in polyphenolic compounds resultant of flour of tomato by-products, which allows all tomato waste (peels, seeds, and pulp) to produce a product with colour, antioxidant properties, dietary fibre and fatty acids.

5.3.4. Impact of freezing and drying on the BC

The samples were frozen to understand the impact on bioactive compounds since the immediate transformation of by-products is limited to the processing conditions, and tomato processing is also seasonal. Fractions in triplicate were frozen at – 20 ºC - 80 ºC for 24 h and compared with fresh bagasse biomass, and results are presented in Figure 5.2. According to the results of total phenolics in general, the ohmic had better results than the conventional method. These results agreed with those obtained previously, where the ohmic potentiate the increase of total phenolics. Also, better results were found in fresh samples, followed by frozen at -20 ºC.

Regarding antioxidant activity, better results were found in ohmic heating conditions with the frozen samples at -20 ºC, followed by -80 ºC. This result means that the frozen process improved the extraction capacity of the followed extraction processes. The freezing process generates the ice crystals within the water molecules inside of cells, and generally, the freezing through time leads to the increment of size of such crystals. The formation of crystals leads to the burst of the cellular membrane and release of inner content, but also release from the cellular membrane of polyphenols and bioactive molecules (Araújo-Rodrigues et al., 2021).

A PCA (Figure 5.3) was performed to understand the impact of frozen treatments on antioxidant capacity, total phenolic compounds, total carotenoids content.

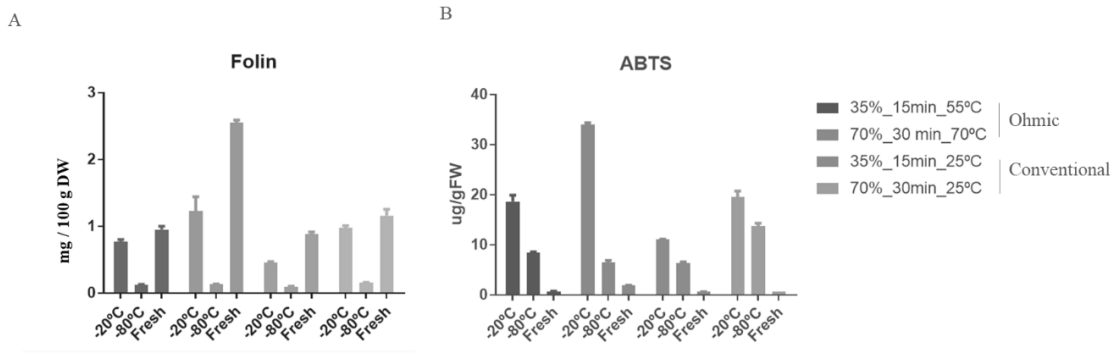


Figure 5.3. Total phenolic compounds (A) and Total antioxidant activity (B) from fresh and frozen samples submitted to ohmic and conventional extractions.

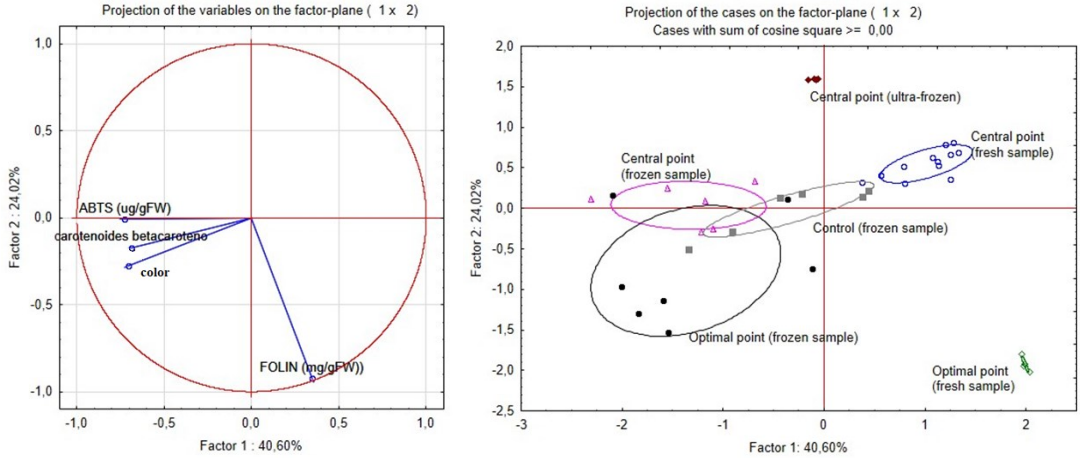


Figure 5.4. PCA analysis to principal variables, ABTS, Folin, carotenoids and colour of fresh and frozen samples.

In the frozen samples, colour and carotenoids are correlated. The fresh samples had better results mainly in terms of phenolic compounds, and this variable has a behaviour less correlated with antioxidant activity (ABTS) and carotenoids and correlated with the behaviour above associated with the freezing process. Nevertheless, the increase in the total amount of phenolics does not directly correlate to higher antioxidant capacity and carotenoids in the extract. It is well reported that different phenolic compounds have different antioxidant activities, and the same has been reported with the different carotenoids. Also, during freezing, cell breakage can occur, leading to enzymatic reactions (Khattab et al., 2015; Oliveira et al., 2016).

5.3.5. Prospective valorization for tomato paste by-products

Considering the research results, evaluating the amount of value-added molecules extracted and valorised would be critical to overview the tomato by-products and natural ingredients markets.

Tomato processing generates around 600 thousand to 2 million tonnes of by-products annually worldwide (Del Valle et al., 2006; Silva et al., 2019). Considering 600 thousand tonnes as the minimum estimate of organic matter disposed of per year, and according to results present in this study, it would be possible to transform the 600 thousand tonnes of tomato by-products into 353.9

thousand tonnes of fibre, 116.9 thousand tonnes of protein,106.2 thousand tonnes of fat, 18.0 thousand tonnes of minerals and 25.2 tonnes of carotenoids are wasted annually with higher economic costs, table 5.7. If specific extraction techniques to recover phenolic compounds, carotenoids, or specific fibres, it would be possible to differentiate and increase the extracted value from tomato by-products.

Table 5.7. Amount of valuable ingredients can be recovered in 600 thousand tomato processing by-products.

Proximal Composition	(g / 1 Kg)	Content (thousand tonnes)
Fibre	590	353.9
Protein	195	116.9
Fat	178	106.9
Minerals	30	18.0
Carotenoids	4	0.025

Nevertheless, further studies must be performed to understand in deep the food properties and the supply value chain of these sources coming from industry, to be able to design an efficient system to improve the performance of the application of emergent technologies, such as OH into the revalorization by-products and place food product development. The introduction of OH in the tomato processing industry's reactor system was regarded as an aseptic process. Coupling of OH treatment with aseptic filling is a straightforward industrial solution to simplify the processing line to prepare food preserves, because of this unnecessary additional thermal management of products after business; the use of OH in the time may be specified and contributes to moving the industrial industry away from the extractive linear structure towards a more circular economy.

5.4. Conclusion

One of the main goals of this research was to valorize by-products from tomato processing and characterize their main bioactive properties. All the data collected is just the beginning of a series of experiments designed to improve an extraction process (on a food-grade basis), followed by a future application as food ingredients.

This vegetable by-product has higher amounts of protein ranging from 16.29 to 19.4 g/100 g, in fibre close to 60.0 g/100 g DW and fatty acids, 17.0 g/100 g DW, including polyunsaturated fatty acids, oleic acid, a monounsaturated fatty acid, and saturated fatty acids, mainly palmitic acid. Secondly, to reduce the environmental impact of extraction processes and process costs, more cleaned extracts of the OH process were introduced than conventional extraction methods. Results showed two different perspectives. With extraction methods, two fractions are obtained, a liquid and solid fraction. In the LF, free compounds (sugars and polyphenols mainly) were obtained, with better total phenolic compounds and antioxidant capacity than the conventional method. The OH presented better results than CONV methods regarding solid samples (obtained after the extractions process). Thus, solid fractions can also be used with high potential as flour, for example, and incorporated as ingredients in food.

Valuable by-products are disposed of annually, and this by-product is promising from a functional point of view as a good source of fibre and protein, fatty acids, carotenoids and polyphenols for food formulations. Therefore, it is necessary to devise strategies for using by-products directly or obtain different ingredients in an integral valorization with zero-waste. Thus, tomato by-products must be revalorized to improve the circular economy in this agro-industrial sector.

PART III

Extraction method optimization

Chapter 6.

Anthocyanin recovery from grape by-products by combining ohmic heating with food-grade solvents: phenolic composition, antioxidant, and antimicrobial properties

Abstract

Usually, wine-making by-products are discarded, presenting a significant environmental impact. However, they can be used as a source of BC. Moreover, consumers' increasing demand for naturally nutritious and healthy products requires new formulations and food product improvement, together with sustainable, environmentally friendly extraction methods. Thus, this work aimed to compare ohmic heating (OH) with conventional methodology (CONV), using food-grade solvents, mainly water, compared to standard methanol extraction of anthocyanins. No significant differences were found between the CONV and OH for total phenolic compounds, which were 2.84 ± 0.037 and 3.28 ± 0.46 mg/g DW gallic acid equivalent, respectively. The same tendency was found for antioxidant capacity, where CONV and OH presented values of 2.02 ± 0.007 g/100 g and 2.34 ± 0.066 g/100 g ascorbic acid equivalent, respectively. The major anthocyanins identified were malvidin-3-*O*-acetylglucoside, delphinidin-3-*O*-glucoside, petunidine-3-*O*-glucoside, cyanidin-3-*O*glucoside, and peonidine-3-*O*-glucoside. These extracts displayed antimicrobial potential against microorganisms such as *Yersinia enterocolitica*, *Pseudomonas aeruginosa, Salmonella* Enteritidis*,* methicillin-sensitive *Staphylococcus aureus*, a methicillin-resistant *Staph. aureus* (MRSA), and *Bacillus cereus.* In conclusion, OH provides similar recovery yields with reduced treatment times, less energy consumption, and no need for organic solvents (green extraction routes). Thus, OH combined with water and citric acid allows a safe anthocyanin extraction from grape by-products, thus avoiding the use of toxic solvents such as methanol, and with high biological potential, including antimicrobial and antioxidant activity.

Keywords: grape by-products; ohmic heating; conventional methods; biological properties; phenolic compounds; anthocyanins; antimicrobial; antioxidant activity

6.1. Introduction

The wine-making process produces a large number of by-products that have a significant environmental impact. This process generates a high amount of solid organic waste, namely stalks, pomace (including skins, seeds, grape pulp l) and lees, which may be disposed of or beneficial use (Lucarini et al., 2020). Nevertheless, these by-products can also be used as a source of BC, such as dietary fibre, grape seed oil, hydrocolloids, and phenolic compounds, which might be applied by the agri-food and feed industries promoting economic value. Its reuse follows the actual circular economy concept imposed by the European Union. According to it, strategies for smart, sustainable, and inclusive growth must be adopted, promoting environmental protection (Chowdhary et al., 2021; Escribano-Bailón et al., 2019; Mendes, Xavier, et al., 2013).

Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 established a legal framework for treating waste in the European Union, emphasizing the importance of proper waste management, recovery, and recycling techniques to reduce the environmental and human health impact (Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on Waste and Repealing Certain Directives (Waste Framework, 2008). Value-added compounds could be isolated from the by-products to be used either as natural ingredients for the formulations of functional foods or as additives. These by-products have drawn the attention of scientists and the food industry. Traditionally, grape pomace has been used to produce wine beverages, nutrient colourants, and grape oil. More recently, research has concentrated on creating different value-added products, e.g., BC, primarily phenols, healing of tartaric acid, and the production of flours (Chowdhary et al., 2021; Hogervorst et al., 2017).

These wine industry by-products are frequently undervalued but represent a possible source of BC, such as polyphenols, that could be applied in many industries. Phenolic substances from grapes, including anthocyanins, are reduced at the skin and seeds, more precisely, the portion that remain as pomace after the processing of grapes (Hogervorst et al., 2017; Maroun et al., 2017).

Anthocyanins belong to the flavonoid class and represent the most significant set of water-soluble plant pigments (S. Silva, Costa, Coelho, et al., 2017b). Anthocyanin's color depends on the solvent's pH; the red-color anthocyanins are stable at lower pH (3.5–4) (Hogervorst et al., 2017; Khoo et al., 2017; S. Silva, Costa, Coelho, et al., 2017a). As they are localized in black grape peels, anthocyanins are usually extracted (30 to 40%) through wine-making operations. However, previous research has shown that the anthocyanin content of a given cultivar is not necessarily positively linked with anthocyanin concentration in the resulting wine. The lack of association was due to the partial preservation by cell-wall polymers of these anthocyanins in the skin cells (el Gengaihi et al., 2014; Hanušovský et al., 2020).

Various methods have been developed to extract bioactive compounds (BC), and their effects on the preparation and functionality of extracts from agro-industrial wastes have been evaluated. These commonly applied methods use harmful and toxic compounds, restricting the use of grape by-product extracts (Hogervorst et al., 2017; Pereira et al., 2020). Nevertheless, given the need for sustainability, the political agenda is fostering the development of "clean label" processes toward the reduction of environmental impacts and a strong bioeconomy. In addition, the conversion of wine-making by-products into added-value products could be possible through the development of environment-friendly technologies. New technologies,

such as microwave-, ultrasound-, and ohmic heating (OH)-assisted extraction, have been used to improve the recovery of BC from food samples (Barba et al., 2016; Maroun et al., 2017). These methods have attracted significant attention from the scientific community. In particular, OH is a thermal process where an alternating electric flow is forced to pass through the food materials. The thermal effect needed to assist the extraction process is rendered internally due to the passage of electric flow through the materials (Joule effect). The general purpose of OH technology is to improve food, cosmetics, and pharmaceutical products that are safe and beneficial to human health (Kumar, 2018). Despite these advantages, the OH application can be impaired by products' physical and chemical properties, such as poor electrical conductivity due to higher fat or sugar contents in their composition (Kumar, 2018).

El Darra and colleagues performed an assisted extraction by pulsed OH from red grape pomace. The authors explored the pulsed OH effects on cell membrane damage to increased polyphenol recovery. They also studied the effects of the electrical field strength, temperature, and the proportion of ethanol/water used. Pre-treatment caused cell membrane permeabilization. In addition, pulsed OH, which was used as a pre-treatment, increased the recovery kinetics of TPC. Other researchers reinforce high yields of recovery of phytochemicals obtained by OH from black rice bran. They suggest OH as a promising technology to extract anthocyanins with a future application in the production of natural colorants (Loypimai et al., 2015).

Pereira et al. (Pereira et al., 2016) explore how ohmic heating (OH) influences phytochemical components recovery from colored potatoes (*Solanum tuberosum L. var. Vitelotte*) using moderate electric fields. Their results reveal that low-energy electrical fields and thermal effects may be integrated and adjusted into a single phase of treatment by recovering anthocyanins and phenolic chemicals from vegetable tissues, which delivers a high recovery rate with lower treatment duration, decreased energy consumption, and no organic solvents (green extraction). More recently, the authors (Pereira et al., 2020) have also shown that OH has the potential to be used as an efficient and environmentally friendly technology toward sustainable food processes; it has been shown that OH can be used as a pre-treatment for enhanced aqueous extraction of anthocyanins from grape skins, particularly when high-temperature short-time (HTST) treatments are applied. There still is scarce information regarding the effects of combining OH pre-treatments with food-grade solvents to enhance the aqueous extraction of phenolic compounds, namely anthocyanins, comparing with conventional methods based on solvent extraction. Accordingly, this study aimed to evaluate the effectiveness of OH pre-treatments in the aqueous extraction of anthocyanins from red grape pomace by-products using food-grade extraction solvents and compared it with traditional solvent extraction methodologies.

6.2. Materials and Methods

6.2.1. Chemicals

The 2, 20-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS diammonium salt), potassium sorbate, sodium carbonate, ethylenediaminetetraacetic acid (EDTA), sodium sulfite, and sodium lauryl sulfate were purchased from Sigma-Aldrich (Sintra, Portugal). Methanol, acetonitrile, and hydrochloric acid were purchased from Fischer Scientific Portugal. Folin–Ciocalteu's reagent, potassium persulfate, citric acid, and lactic acid were purchased from Merck (Algés, Portugal). Standards of ascorbic acid, Trolox, and gallic acid were purchased

from Sigma-Aldrich (Sintra, Portugal), while delphinidin-3-*O*-glucoside, cyanidin3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside were purchased from Extrasynthese (Lyon, France).

6.2.2. Samples

The red grape pomace, obtained from a wine manufacture using Vinhão cultivar, was used for the study. Grape pomace includes a mixture of pulp, skins, and seeds, which were separated randomly in aliquots of 50 g and dried in an oven at 50 °C ± 3 °C. After samples were milled in a cuisine robot (Bimby Vorwerk, TM5), the powder was sieved manually at 150 µm. The powder was used for the following phenolic extractions.

6.2.3. Extraction Procedures

One of the most critical factors that affects the BC recovery yields is the solvents used in the extraction method. Solvents differ in polarity and comprise methanol, hexane, acetone, chloroform, and diethyl ether (Costa et al., 2019). According to the literature, the traditional solvent for anthocyanins recovery is acidified methanolic solution (Grumezescu & Holban, 2017; Rodriguez-Saona & Wrolstad, 2001).

6.2.3.1. Pre-Treatments

The grape by-products are non-conductive samples; thus, 2.5 g of grape by-products were placed in 5 mL sodium chloride (NaCl) 0.1 M solution to increase the conductivity to 4.6 mS/cm at room temperature. Three methodologies were performed with each solvent described before—ohmic heating (OH), which reaches 100 °C in 13 sec; a control negative (CN) reaches 100 °C in 20 min; and the control positive (CP) used at room temperature (Figure 7.1.) —to understand if the effect came from the ohmic heating (OH) and not from the temperature and solvent during the extraction process. After, all samples were cooled in ice to stop the reactions and proceed with the solvents' extractions.

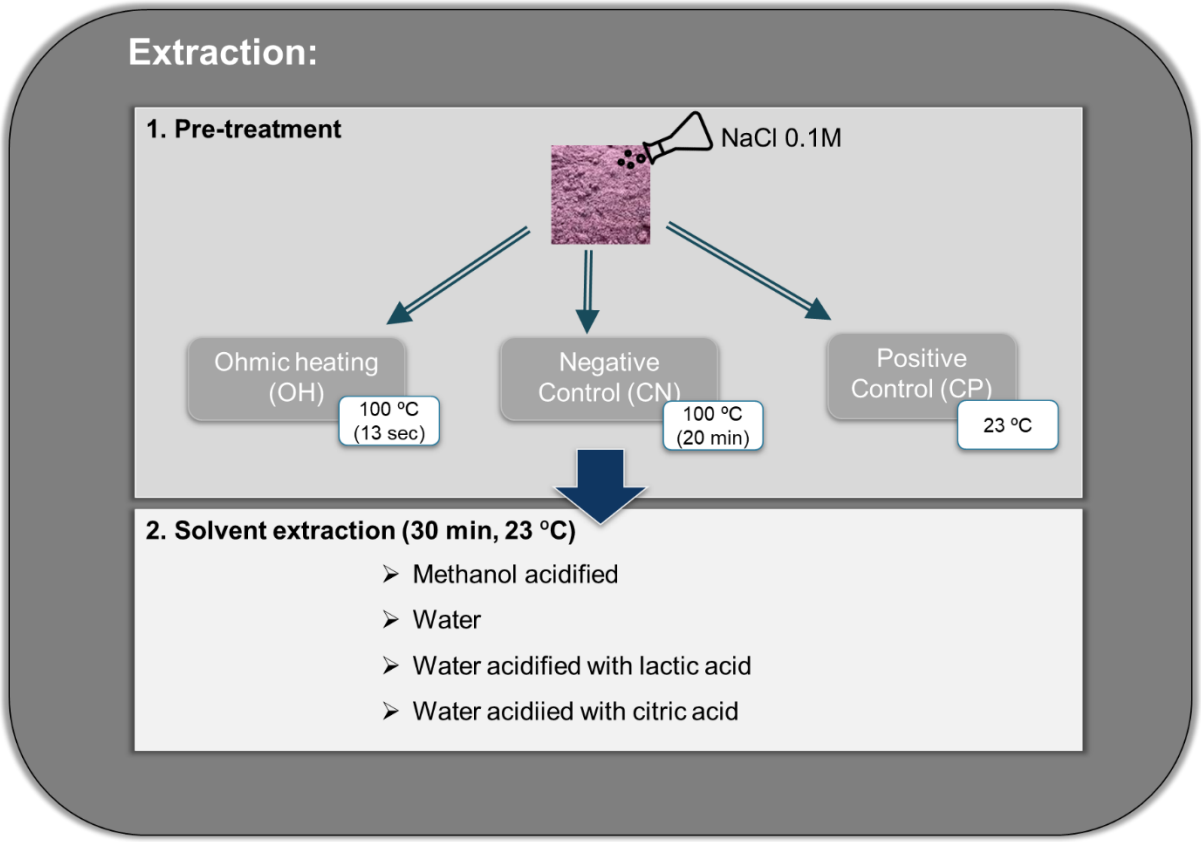


Figure 6.1. Scheme of experimental analysis.

OH Pre-treatment

The OH was carried out as a pre-treatment before solvent extraction. A high frequency of 25 kHz and an electric field of ≈30 V/cm was applied. Within these conditions, the temperature reached 100 °C in 13 s, after which the system was turned off; then, samples were placed on ice to stop the residual temperature effect and promote a fast decrease toward room temperature (23 °C).

Control Negative

Control of temperature was also performed to observe the temperature effects. It consisted of placing 2.5 g of grape by-products (placed in the same sodium chloride solution described before) in a bath, and when samples reached 100 °C (≈ 20 min), they were placed on ice to stop the reaction and also promote a fast decrease toward room temperature (23 °C).

6.2.3.2. Solvent Extraction

Water, acidified water (lactic and citric acid 1%), and methanol/water solution (80:19:1 v/v) acidified with hydrochloric acid usually are used as a conventional method (CONV) for anthocyanins extractions, as described by Silva et al. [8]. Both lactic and citric acid are food-grade acids usually used in the food industry.

Extraction solvents (25.0 mL) were added to pre-treated samples (OH and CN) and directly to 2.5 g of grape by-products pre-treated with NaCl and CP, and the material suspensions were kept under gentle stirring at

room temperature (23 °C) for 30 min, allowing the bioactive compound recovery. Water solution and water acidified with two food-grade acids (lactic, citric) were used to decrease the pH (pH 3) to favor extraction and stabilize the color of anthocyanins.

Then, the extract was centrifuged at 4000× *g*, 4 °C for 10 min, and the supernatant was filtered through a 0.45 mm cellulose acetate filter (Orange Scientific, Braine-l'Alleud, Belgium), and the pellets were stored at −80 °C. This procedure was used for total activities measurement.

6.2.4. Total Antioxidant Capacity, Phenolic and Anthocyanins Content

6.2.4.1. Total Antioxidant Activity

After the extraction's procedure, the extracts were evaluated in terms of antioxidant activity to understand the effects of treatments and solvents extraction.

AA was performed using the ABTS method, according to (Gião et al., 2007). The sample was added to a colored solution of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation) ($ABTS^{\bullet+}$), with an optical density (OD) measured at 734 nm and adjusted to 0.700 ± 0.020 in a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). After 6 min of reaction, the final OD was read, and the results were given in ascorbic acid equivalent. The standard was ascorbic acid (0–500 mg/L), and the regression equation for ascorbic acid and samples was calculated (R^2 = 0.999).

The Oxygen Radical Absorbance Capacity (ORAC-FL) was also measured to evaluate the AA, according to (Ubeda et al., 2011). Briefly, 20 µL of OH, CN, and CP extracts were mixed with 120 µL of fluorescein (70 nM) in black, untreated 96-well microplates (Nunc, Roskilde, Denmark) and incubated at 40 °C for 10 min. Then, 60 µL of 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride (APPH) solution (12 mM, final concentration), Sigma-Aldrich, AAPH solution (60 µL; 12 mM, final concentration) was rapidly added using a multichannel pipet. The microplate was immediately placed in the multidetector plate reader (Synergy H1, Vermont, USA), and the fluorescence was recorded at intervals of 1 min for 140 min. The excitation wavelength was set at 485 nm, and the emission wavelength was set at 528 nm (Yoon et al., 2002). The microplate was automatically shaken before each reading. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. Trolox (10–80 µM) was used as the standard, and regression equations for Trolox and samples were calculated. The ORAC-FL values were calculated by the ratio of sample slope to Trolox slope obtained in the same assay. Final ORAC-FL values were expressed as micromol of Trolox equivalent (TE) per mg of sample.

6.2.4.2. Total Phenolic Content

The TPC of extracts was evaluated through the Folin–Ciocalteu spectrophotometric method as described by (Ferreira-Santos et al., 2019). A mixture of the sample (5 µL), Folin–Ciocalteu reagent (15 µL), sodium carbonate at 75 g/L (60 µL, Sigma-Aldrich) and distilled water (200 µL) were performed, and the solutions were mixed. Afterwards, samples were heated at 60 °C for 5 min, and the OD was read at 734 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). The gallic acid standard (0–500 mg/L) was used to measure TPC, and regression equations for gallic acid and samples were calculated (R^2 0.997) and expressed as a milligram of gallic acid equivalent per dry weight material (mg GAE/100 g). The analyses were performed in triplicate, and a standard deviation was calculated.

6.2.4.3. Total Anthocyanins

Total anthocyanins (TA) were assessed using a spectrophotometric analysis, as Pereira et al. [25] described. The results are expressed in equivalent cyanidine-3-glucoside equivalents (Cy-3-GE) and compared to a range of standards prepared to start from a stock ethanolic solution of cyanidin-3-glucoside. The analysis was performed in triplicate.

6.2.5. High-Performance Liquid Chromatography (HPLC) Analysis

Polyphenol profiles (quantitative and qualitative) were assessed according to Coelho et al. (Oliveira et al., 2015). The analysis was conducted on HPLC-DAD (Waters Series 600. Mildford MA. USA). A Symmetry® C18 column, 250 × 4.6 mm i.d. 5 µm particle size, and 125 Å pore size with a guard column (waters) was used, and solvents elution consisted of solvent A—acetonitrile (100%) with 0.2 % TFA; Solvent B—acetonitrile/water (5:95 *v/v*) (Merck pure grade and pure water) with 0.2% TFA (Sigma-Aldrich, Germany). A linear gradient at a flow rate of 1 mL/min was applied: 0–20 min (100%B); 30–60 min (60% B); and 10 min (100% B). Samples were analyzed in triplicate. Calibration curves were obtained at a detection wavelength of 520 nm. Standards solutions over the concentration range from 0.10 to 100.00 mg/L were prepared for the identification and quantification of the following compounds: delphinidin-3-*O*-glucoside; cyanidin-3-*O*-glucoside; petunidin-3-*O*-glucoside; peonidin-3-*O*-glucoside; and malvidin-3-*O*-glucoside expressed as µg per mL of dry weight (DW) biomass of grape. All calibration curves were linear over the concentration ranges tested with correlation coefficients of 0.999.

6.2.6. Antimicrobial Analysis

6.2.6.1. Microorganisms

A few pathogenic microorganisms were utilized in this study. Clinical bacterial isolates from urine were kindly given by CHTMAD—Hospital Center of Trás-os-Montes e Alto Douro (through PhD Maria José Alves). The isolated strains comprised E. coli (E. coli CI resistant to ampicillin, nalidixic acid, norflaxin, and ciproflaxin), a *P. aeruginosa* (Ps. aeruginosa CI intermediately resistant to cefotaxime), a methicillin-resistant ((MRSA) resistant to oxacillin, ciprofloxacin, and levofloxacin), and a methicillin-sensitive *Staph aureus* (MSSA) [60]. Additionally, there were three references (R) strains of food isolate from ESB collection: *S.* Enteritidis ATCC 13076, *Y. enterocolitica* NCTC 10406, and *L. monocytogenes*. An inoculum of each bacteria was prepared at a density equivalent to 0.5 on the McFarland scale (~ 1.5×10^8 CFU mL^{-1}). Next, serial decimal dilutions were performed in saline solution, obtaining suspensions with about 1.5×10^6 CFU mL^{-1}.

6.2.6.2. Plate Test

The different extracts at (1 $mg.mL^{-1}$) were used to evaluate their effects on antimicrobial properties. The assays were performed after 48 and 72 h of sample preparation. To perform these assays, 100 µL of inoculum was transferred to Petri dishes containing the Nutrient Agar medium by the spread plate method, and each plate was incubated at 37 °C for 48 h. As a positive control, nutrient agar plates containing bacterial suspension and saline solution were used as well as plaques containing the bacterial suspension, and the solvents were used to perform the extracts (methanol, water, water acidified with citric and lactic acid). All tests were performed in triplicate according to the method described by Ramos et al. (2012).

6.2.7. Cytotoxicity

The colorimetric method using the XTT was performed to assess cell viability as a function of redox potential, according to Jiang et al. [63]. In the presence of metabolic activity, the water-soluble XTT is converted to a water-soluble, orange-colored formazan product. Shortly, 100 µL cell suspension aliquots were seeded in a 96 micro-plating well (1 µL to 105 cell/mL) (Nucleon Delta Surface, Thermo Scientific, Roskilde, Denmark). Then, after seeding, the cultivated media was carefully changed and incubated in the dark by the various test solutions. After 24 h, cell viability was tested as follows: a 10 mmol/L of PMS solution was prepared in phosphate-buffered saline (PBS, 0.01 M; pH 7.4), and a 1 mg/mL XTT solution was prepared using the appropriate culture media, previously heated to 37 °C. OH, CN, and CP extracts were used at concentrations of 1.0 mg/mL. Both solutions were sterilized using sterile membrane filters of 0.22 µm (Millipore, Billerica, MA, U.S.) and combined (2.5 µL PMS per mL XTT solution just before application). In each well, aliquots (25 µL) of mixture were added, and cells were incubated for about 2 h in the dark. The optical density was measured with a microplate reader at 485 nm (FluoSTAR, OPTIMA, BMG Labtech, Ortenberg, Germany). The findings were shown as the percentage of inhibition of cell metabolism. Plain culture media was used as a negative control. All assays were performed in quintuplicate.

6.2.8. Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation. The SPSS v. 19 (Chicago IL, USA) software was used to evaluate the statistical differences within different treatments determined by ANOVA, using the Shapiro–Wilk for variance homogeneity and Tukey's test for multiple comparisons. Differences were considered significant at a 5% confidence level ($p < 0.05$).

6.3. Results

6.3.1. Total Phenolic Compounds and Antioxidant Capacity

Concerning total phenolic compounds (TPC) (Figure 6.2.), a better recovery yield was obtained with MeOH acidified in all extraction methods ohmic (OH), negative control (CN), and positive control (CP). Comparing the extraction methods, OH presented higher values than CP for all solvents tested. In addition, higher amounts of TPC were obtained when combined OH with MeOH acidified. Significant differences between extraction methods were only verified for MeOH extraction ($p < 0.001$). In addition, values of 423 ± 0.2 mg/100 g DW gallic acid equivalent and 360 ± 0.8 mg /100 gallic acid equivalent were observed for MeOH and citric acid, respectively, with OH extraction. These results are corroborated by results from antioxidant analysis measured by ABTS, where citric acid showed the highest values of antioxidant capacity (AA) when compared with other solvents (Figure 6.3.).

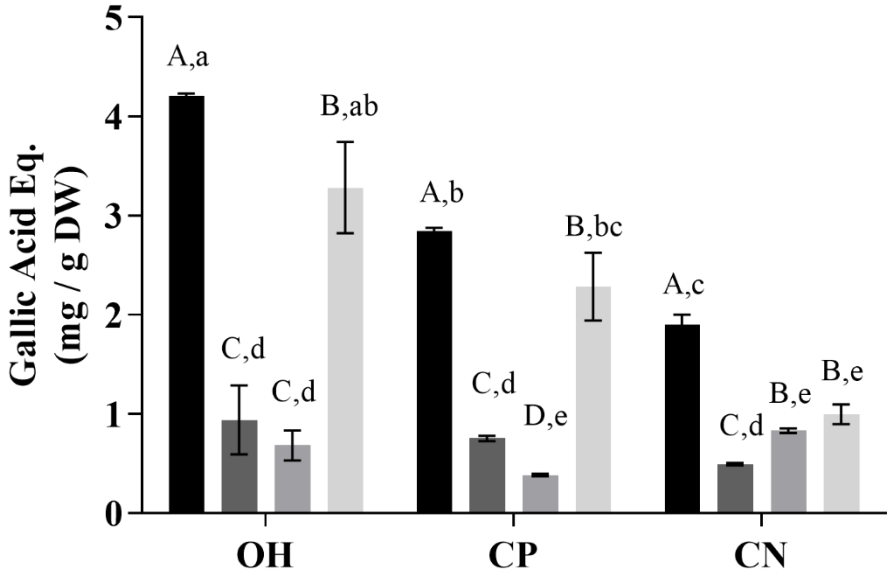


Figure 6.2. Total phenolic content of extracts performed with methanolic and aqueous solutions from grape wine-making by-products (gallic acid equivalent mg/g DW). OH—ohmic heating; CP—positive control; CN—negative control. Extraction solvents. ▪ MeOH with 1% HCl, ▪ H_2O, ▪ lactic acid, ▪ citric acid. Different capital letters in the same extraction method indicate a statistically significant difference ($p < 0.01$). The small letters represent statistical differences between solvents and extraction methods ($p < 0.05$).

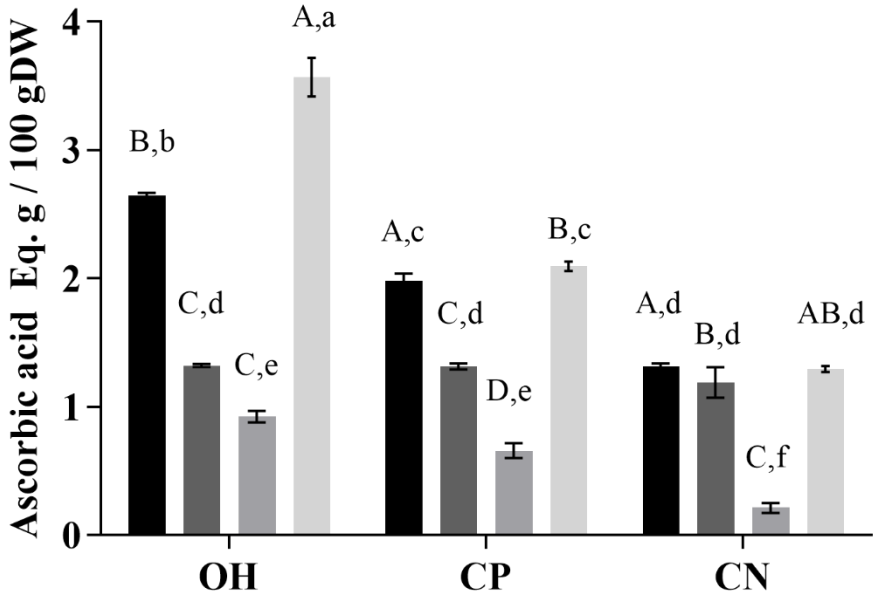


Figure 6.3. Antioxidant activity by ABTS test of extracts from grape by-products (g ascorbic acid equivalent/100 g DW). OH—ohmic heating; CP—positive control; CN—negative control. Extraction solvents. ▪ MeOH with 1% HCl, ▪ H_2O, ▪ lactic acid, ▪ citric acid. Different capital letters in the same extraction method indicate a statistically significant difference ($p < 0.01$). The small letters represent statistical differences between solvents and the extraction method ($p < 0.05$).

AA was also analysed by the ORAC method. According to the results of AA with the ORAC method presented in Figure 6.4, this method resulted in different patterns compared with ABTS. Two-way ANOVA showed that

there is a significant interaction between solvent and treatment, $p < 0.0001$. Methanol CP extract showed ORAC quantities of 0.489 ± 0.0443 µmol/g Trolox equivalents, which were analogous to those in citric extracts with OH (0.351 ± 0.022 micromol/g Trolox equivalents).

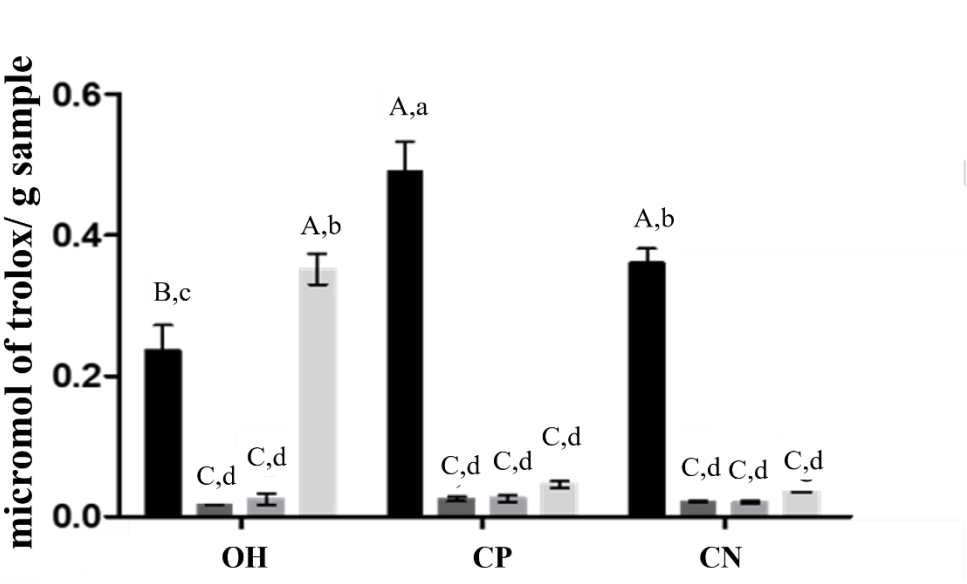


Figure 6.4. Antioxidant capacity by the ORAC method. Results are expressed in micromol of Trolox equivalent per gram of sample. OH—ohmic heating; CP—positive control; CN—negative control; extraction solvents. ▪ MeOH with 1% HCl, ▪ H_2O, ▪ lactic acid, ▪ citric acid. Different capital letters in the same extraction method indicate a statistically significant difference ($p < 0.01$). The small letters represent statistical differences between solvents and the extraction method ($p < 0.05$).

6.3.2 Total and Individual Anthocyanins Content

Results showed an increase of anthocyanin recovery (Figure 6.4) in OH samples when compared with the CN method for all solvents extraction ($p < 0.05$), while when compared with CP, similar results were obtained. This increase could be explained by non-thermal effects on plant cell permeabilization, which were probably due to electrical disturbances in the membranes of cells or by electroporation impacts (Pereira et al., 2020; Rocha et al., 2018). However, it is essential to highlight that compared with CN, OH treatment resulted in a reduced thermal load. This may have contributed to less degradation of extracted anthocyanins and justify the presented results. Regarding individual compounds, the malvidin-3-*O*-glucoside is the main anthocyanin present for all extracts (Table 6.1. and Figure 6.5).

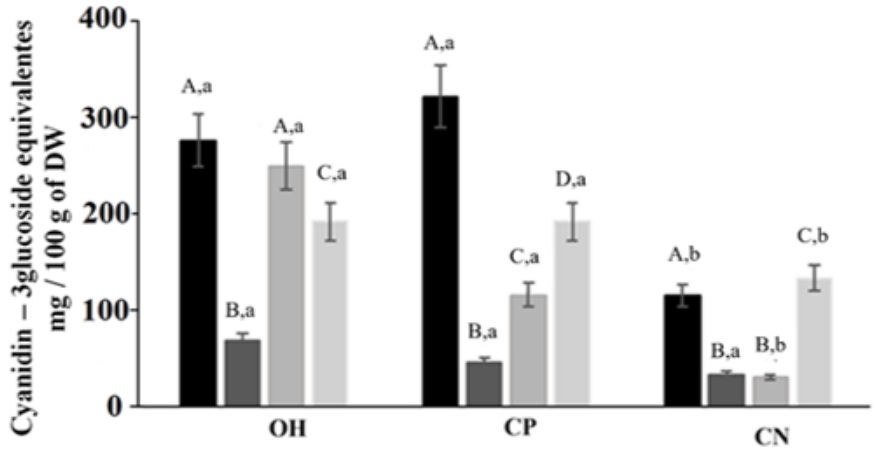


Figure 6.5. Total anthocyanins for all extraction process of grape by-products. OH—ohmic heating; CP—positive control; CN—negative control. extraction solvents. ▪ MeOH with 1% HCl, ▪ H2O, ▪ lactic acid, ▪ citric acid. Different capital letters in the same extraction method indicate a statistically significant difference ($p < 0.05$). Different small letters represent a statistical difference between methods of extraction ($p<0.05$).

Anthocyanins profile from OH, CONV, and CT methods for each solvent (µg/g DW).

Table 6.1. Anthocyanins profile from OH, CONV, and CT methods for each solvent (µg/g DW).

	Extraction Solvent											
	MeOH			Water			Water with Citric Acid			Water with Lactic Acid		
Compounds	OH	CP	CN	OH	CP	CN	OH	CP	CN	OH	CP	CN
delphinidin-3-*O*-glucoside	48.30±0.36A,a	48.82±0.20A,a	20.45±0.09D,b	8.14±0.03F,a	4.90±0.01GH,b	5.08±0.07G,b	36.7±0.81B,a	20.06±0.25D,b	4.71±0.08H,c	29.47 ± 0.49C,a	29.43 ± 0.29C,a	11.87± 0.08E,b
cyanidin-3-*O*-glucoside	2.18±0.02C,a	2.94±0.02B,a	0.37±0.07G,b	0.15±0.01H,c	0.30±0.01G,b	0.49±0.01F,a	1.10±0.08D,a	0.58±0.03E,c	0.70±0.02E,b	0.03 ± 0.003I,b	0.04 ± 0.001I,b	9.79 ± 0.01A,a
petunidine-3-*O*-glucoside	34.24±0.26B,a	38.34±0.16A,b	14.31±0.06D,c	6.66±0.05F,a	4.49±0.06G,b	6.24± 0.05F,a	27.46±0.92B,a	0.17±0.01I,b	3.45±0.07H,c	22.10 ± 0.45C,a	22.07 ± 0.45C,a	12.04± 0.05E,b
peonidine-3-*O*-glucoside	12.65±0.10B,a	16.86±0.07A,b	5.07± 0.02E,c	3.37±0.03F,a	2.32±0.02G,b	1.36±0.02H,c	12.56±0.07B,a	6.43±0.12D,b	0.92 ± 0.06I,c	9.26 ± 0.41C,a	8.92 ± 0.31C,a	2.19 ± 0.20G,b
malvidin-3-*O*-glucoside	128.88±0.94B,b	151.96±0.62A,a	53.41±0.22F,c	37.08±0.06G,a	24.87±0.09H,b	17.34±0.05I,c	125.94±1.25B,a	66.93±1.23E,b	15.57±0.29I,c	101.59±0.39C,a	97.32 ± 0.42C,a	73.02±0.70D,b
Total anthocyanins	224.06±1.25B,b	258.93±2.34A,a	93.62±1.87G,c	55.40±0.99H,a	36.89±1.38I,b	30.51±1.11J,c	203.83±4.21C,a	94.17±1.47G,b	25.35±1.26K,c	162.45±2.14D,a	157.81 ± 1.37E,b	108.90±1.96F,c

Capital letters in rows indicate the significance between treatments ($p < 0.05$). Small letters in rows indicate the differences between solvents of extraction ($p < 0.05$). treatments: OH—ohmic heating; CP—positive control; CN—negative control.

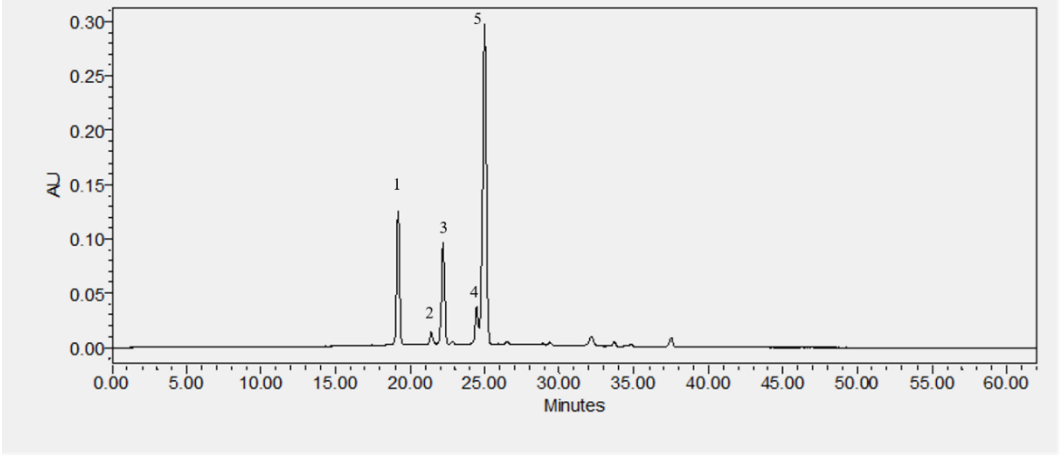


Figure 6.6. Chromatogram example of anthocyanins profile detected in samples by HPLC-DAD. (1) delphinidin-3-*O*-glucoside; (2) cyanidin-3-*O*-glucoside; (3) petunidin-3-*O*-glucoside; (4) peonidin-3-*O*-glucoside; and (5) malvidin-3-*O*-glucoside.

6.3.2. Antimicrobial Properties

We tested the antimicrobial effects of the extracts using the disk diffusion test and performed a screening of the inhibitory effect on halo formation. The results are presented in Table 6.2.

Both CP and OH with citric acid extracts exhibited significant higher inhibitory activity against *Escherichia coli (E. coli)*, *Salmonella* Enteritidis (*S.* Enteritidis), a methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staph. aureus* (MSSA), *Bacillus cereus* (*B. cereus*), *Pseudomonas aeruginosa* (*P. aeruginosa)*, and *Yersinia enterocolitica* (*Y. enterocolitica)*. It is noteworthy that there are varietal differences involving the phenolic compounds content of grape by-products and their antioxidant and antimicrobial attributes.

Table 6.2. Antimicrobial activity of extracts (1 mg/mL).

Extraction	Microorganism	OH	CP	CN
MeOH	*Y. enterocolitica*	0	0	0
	P. aeruginosa	0	+	0
	E. coli	0	0	0
	S. Enteritidis	+	0	0
	MRSA	+	0	0
	MSSA	0	0	0
	Listeria monocytogenes	0	0	0
	B. cereus	+	0	0
H_2O	*Y. enterocolitica*	0	0	0
	P. aeruginosa	0	0	0
	E. coli	0	0	0
	S. Enteritidis	0	0	0
	MRSA	+	*0*	0
	MSSA	0	0	0
	Listeria monocytogenes	0	0	0
	B. cereus	0	0	0
Lactic acid	*Y. enterocolitica*	0	0	+
	P. aeruginosa	0	0	0
	E. coli	0	0	+
	S. Enteritidis	0	0	+
	MRSA	0	*0*	+
	MSSA	0	*0*	0
	Listeria monocytogenes	0	0	0
	B. cereus	0	0	0
Citric acid	*Y. enterocolitica*	+	0	+
	P. aeruginosa	+	0	+
	E. coli	+	0	0
	S. Enteritidis	+	0	+
	MRSA	+	0	+
	MSSA	+	0	+
	Listeria monocytogenes	0	0	0
	B. cereus	++	0	+

Extracts halos for each bacterium (mg/mL) and its inhibitory effect upon disk diffusion test. 0—no halo formation; +—moderate halo formation; ++—strong halo formation.

Other authors also described that grape extracts at 2% have antibacterial action toward *P. aeruginosa*, *Staph. aureus*, and *E. coli*(García-Lomillo et al., 2014; Luchian et al., 2019; D. A. Oliveira et al., 2013).

6.3.3. Cytotoxicity

The cell viability test XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2*H*-tetrazolium, monosodium salt), a mucus-secreting line HT29-MTX, was utilized to evaluate the potential cytotoxicity effect of grape extracts (OH, CP, and CN). The results showed that the highest concentration of 1 mg /mL of water extracts tested did not inhibit the cellular metabolism (negative values of metabolism inhibition), thus not showing cytotoxicity for these cells (Figure 6.7). Only citric acid presented an inhibition above 10% for the CN, which indicates some inhibition of cell viability, but also with no relevance, since values are lower than 30% cell metabolism inhibition. All extracts obtained by OH and some from the CN extracts presented negative values, suggesting that they promote cell growth, mainly the extracts produced by OH with citric acid.

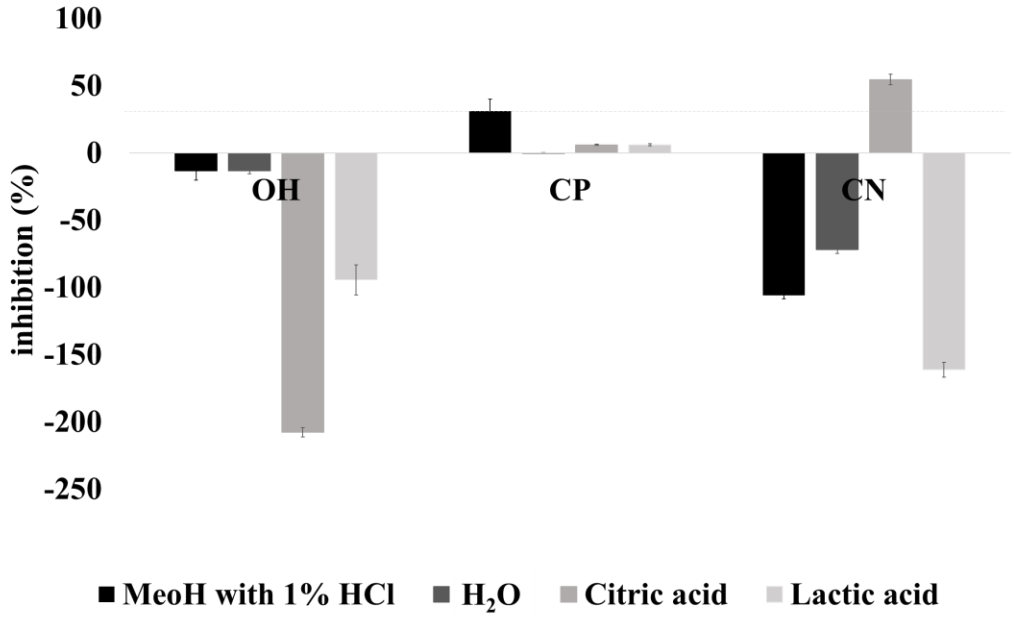


Figure 6.7. Cytotoxicity analysed by the XTT method. The line represents the non-toxic extracts.
OH—ohmic heating; CP—positive control; CN—negative control. The extraction solvents. ▪ MeOH with 1% HCl, ▪ H_2O, ▪ lactic acid, ▪ citric acid.

6.4. Discussion

6.4.1. Total Phenolic Compounds and Antioxidant Capacity

The results showed that all treatment and solvents influenced the total polyphenols extraction. Significant differences were found between OH and the CN. A decrease in TPC was observed in CN when compared with OH and the CP method. Although OH and CN treatments use high temperatures in the OH, a maximum of 2 s was required for the temperature to reach 100 °C, while in the CT method, 20 min were necessary to reach the same temperature.

Both heating processes and solvent used affected the TPC. It can cause leaching, and consequently, the TPC can decrease, or it could affect the rupture of the cell wall and, as a result, the release of cell-bound polyphenols. Several studies reported the effect of heating processes on polyphenols content (Achir et al., 2016; Marra et al., 2009; Oliveira et al., 2015; Pereira et al., 2020; Rocha et al., 2019). A study carried out by Pereira et al. (2020) showed that it is possible to obtain higher extraction yields of TPC and anthocyanins with aqueous extracts with an OH pre-treatment. The fast heating prevents the increase of compounds degradation. Additionally, the polarity of the solution used for polyphenols extraction affects its cell availability, and it also changes the recovery of the TPC. In moderate settings (23 °C), pre-treatments with limited permeabilization effects and no organic solvent have only encouraged the diffusion of small molecular weight hydrophilic components. This explains the differences in the TPC levels across pre-treated OH and CP (p>0.05) samples. In general, the flavonoids in which anthocyanins are contained are low molecular weight molecules and hence easily extracted due to further OH-induced permeabilization.

A significant impact of the treatment was observed when compared to the impact of the solvent by the application of the two-way ANOVA and Tukey's tests. The treatment accounts for 14.94% of the total variance, with significance p<0.001, while the solvent account for 66.64% of the total variance. The same was observed for AA, according to the ABTS method (Figure 2). Regarding AA, there were differences between results obtained by ABTS and ORAC methods, which could be explained by the sensitivity of methods and the compounds recovered during the extraction processes (Abreu et al., 2019; Coscueta et al., 2019). The ORAC evaluates the AA and determines the antioxidant status in biological systems, while ABTS measures the reduction of the specific force. In addition, different solvents and extraction methods could recover different BC, which could justify the differences in values.

This result suggested that for TPC and AA, the solvent has more impact than the treatment used. Mostly, according to several studies, the inhibition of polyphenol oxidases and acid hydrolysis could occur; as a result, the TPC increases, and consequently, the AA also increases (Fallis, 2013; Pereira, et al., 2016). The differences found in TPC and AA when citric acid ($C_6H_8O_7$) or lactic acid ($C_3H_6O_3$) were used could be explained by its chemical structure and its pH solution, 2.55 and 2.98, respectively. Studies have shown that the nature of the acid employed, along with its pka, influences the selectivity of the extracting medium toward phenolic compounds (Tzima et al., 2015). Furthermore, the chemical properties of anthocyanins make them susceptible to reactive oxygen molecules.

6.4.2. Total and Individual Anthocyanin Content

Significant differences in total anthocyanin in OH extracts were observed compared to the CP method for food-grade solvents, indicating the greater anthocyanin recovery capacity of red pigments with OH. Anthocyanins are water-soluble glycosides of polyhydroxy and polymethoxy derivatives of 2-phenyl-benzopyryliurn. Furthermore, the pH solution influences the anthocyanin's colour due to its ionic structure. They present a flavylium form in an acidic condition, being highly soluble in water, and they are also more stable (Khoo et al., 2017; Lee & Shibamoto, 2002). In addition, some studies

have shown a disintegration of cell wall caused by electric fields during OH, which could increase the compound's availability and, consequently, their extraction capacity [15,17,34].

Regarding CN, the time to reach 100 °C is significant. In this work, higher levels of anthocyanin degradation were observed in CT samples (exposed to high temperatures for an extended time). These results showed that higher temperatures could promote the degradation of anthocyanins compounds. According to results reported with the literature, the anthocyanins degradation levels could reach 55% with higher temperature usage (Queiroz et al., 2009). Furthermore, some studies also indicate that (Loypimai et al., 2015; Moongngarm et al., 2019) the heating process and the electric field could potentiate synergies affecting enzymatic activities, such as the polyphenol oxidase activity, which indirectly degrades the monomeric anthocyanins during enzymatic browning (Minatel et al., 2017; Turfan et al., 2011). Eventually, the electric effects can activate or inactivate enzymes, since there are studies that report effects on the enzymes (Brochier et al., 2018; Funcia et al., 2020; Kubo et al., 2020; Leite et al., 2019). However, further studies would be needed to confirm whether the same is true in this case. In addition, the electric fields can promote a selective extraction or even a change in the structure, similar to what happens with proteins (Ferreira-Santos et al., 2020; Pereira et al., 2018, 2021; Rakic et al., 2015). However, it is not known whether the same is true for anthocyanins, and further studies are needed to understand at a fundamental level the interactions between electric fields and anthocyanins molecules.

Regarding individual anthocyanins content, Pereira et al. (2020) used water to recover phenolic compounds, and they obtained higher rate yields using OH as pre-treatment to anthocyanins extraction. They only compared OH extraction with the heating process without comparing CONV methods (organic solvents). The authors extracted anthocyanins with OH, which avoids chemical solvents, by using water and reduced treatment times. The main anthocyanins found in this study were malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, and delphinidin-3-*O*-glucoside, which is in agreement with our results. In addition, they present higher cyanidin-3-*O*-glucoside values than delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside, while we present lesser cyanidin values compared with delphinidin. The differences found between anthocyanins content could be explained by the extraction method and solvents used. Rackic et al. (2015) showed that an alkaline pH increases the cyanidin contents in the extracts, while the opposite happens for pH values ranging from 2.0 and 4.0.

6.4.3. Antimicrobial Properties

Polyphenolic compounds, including anthocyanins, have antimicrobial activity against a wide variety of microorganisms, particularly in inhibiting the development of food-borne pathogens. Anthocyanins demonstrate antimicrobial action through various mechanisms, e.g., causing cell damage by damaging the cell wall, membrane, and intercellular matrix (Khoo et al., 2017). The amounts of phenolic compounds showing antibacterial action correspond to those previously reported. Polyphenols from Touriga Nacional and Preto Martinho wine by-products were isolated by (V. Silva et al., 2018), and they showed strong antibacterial action against different pathogens. These are similar to the findings obtained for the non-conventional extraction OH with water acidified with citric

acid, presenting significant differences compared with CN (p<0.05). The CP method displayed similar results regarding antibacterial activity. These microorganisms are usually linked with food as indicators of pathogenic microorganisms. The extraction process of BC affects the antimicrobial activity of the recovered compounds (Pourhashemi et al., 2019; Valle et al., 2016).

Additional investigations with *Staph. aureus* showed that doses as low as 1.6 g/100 g of total phenolics might have a large inhibitory impact on the development of MRSA and MSSA biofilm, but chlorogenic acid was the primary component in this case rather than anthocyanin (Khoo et al., 2017). The possibility that the OH method could be a selective extraction method for certain compounds, as mentioned above, may potentiate this antimicrobial effect when compared to CN. According to Table 1, the main anthocyanin present in this extract is the malvidin 3- *O*-glucoside. The antibacterial activity of anthocyanin-containing extracts may be caused by the diverse processes and synergy effects of distinct extract phytochemicals such as anthocyanin, phenolic acids, and their chemical combinations (Khoo et al., 2017).

In addition, CN uses a longer thermal effect (100 °C, 20 min). Therefore, it can degrade some more sensitive compounds, including anthocyanins, which have this antimicrobial effect, as described in the literature (Ghada et al., 2020; Silva et al., 2016). Other studies have shown that Gram-negative bacteria but not Gram-positive bacteria are inhibited in anthocyanin-rich extracts. This may be related to the distinct cell wall structures between Gram-negative and Gram-positive bacteria in which the outer membrane of Gram-negatives functions as a preventative barrier for hydrophobic compounds but not on hydrophilic compounds (Khoo et al., 2017). Côté et al. (2011), showed the antibacterial activity of cranberry extract in vancomycin-resistant *Enterococcus faecium*, *P. aeruginosa*, *Staph aureus*, and *E. coli*. The antibacterial action of cranberries extracts is not related to their low pH, but it is likely to be attributable to bioactive elements, such anthocyanin and flavonols, in pH-adjusted cranberry extracts.

The results show the potential use of OH extracts against both Gram-negative and Gram-positive bacteria.

6.4.4. Cytotoxicity

The XTT method is an excellent technique for measuring cell viability. Only methanol extracts presented inhibition of cell viability in the case of the CP samples higher than 30%, demonstrating a cytotoxic effect. The results are following the literature, which reports the cytotoxicity of methanolic extracts [54]. In addition, no evidence of cytotoxicity was found with water grape extracts (Bozkurt et al., 2012).

6.5. Conclusion

The present study assessed the recovery of anthocyanins based on thermal and solvent treatments of grape by-products by OH, CP, and CT extraction methods. The present study assessed for the first time the recovery and characterization of anthocyanins based on thermal treatments of grape by-products by OH combined with acidified food-grade solvents. OH with water acidified with citric acid allowed higher extraction yields of total polyphenols content when compared with other

methods. Furthermore, it is possible to obtain extracts with higher AA in the case of OH with water acidified with citric acid than obtained with MeOH. Total anthocyanins recovery was higher with OH and citric acid application. This treatment yielded similar results when compared with the CP method. The main anthocyanins recovery was of malvidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, and petunidin-3-*O*-glucoside. No cytotoxicity was found for OH extracts obtained with citric acid at 1 mg/mL. On the other hand, for the CP method with MeOH at the same concentrations, there was an inhibition of cell viability of 80%. Additionally, OH with citric acid at 1 mg/mL exhibited antimicrobial properties against pathogens, namely *P. aeruginosa*, *Y. enterocolitica, S.* Enteritidis, MSSA, MRSA, and *B. cereus*.

OH combined with food-grade solvent (water and citric acid) allows the recovery of stable anthocyanins, which is in line with the European Union Directive 2009/32/EC. These results demonstrate a relevant opportunity to valorize red grape by-products in a circular economy context.

Chapter 7.
Extraction of tomato by-products' bioactive compounds using ohmic technology

Abstract

Tomato peels and seeds are the main by-products of the tomato industry and represent an interesting source of bioactive compounds (BC) including carotenoids, which can be then used as colorant to commercial aquaculture. The aim of the present work was to optimize the extraction of BC from tomato by-products using Ohmic heating (OH) technology. OH extraction experiments were done in the presence of moderate electric fields (MEF) of different intensity (i.e. 4, 6 and 11 V.cm-1) to identify the presence of non-thermal effects on the extraction process and its influence on bioactive properties of the extracted compounds. Polyphenol extraction using OH was successfully optimized with the best extraction conditions being 70 ºC for 15 min using 70 % ethanol as a solvent, which exhibited rutin recovers of 77 % higher than control samples. It allowed to recover up to 4.93 µg/gFW lycopene from tomato by-products without resorting to organic solvents. OH can be used as an environmental-friendly, fast and economic process to polyphenols recover from industrial tomato by-products. In addition, the use of different MEF during extraction shows to have a high potential to cause different levels of permeabilization and cell stress that can help to define a selective extraction process of valuable components from tomato by-products.

Keywords: *Lycopersicon esculentum*; Tomato by-products; Ohmic heating; Extraction optimization; By-products valorization; New extraction method; Polyphenols; Carotenoids

7.1. Introduction

Annual tomatoes (Lycopersicon esculentum) production worldwide is 170 million tons, which 127.5 million are for the fresh consumption and 42.5 million are for the industrial processing, being one of sectors with the greatest impact in the agro-food industries (FAO, 2017; Heuvelink, 2018). Processed tomato products often result in higher amounts of by-products, namely, skin, pulp and tomato seeds. They are excellent sources of bioactive compounds (BC), such as vitamins, β-carotene, lycopene, flavonoids, which can be used as synthesis of pharmaceutical, colorants and food products (Figueiredo-González et al., 2016; Galanakis, 2017).

However, current extraction treatments involve application of solvents and leaching, which besides being a hazard to the environment, can cause degradation of these compounds, promote their toxicity and reduce their biological properties and health benefits, thus hampering their added value (Ameer et al., 2017; Caldas et al., 2018; Tommonaro et al., 2008). Therefore, it is imperative to search for new alternatives for extraction methods that can bring added value to these by-products. OH is one of these emergent extraction method, and is based on passage of alternating electrical current through semi-conductive materials allowing the generation of internal heat due to inherent electric resistance of the product to be treat (Brochier et al., 2018; Gavahian et al., 2016; Pereira et al., 2016; Seidi Damyeh & Niakousari, 2017).

OH has great potential for achieving rapid, uniform and precise heating in foods, providing a wide range of food processing applications including pasteurization, sterilization, microbial inactivation, fermentation, cooking, blanching, thawing, starch gelatinization, enzyme stabilization (Anderson & Finkelstein, 1919; T. Kumar, 2018; Pereira et al., 2018; Pereira, Rodrigues et al., 2016).

It may be very attractive to enhance the extraction of BC with lower energetic costs (> 95% of energetic efficiency) and better product quality (Brochier et al., 2018; El Darra et al., 2013; Hogervorst Cvejić et al., 2017; Pereira et al., 2018). Nevertheless, there is a great gap in knowledge between the interaction of the electric field and the BC, e.g. if the electric fields are sufficiently to cause bound-breaks, change chemical structures and BC degradation, or regarding cell wall can cause differences in its permeability and increases extraction, being imperative studies to verify the effect of the OH in these compounds.

This technology is not possible to apply in non-conductive food systems, as well as, it is also difficult to apply it in non-homogeneous food systems. Furthermore, many systems/foods are rich in proteins can lead to deposit formation on the surface of OH electrodes, that if not properly cleaned can result in an electrical arcing. The disadvantages aforementioned can be easily controlled (Kumar, 2018; Ramaswamy et al., 2014).

Although OH has been applied for some time, there are few studies on its influence as an extraction method of bioactive polyphenols and carotenoids from tomato by-products (Hogervorst Cvejić et al., 2017; Rajha et al., 2014).

Hence, in the present work, the influence of OH and moderate electric fields (MEF) of different intensities, in the extraction of BC from tomato by-products were evaluated in order to value this fraction in a sustainable industry system.

7.2. Materials and methods

7.2.1. Samples

Fresh samples of industrial tomato by-products (seeds and skins) were collected from the company Sugalidal in Santarém, Portugal. After being collected, the dry matter of samples was measured, and by-products were stored at -80 ºC until further analysis.

7.2.2. Extraction strategy evaluation

In order to understand best approach to OH technology (using as pre-treatment step, A and B, followed by extraction at room temperature or by assisting thermal-extraction, C) an initial set of tests were performed –. For pre-treatment A, OH was applied directly to 2.5 g of tomato by-products; in this case the maximum temperature which system was able to achieve was of 55 ºC (with a holding time of 1 min). This temperature was then used for the remaining treatments for a proper comparison. For pre-treatment B, 2.5g of tomato by-products were previously washed in a 0.1 M sodium chloride solution (NaCL) before OH application in the solid residue (with a holding time of 1 minute) this treatment allows the increase of extract conductivity and also allows reach at 55 ºC in short time; in both pre-treatment A and B the extraction was then performed with 12.5 mL (1:6 w/v) of 70% water:ethanol absolute solution (during 0, 10, 20 and 30 minutes) at 55 ºC. For C, an OH assisted extraction was performed by combining the same solvent solution prepared before (70% of water:ethanol absolute (v/v) with OH at 55 ºC. For all the water:ethanol extraction was performed for 0 (only the time to reach target temperature), 10, 20 and 30 minutes.

7.2.2.1. Ohmic heating extraction

A cylindrical glass reactor with 30 cm total length, an inner diameter of 2.7 cm and two stainless steel electrodes placed at each edge isolated by polytetrafluoroethylene (PTFE) caps, were used to OH assisted extraction from tomato samples, as referred by Pereira et al. (2016). The supplied voltage ranged from 60 to 280 V, during 30 min, with different temperatures (0 to 100 ºC). Electrodes used stainless steel 316 and OH was performed at 25 kHz. This combination avoid corrosion and electrochemical reactions (Pataro et al., 2014). A function generator (Agilent 33,220 A, Bayan Lepas, Malaysia; 1 Hz- 25 MHz and 1-10 V) connected to an amplifier system (Peavey CS3000, Meridian, MS, USA; 0.3 V-170 V) was used to control system.

7.2.2.2. Conventional heating (CONV)

A reactor vessel with double-walled (3 mm of internal diameter and 100 mm height) was coupled with a circulating thermo-stabilized water bath to perform conventional extraction at controlled temperature, as reported previously (Pereira et al., 2010).

Both methods (OH and conventional) presented a similar thermal history as well as the same solutions were used. All procedures were recorded to ensures having exactly similar conditions for both methods.

7.2.3. Total Antioxidant Capacity and Phenolic Content

Total antioxidant activity (AA) was determined using the ABTS method as described by Gião et al. (2007). The results were given in ascorbic acid equivalent.

The total content of polyphenol compounds (TPC) in the extracts was evaluated through the Folin – Ciocalteu spectrophotometric method as described by Singleton & Rossi J A Jr. (1965) and expressed as milligram of gallic acid equivalent.

7.2.4. Total Carotenoids

Total Carotenoids (TC) was assed using a spectrophotometric analysis, as described by Kimura, Rodriguez-Amaya, & Godoy (1990) and are expressed in equivalent β-carotene.

7.2.5. Color

The nonspecific turbidity was determined by optical density at 600 nm using a Versamax Elisa microplate reader with polystyrene 96-well microplates (Nunc, Denmark) as described by Hodges, DeLong, Forney, & Prange (1999).

7.2.6. HPLC-analysis

Qualitative and quantitative profiles of polyphenols were carried out according to the method proposed by Oliveira, Ferreira, & Silva (2015), with slight modifications. Analysis was conducted on HPLC-DAD (Waters Series 600. Mildford MA. USA). A Symmetry® C18 column, 250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size with a guard column (waters), was used and solvents elution consisted of solvent A – Acetonitrile (100%) with 0.2 % TFA; Solvent B: acetonitrile/water (5:95 v/v) (Merck pure grade and pure water) with 0.2% TFA (Sigma-Aldrich, Germany); Samples were analyzed in triplicate. Calibration curves were obtained at a detection wavelength 280 nm. Standards solutions over the concentration range from 0.10 to 100.00 mg/L were prepared for the identification and quantification of the following compounds: rutin, naringenin and Kaempferol (Sigma, Sintra, Portugal) expressed as µg per mL of fresh weight (FW) biomass of tomato. All calibration curves were linear over the concentration ranges tested, with correlations coefficients of 0.999.

Carotenoids content was also analysed by HPLC-DAD (Vydac 201TP54 C-18 column, 250 mm - 4.6 mm), equipped with a C-18 precolumn.

Chromatographic separation was performed as described by Oliveira et al., (2004). Solvent A with ethyl acetate (Merck pure grade) and solvent B 90:10 acetonitrile:water (Merck pure grade and pure water, 1.0 ml/min flow rate, at room temperature.

The UV–vis detector was set between 270 and 550 nm. Individual carotenoids were quantified and from a calibration curve built with pure standards: β-Carotene, lycopene, zeaxanthin and lutein (Extrasynthese, Genay Cedex, France) and expressed as milligrams per kilogram of fresh product.

7.2.7. Extraction optimization

Based on results obtained according methodologies applied in 2.2.1 and 2.2.2 and to better understand both temperature, organic solvents ratio and time effects on BC extraction a design of experiments (DOE) was now applied. Twelve experimental combinations were determined according custom design, n=23, consisting of a 3^3 factorial design with three levels (−1, 0, +1). The independent variables were as follows: time (0-30 minutes); temperature (35 °C-100 °C); and percentage of solvent (0-70%). Total phenolic compounds (TPC), antioxidant activity (AA), total carotenoids (TC) and colour were used as dependent variables. Table 1 shows the combination of the different independent experimental variables.

Based on data from phenolic, antioxidant and carotenoids compounds, and overall linking, a response surface was fitted to data, by adjusting a quadratic polynomial equation (equation 1):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_2x_3 + b_{13}x_1x_2 + b_{23}x_1x_3$$

Equation 1

Were b0 represents the constant term, b1, b2 and b3 linear effects, b21, b22 and b33, represents quadratic effects and b12, b13 and b23 the interaction effects, namely, time (x1) temperature (x2) and ethanol (x3). ANOVA was performed in order to assess the regression coefficients used.

7.2.8. Moderate Electric fields (MEF) effects

The OH in concomitance with MEF can causes changes on polyphenols and carotenoids. Thus, after determining the best extraction process with the experimental design, the impact of temperature and MEF intensity on BC extraction was assessed. Three sodium salt concentrations (0.514, 0.257 and 0.120 mol/L) were added to the extraction solutions to increase the electrical conductivity and to allow working at different electric field strengths – i.e. 4, 6 and 11 V.cm-1, respectively-, thus ensuring the generation of heat within the mixture (Table 7.2). Three levels of MEF were used at the same temperature profile in order to evaluate possible non-thermal effects of the OH. An experiment was also performed where a maximum voltage was applied on solution with moderate electric conductivity (0.257 mol/L) in order to create a fast OH extraction and decrease the thermal load of OH (by this procedure, the extraction temperature was reached very fast, in 2 seconds; the voltage was immediately reduced to maintain the temperature). The conventional extraction method (without the presence of electrical variables) was performed in the solutions of different electrical conductivities benchmarking all OH performed. All experiments were done in triplicated.

7.2.9. Statistical analysis

Data analyses were performed using STATISTICA software v.12 (StatSoft, Inc., USA) and STATGRAPHICS Centurion software v. XVI.I. A descriptive statistics (mean and standard deviation) were performed, and a two-way ANOVA was applied at a 95 % confidence level.

The Multiple Factor Analysis (MFA) was applied to assess the consensus among the different variables analysed: total phenolic compounds, antioxidants, colour and carotenoids. Additionally, aggregated data was used to perform a PCA, identifying major relationships between variables.

A screening of DOE was executed considering the responses obtained. Moreover, to elaborate the predictive statistic model, according to Rsquare and Rsquare adjusted values, the independent variables that influenced the dependent variables behaviour were selected. Finally, optimal conditions were obtained by settings desirability values to each responses type to obtain maximum desirability. Additional trials at the optimum predicted conditions were performed in order to validate the models obtained and experimental results were compared with the predicted ones.

All analysis was performed in triplicated, and a standard deviation was calculated.

7.3. Results and Discussion

7.3.1. Influence of extraction conditions

In this study, a solvent system based on water-ethanol was used to attain a green extraction of the BC polyphenols and carotenoids from tomato by-products. In the first part of this study, OH was used as pre-treatment preceding the extraction with water/ethanol at 55 ºC in an in attempt to soften tomato tissues, but an increase in the contents of total carotenoids and total phenolics of the extracts were only observed during OH-assisted extraction with water/ethanol (Figure 7.1).

The application of OH pre-treatments was reported to induce the permeabilization of cell membranes and facilitate the polyphenolic extraction with ethanol addition (El Darra et al., 2013). Nevertheless, this sample is solid and poorly conductive, which requires applying higher electric fields to the temperature reaching 55 ºC, leading to a degradation of the most sensitive compounds such as carotenoids. During this process, several mechanisms may be occurred, including a highest internal heating of samples, electrode reactions and the electrolysis of samples, thus the BC degradation was caused by heat and by electrochemical degradation. In addition, both degradation reaction mechanisms and kinetic parameters may be influenced by reactions between the electrode materials and the electrolysis products (Kaur et al., 2016). In this way we consider the treatment C an opportunity to develop a new method for BC extraction. Furthermore, several studies, have reported that the recovery of different BC, such as polyphenols and carotenoids, depends both on the treatment time and temperature, as well as solvents used (Lapornik, Prošek, & Golc Wondra, 2005). This information together with the results obtained was decisive to study the potential of OH for BC recovery, due its ability to achieves fast and relatively uniform heating rates. Thus, the treatment C was used for the following experiments.

Table 7.1. Experimental combinations of temperature, time and % of ethanol of tomato wastes obtained from experimental design and the total carotenoids equivalent of β-carotene and lycopene in extracts from tomato by-products.

Temperature (ºC)	Time (min)	Ethanol (%)	Total carotenoids (mg eq. β-caroten**E**/g FW)	Lycopene (µg/gFW)
40.0	0.0	0.0	0.080 ± 0.012 ^{d}	2.253 ± 0.002 ^{c}

40.0	15.0	70.0	0.178 ± 0.011[b]	n.d.
40.0	30.0	35.0	0.195 ± 0.020[a,b]	n.d.
55.0	0.0	70.0	0.126 ± 0.017[c]	n.d.
55.0	15.0	35.0	0.237 ± 0.085[a]	4.926± 0.003[b]
55.0	30.0	0.0	0.093 ± 0.007[c]	2.339 ± 0.001[c]
70.0	0.00	35.0	0.096 ± 0.005[c]	4.772 ± 0.011[b]
70.0	15.0	0.0	0.163 ± 0.031[b,c]	n.d.
70.0	30.0	70.0	0.099 ± 0.003[c,d]	2.939 ± 0.002[c]
55.0	15.0	35.0	0.116 ± 0.027[c]	2.566 ± 0.003[c]
55.0	15.0	35.0	0.115 ± 0.010[c]	7.108 ± 0.122[a]
55.0	15.0	35.0	0.133 ± 0.040[c]	2.521 ± 0.001[c]
70.0	15.0	35.0	0.240 ± 0.006[a]	n.d.

Values were present as mean ± SD. [a] Different letter represent significant differences ($p < 0.05$) in comparison to the original contente

Table 7.2. Ohmic and conventional treatments applied on by-products from tomato (temperature, time, ethanol, conductivity, salt concentration and electric field)

Assay nr.	Treatment	Temperature (ºC)	Time (min)	Ethanol (%)	Conductivity ($ms.cm^{-1}$)	Salt concentration (mol/L)	Electric Field ($V.cm^{-1}$)
1	Conventional	70	15	70	1.00	0.12	n/a
2	Conventional	70	15	70	3.99	0.25	n/a
3	Conventional	70	15	70	8.17	0.51	n/a
4	OH	70	15	70	1.00	0.12	11
5	OH	70	15	70	3.99	0.25	6
6	OH	70	15	70	8.17	0.51	4
7	OH	70	1	70	3.99	0.25	6
8	Control	25	15	70	1.00	0.12	n/a
9	Control	25	15	70	3.99	0.25	n/a
10	Control	25	15	70	8.17	0.51	n/a

7.3.2. Extraction optimization

The agro-industrial sector is interested in the development of affordable green extraction processes, that reduce/eliminated the organic solvents and elevated temperatures (Ribeiro et al., 2015). Furthermore, the extraction efficiency of BC, is affected by their chemical nature, particle size, solvent and extraction method (type of solvent, polarity, pH, temperature, extraction time) used and the synergy due the presence of interfering substances (Do et al., 2014). Accordingly, for the second part of this work, the effect of OH with various treatment times, temperatures and concentrations of ethanol on the antioxidant activity, polyphenols and carotenoids were evaluated. An experimental design was constructed to reduce the number, time and cost of the experiments.

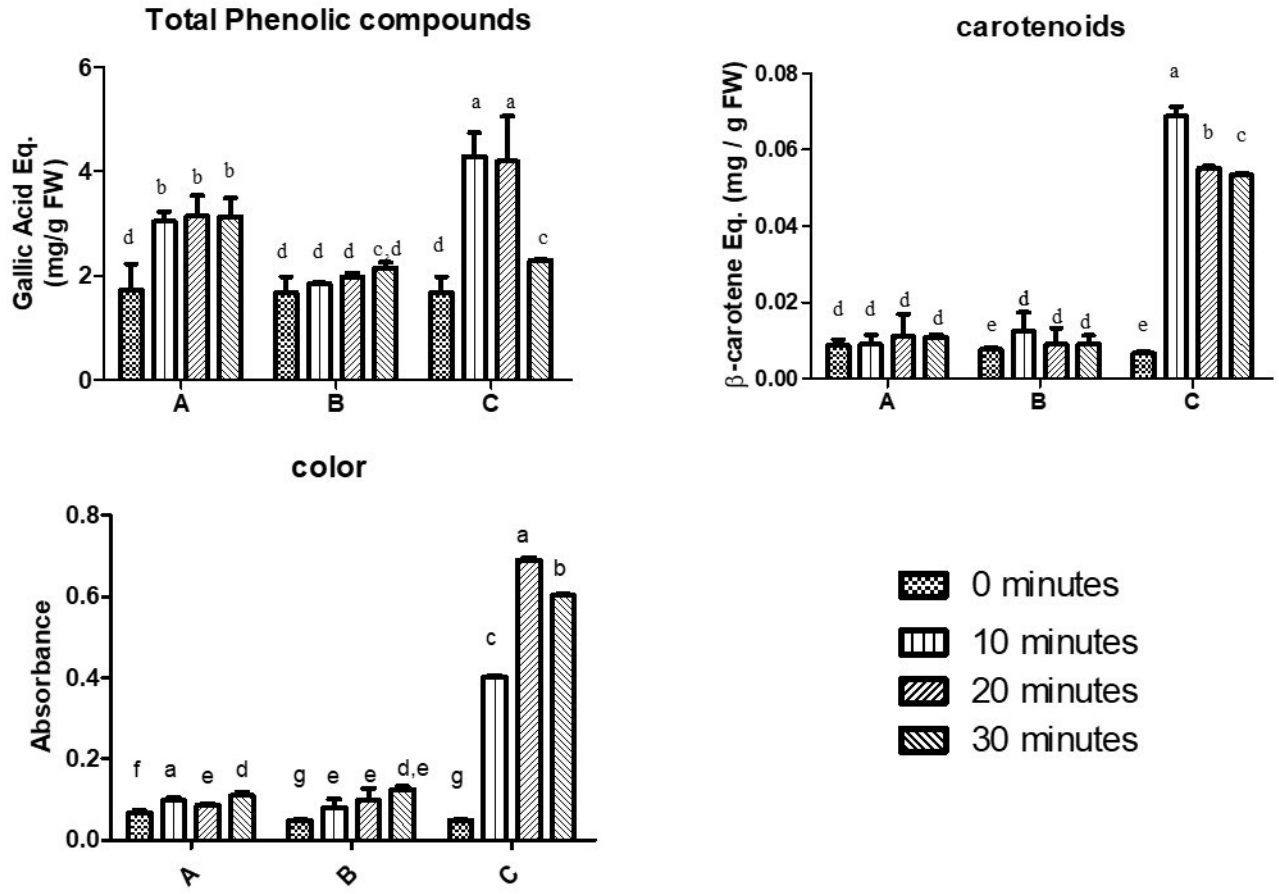


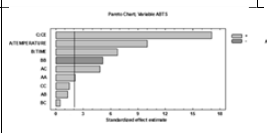
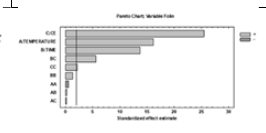
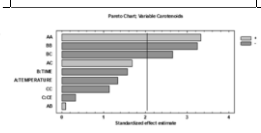
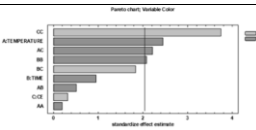
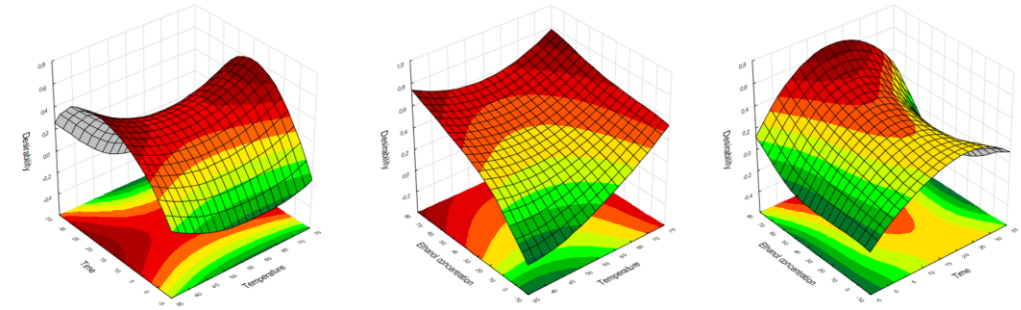
Figure 7.1. Influence of different ohmic treatment (same electric field applied, 6 V.cm-1)approaches on color, carotenes and total phenolic compounds. A) OH treatment directly applied on tomato by-products; B) OH applied to samples of tomato by-products previously merged in NaCl; C) OH treatment on samples constituted by tomato by-products samples and a solution of water:ethanol. [a] Different letters represent significant differences ($p < 0.05$) in comparison to the original content.

7.3.3. Experimental Modelling and Statistics

All response values were demonstrated by statistical analysis to fit best the quadratic order polynomial equations expressing the relation between the experimental parameters and the response variables. The regression models were used to fit a linear model and consequently the data. Furthermore, the adjusted model was adequate and a good representation of the behaviour of the system, with R2 values for antioxidant capacity, total polyphenols, total carotenoids and colour of 0.97, 0.98, 0.53 and 0.76, respectively. Significant parameters affecting thermal extraction process are highlighted in Table 7.1.

According experimental model, the better conditions to obtain higher antioxidant capacity with application of 70 ºC, 70% of ethanol during 15 min of extraction (Table 7.3).

Table 7.3. Statistical Analysis of the effects of temperature, time and ethanol percentage on antioxidant capacity, phenolic content, total carotenoids and colour of tomato by-products.

Variables	Antioxidant capacity	Total phenolic content	Total carotenoids	Colour
Statistical analysis (pareto diagram)				
Desirable Analysis (Spline Fit) Optimal values for all dependent variables > 0,6 < 0,6 < 0,4 < 0,2 < 0 < -0,2				

[a] Vertical line in the pareto diagram represent significant differences ($p < 0.05$). CE is the ethanol concentration.

To total polyphenolic content R-quadratic, explain 98.0% of total polyphenols variability. The standard error of the estimate shows that the standard deviation of the residues is 0.084. The absolute mean error (MAE) of 0.055 is the average value of the residuals.

The R-Squared statistic indicates that the model, thus adjusted, explains 76.4% of the variability in color. The adjusted R-squared statistic, which is more adequate to compare models with different number of independent variables, is 69.0%. The standard error of the estimate shows that the standard deviation of the residues is 0.05. The absolute mean error (MAE) of 0.032 is the average value of the residues.

Results showed a high degree of correlation between all observed and predicted values, indicating a reasonable agreement of the corresponding model with the experimental results was found.

The results showed that, the variables have different weights for each parameter studied; for instance, the ethanol concentration is the main parameter controlling color intensity and extraction of AA, TPC, while, for carotenoids extraction the most significant variable is the temperature. This may be attributable to the higher content of non-phenolic compounds present in tomato by-products. Furthermore, these extracts are rich in lipophilic molecules, mainly sterols, terpenes and carotenes, which are more soluble in organic solvents (ethanol) (Do et al., 2014). To better understand the results and to obtain the optimum condition, of temperature, holding time and ethanol percentage, to extract simultaneously the maximum concentration of variable dependents (antioxidant activity, total and individual phenolic compounds, carotenoids) a desirability function was applied (Table 7.3). This function is based on transformation of the original each response in a dimension desirability scale, assigning values between 0 (undesirable value) and 1 (completely desirable/ ideal response value) (Coelho et al., 2017). The optimum extraction conditions found is described below.

7.3.3.1. Total antioxidant activity and polyphenols content

To determine the best conditions of extraction, as previously described, an experimental design was performed with different conditions of time, temperature and ethanol concentration using an OH system. The results of antioxidant activity show that ethanol concentration influences the antioxidant activity, followed by the temperature and time ($p<0.05$) (Table 7.3). A significant difference was observed between the effects of temperature and ethanol concentration ($p < 0.05$).

In accordance with different conditions of DOE the AA ranged from 0.106 to 1.920 mg/ g FW, and this is in accordance with the published literature. Toor & Savage reported concentrations of AA ranging from 15.7 and 0.186 mg/ g FW in skins of tomatoes (Toor & Savage, 2005). For TPC they found concentrations ranging from 0.244 to 2.558 mg GAE/g FW which is in accordance with our results. Higher values of TPC and AA may be found in ethanol extracts when compared with water extracts, due the lower polarity of ethanol than water. Cell walls and seeds have unipolar character and for this reason ethanol is more efficient in recovery of polyphenol compounds (Lapornik et al., 2005). An increase of TPC was also revealed with temperature and percentage of ethanol; extraction performed at 70 ºC with 70% of ethanol during 15 min gave rise to 2.821 ± 0.211 mg gallic acid equivalents/g Fw, a much higher value than the value of 0.403 ± 0.019 mg gallic acid equivalents/ g Fw in the control (40 ºC, 0 min, 0% ethanol).

In this way, the response surface analysis showed that the percentage of ethanol influenced both antioxidant activity and phenolic compounds extraction (Table 3), being in accordance with results reported by Lapornik et al. (2005).

Temperature and time have also significant effects on total polyphenols extraction ($p < 0.05$); polyphenolic content increases with increasing temperature. As mentioned before, these by-products extracts are rich in lipophilic compounds that could form complexes with polyphenolic compounds thus enhancing their solubility in ethanol (Do et al., 2014).

The results obtained indicate that treatment temperatures of 70 ºC are suitable for the extraction of compounds (higher content of phenolics), with higher antioxidant capacity and distinctive phenolic composition when compared to the extraction at 40 to 55 °C ($p<0.05$). The combination of the different factors showed that to obtain higher levels of extraction of total phenolics treatment conditions should be as follow: 70 ºC, during 15 minutes with 69.9 % of ethanol. The combination of OH and temperature can be an effective alternative method for extraction of phenolic compounds, which supports other studies (El Darra et al., 2013; Khajehei et al., 2017; Loypimai et al., 2015; Pereira et al., 2016).

7.3.3.2. Carotenoids content

In accordance with results of total carotenoids, temperature and time had a positive and a negative quadratic effect, respectively, resulting in the increase of carotenoid levels with increasing temperature and a maximum recovery capacity of these compounds (mainly lycopene and β-carotene) is reached independently of extraction time. In addition, the results for individual carotenoids showed that depending on the conditions the lycopene may be or not extracted (Tables

7.1 and 7.3). Both temperature and time are key for non-degradation of lycopene (Aguilar-Machado et al., 2017). According desirable analysis the best conditions to extract carotenoids are 55 ºC with 70% of ethanol during 15 minutes. The type of solvent and the number of extractions have a significant impact on lycopene extraction due the draw water capacity, while time and solvent to sample ratio have no significant effect (Nunes & Mercadante, 2004). Nunes and Mercadante (2004), evaluated lycopene extraction with ethanol and ethyl acetate, with ethyl acetate being more efficient. The carotenoid concentration ranged from 0.0284 to 0.0935 mg/g FW.

Low temperatures (ambient 23 ºC), may cause degradation due to oxidation without isomerization (Aguilar-Machado et al., 2017; Hackett et al., 2006), while at higher temperatures (125 ºC) isomerization of lycopene may occur with associated losses (Oliveira et al., 2015; Seybold et al., 2004). Dewanto et. al., 2002 refers that at 88 ºC lycopene bioavailability is improved when compared with lycopene in fresh tomatoes. Furthermore, lycopene forms complexes with insoluble fibre located in the outer pericarp and the skin (Dewanto et al., 2002). These results are very interesting and open a door for new studies on the effect of time and temperature on lycopene degradation.

7.3.3.3. Colour

Red colour intensity mainly depends on ethanol concentration and temperature of extraction (Table 3). A positive quadratic effect was shown with increasing of ethanol, while increasing temperature extraction has a significant negative linear effect ($p < 0.05$). In addition, a negative quadratic effect related with the time extraction was also significant ($p < 0.05$). These results suggest a saddle point concerning the effects of temperature, time and ethanol concentration on colour intensity. These results were somehow expected due the relationship between colour and the main responsible by the colour present in tomato, the carotenoids (Kim & Chin, 2016).

7.3.4. Electric Field effects on BC extraction from tomato

Electrical processes are gaining an increasing interest for its application in food processing, including the extraction of polyphenols (Kaur & Singh, 2016). Nevertheless, little is known about the impact of electric fields on structural changes of by-products samples or BC, which also may compromise its bioavailability and extraction. Furthermore, some BC are very thermally sensitive and unstable, such as flavonoids and carotenoids and can degrade with the voltage application. Thus, distinct levels of electric field intensity were applied during thermal extraction, namely 4, 6 and 11 V cm-1 and optimal conditions determined before (70 ºC for 15 min using 70% ethanol). A multivariate analysis was performed to better understand the experimental data, with significant differences being observed between treated and control samples (Figure 7.2). Results showed significant differences between conditions applied, namely the heating mode (OH or conventional) or the intensity of the electric field during OH.

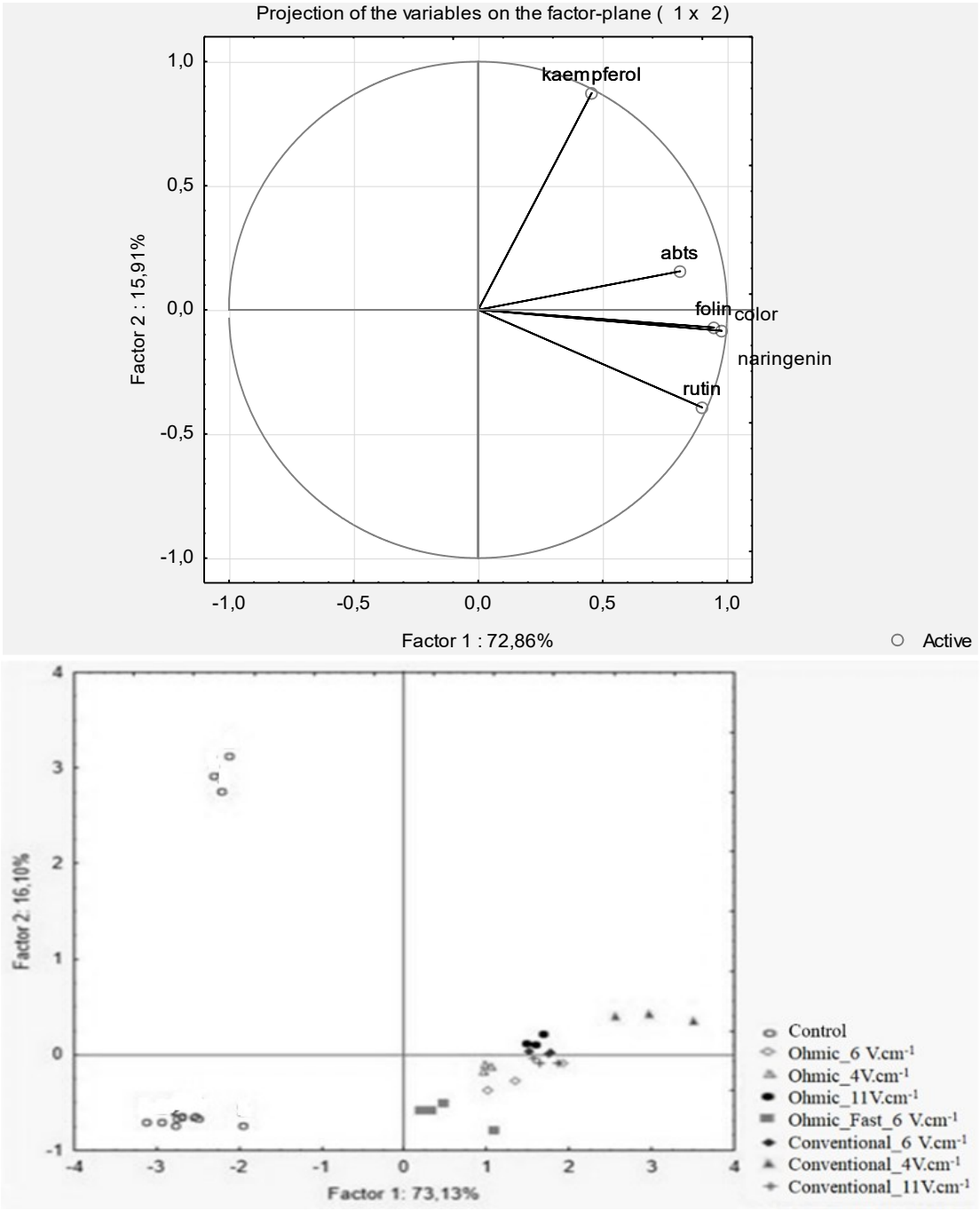


Figure 7.2. PCA analysis to variables studied..

According to the obtained results, no significant differences ($p > 0.05$) were found between control samples with different conductivities, suggesting that salt addition has no impact on extracted antioxidant activity (results not shown).

Concerning polyphenolic content, the method of extraction is more important on the recovery of these compounds, with a more significant effect than with electric field effect (Figure 2). The complexity and diversity of polyphenols (molecular size and structure) makes it difficult to anticipate the effect of electric field (Elez-Martínez et al., 2017). Therefore, the following results consider the extraction

of individual polyphenols, mainly three relevant polyphenolic compounds that were found to be present: naringenin, rutin and kaempferol.

Electric fields have different effects on the recovery of each individual polyphenol compound (Figure 2); moreover significant differences were observed between conventional and OH extractions ($p < 0.05$). According PCA analysis a correlation between antioxidant activity and kaempferol extraction were observed. OH at 11 V. cm-1 influence both antioxidant activity and kaempferol extraction, while OH at moderate (6 V. cm-1) and low (4 V. cm-1) electric fields influences color, naringenin and rutin extraction. Results suggests that at higher electric fields application, there are glycosides break bonds, in chemical structural of rutin with kaempferol formation, as Khan et al. (2018), refers the electromagnetic fields could have the capacity to break down, both -ester and glycoside-bound phenolic compounds.

OH and conventional extractions at 6 V.cm-1 gave rise to an increase in rutin concentration of 82 and 79 %, respectively, when compared to control sample. At this frequency pores can be formed across cell wall altering the membrane permeability and consequently to accelerating the diffusion kinetics (Kaur et al., 2016; Maroun et al., 2017). In addition, the OH extraction used to achieve a high temperature in few seconds, allowed an increase of 77 and 61% in rutin and naringenin extraction, respectively, compared to the control sample. No significant differences were obtained between conventional and fast OH extraction ($p>0.05$). However, it is important to highlight that it was possible to reduce total extraction treatment time for approximately 16% through OH, as less time was required to reach the target temperature, reducing the overall processing time.

Both electric field effects and methods of extraction have significant effects on kaempferol and rutin recovery. OH application allows the temperature of extraction to be reached faster than the conventional method, reducing the overall processing time. The application of a fast heating process may be important on the preservation of heat sensitive phenolic compounds. At the same time, the fast heating and electroporation effects can cause cellular heat stress and consequently structural damages, enhancing the release of certain phenolic compounds (Khajehei et al., 2017; Sakr & Liu, 2014; Sastry, 2008).

Therefore, for a better understanding of the electric field influence, the results were normalized according to equation 2, as shown in figure 3.

$$Electric\ field\ effect = \frac{(OhmicA - ControlA)}{(ConventionalA - ControlA)}$$

Equation 2

Normalization of the experimental data revealed that significant differences were found between 4 and 11 V.cm-1 and 6 and 4 V.cm-1 for all individual compounds detected. The application of 6 and 11 V.cm-1 electric fields showed higher individual compounds recoveries from tomato extracts when compared with 4 V.cm-1 (see Figure 7.3), being this difference significant ($p < 0.05$). Relatively to rutin and kaempferol extraction, no significant difference was detected between 6 and 11 V.cm-1,

while to naringenin there was significant differences between the electric fields applied. Joule effect can induce an electro and thermal-permeabilization of cell membranes, causing disturbances on their permeability, structural alterations, contributing in release of higher amounts of polyphenol compounds (Khajehei et al., 2017). In addition, it was detected two groups of polyphenols (rutin and kaempferol are flavonols and naringenin is a flavanone) with different structural comportments. It means, the increased of naringenin with the electric field application could be attributed to the induction of flavanone synthase when OH treatments were applied (Vallverdú-Queralt et al., 2012).

Regarding carotenoids, see Figure 7.3, significant differences were found at different levels of MEF applied ($p > 0.05$). The effect of electrical fields on BC such as carotenoid needs to be better understood through systematic and fundamental studies. Notwithstanding the low quantities of carotenoids recovered (5 to 10 µg), OH may be a useful source to extract these compounds without their degradation.

In addition, a selective extract can be obtained depending on the treatment applied, as OH can be used to selectively extract some compounds without reducing the efficiency of the extraction process. This is the case for rutin extraction when a fast OH is applied. For kaempferol extraction, both OH with high electric field and conventional treatments with moderate conductivity may be applied. Nevertheless, if the objective is to obtain extracts rich in polyphenolic content, or high in rutin and naringenin, an ohmic heating with 6 and 11 V.cm-1may have shown to be promising approaches. These results may suggest a selective extraction of certain compounds depending on the electric field applied. Thus, OH seems to be an interesting processing tool to improve the extraction of valuable components from vegetable tissues (Medina-Meza & Barbosa-Cánovas, 2015; D. Xue & Farid, 2015).

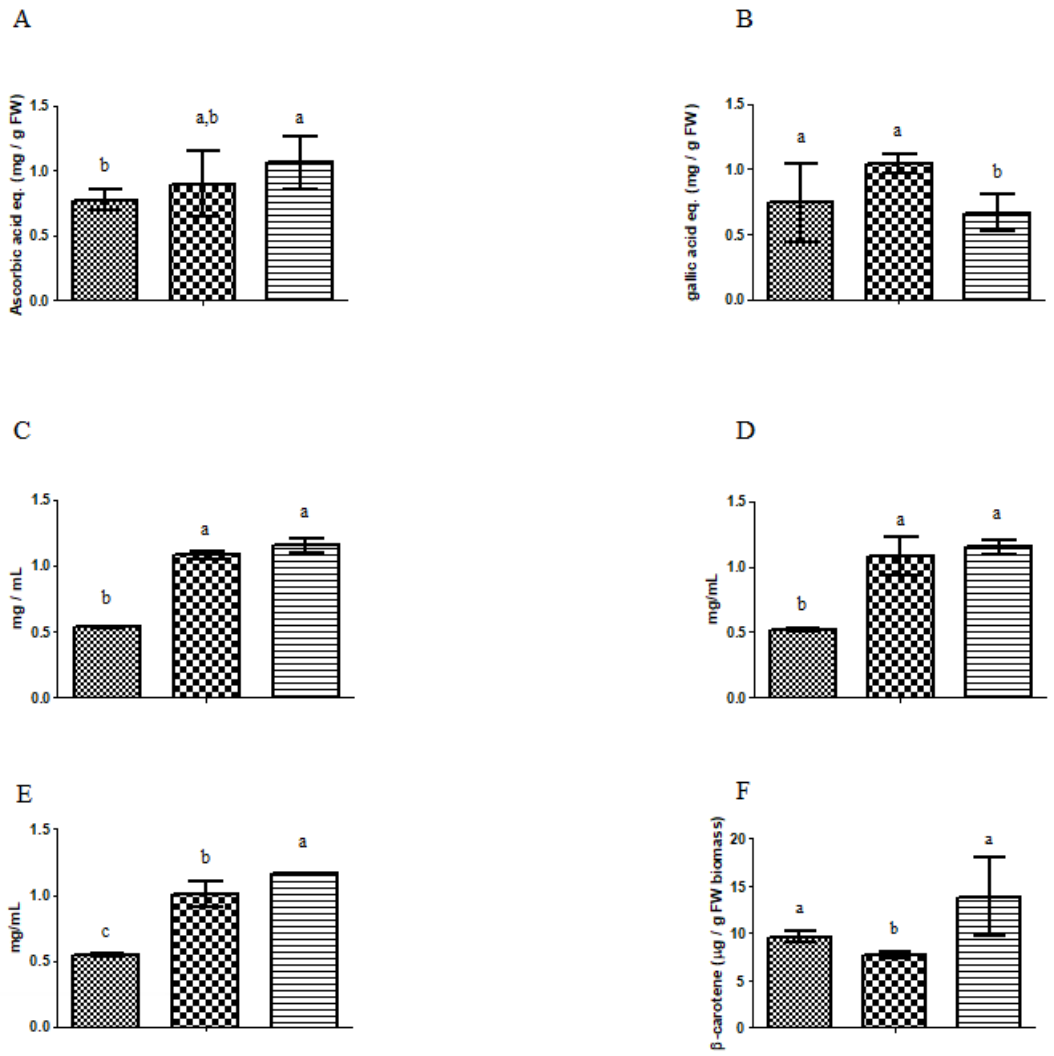


Figure 7.3. Electric fields effects on A) antioxidant activity, B) total polyphenolic content, C) rutin, D) kaempferol, E) naringenin and F) total carotenoids content. 4 V.cm^{-1}, 6 V.c, 11 V.cm^{-1}. [a] Different letters represent significant differences ($p<0.05$) in comparison to the original content.

7.4. Conclusion

Results suggest that OH is an efficient extraction process for polyphenols from tomato by-products with a 58% higher recovery rate than control samples. Relatively to the lycopene and β-carotene compounds the optimization process allows their extraction from tomato by-products without addition of organic solvents (usually useful to obtain these lipophilic compounds). Nevertheless, their extraction yields were lesser when compared with conventional method and new approaches were needed. The Joule effect and fast heating in association with high electric field intensities allowed a significant recovery of polyphenols and carotenoids compounds. In addition, there are a resourceful polyphenols and carotenoids recovery when 6 and 11 V.cm^{-1} electric fields were applied.

In conclusion, OH may be an efficient - as well as selective - technique for the extraction of compounds from tomato by-products that allows same yields to be achieved as in conventional processes but at a higher extraction rate (less time). Furthermore, this technique can be used as an

alternative to conventional organic solvents methods to extract both polyphenols and carotenoids compounds.

PART IV

Bioactives from Tomato bagasse fractions: gastrointestinal digestion

Chapter 8.

Bioaccessibility of bioactives and antioxidant activity of ohmic's tomato (Lycopersicon esculentum) bagasse liquid extracts throughout gastrointestinal digestion

Abstract

A nutrient-rich diet is a key to improving chemical signals, such as antioxidants, which modulate the resistance in the gut and prevent diseases. A current industrial problem is the generation of agri-food by-products with high biological potential, such as tomato bagasse, rich in bioactive compounds (BC), e.g., carotenoids and phenolic compounds, impacting human health. Thus, this study focused on understanding how gastrointestinal digestion (GID) modulates the bioaccessibility of carotenoids, polyphenols and their related bioactivities of two tomato bagasse extracts, one obtained by ohmic-heating (OH) technology and the other by conventional (CONV) methodology based on organic solvents. Results showed that the main phenolic compounds identified in both tomato bagasse extracts by UPLC-qTOF-MS were p-coumaric acid (163 m/z), naringenin (271 m/z) and luteolin (285 m/z). A higher recovery index for total phenolic compounds throughout the GID was observed for OH, while for carotenoids, a strong reduction after stomach conditions was observed for both extracts. Furthermore, colon-available fraction exhibited prebiotic effect upon different *Bifidobacterium* and *Lactobacillus casei*, but showing to be strain-dependent with more emphasis in the case of OH. Thus, the extraction technology highly influences bioaccessibility and bioavailability of BC, with OH demonstrating a positive impact on BC recovery and related health benefits, such as antioxidant, anti-hypertensive, prebiotic, and anti-inflammatory properties, the last being demonstrated for the first time.

Keywords: by-products; ohmic-heating extraction; bioaccessibility; BC; anti-hypertensive; anti-inflammatory

8.1. Introduction

Epidemiological studies and associated meta-analyses strongly suggest that long-term eating of fruits and vegetables is critical to help increase immune well-being and prevent diseases (Galanakis, 2020). Currently, and especially during the COVID-19 epidemic, BC may create a good health state preventing disease through the improvement of immunity, prevent transmission, and minimizing the effect of the virus at its initial stages (Galanakis, 2020).

BC need to undergo enzymatic hydrolysis in the digestive tract or be metabolized by the bowel microflora to be absorbed (Ribeiro, et al., 2020). Their bioavailability and bioaccessibility could depend on the capacity of extraction methods to improve their recovery (Ribeiro et al., 2020).

Also, the ever-growing demand to recover BC from by-products encourages a constant search for accessible extraction methods (Galanakis, 2020). A few studies have applied green technologies as a new extraction method. An example is OH which is used in the food industry to pasteurise (Coelho et al., 2020).

Barba et al. (2015) applied a pulsed electric field, which uses electric voltage pulses, being also solvent-free. The main reasons for the increased bioaccessibility verified in this study are unknown. However, it may be due to the electroporation phenomenon causing electrical breakdown and allowing the perforation of the cytoplasmic membrane promoting leakage of cell content, improvement of BC, and probably solubilization and digestion (Barba, Brianceau, et al., 2015). However, the use of extracts rich in BC does not mean that all of them, when ingested, can pass into the bloodstream and cause beneficial health effects (Ribeiro et al., 2020).

The tomato paste industry generates significant amounts of bagasse, a valuable by-product rich in health-promoting compounds, including polyphenols and carotenoids (Coelho et al., 2021).

The tomato fruit bioaccessibility (Bot et al., 2018; Coelho et al., 2021) has been studied by some authors; nevertheless, there is no straightforward correlation between process-induced matrix delay and BC extractability and bioavailability. Indeed, there are no studies to compare the impact of new extraction technologies, such as OH, with CONV extractions methods on bioavailability and bioaccessibility of BC throughout GIT.

Therefore, the purpose of this study was to evaluate the in vitro GID on the stability, recovery, and biological properties of carotenoids and phenolic compounds, isolated by OH technology and CONV method (organic solvents) from tomato by-products. A static in vitro GID model was used to simulate the digestion (categorized into salivary, gastric and intestinal) to respond i) whether the mouth, stomach or intestine compartment plays a role in the bioaccessibility of both groups of compounds; ii) how the extraction conditions (OH and CONV extract) affect the bioaccessibility and related biological properties of extracted BC throughout the GID.

8.2. Materials and methods

8.2.1. Chemicals and reagents

The 2, 20-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS diammonium salt), potassium sorbate, sodium carbonate, ethylenediaminetetraacetic acid (EDTA), sodium sulfite, and sodium lauryl sulfate, lipopolysaccharide from O111:B4, and E. coli were purchased from Sigma-Aldrich (Sintra, Portugal). Hexane, ethanol, Folin–Ciocalteu's reagent, and potassium persulfate were purchased from Merck (Algés, Portugal). The fetal bovine serum, RPMI 1640, glutamine, Pen/Strep (Gibco, US), ELISAs from Biolegend, US. Standards of ascorbic acid, trolox, gallic acid, rutin, p-coumaric, and 4-hydroxybenzoic acid, were purchased from Sigma-Aldrich (Sintra, Portugal), while kaempferol, β-carotene, lycopene, zeaxanthin, and lutein (Extrasynthese, France) were purchased from Extrasynthese (Lyon, France).

8.2.2. Samples

This experimental work used the extracts recovery from tomato (H1015 Heinz seeds) bagasse (seeds, skins, and pulp) from the south of Portugal. OH and CONV extraction methods were used in the recovery process, as indicated in chapter 7. Briefly, the CONV method with organic solvents, such as ethanol (70%) and hexane, extracted phenolic compounds and carotenoids, respectively (Oliveira et al., 2015). In OH, hydroethanolic solutions of 70% ethanol were used as selected solvent extraction, during 15 min, at 70 and 55 ºC for phenolic and carotenoids compounds, respectively, as described by Coelho et al. (2019). The obtained liquid fractions (LF) were submitted to freeze-drying to obtain two LF powders (LF-OH and LF-CONV) replicates.

8.2.3. *In vitro* GID

Simulated complete digestion of the LF (LF-OH and LF-CONV) was performed according to the method described by Coelho et al. (2021). This procedure mainly comprised sequential phases simulating different conditions along the GID. In the oral solution simulation, 2 g of each sample were diluted in 2 mL of PBS solution and incubated for 5 min at 37 ºC under agitation (200 rpm). After, the gastric stomach phase was simulated with the pepsin solution at pH 2.3 following incubation for 2 h at 37 ºC at 130 rpm orbital agitation. Finally, the small intestine phase was simulated with the addition of 20 mL of a solution composed of 1.98 mg of pancreatic and bile extract solution at pH 7 and incubated for 3 h at 37 ºC with 45 rpm. After, the samples were placed in a dialysis membrane, closed in vials with a solution of PBS to simulate the colon (non-absorbable sample) and the liquid outside the membrane or blood, basolateral part. Both volumes, inside and outside the membrane, were measured. After the gastrointestinal simulation, the basolateral fraction was freeze-drying for subsequent analysis.

During the GID simulation, samples were collected in each step: mouth, stomach, small intestine, colon and basolateral fraction to analyse total phenolic compounds, total carotenoids, and qualitative and quantitative profiles of both polyphenols and carotenoids by HPLC and UPLC-q-TOF MS.

After gut digestion, the digested OH and CONV extracts and their bioactive properties were measured (antioxidant, prebiotic, and IACE and anti-inflammatory activities). All analyses were performed in triplicate.

8.2.4. Recovery and bioaccessibility indexes of polyphenolic and carotenoids compounds throughout *in vitro* GID digestion.

The recovery percentage determines the compound amount in each step of GID following the equation:

Recovery index (%) = (BCDF/BCTF) × 100,

BCDF represents the digested's bioactive content (mg), and BCTF is the bioactive (mg) quantified in the test matrix.

Bioaccessibility is defined as the percentage of the BC solubilized after intestinal dialysis step; this index defines the proportion of the bioactive compound that could become available for absorption into the blood system:

Bioaccessibility index (%) = (BC/BCDFE) × 100

where: BC is the bioactive content (mg) in the digested sample after the dialysis step (OUT) and BCDF is the bioactive content (mg) in the digested sample after the intestinal step (IN + OUT) –end of digestion.

8.2.5. Analysis of gastrointestinal fractions

8.2.5.1. Total phenolic content (TPC)

The total content of polyphenol compounds present in the extracts was evaluated through Folin – Ciocalteu spectrophotometric method as described in chapter 7.

8.2.5.2. Total carotenoids content (TCC)

The TCC present in the OH and CONV extracts were measured through the spectrophotometric method and read at 454 and 536 nm using a microplate reader (Sunrise Tecan, Grödig, Austria). The content of total carotenoids was expressed as milligram gallic acid equivalent per dry weight material (mg GAE/100g). The analyses were performed in triplicate, and a standard deviation was calculated.

8.2.5.3. HPLC-analysis (polyphenols and carotenoids)

Qualitative and quantitative profiles of polyphenols were carried out according to the method proposed by Oliveira et al. (2015) with slight modifications, as described in chapter 9.). The phenolic compounds were analysed in a Waters Liquid Chromatograph (Waters Series 600. Mildford MA. USA) with A C18 guard column (Symmetry® C18) and an Alltech adsorbosil C18 reversed-phase packing column (250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size) for compounds separation throughout this study. The PDA acquisition wavelength was set in the range of 216 – 600 nm, analogue output channel A at wavelength 280 nm and analogue output channel B at 360 nm both with a band with 2 nm. Calibration curves were obtained at a detection wavelength 280 nm. Standards solutions were prepared to identify and quantify phenolic compounds over the

concentration range from 0.10 to 100.00 mg/L and expressed as micrograms per mL of dry weight (DW) biomass of tomato. All calibration curves were linear over the concentration ranges tested, with correlations coefficients of 0.999.

8.2.5.4. UPLC-qTOF MS analysis (polyphenols)

The UPLC-qTOF MS allows an analysis of the complete compound profile and its derivatives, which is not possible by HPLC-DAD. This analysis was carried out according to the method described in chapter 9. Briefly, an Ultimate 3000 Dionex UHPLC coupled to an ultra-high resolution Qq-time of flight (UHR-QqTOF) mass spectrometer (Impact II, Bruker Daltonics, Germany) was used to analyse the phenolics and carotenoids. For polyphenols, data was acquired in negative mode (50 to 1500 amu) and for carotenoids (50 to 2000 amu) in the positive mode with a scan duration of 0.3 s in centroid mode. The MS parameters were: capillary voltage: 4.5 kV; drying gas temperature: 200 ºC; drying gas flow: 8 mL/min; nebulising gas pressure: 2 bar; collision RF: 300 Vpp; transfer time: 120 µs and pre-pulse storage: 4 µs. Mass calibration was performed by the external injection of a sodium formate solution.

8.2.6. Biological properties

8.2.6.1. Antioxidant capacity

Both antioxidant activity (AA) and total phenolic compounds content were measured in samples before and after in vitro digestion. Each sample's in vitro antioxidant activities were directly evaluated in the lyophilized powdered fractions using the ABTS and ORAC methods, as described by chapter 7.

8.2.6.2. Prebiotic effect

Based on the most common group of bacteria used to test this property, a group of four commercial probiotic bacteria were selected for the present work, namely *Bifidobacterium animalis* subsp. *lactis* BB-12; *Bifidobacterium longum* BG3; *Bifidobacterium animalis* Bo; *Lactobacillus acidophilus* LH5; *Lactobacillus casei* LC1; *Lactobacillus rhamnosus* R11.

The effect of tomato bagasse extracts on the growth of the target probiotic microorganisms was evaluated. Solutions prepared using tomato bagasse extract (before and after digestion) and basal media were prepared at 2, 4 and 6% (w/v) and inoculated, using a 24 h inoculum, at 10% (v/v). These bacteria were then incubated for 24 h at 37 °C, and *Bifidobacterium* strains were inoculated under an anaerobic environment. After this period, the cell growth was assessed by plating, using the spread plate method in de Mann, Rogosa, and Sharpe agar (MRS) enhanced with 0.5 g/L of L-cysteine hydrochloride. After 48 h incubation at 37 ºC under anaerobiosis, the colonies were enumerated, and the outcomes were plotted as log CFU/mL. All inoculations were performed in triplicate, and the plain inoculated basal media was utilized as a control.

8.2.6.3. ACE-inhibitory activity assay (iACE)

The ACE-inhibitory activity was measured by fluorescence using the method described by Coscueta, Campos, Osório, Nerli, & Pintado (2019).

8.2.6.4. Cytokines inhibition – anti-inflammatory activity

Peripheral blood mononuclear cells (PBMCs), namely lymphocytes and monocytes, were isolated from whole blood obtained from healthy donors of whom informed consent was obtained that their donated blood would be used for scientific purposes. Separation of blood cells was performed using density centrifugation. After isolation, PBMCs were washed twice in cold phosphate-buffered saline (pH=7.4) containing 3% heat-inactivated fetal bovine serum. The viability of the PBMCs was evaluated by the trypan blue exclusion test with a Neubauer counting chamber.

Isolated PBMCs were then plated at a density of 1.0 × 106 cells/ml in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 100 U/mL of Pen/Strep in a humidified atmosphere containing 5% CO_2 for 24 h. Tomato absorbed fraction treated by CONV, and OH technology was tested for its pro- or anti-inflammatory properties on PBMCs simulated or not with lipopolysaccharide at 100 ng/mL. After 24 h incubation, supernatants were harvested by centrifugation and different pro-inflammatory cytokines concentrations (TNF-α and Il-6) were measured by commercially available ELISAs according to the manufacturers' instructions.

8.2.7. Statistical analysis

Statistical analysis was done using IBM SPSS Statistics v21.0 (IBM, Chicago, USA). The normality of the data's distribution was evaluated through Shapiro-Wilk's test. As the data followed a normal distribution, One-way ANOVA, coupled with Tukey's post hoc test, was used to determine the differences of the mean values between BC or bioactivities concentrations along with digestion. The correlation between total phenolic compounds, carotenoids, individual compounds and bioactivities were assessed by Pearson's test. Tomato bagasse biomass' effect on bacterial populations, at each time point. Repeated Measures ANOVA was used to evaluate the effect of Tomato bagasse biomass on the bacterial population over time. Differences were considered significant for p-values≤0.05.

8.3. Results and Discussion

8.3.1. Extracted phenolic and carotenoids characterization

8.3.1.1. Phenolic characterization

The results showed significant differences between the OH and CONV methods for most phytochemicals analysed (Fig. 8.1). The TPC of OH extract was 2.15 ± 0.049 g / Kg DW while the CONV presented higher values 4.23 ± 0.064 g /Kg DW compared with OH, almost double. Similar values were found in chapter 6, where the authors applied an experimental design to optimize the polyphenols and carotenoids extraction yields with OH. They found TPC values ranging from 0.480 and 2.83 g GAE / Kg DW and concluded that temperature, solvent (ethanol : water ratio) and time are essential to obtain higher extraction yields of BC. Besides, this method could be selective, i.e., different conditions allow the extraction of different BC such as polyphenols and, or carotenoids compounds. Other studies reported TPC values for peels ranging from 0.44 and 6.10 g GAE / Kg (Peschel et al., 2006). The discrepancies found in the results indicated that an ethanol-water solution

did not recover the major phenolic compounds present in tomato bagasse. These differences may occur because tomato bagasse contains mainly peel and seeds, and the insoluble polyphenols are covalently bound to cell wall components, such as hemicellulose, cellulose, lignin, structural proteins and pectin. Also, the various extraction processes release the phenolic compounds from the matrix in which they are contained differently (Peschel et al., 2006).

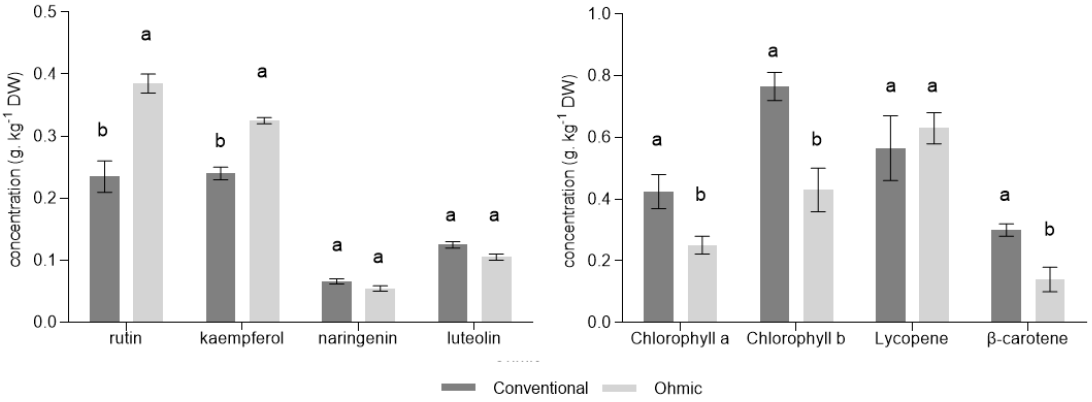


Figure 8.1. The concentration of (A) principal polyphenols (B) chlorophylls and carotenoids present in CONV and OH extracts. *p<0.05 significant differences between methods of extraction represent by letters.

The same was found in AA, where the OH presented lower values than CONV (2.72 ± 0.02 and 3.45 ± 0.08 g AAE / Kg DW, respectively), but the difference was much lower. The results are comparable with those reported by other authors (Ćetković et al., 2012; Szabo et al., 2019). OH could be a promising technology over the CONV method to reuse all tomato bagasse despite the results. Previous studies reported a significant influence on BC recovery by setting temperature, time and ethanol concentration (Coelho et al., 2019; Oliveira et al., 2015). Furthermore, a synergetic effect between temperature and electric fields caused by OH has been verified, facilitating the cell walls breakdown and allowing polyphenols' bioaccessibility with higher recoveries in short periods.

Regarding HPLC-DAD and UPLC -q-TOF analysis, the most abundant phenolic compound in both extracts from tomato by-products is rutin, followed by kaempferol, luteolin and naringenin (Fig. 8.1 A). However, the amount of phenolic compounds changes with the type of extraction used. Other compounds were also found, such as phenolic acids, *p*-coumaric acid, caffeic acid, hydroxycinnamic acid. These discrepancies could be explained by the type of extraction used, the tomato cultivar and the moment of tomato ripening, which influence the individual compounds (Preedy, 2009). The effect of the extraction process was evidenced in the different parameters evaluated in this study. Regarding OH extracts obtained, it presented higher rutin content than the CONV samples ($p<0.05$), while for other polyphenols, the concentrations between extraction methods were very similar. Rutin is better extracted in hydrophilic solutions than hydrophobic, like the CONV method used, which promote its higher recovery yield with OH application (Preedy, 2009).

UPLC-q-TOF MS only identified some compounds. These results express the enormous potential of tomato bagasse in diverse polyphenolic compounds. Excellent mass accuracies were observed for all molecular ions, presenting differences between experimental m/z values and calculated m/z

values below 2 mDa. The most representative polyphenols and derivatives profiles from OH and CONV extracts are present in Table 8.1. and revealed the similarity of compound profile between OH and CONV, differing in the peak intensity. The OH extract differs in the presence of sucrose, which proves the affinity of hydrophilic compounds in the OH extraction. Also, for the first-time, derivatives of kaempferol, such as kaempferol 3-sophorotrioside, and other compounds, including phene-di-hexoxe and N-acetyl-D-tryptophan, were found and described in the literature. The results showed the presence of flavonol and derivates such as rutin at 609 m/z, kaempferol 3 - sophorotrioside at 771 m/z; quercetin-3-O-neohesperidoside at 609 m/z (Vallverdú-Queralt et al., 2010). Naringenin at 271 m/z, a flavanone and the primary polyphenolic compound present in tomatoes by-products, was also reported by other authors (Vallverdú-Queralt et al., 2010). Hydroxycinnamic acids were also present, namely p-coumaric acid at 163 m/z. The phloridzinyl glucoside at 597 m/z was also present. It is a dihydrochalcone, a group of polyphenols often forgotten but essential due to its biological properties (Choi, 2019; Zidorn, 2015).

Table 8.1. UPLC-qTOF-MS compounds profile of OH and CONV liquid extracts from tomato bagasse.

Name	RT (min)	DAD	Formula	m/z Experimental	m/z Calculated	MSMS fragments					err [mDa]
ohmic samples											
Not identified	**7.7**	295	$C_{15}H_2ON_2O_4$	291.1355	291.1350	291.1 (22)	171.1 (51)	145.0 (51)	119.1 (100)	145.0 (19)	**-0.5**
Caffeoyl-glucose	**8.3**	370	$C_{15}H_{18}O_9$	341.0897	341.0878	341.1 (6)	179.0 (100)				**-1.9**
Quinic acid	**8.5**	330	$C_7H_{12}O_6$	191.6634	192.0634	191.0 (100)	146.9 (33)	119.0 (54)	102.9 (75)		**-0.4**
Kaempferol 3-sophorotrioside	**8.7**	266/360	$C_{33}H_{40}O_{21}$	771.2004	771.1989	771.2 (100)	609.1 (25)	463.1 (6)	301.0 (4)		**-1.4**
Phene-di-hexoxe	**9.8**	285	$C_{20}H_{30}O_{11}$	445.1709	445.1715	445.1 (12)	267.1 (31)	221.1 (28)	179.0 (100)		**0.6**
Quercetin O-sophoroside	**10.5**	266/366	$C_{27}H_{30}O_{17}$	625.1421	625.1410	300.0 (74)	179.0 (6)	741.2 (100)			**-1.1**
Quercetin 3-(2G-xylosylrutinoside)	**10.9**	266/356	$C_{32}H_{38}O_{20}$	741.0956	742.19564	714.2 (0.01)	300.0 (22)				**-1.1**
Malvidin 3-(6-malonylglucoside) 5-glucoside	**11.1**		$C_{32}H_{38}O20$	741.1895	741.884						**0.7**
Quercetin-3-*O*-neohesperidoside	**11.3**	262	$C_{27}H_{30}O_{16}$	609.1474	609.1461	609.1 (100)	429.1 (3)	284.0 (53)	179.0 (4)		**-1.3**
***p*-Coumaric acid**	**11.3**	314	$C_9H_8O_3$	163.04	163.0401	163.0 (14)	119.0 (100)				**0.1**
Rutin	**11.8**	262/356	$C_{27}H_{30}O_{16}$	609.1442	609.1461	609.1 (100)	301.0 (34)	300.0 (38)	179.0 (2)	151.0 (1)	**1.9**
Phloridzinyl glucoside	**12.2**	290	$C_{27}H_{34}O_{15}$	597.1825	597.1824	597.2 (100)	477.1 (33)	417.1 (24)	387.1 (76)	357.1 (100)	**-0.1**
N-Acetyl-D-tryptophan	**12.4**	281	$C_{13}H_{14}N_2O_3$	245.0936	245.0932	245.1 (53)	203.1 (100)	159.1 (5)	116.0 (29)	98.0 (7)	**-0.5**
Sucrose	**12.5**	310	$C_{12}H_{22}O_{11}$	341.109	341.1093	221.0665 (22)	179.0562 (100)	113.0243 (33)	89.0244 (44)		**-0.3**
Naringenin-7-*O*-glucoside	**13.7**	290	$C_{21}H_{22}O_{10}$	433.1	433.114	433.1 (2)	271.1 (100)	151.0 (8)			**0.5**
cis-2-Coumarate	**16.2**		$C_9H_8O_4$	163.0396	163.0396						**-0.5**
Flavimycin A	**17.3**		$C_{18}H_{18}O_9$	377.0862	377.0862						**1.6**
Naringenin	**18**	290	$C_{15}H_{12}O_5$	271.0608	271.0612	271.1 (84)	177.01 (13)	151.0 (100)	119.0 (32)		**0.4**
CONV method											
No identified	**7.7**	295	$C_{15}H_{20}N_2O_4$	291.1345	291.1350	291.1 (22)	171.1 (51)	145.0 (51)	119.1 (100)	145.0 (19)	**-0.5**
Caffeoyl-glucose	**8.3**	370	$C_{15}H_{18}O_9$	341.0859	341.0878	341.1 (6)	179.0 (100)				**-1.9**
Quinic acid	**8.5**	330	$C_7H_{12}O_6$	192.063	192.0634	191.0 (100)	146.9 (33)	119.0 (54)	102.9 (75)		**-0.4**
Kaempferol 3-sophorotrioside	**8.7**	266/360	$C_{33}H_{40}O_{21}$	771.1975	771.1989	771.2 (100)	609.1 (25)	463.1 (6)	301.0 (4)		**-1.4**

Phene-di-hexoxe	**9.8**	285	$C_{20}H_{30}O_{11}$	445.1721	445.1715	445.1 (12)	267.1 (31)	221.1 (28)	179.0 (100)		**0.6**
Quercetin O-sophoroside	**10.5**	266/366	$C_{27}H_{30}O_{17}$	625.1399	625.1410	300.0 (74)	179.0 (6)	741.2 (100)			**-1.1**
Quercetin 3-(2G-xylosylrutinoside)	**10.9**	266/356	$C_{32}H_{38}O_{20}$	742.19454	742.19564	714.2 (0.01)	300.0 (22)				**-1.1**
Malvidin 3-(6-malonylglucoside) 5-glucoside	**11.1**	510	$C_{32}H_{38}O_{20}$	741.8851	741.884						**1.1**
Quercetin-3-*O*-neohesperidoside	**11.3**	262	$C_{27}H_{30}O_{16}$	609.1448	609.1461	609.1 (100)	429.1 (3)	284.0 (53)	179.0 (4)		**-1.3**
***p*-coumaric acid**	**11.3**	314	$C_9H_8O_3$	163.0	163.0401	163.0 (14)	119.0 (100)				**0.1**
4-Coumarate	**11.5**		C_9H8O_3	163.0401	163.0406						**-0.5**
Rutin	**11.8**	262/356	$C_{27}H_{30}O_{16}$	609.148	609.1461	609.1 (100)	301.0 (34)	300.0 (38)	179.0 (2)	151.0 (1)	**1.9**
Phloridzinyl glucoside	**12.2**	290	$C_{27}H_{34}O_{15}$	597.1823	597.1824	597.2 (100)	477.1 (33)	417.1 (24)	387.1 (76)	357.1 (100)	**-0.1**
N-Acetyl-D-tryptophan	**12.4**	281	$C_{13}H_{14}N_2O_3$	245.0927	245.0932	245.1 (53)	203.1 (100)	159.1 (5)	116.0 (29)	98.0 (7)	**-0.5**
Naringenin-7-*O*-glucoside	**13.7**	290	$C_{21}H_{22}O_{10}$	433.1145	433.114	433.1 (2)	271.1 (100)	151.0 (8)			**0.5**
Not identified	**16.2**		$C_{54}H_{67}O_{10}$	875.4739	875.4723						**1.6**
Naringenin	**18**	290	$C_{15}H_{12}O_5$	271.0616	271.0612	271.1 (84)	177.01 (13)	151.0 (100)	119.0 (32)		**0.4**
(2S)-2-[[2-(diethylamino)-5-[ethyl(piperidine-1-carbonyl)amino]pyrimidin-4-yl]amino]-3-[4-(pyrrolidine-1-carbonyloxy)phenyl]propanoic acid	**20.7**		$C_{30}H_{42}N_7O_5$	580.3253	580.32474						**-0.6**

RT-retention time

8.3.1.2. Carotenoids

Figure 8.1 B, compares carotenoid content in extracts obtained by CONV and OH methods. As can be seen, individual carotenoids extracted were influenced by the method used, which their lipophilic characteristics and solvent affinity can explain.

Although chlorophylls and carotenoids have presented higher values in the CONV relatively to the OH method, the values obtained in OH were reasonable, considering the lipophilic structure of these compounds and the solvent used (70% ethanol).

As expected, significant differences were found in total carotenoid content extracted by CONV and OH methods, $p<0.05$ (Table 8.1.). Although the OH was carried out using 70% ethanol, the total carotenoids recovery was 3.75 ± 0.09 g / kg DW against 6.98 ± 0.06 g / Kg DW in the CONV method that uses organic solvents. Nonetheless, when the carotenoids profile was analyzed, it was interesting to observe that the impact of the system on each compound was different. While for β-carotene, the CONV method showed slightly better extraction (and significant $p<0.05$), for lycopene, OH promoted the best extraction, with a significant difference ($p<0.05$). Both compounds have different chemical structures, which may influence their hydrophobicity, solubility, and, consequently, their thermal stability. Lycopene is a linear molecule with a more stable structure, which may form multilayers and aggregates resisting further structural changes, while β-carotene and lutein have unstable β-ionone rings and are not available for molecular self-assembling (Coelho et al., 2021; D'Evoli et al., 2013; Nguyen et al., 2001). In OH, a temperature of 55 ºC was applied on tomato bagasse, not enough to degrade lycopene, but enough to break down protein/polysaccharide-lycopene complexes, releasing more lycopene into the solution. Also, ethanol, the solvent used in OH, possibly leads to a better penetration inducing a selective and physical cell disruption, allowing the recovery of lycopene. Thermal treatments cause oxidation and isomerization of β-carotene (D'Evoli et al., 2013; Nguyen et al., 2001; Oliveira et al., 2016). It is the first time that OH was proven to preserve lycopene compared with CONV methods.

Calvo et al. (2007) studied the influence of ethanol and ethyl acetate and temperature on carotenoids extraction from tomato peel powder. They observed an increase of carotenoids recovery with temperature increase to 50 ºC and a decrease when the temperature reached 60 ºC, suggesting an oxidative degradation of carotenoids. This observation is consistent with the results reported by Nguyen and colleagues (2001). These authors studied the thermal isomerization of carotenoids from different tomato varieties, and they also attribute the structural lycopene stability relative to β-carotene to thermodynamic nature due to the chemical structure difference and the intracellular localization of lycopene (chloroplasts). Besides, they observed better extraction yields for ethanol than ethyl acetate. Both polyphenols and carotenoid recoveries are influenced by the solvent type, their concentration used to extract, and their polarization.

Regarding CONV and OH methods used to extract BC from tomato bagasse, significant differences between methods were only observed with β-carotene. No differences were found between CONV and OH methods for other carotenoids. Even though the three compounds (β-carotene, lycopene, and lutein) are structurally similar, they perhaps present different solubilities in the solvents and

specific solvency conditions of temperature. Different studies (Coelho et al., 2021; Nguyen et al., 2001; Oliveira et al., 2016; Sabio et al., 2003) have described that expanding the temperature builds the solvency of the carotenoids. The explanation behind this behaviour could be: (i) the carotenoids concentrations in the different segments of the vegetable tissues are distinct (ii) the manner how lycopene crystallizes inside chromoplasts. This reality hampers lycopene extraction since the chromoplast cell wall acts as a boundary. Furthermore, the disintegration of a crystallized substance is slower than that of an amorphous one, while the solid: liquid ratio is lower, and the intermolecular forces in the crystal are most powerful (Sabio et al., 2003). An explanation is that temperature improves lycopene extraction by expanding the solvent diffusivity (and the capacity to enter into the robust matrix).

8.3.2. Recovery and bioaccessibility indexes of polyphenolic and carotenoids compounds throughout in vitro GID

8.3.2.1. Phenolic compounds

The TPC and individual phenolic compounds detected during the in vitro GID are presented in Table 8.2. and Figure 8.2. The individual compounds were identified by UPLC-qTOF-MS (Table 8.2.) and quantified by HPLC-DAD.

A statistically significant increase ($p<0.05$) was observed regarding TPC between undigested (extract) and digested fractions of OH extracts. As noted earlier, OH extracts contain lower total phenolics than those obtained by the CONV method ($p<0.05$). Following the digestion, the OH samples presented lower TPC than CONV samples in the mouth ($p<0.05$), nevertheless, the OH extracts allowed the greater release of compounds than the CONV method (RI 131% and RI 92%, respectively).

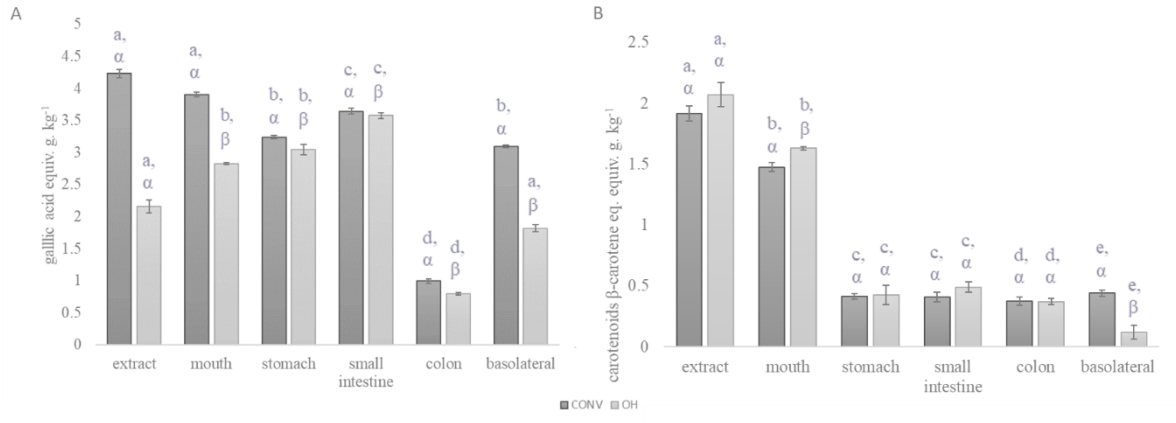


Figure 8.2. TPC (A) and TC (B) during the digestion process. TPC (A) and total carotenoids (B) assays. Significant differences ($p<0.05$) among gastrointestinal for each extract are indicated by Latin letters. Significant differences ($p<0.05$) among extracts for each digested fraction are indicated by Greek letters.

These results showed that the oral digestion phase affected tomato extracts and, consequently, their BC differently since the release of polyphenols from the extracts matrix depended on the type of OH and CONV extraction ($p<0.05$). Thus, the different compounds of tomato bagasse extract under the mouth conditions, namely the soluble fibre content (data are not shown), which is higher in OH than

in CONV, certainly influences the bioavailability of polyphenols and the interaction of α-amylase and polyphenolic compounds. Specific BC, such as phenolic compounds, could be linked to soluble fibre, and the digestive enzymes could hydrolyze and release the phenolic compounds in ester and glycoside forms present in a higher amount in OH than the CONV extracts. Also, the use of OH during the extraction process could improve the release of compounds since this method uses the electric fields, which could also change the cell wall and the molecular structure and consequently could improve the release of polyphenols towards the matrix (Barba et al., 2015; Coelho et al., 2019), leading to these differences in the recovery index (Lucas-González et al., 2018). Also, the TPC amount of OH extracts may be explained by the higher content of hydroxybenzoic acid, rutin, naringenin caffeic acid, and luteolin compared with CONV samples.

No differences were found for the TPC in the stomach and small intestine for both extraction methods, presenting similar TPC ($p>0.05$). Nevertheless, the stomach presented a higher impact on the TPC of OH extracts leading to a higher TPC released observed in the small intestine. The TPC recovery in OH extract is higher than in the CONV samples (RI 141% and RI 76%, respectively). The acidic pH in the gastric step promotes the breakage of bonds between BC and other extracts components, like dietary fibre or protein, which release polyphenols into the matrix. Other authors reported the increase of phenolic compounds recovery in the gastric step(Lucas-González et al., 2018; Pavan et al., 2014; Ribeiro et al., 2020).

Regarding the intestinal fraction, the OH also showed a higher percentage recovery of TPC than the CONV samples (RI 33% and RI 23%, respectively). The differences obtained between extracts may be explained by (i) the presence of lutein in OH extracts in this fraction (ii) the interactions between carbohydrates, dietary fibres or proteins (Gullón et al., 2015) (iii) the chemical reactions, such as oxidation and polymerizations of BC, which lead to the formation of other derivatives, such as chalcones and (iv) the structural changes associated to enzymatic actions, which cause influence in the solubility (Ribeiro et al., 2020).

In the basolateral fraction, the OH extracts showed lower TPC than the CONV samples ($p<0.05$), but OH presented a recover index of TPC higher than the CONV extracts (RI 84% and RI 73%, respectively). This difference could be promoted by the presence of caffeic acid in OH extracts at this step. Possibly, one of the reasons for the higher TPC content of CONV is that this process is more aggressive upon other structures present in the extracts that facilitate the increasing availability of polyphenols and consequently higher absorption capacity towards the bloodstream (here represented by a basolateral fraction) (Ribeiro et al., 2020). Also, the recovery index differences could be explained by the type of extraction used and the BC present in the extracts (Barba, Brianceau, et al., 2015). The more polar compounds, such as benzoic acids or hydroxycinnamic acids, are not conveniently extracted by CONV methods, which mainly uses organic solvents, and at the same time, the OH uses electric fields, which makes polyphenols more accessible in the extracted matrix (Barba et al., 2015; Coelho et al., 2019).

Globally, the highest TPC recovery values along the digestion process were observed in the oral phase and gastric digestion of OH extracts ($p<0.05$). The recovery indexes were consistently lower

in the case of the CONV extracts. In the stomach, the TPC value increased for both extracts ($p<0.05$) and decreased in the intestine phase ($p<0.05$).

As previously mentioned, variations such as pH value and acidity in the gastric phase promote the breaking of bonds between BC and nutrients, such as fibres, proteins, and carbohydrates. Also, the acidic conditions of the stomach protect the polyphenols from degradation (Lucas-González et al., 2018; Ribeiro et al., 2020).

The decrease in the colon fraction ($p<0.05$) is aligned with the increase in the basolateral fraction ($p<0.05$) and also with the large intestine' absorption of phenolic compounds. This fact is confirmed by the results obtained for individual phenolic compounds, namely, naringenin, rutin, hydroxybenzoic acid and caffeic acid (Table 8.2). Furthermore, the additional contact time (24 h) of the extract and intestinal fluids (including the splitting enzymes, namely lipolytic, amylolytic, proteolytic) allows the release of polyphenols (Barba, Brianceau, et al., 2015; Gullón et al., 2015). The same results were obtained with tomato sauce (Martínez-Huélamo et al., 2016) and olive pomace (Ribeiro et al., 2020).

Table 8.2. .Recovery index and bioaccessibility of total phenolic compounds, carotenoids and antioxidant activity by spectrophotometric analysis and individual compounds by HPLC-DAD analysis from LFCONV and LFOH samples throughout digestion.

Compounds	Recovery index (%) Mouth		Stomach		small intestine		colon		basolateral		Bioaccesibility index (%)	
	CONV	OH	CONV	OH	CONV	OH	CONV	OH	CONV	OH	CONV	OH
Total phenolic compounds	92.32 ± 1.78a	131.05 ± 1.25ª	76.53 ± 2.23b	141.19 ± 7.25b	86.12 ± 2.14ª	165.98 ± 3.51c	23.44 ± 1.25d	36.87 ± 1.78d	73.10 ± 2.62b	84.25 ± 1.89e	75.72 ±1.45α	69.56± 1.53β
4-Hydroxybenzoic acid	10.69 ± 1.25a	94.46 ± 1.84ª	1.35 ± 0.09b	105.94 ± 3.28b	2.50 ± 0.56b	44.00 ± 1.98c	3.95 ± 1.25c	9.54 ± 1.33d	8.18 ± 1.44d	4.94 ± 0.78e	67.41 ±2.73α	34.13 ± 1.23β
Hydroxymethylfurfural	n.d.	173.13 ± 7.81ª	n.d.	240.79 ± 2.84b	n.d.	322.70 ± 4.27c	n.d.	n.d.	n.d.	n.d.	n.d	n.d
Trans-cinnamic acid	92.19 ± 4.13a	122.70 ± 5.67ª	n.d.	296.89 ± 8.23b	n.d.	197.20 ± 3.63b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Vanillin	n.d.	65.51 ± 2.59ª	n.d.	175.37 ± 4.17b	n.d.	59.83 ± 1.88a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Caffeic acid	96.54 ± 3.32a	138.16 ± 1.61ª	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	47.73 ± 9.85b	n.d.	10.00 ± 1.23α
Luteolin	n.d.	167.40 ± 4.21ª	n.d.	143.45 ± 3.78b	n.d.	57.91 ± 3.21c	n.d.	101.99 ± 4.45d	n.d.	n.d.	n.d.	n.d.
Rutin	59.71 ± 2.24a	145.44 ± 2.13ª	3.40 ± 0.78b	236.33 ± 5.45b	41.90 ± 2.32c	131.40 ± 2.41ª	35.72 ± 1.69d	32.99 ± 2.44c	33.01 ± 1.12d	13.72 ± 2.56d	48.03 ± 3.24 β	29.37 ± 4.71α
Naringenin	n.d.	107.49 ± 3.27ª	n.d.	149.67 ± 2.98b	n.d.	224.15 ±7.42c	62.22 ± 4.44a	67.60 ± 1.94d	52.50±2.41a	12.95 ± 1.76e	45.76 ± 1.85 β	16.08 ± 1.89α
Total carotenoids	77.03 ± .67a/α	78.75 ± 2.25a/α	21.58 ± 2.85a/β	20.65 ± 2.43a/β	21.27 ± 4.25a/β	23.67 ± 2.81a/β	19.38 ± 0.97a/β	17.92 ± 1.71/β	22.967 ± 2.43a/β	5.76 ± 3.12a/y	93.62 ± 1.41α	25.86 ± 1.12β
Total antioxidant activity	92.32 ± 2.01	131.05 ± 12.99	76.53 ± 4.67	141.19 ± 12.01	86.12 ± 3.21	169.26 ± 11.84	23.44 ± 1.24	37.62 ± 2.78	73.10 ± 5.87	85.92 ± 3.32	84.89 ± 2.98α	50.76 ± 2.44β

n.d. – not detected;. Results are the average of three determinations ± standard deviation. Significant differences ($p<0.05$) between digestive fractions (mouth, stomach, small intestine, colon nad basolateral) for each extraction method are indicated by greek letters. Significant differences between the extraction methods (OH nad COMV) for each digestive fraction are indicated by roman letters, as determined by one-way ANOVA test ($p<0.05$), respectively

Regarding the individual phenolic compounds present in both extracts, the results obtained by HPLC followed the TPC results. High correlation coefficients ($r^2 \geq 0.73$) were obtained with the GID for TPC and individual compounds for both extractions. The individual compound with a higher correlation coefficient in OH extracts is naringenin ($r^2 \geq 0.91$), followed by trans-cinnamic acid ($r^2 \geq 0.79$) and rutin ($r^2 \geq 0.73$); on the other hand, the best correlation of the CONV extract was observed for 4-hydroxybenzoic acid ($r^2 \geq 0.95$). This correlation between individual compounds and TPC is evident for the recovery index in each step. Regarding the naringenin content for OH extracts, it increased in the mouth (RI 107%), followed by an increase in the stomach and small intestine (RI 150% and RI 224%, respectively) and a decrease in the basolateral phase (RI 13%), which represents the bioaccessible blood. The non-absorbed fraction interacts with gut microbiota. For the CONV method, the recovery index of 4-hydroxybenzoic acid increased in the mouth fraction (RI 11%), followed by a decrease in the stomach (RI 1%) and an increase after the gastric step (RI 2.50%) as well as in the intestinal process (RI 8%) with values far from the OH extract.

UPLC-qTOF-MS identified other individual compounds and their metabolites during the GID step. Principal component analysis (PCA) was performed to identify a global pattern throughout the GID of tomato extracts under the extractions conditions applied, OH and CONV (Figure 8.3). According to PCA, the two main components (first present in the horizontal axis and the second the vertical axis) accounted for 77% of the variability in the data assessed, representing a satisfactory analysis. The OH and CONV extracts confirmed their differences in the type of compounds content, and also, they are similar in terms of compounds absorbed fraction.

Furthermore, applying the PCA and co-expression analysis (fold change analysis), applying the Pearson correlation coefficient allowed to find the correlations between compounds in the dataset, eliminating the biased results. Thus, looking for correlation coefficients, it was possible to understand the main compounds/metabolites, which interfere throughout the GID in OH and CONV extracts. The results obtained showed that the main compounds which interfere with gastrointestinal conditions are *p*-coumaric acid (163 m/z), naringenin (271 m/z), and 4-coumarate (601 m/z). The impact of extraction methods in each compound recovery is also presented in Fig. 8.3, with OH significantly affecting the naringenin and chalcone, and in the CONV extraction, the quercetin (271 m/z) was the most affected. Analysing the results following the literature, the OH allows the recovery of more hydrophilic compounds than the CONV method since the former uses more lipophilic solvents (Coelho et al., 2019).

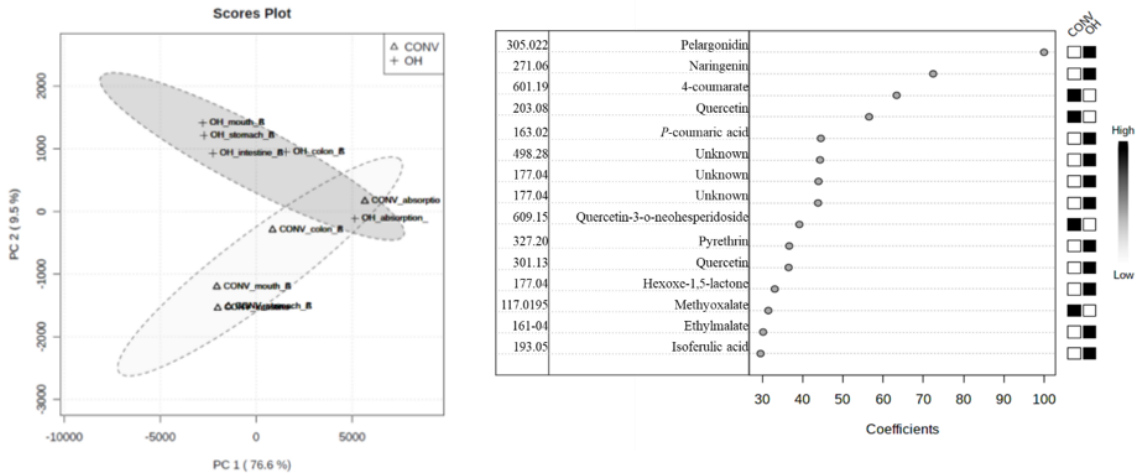


Figure 8.3. PCA, compounds with significant impact by co-expression analysis on OH and CONV extracts by PLS-DA analysis.

The intestinal phase represents the most significant step in GID, where the main BC are absorbed in the epithelium. Thus, it is crucial to define bioaccessibility as the amount of an ingested BC available for absorption.

The digested fractions obtained after the GID process generate different soluble and insoluble compounds, which may be bioaccessible and bioavailable molecules (Pino-García et al., 2016).

The results showed that 4-hydroxybenzoic acid is the most bioavailable compound for OH extract, and also with higher bioavailability than when present in the CONV extract (24.32 and 3.27%, respectively), followed by rutin, which presented much lower bioavailability than in the CONV extract (10.41 and 78.78%, respectively). Naringenin was only bioavailable in the OH extract (6%). Thus, the polyphenols' chemical structure defines their rate and extent of intestinal absorption and the type of metabolites circulating in the blood (Lucas-González et al., 2018). These compounds have been reported with beneficial properties like antioxidant and antidiabetic properties, among other properties (Coelho et al., 2020).

8.3.2.2. Carotenoids

Concerning the total carotenoid content along the GID present in both extracts, OH and CONV (Table 8.2, Fig.8.2 B), a significant decrease between extracts and mouth was observed for OH extracts (21.25%) and CONV (22.97%). The high sensitivity of carotenoids to external factors may explain the higher carotenoid losses, which led to similar values of recovery in OH (79.35%) and in CONV (78.42%) extracts after stimulation of enzymatic digestion at acidic conditions of the stomach (Courraud et al., 2013; Scrob et al., 2019).

After intestinal digestion, the total carotenoid decrease is probably due to interactions with other compounds or changes produced by the hydrolysis of enzymes present in the digestion process and pH changes in each compartment. Besides, carotenoids are lipophilic and have reduced

bioavailability in the GID because of poor absorption in the gastrointestinal fluids (Salter-Venzon et al., 2017).

Moreover, although the OH extract presented similar values to the CONV in terms of total carotenoids content along the digestion process, the recovery index of carotenoids in the basolateral fraction was 5.76 ± 2.43% for OH extract compared to 22.97 ± 3.12% from CONV extract. The more hydrophobic profile of extracts, hydroxyl groups and type of carotenoids may explain the higher CONV extracts bioavailability (Reboul, 2019).

After intestinal digestion, the total carotenoid decrease is probably due to interactions with other compounds or changes produced by the hydrolysis of enzymes present in the digestion process and pH changes in each compartment. Besides, carotenoids are lipophilic and have reduced bioavailability in the GID because of poor absorption in the gastrointestinal fluids (Salter-Venzon et al., 2017).

8.3.3. Bioactive properties of liquid extracts after simulated enzymatic GID

8.3.3.1. Antioxidant activities of liquid digest extracts throughout in vitro GID

The results obtained from ABTS and ORAC assays (Figure 8.4, A and B, respectively) showed significant differences concerning total AA regarding the two extraction methods and the digested fractions analyzed ($p<0.05$).

Regarding the antioxidant capacity measured by ABTS, before digestion (extract), OH exhibited lower AA than CONV extracts. These results were almost certainly due to the differences among tomato extracts in terms of compounds, hydrophilicity, solubility, and accessibility (Del Pino-García et al., 2016).

OH extracts showed higher AA than the CONV extracts ($p<0.05$). These results indicate that OH allowed the release of compounds from the matrix to the gastrointestinal fraction (Table 8.2) (Barba et al., 2015; Coelho et al., 2019).

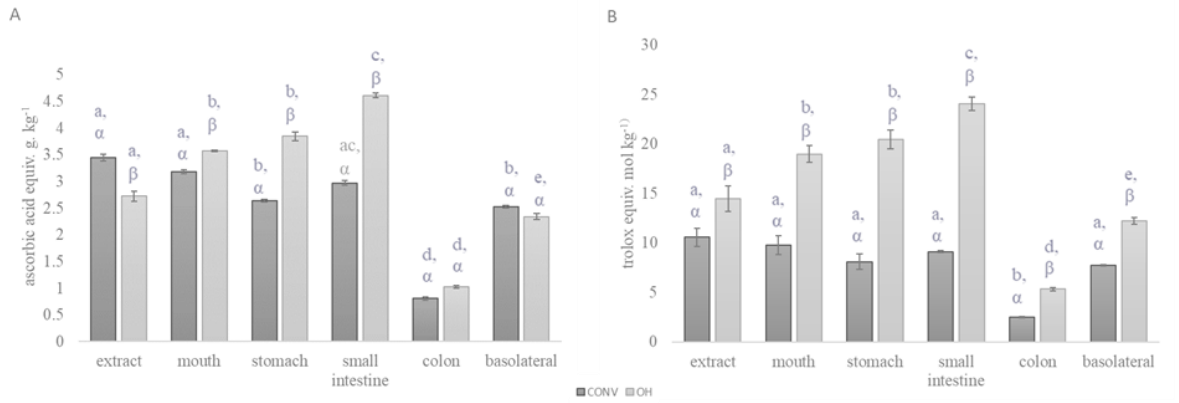


Figure 8.4. Total antioxidant capacities were determined using the ABTS (A) and ORAC (B) assays of the *in vitro* digested fractions derived from tomato liquid extracts (obtained by OH and CONV extraction). Significant differences ($p<0.05$) among gastrointestinal for each extract are indicated by Roman letters. Significant differences ($p<0.05$) among extracts for each digested fraction are indicated by Greek letters.

After mouth and gastric simulations, the AA of OH samples increased while CONV samples decreased. Both extracts displayed an increase in the total AA following enzymatic GID. After releasing and absorbing bioavailable antioxidants in the small intestine, the AA exhibited by the compounds that reach the colon was again higher for OH than for CONV. The highest AA was provided by OH extracts in the colon, despite the slightly lower fermentability estimated for this extract (water ethanolic extract, which contains lower dietary fibre) (Domínguez-Avila et al., 2017). Also, in general, the digested fractions showed significantly higher AA than their respective undigested extracts. The enzymatic GID phase produced a marked increase in the antioxidant capacity of the OH extracts. The antioxidant effects depend not only on their polyphenols and carotenoid concentrations in foods but also on bioaccessibility and bioavailability after ingestion (Coelho et al., 2019; Pavan et al., 2014).

Regarding phenolic compounds, metabolism from liquid extracts, which contains lower dietary fibre, practically begins within the lumen of the small digestive system and can pass through the intestine wall into the bloodstream (Del Pino-García et al., 2016). In contrast, post absorption suffers modifications within the liver and other organs. For example, to the flavonoids retention from the small digestive tract occur, the glycosides (sugars) that are linked to the flavonoid skeleton must be the first broken by the enzymatic activity of the small intestine (e.g. β-glucosidase) (Palafox-Carlos et al., 2011; Pérez-Jiménez et al., 2009) and, consequently are potentially bioavailable and in condition to promote their antioxidant capacity (Tagliazucchi et al., 2010).

The results showed the highest values of hydroxycinnamic acids and hydroxybenzoic acids in OH extracts than in CONV (Table 8.1 and 8.2). These compounds are frequently present as bound phenolics in the form of glycosides, amides, esters, and rarely in free form and may be released by enzymes that could break these bonds (Pérez-Jiménez et al., 2009). An example of hydroxycinnamic acid is chlorogenic acid, which results from the linkage of caffeic acid and quinic acids, and the bond is broken by gastric enzymes action (Kumar & Goel, 2019). The free hydroxycinnamic acids are better released throughout GID, increasing the AA (Kumar & Goel, 2019). Table 8.2 confirms the increase of caffeic acid and hydroxybenzoic acid during the digestion process, rapidly absorbed in the stomach and small intestine (Kumar & Goel, 2019) and consequently increase the antioxidant capacity, as Figure 8.4 confirms. Also, during the digestion process, these soluble phenolic acids reduce the hydroxyl group (Kumar & Goel, 2019). The AA also depends on the carotenoids release throughout the GID, and the recovery index differences found in the OH and CONV samples could be explained by its hydrophobicity differences (Palafox-Carlos et al., 2011). The CONV extract is hydrophobic, allowing the carotenoids to dissolve in dietary lipids and bile acids and enzymes; the action led to its incorporation into micelles and solubilization in the system (Palafox-Carlos et al., 2011).

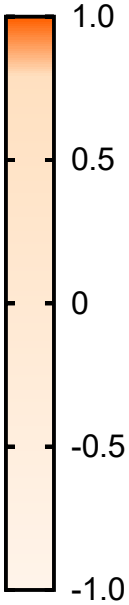
The differences between ORAC and ABTS are shown in Figure 8.4. In ORAC, the AA of OH extract is slightly higher than when measured by ABTS. Unlike the ABTS, which measures the antioxidant activity and the specific reducing power, the ORAC allows assessing the antioxidant scavenging activity and determines the antioxidant status in biological systems (Coscueta et al., 2019).

In each phase of digestion, the OH presented higher values than the CONV extracts ($p<0.05$) for both methods, associated with the higher release of polyphenols.

The AA of OH extracts increased during digestion. The tomato extracts are rich in BC, including carotenoids and phenolics, released throughout digestion and increase the AA (Coelho et al., 2019; Pavan et al., 2014). This increase is supported by the direct correlation between AA measured by ABTS and total phenolic compounds, carotenoids and individual compounds $r^2> 0.84$ (Table 8.3). Besides, the increase of AA may be derived by the formation of new BC with antioxidant properties, which may consist of bioactive metabolites generated from modifications of compounds, broken bounds, and others that may be originated from metabolic reactions (Del Pino-García et al., 2016). The increasing recovery index confirms this throughout the GID. The OH samples presented a recovery of 130% index in the mouth, and an AA increase between undigested and digested OH samples was also shown. In the small intestine, the recovery index of OH increased up to 160%, which confirms the saliva, gastric enzymes action, and the bile salts effects on compounds and, thus, its better release throughout the GID system, confirmed by the antioxidant increased. According to the results, the OH presented lower bioaccessibility than the CONV samples, 51% and 89%, respectively.

Table 8.3. Pearson's correlation between principal compounds determined in the basolateral fraction and the bioactivities; TPC – total phenolic compounds; TC- total carotenoids; IACE – anti-hypertensive activity; IL6- interleukine 6

	TPC	TC	rutin	naringenin	lutein	β-carotene	ABTS	ORAC	IL6	IACE
TPC	1.00	0.93	-0.94	0.87	0.80	0.93	0.98	-0.94	-0.98	-0.97
TC	0.93	1.00	-0.99	0.96	0.97	0.99	0.94	-0.99	-0.98	-0.99
rutin	-0.94	-0.99	1.00	-0.98	-0.95	-1.00	-0.97	0.98	0.97	0.98
naringenin	0.87	0.96	-0.98	1.00	0.96	0.98	0.93	-0.92	-0.91	-0.92
lutein	0.80	0.97	-0.95	0.96	1.00	0.95	0.84	-0.94	-0.89	-0.91
β-carotene	0.93	0.99	-1.00	0.98	0.95	1.00	0.97	-0.97	-0.97	-0.97
ABTS	0.98	0.94	-0.97	0.93	0.84	0.97	1.00	-0.93	-0.97	-0.96
ORAC	-0.94	-0.99	0.98	-0.92	-0.94	-0.97	-0.93	1.00	0.99	0.99
IL6	-0.98	-0.98	0.97	-0.91	-0.89	-0.97	-0.97	0.99	1.00	1.00
IACE	-0.97	-0.99	0.98	-0.92	-0.91	-0.97	-0.96	0.99	1.00	1.00



As explained above, the compounds present in the matrix are digested differently (Kumar & Goel, 2019; Palafox-Carlos et al., 2011), translating into the compounds' bioavailability. In this case, the compounds are mainly phenolic compounds and carotenoids and confer antioxidant activity throughout the GID (Palafox-Carlos et al., 2011).

Both digested extracts, OH and CONV, showed a significant decrease in AA in ORAC and ABTS in the colon (large intestine) and a statistical increase in the basolateral fraction. The results suggest the solubilization of the compound during the GID and diffusion out of the dialysis tube, being bioavailable (Ribeiro et al., 2020), reducing the level maintained in the colon.

8.3.3.2. Prebiotic effect of digested OH and CONV extracts

The effect of tomato extracts after GID simulation, representing the fraction non absorbed and collected in the colon, on the growth of the potential prebiotics can be seen in Figure 8.5.

Bifidobacterium and *Lactobacillus*, found in the human gastrointestinal microbiota, play an essential role in health promotion. These bacteria are often used in probiotic preparations or considered target microorganisms for prebiotic substrates, taking into account their beneficial effects (Carvalho et al., 2019).

The *in vitro* evaluation of the impact of the tomato bagasse extracts (OH and CONV), after GID passage, upon *Bifidobacterium* strains showed two different behaviours. Both bacteria growths measured by turbidity at 660 nm under the effect of both digested extracts tested and the fructooligosaccharides (FOS) (used as control) at the same concentrations were tested and presented in Table 8.2.

For *B. animalis* subsp. *lactis* BB-12 (Figure 8.5 A), the OH extracts promoted less growth increment (growth rate in the exponential phase) than the CONV extracts ($p>0.05$). But that is without significant difference for the maximum growth rate reached. Both extracts appeared to promote the growth of the microorganisms, with total biomass after 24 h incubation being higher than the negative control and even with better performance than FOS. The OH also presented a lower growth rate With *B. longum* (Figure 8.5 B) than the CONV samples. Furthermore, the OH showed higher absorbance (biomass) than the CONV samples ($p<0.05$) and presented similar results to the positive control. To *B. animalis* subsp. B0 (Figure 8.5 C), there is an initial lag phase for all the samples, particularly FOS, maintaining for 20 h. Also, the presence of both extracts had no impact on this bifidobacteria growth comparatively with the positive control (FOS), presenting similar results to the negative control (bacteria growth). Different *Bifidobacterium* strains may have different carbohydrate utilizing

abilities, as established by some studies (Costa et al., 2019; Carvalho et al., 2019b)

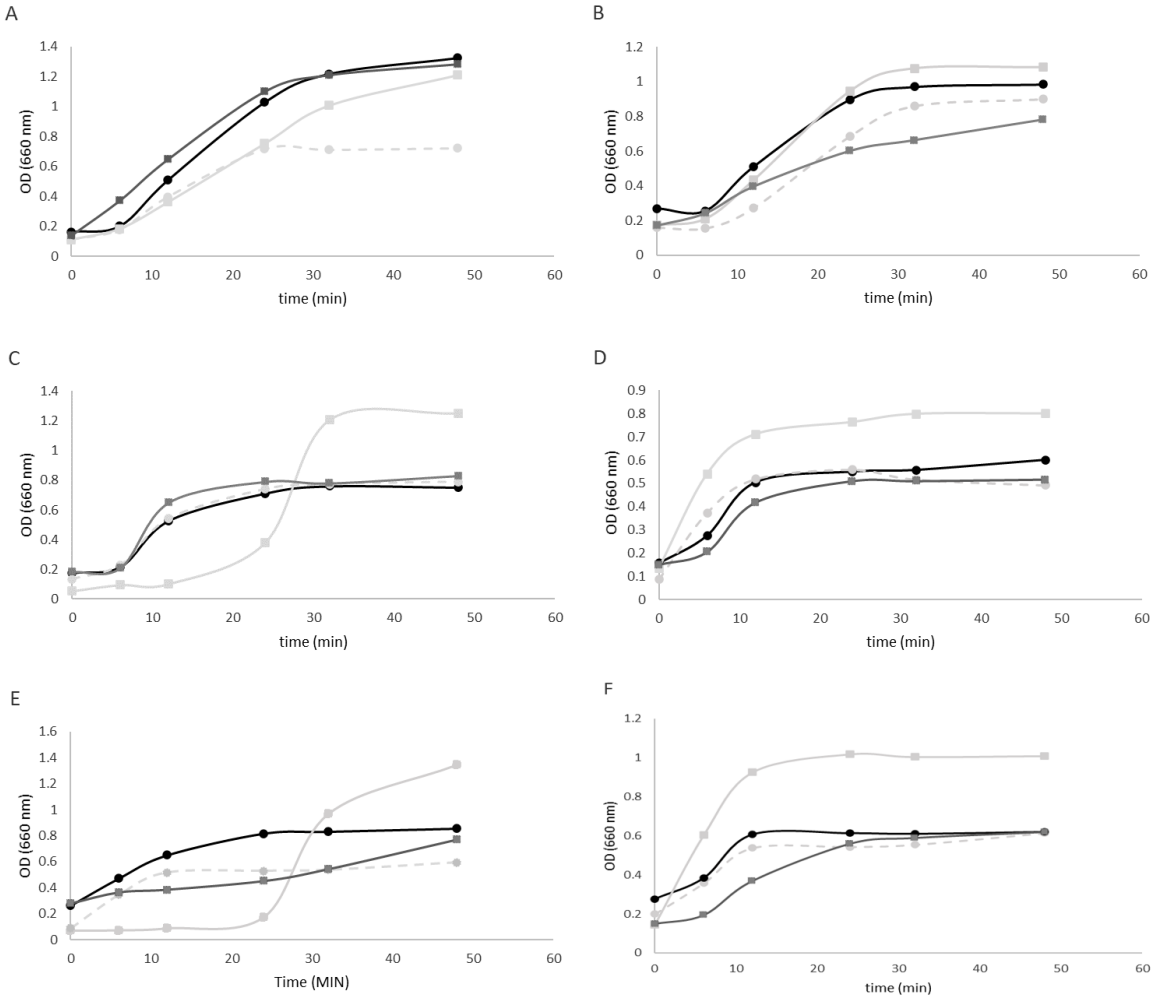


Figure 8.5. Growth curves of A-*B. animalis* subsp. *lactis* BB-12; B-B. *longum* BG3; C-*B. animalis* Bo; D-*L. acidophilus* LH5; E-*L. casei* LC1; F-*L. rhamnosus* R11 in the presence of 1% extract obtained by OH process. (●), 1% extract obtained with CONV process (■), positive control – FOS (●) and negative control without sugar source (●).

Regarding *L. acidophilus* the OH extracts promoted slightly more growth than CONV samples; also, it presented higher absorbance than the CONV extracts (Figure 8.5 D). Nevertheless, although the OH presented higher absorbance at 6 h when compared with the negative control, after 15 h, the extracts displayed similar results ($p>0.05$). For *L. casei* the OH sample promoted a faster growth (higher growth rate during 24 h) than CONV one and negative control (Figure 8.5 E). The OH presented higher absorbance in the stationary phase than the CONV extracts ($p<0.05$). For FOS, there is a prolonged lag phase, but after 24 h, it induced recovery, promoting higher maximum growth than the other samples. For *L. rhamnosus* the OH and CONV samples presented a slower growth rate than the positive control (Figure 8.5 F). Also, the OH extract showed a higher growth rate than the CONV one. These results agree with the results presented before, where the OH possesses more glucosides compounds that can promote microorganism growth (Costa et al., 2019). The OH presented lower absorbance (biomass) compared to the positive control ($p<0.05$), but higher than

the negative control and especially than the CONV extract. The results indicate that tomato extracts may be used as potential prebiotic by this probiotic bacterium.

These results indicated that OH extracts promote different probiotic strains growth, probably due to the presence of saccharides, polyphenols and carotenoids. Thus, the extract impact is strain-dependent, as mentioned in the literature (Costa et al., 2019; Gullón et al., 2015). Gullón et al. (2015) studied the arabinoxylooligosaccharides from wheat bran, and they found different probiotic growth profiles to the same extract.

Costa et al. (2019) observed grape flour's prebiotic and antimicrobial effect, rich in xylooligosaccharides. The authors have also shown that extracts stimulate the growth of probiotics differently. On the other hand, the same extract has selective antimicrobial effects for Gram-positive and Gram-negative bacteria. This result reinforces those extracts present a selective capacity to stimulate bacteria. *L. casei, B. longum* BG3 and *B. animalis* subsp. OH extracts enhanced *B. animalis* subsp. *Lactis* BB-12 growth.

Furthermore, this extract promoted a faster growth rate for *L. casei* and *B. animalis* subsp. *lactis* BB-12 than the positive control. The extracts did not affect the *L. acidophilus*, *L. rhamnosus* and *B. animalis* B0 growth.

8.3.3.3. Inhibition of angiotensin-converting enzyme activity (IACE)

Angiotensin-converting enzyme (ACE) is one of the leading regulators of blood pressure. Some anti-hypertensive agents act through the suppression of the enzyme. The *in vitro* test that assessed ACE inhibitory action can determine the potential anti-hypertensive activity. The basolateral fraction (corresponding to the absorbed samples) evaluated the ACE inhibition since this fraction will be distributed through the bloodstream.

Both OH and CONV tomato bagasse extracts inhibited ACE (Figure 6 A). Statistical results indicated that there was no significant difference in the inhibitory action between both extracts. These extracts contain polyphenols, mainly rutin, with a positive correlation to IACE, r^2<0.98. Several studies have confirmed the rutin inhibitory potential against the ACE activity by its capacity to bind to active sites of the enzyme, competing with the substrate (Ang I) (Ciumărnean et al., 2020; Moayedi et al., 2017). The ACE inhibitory capacity of flavonoids seems to depend on their total flavan-3-ol content, depending on the mean degree of polymerization (mDP) of proanthocyanidins, since the monomers like epicatechin and catechin do not inhibit. Besides, as ACE is a membrane protein, the capacity of procyanidins to be adsorbed on the membrane surface is dependent on the hydroxyl groups on the procyanidins (Guerrero et al., 2012).

Other authors found IC50 values of 1.5 mg/mL ACE-inhibitory activities or tomato processing by-products (Moayedi et al., 2017). Nevertheless, they only studied the total fraction of tomato, with no result reported to the digested fraction and, consequently, the absorbed fraction.

8.3.3.4. Cytokines inhibition – anti-inflammatory activity

The results showed (Figure 8.6 B and C) inhibition of the release of pro-inflammatory cytokines by peripheral blood mononuclear cells (B and T lymphocytes and monocytes) from healthy donors.

Acute inflammation is a short-term activity, usually appearing within a couple of minutes or times and stopping upon removing the harmful stimulus. It requires the coordinated and general mobilization response locally of several immunes, endocrine, and neurological mediators of the acute-phase reaction of inflammation (Fernandes et al., 2010; Ghavipour et al., 2013). Interleukine- 6 (IL-6) and tumour necrosis factor-alpha (TNF-α) are examples of cytokines involved in systemic inflammation (Ghavipour et al., 2013). The typical good reaction turns into activated, clears the pathogen, starts the repair process, and then ceases. The OH and CONV samples' anti-inflammatory activity was assessed by measuring their ability to reduce the level of Il-6 and TNF-α (tumour necrosis factor) after stimulation with a mixture of lipopolysaccharides (LPS), as showed the Figure 8.6. The positive control results (cells stimulated only with LPS) are also included in the figure. *In vitro* results strongly suggest that OH and CONV inhibited LPS-stimulated pro-inflammatory cytokines Il-6 and TNF-α. Both absorbed fractions, OH and CONV, decreased the IL-6 ($p<0.05$) and TNF-α concentrations ($p>0.05$). These results could be explained mainly by compounds absorbed in the blood, such as carotenoids (lycopene), phenolics (naringenin, quercetin), which presented anti-inflammatory properties (Fernandes et al., 2010; Ghavipour et al., 2013). This bioavailability and bioaccessibility of ingredients are exceptionally contingent on the composition of the nutrients array. Indeed, studies, up to now, suggest a process impact on the ingredients bioaccessibility and bioavailability, which is contingent on this type of compound, the structure and arrangement of the food matrix-matrix and the extraction solvents method used. Depending on BC solubility in the water, they can be separated into lipophilic and hydrophilic. The results showed that the CONV samples contain more carotenoids and flavonoids than the OH samples, presenting a slightly higher anti-inflammatory activity. Also, the samples could be encapsulated to withstand the stomach acidity and degradation by enzymes to eventually enhances its absorption.

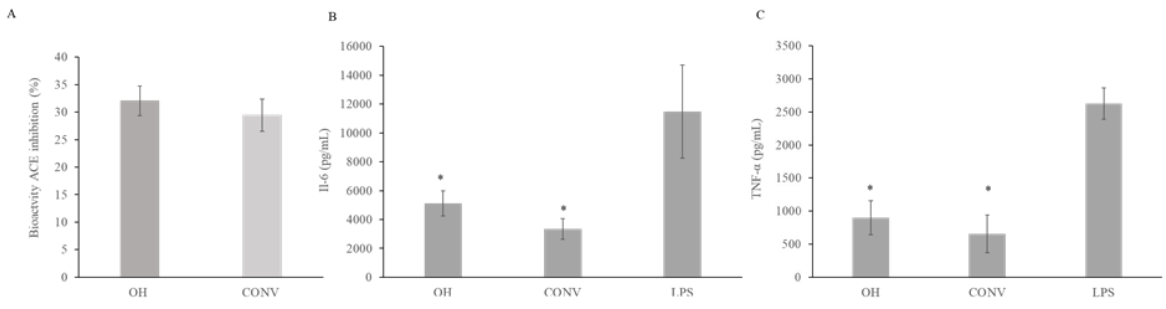


Figure 8.6. Bioactivity ACE inhibition (%), graph A; Interleukin-6 (IL-6), graph B, tumour necrosis factor-alpha (TNF-α), graph C, concentration in the supernatants from cells stimulated with an absorbed fraction of OH and CONV extracts in combination with 2.5 µg/ mL lipopolysaccharide (LPS) per well. The data are expressed as the mean ± SD (*$p<0.05$ vs LPS control).

These results agree with others reported with literature, in which authors attributed the anti-inflammatory activity mainly to lycopene from the tomato juice. Riso et al.(2006) exhibited modest

effects on the production of TNF-α, due to tomato drink by young, healthy volunteers. Mohri et al. (2018), studied the anti-inflammatory activity of different compounds extracted from tomato on obese people, and they concluded that tomatoes containing diverse anti-inflammatory compounds could help respond to the chronic inflammation in obese adipose tissue.

Concluding, the OH extracts from tomato by-products likewise demonstrated anti-inflammatory activity, antioxidant, prebiotic activity stain dependent, and moderate anti-hypertensive action.

8.4. Conclusion

The BC extracted from tomato bagasse and present in two LF, one using a green (OH) and the other a CONV technology, were studied regarding their stability throughout the GID tract, demonstrating the predicted bioaccessibility and bioavailability of the main compounds present – carotenoids and polyphenols. Both extracts are rich in carotenoids, mainly lutein, lycopene, and β-carotene, as well as polyphenols, mainly hydroxycinnamic acids and benzoic acids. Although the CONV extract is richer in polyphenols, the OH extract presented a higher TPC recovery index throughout the digestion process, reaching similar concentrations to CONV only in the small intestine. The main phenolic compounds contributing to OH's higher TPC recovery index are 4-hydroxybenzoic acid, rutin, naringenin, and luteolin. Regarding the total carotenoids content, the content is slightly higher in OH extract, but very similar recovery indexes throughout the GID were observed for both extracts, with a significant decrease after the gastric step. In any case, a higher bioavailability was observed for carotenoids present in CONV extract than for OH extract. Furthermore, the changes caused throughout the digestion process on BC also contributed to their bioactivities.

Regarding AA, the OH bioaccessibility is also higher than in CONV samples and well correlated with polyphenols. Furthermore, the OH extracts showed a potential prebiotic effect upon *B. animalis* subsp. *lactis* BB-12*, B. longum* BG3 and *L. casei*, while the CONV showed a prebiotic effect to *B. animalis* subsp. *lactis* BB-12. Both extracts also presented anti-hypertensive activity in their bioavailable fraction, although OH bioavailable compounds showed slightly higher ECA inhibitory capacity (32%) than the CONV ones (28%). Both OH and CONV extracts also demonstrated for the first time anti-inflammatory properties in their bioavailable compounds.

In conclusion, the type of extraction profoundly influences the bioaccessibility and bioavailability of polyphenols and carotenoids of tomato bagasse LF and related biological properties. The OH extraction, a green alternative technology, improved the bioaccessibility of polyphenols compared to CONV and improved the antioxidant bioavailability. Furthermore, OH extract showed a potential prebiotic effect and anti-hypertensive activity and anti-inflammatory activity in general with better performance than CONV.

Chapter 9.

In vitro gastrointestinal digestion impact on the bioaccessibility and antioxidant capacity of BC from tomato flours obtained after conventional and ohmic heating extraction

Abstract

In times of pandemic and when sustainability is in vogue, the use of by-products, such as fibre-rich tomato by-products can be an asset. Therefore, there are still no studies on the impact of extraction methodologies and the gastrointestinal tract action on bioactive properties. Thus, this study used a solid fraction obtained after the conventional method (SFCONV) and a solid fraction after the ohmic method (SFOH) to analyze the effect of the gastrointestinal tract on bioactive compounds (BC), and bioactivities. Results showed that the SFOH present higher total fibre than SFCONV samples, 62.47±1.24 - 59.06±0.67 g / 100 g DW, respectively. Both flours present high amounts of resistant protein, representing between 11 to 16% of insoluble dietary fibre. Furthermore, concerning the total and bound phenolic compounds, the related antioxidant activity measured by ABTS assay presented significantly higher values for SFCONV than SFOH samples ($p<0.05$). The main phenolic compounds identified in the two flours were gallic acid, rutin, and p-coumaric acid, and carotenoids were lycopene, phytofluene, and lutein, all known as health promoters. Despite the higher initial values of SFCONV polyphenols and carotenoids these BCs' OH flours were more bioaccessible and presented more antioxidant capacity than SFCONV flours, throughout the simulated gastrointestinal tract. These results confirm the potential of OH to modify the bioaccessibility of tomato BC, enhancing their concentrations and improving their antioxidant capacity.

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Keywords: *Lycopersicum esculentum*; tomato; by-products; ohmic heating; bioaccessibility; bioavailability; carotenoids; phenolic compounds

9.1. Introduction

Tomato pomace is a major by-product worldwide (Coelho et al., 2019; FAO, 2017) and is well known for its bioactive compounds, e.g., fibres, phenolic compounds (Navarro-González et al., 2011b), and carotenoids, which have a positive impact upon human health , including gastrointestinal health (T. B. Ribeiro, Oliveira, Campos, et al., 2020; Szabo et al., 2018). Nevertheless, the health benefits provided by dietary bioactive compounds depends on their bioavailability (Ribeiro, et al., 2020). According to the Food and Drug Administration, bioavailability "is the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of drug action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by scientifically valid measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of drug action". Components present in food pass through the mouth, stomach, and intestine before reaching the blood (Dima et al., 2020). Moreover, bioactive compounds are liberated from food matrices, with modifications within the gastrointestinal tract, solubilization at the intestinal fluids, and permeation through the gut (Dima et al., 2020; Ribeiro et al., 2020). The remaining non-bioaccessible fraction is directed to be used by gut microbiota.

One example is the group of carotenoids that are lipophilic food pigments, including precursors of Vitamin A, a nutrient needed for cell differentiation, vision, and immunity (Cabezuelo et al., 2020; Maoka, 2020). Humans cannot synthesize carotenoids, so they are usually consumed with natural sources like fruits and vegetables. In order to exert their function, carotenoids and their metabolites must be assimilated for distribution to tissues and organs (Maurya et al., 2020). Unlike some other dietary lipids, the bioavailability of carotenoids is impacted by several factors, including food matrix, kind of process, different dietary elements, and nutritional and physiological status (Moran et al., 2018). Carotenoids are extremely sensitive to heat and light so they can easily undergo thermal degradation and photodegradation. Therefore, a reliable assessment of carotenoid bioavailability is difficult. The traditional methods used to extract both polyphenols and carotenoids compounds from food matrices require organic solvents, most of them being toxic to human health, and requiring purification methods to be used in the food industry. Currently, several authors propose the use of alternative methodologies to recover these bioactive compounds, like Ohmic Heating (OH) (Coelho et al., 2019; Coelho et al., 2020).

OH is a process where the electrical current passes through a conductor matrix (e.g. food), generating heat through the food's electrical resistance (Coelho et al., 2020). Coelho et al. (2019) applied this technology to tomato by-products to extract bioactive compounds. The authors showed that this method could be a selective method to extract both polyphenols and carotenoids using optimized conditions. Furthermore, they only used ethanol:water as a solvent to recover these bioactive compounds, highlighting the potential of this technology to substitute the organic solvents usually used in conventional methodologies.

Although the technology has been successfully applied previously (Coelho et al., 2019), many of the bioactive compounds are not extracted, remaining bound to dietary fibres, which confer biological properties to the solid residue resulting from the extraction (Ribeiro et al., 2020). However,

the potential antioxidant properties of these solid residues and how their main bioactive compounds are affected by the gastrointestinal tract during digestion were never studied until now.

Thus, this study aimed to assess the impact of gastrointestinal conditions on bioactives composition and antioxidant activity from tomato flours obtained after two methods of extraction (OH and CONV).

The bioaccessibility of the major polyphenols and carotenoids present were also evaluated, as well as the cytotoxicity of the digested fractions to guarantee a safe food ingredient with biological properties.

9.2. Materials and methods

9.2.1. Chemicals

The 2, 20-azo-bis-(2-methylpropionamidine)-dihydrochloride (AAPH), fluorescein, 2, 2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS diammonium salt), potassium sorbate, sodium carbonate, ethylenediaminetetraacetic acid (EDTA), sodium sulfite, and sodium lauryl sulfate were purchased from Sigma-Aldrich (Sintra, Portugal). Hexane, Folin–Ciocalteu's reagent, and potassium persulfate were purchased from Merck (Algés, Portugal). Standards of ascorbic acid, trolox, gallic acid, rutin, p-coumaric, and 4-hydroxybenzoic acid, were purchased from Sigma-Aldrich (Sintra, Portugal), while kaempferol, β-carotene, lycopene, zeaxanthin, and lutein (Extrasynthese, France) were purchased from Extrasynthese (Lyon, France).

9.2.2. Preparation of tomato bagasse flours

Tomato bagasse was kindly provided by Sugal and Italagro group, from the centre of Portugal. The two samples were collected and transported immediately to the laboratory. Each sample was characterized for nutritional composition, and a mixture of samples was performed to guarantee a more representative global sample. From there, homogenous samples were immediately dried at 55 ºC in a convection oven for 24 h until levels of water activity (a_w) reached 0.4. The dried solid sample was milled with a kitchen robot (Bimby Vorwerk TM5) and sieved (particle size distribution study using a sieve shaker with a series of sieves, mesh No. 10, 18, 30, 40, 60, 100, and 200) (Ribeiro, Oliveira, Coelho, et al., 2020). The fractions were combined to obtain tomato bagasse flour (TBF). The TBF represents 86.8% dry weight (DW) of the total solid fraction, and its particle size ranged between 75 to 400 μm. After this, TBF was used to perform the extractions throughout OH using green solvents (70% ethanol, 15 min, 55 ºC) and CONV (hexane for carotenoids extraction) (Navarro-González et al., 2011b). The carotenoids extraction was performed following (Oliveira et al., 2017). Briefly, cold ethanol, 5 mL, was added to 2.5 g of TBF. After the hexane (4 mL) was added and the resulting mixture was centrifuged (10 min at 4000×*g* under 4 ºC), and the top layer was removed to polypropylene tube. The extraction was repeated with 2.5 mL of saturated NaCl and 4 mL hexane. In this process two fractions were obtained, the liquid fraction (LF), which corresponds to the supernatant, and the solid fraction (SF) to the residue, respectively. The SF was oven-dried at 55 ºC for analysis for 24 h, constituting the solid fraction equivalent to a homogeneous flour. To

simplify the designations, the SF of OH tomato by-products is abbreviated as SFOH, and SF of CONV samples is SFCONV.

9.2.3. Chemical composition of TBF

9.2.3.1. Proximate composition of tomato bagasse flours

Determination of moisture, ash, protein, dietary fibre (TDF), insoluble dietary fibre (IDF), and soluble dietary fibre (SDF)was achieved following the recommendations of the Official Methods of Analysis Chemists (Chemists, 2000). The protein determined by the Kjeldahl method was calculated using a conversion factor of 6.25. All analyses were done in triplicate and expressed as g/ kg DW. The moisture content was determined following the method as specified in (Chemists, 2000). The total fatty acids were analyzed according to Coelho et al. (2018), with slight modifications. The internal standard used was glyceryl tritridecanoine, TG-C13, and methanol and sodium methoxide were added to 50 mg of sample in the amounts of 2.26 mL and 240 µL, respectively in the derivatization process. TDF content was estimated using the enzyme-gravimetric method, (Association of Official Analysis Chemists International (AOAC), 2011), with some modifications according to (Deng et al., 2011d). The results were expressed as TDF, insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) g /100 g DW. Free sugar content was determined by Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to RI and UV detector using Aminex 37-H column (Bio-rad, Berkeley, USA) at 55 °C and 35 mM H_2SO_4 as mobile phase (flow rate: 0.5 mL/min) Monforte, Martins 6 Ferreira (2019). The quantification was achieved using standard calibration curves (0.2 - 2.0 mg/mL) of the following compounds, glucose, xylose, galactose, arabinose, and mannose. All measurements were done in triplicate and expressed as g/ 100 g DW.

9.2.4. Bioactive phytochemicals

9.2.4.1. Phenolic compound quantification: total, free and bound profiles

Both SFOH and SFCONV were evaluated in terms of free and bound polyphenols to fibres. The SFOH and SFCONV samples obtained after extraction was washed with absolute ethanol 3 times to extract the soluble free polyphenols (SFPC) and centrifuge for 5 min at 10,000 x g. Then, for 1 g of washed SFOH and SFCONV, a reaction of 4 h was carried out with 20 mL of NaOH (4 M). The solution obtained was acidified with HCl (6 M) at pH 1.5 to 2.0 and then centrifuged for 30 min at 10,000 x g. An extraction was then performed with ethyl-acetate, for 15 min, five times (Xie et al., 2015). The supernatant was concentrated in a vacuum evaporator, resuspended with 10 mL of ethanol, and the polyphenols obtained (bound phenolic compounds, BPC) were analyzed by HPLC-DAD. Results were expressed as mg gallic acid equivalents (GAE)/ 100g DW.

The phenolic compounds were released from IDF and SDF fractions using the same hydrolysis process described above. The BPC extract obtained was used to measure the total phenolic compounds (TPC), and to determine the profile of phenolic compounds by LC-ESI-UHR-QqTOF-MS (methodology described below in section 2.3.3). The TPC was evaluated through the Folin – Ciocalteu (TPC) spectrophotometric method as described elsewhere (M. Coelho et al., 2019b). To 5

µL of a sample, diluted when needed, 15 µL of Folin – Ciocalteu reagent, 60 µl of sodium carbonate at 75 g/L (Sigma) and 200 µl of distilled water were added, and the solutions were mixed. After samples were heated at 60 ºC for 5 min and the OD was read at 700 nm using a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria). The content of total polyphenols was expressed as milligram gallic acid equivalent per DW (mg GAE/g). The analyses were performed in triplicate, and a standard deviation was calculated.

9.2.4.2. Total Antioxidant Activity, and Total Carotenoids Content

Total antioxidant activity (AA) was determined using the ABTS method as described by Gião et al. [28]. The sample was diluted when needed and added to a coloured solution of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation) ($ABTS^{\bullet+}$). The initial optical density (OD) of the $ABTS^{\bullet+}$ solution, measured at 734 nm using a UVmini 1240 UV-Vis spectrophotometer (Shimadzu, Japan), was adjusted to 700 ± 0.020. After 6 min the final OD was measured and the results were given in ascorbic acid equivalent per 100 g DW (ascorbic acid eq. / 100 g DW).

Oxygen radical absorbance capacity (ORAC) assay was performed according to described by Coscueta et al. (Coscueta et al., 2019), with some modifications in black polystyrene 96-well microplates (Nunc, Denmark). Antioxidant (20 µL) and fluorescein (120 µL; 70 nM, final concentration in well) solutions were placed in the well of the microplate at 200 µL final volume. A blank (fluorescein + AAPH) using 75 mM phosphate buffer (pH 7.4) instead of the antioxidant solution and eight calibration solutions using Trolox (1–8 µM, final concentration in well) as antioxidant were also carried out in each assay. The mixture was preincubated for 10 min at 37 ◦C. After an AAPH solution (60 µL; 12 mM, final concentration in well) was added. The microplate was immediately placed in the reader and the fluorescence was recorded at intervals of 1 min during 80 min with an excitation wavelength at 485 nm and the emission wavelength at 528 nm. The microplate was automatically shaken before each reading. This assay was performed with a multidetector plate reader (Synergy H1, Vermont, USA) controlled by the Gen5 Biotek software version 3.04. Both AAPH and Trolox solutions were prepared daily and fluorescein was diluted from a stock solution (1.17 mM) in the same phosphate buffer. Antioxidant curves (fluorescence versus time) were first normalized to the curve of the blank corresponding to the same assay by multiplying original data by the factor $\text{fluorescence}_{\text{blank,t=0}}/\text{fluorescence}_{\text{control,t=0}}$. Results were expressed as µmol TE (Trolox equivalent)/ 100 g DW.

Total carotenoid content were Total carotenoids (TC) was assessed using a spectrophotometric analysis, as described by Kimura et al. (Kimura et al., 1990) and are expressed in milligram equivalent β-carotene per DW (mg β-carotene eq. / g DW).

9.2.4.3. HPLC analysis

Qualitative and quantitative profiles of polyphenols were carried out according to the method proposed by (C. M. Oliveira et al., 2015) with slight modifications. Analysis was conducted on a Waters Liquid Chromatograph (Waters Series 600. Mildford MA. USA). A C18 guard column (Symmetry® C18) and an Alltech adsorbosil C18 reversed-phase packing column (250 x 4.6 mm i.d.

5 µm particle size and 125 Å pore size) were used for separation throughout this study. The PDA acquisition wavelength was set in the range of 216 – 600 nm. Mobile Phase: Solvent A with acetonitrile /pure water (5:95 v/v), and 0.2% TFA (Sigma-Aldrich, Germany); Solvent B (acetonitrile (100%), Merck pure grade, with 0.2% TFA; flow rate = 1 mL/min. The gradient 0–2 min (100% B); 2–28 min (60% B); and 28–30 min (100% B).

All the prepared solutions were filtered through 0.45 µm membranes (Fisher Scientific) and the mobile phase was degassed before injection onto HPLC. The volume of injection was 20 µl and samples were analyzed in triplicate. Calibration curves were obtained at a detection wavelength 280 nm (gallic acid, and 4-hydroxybenzoic acid), 320 nm (*p*-coumaric), 360 nm (rutin). Standards solutions over the concentration range from 0.10 to 100.00 mg/L were prepared for the identification and quantification of the following compounds: 4-hydroxybenzoic acid (y= 10.0 x 10^4x – 31507; R2 0.998), *p*-coumaric acid (y= 12.6 x 10^4x – 4232; R^2= 1); rutin (y = 29.5 x 10^4x + 8065; R^2 0.999), and gallic acid (y = 48.7 x 10^3x – 4520; R^2 = 0.999) expressed as milligrams per mL of DW. All calibration curves were linear over the concentration ranges tested, with correlation coefficients of 0.999.

Individual carotenoids content was analyzed by HPLC-DAD, in a Vydac 201TP54 C-18 column (250 mm - 4.6 mm), equipped with a C-18 reversed-phase packing column (250 x 4.6 mm i.d. 5 µm particle size and 125 Å pore size). The carotenoid profile was performed by chromatographic separation as described by (C. Oliveira et al., 2004). Solvent A with ethyl acetate (Merck pure grade) and solvent B 90:10 acetonitrile:water (Merck pure grade and pure water, 1.0 ml/min flow rate, at room temperature.

The UV–vis detector was set between 270 and 550 nm. Individual carotenoids were quantified based on a calibration curve built with pure standards: β-carotene, lycopene (y = 2E+08x – 44717, R^2= 0.999), zeaxanthin (y = 3E+08x - 3E+06; R^2 = 0.999) and lutein (y = 2E+08x + 2E+06, R^2 0.998) (Extrasynthese, France) and expressed as milligrams per kilogram of DW (mg / Kg DW).

9.2.4.4. UPLC -qTOF MS analysis

The UPLC -qTOF MS allows an analysis of the complete compound profile and its derivatives, which it is not detected by HPLC- DAD. Before analysis, samples were filtered with 0.2 µM PTFE filters and placed in 2 mL vials.

The phenolic and carotenoid analysis was performed on an Ultimate 3000 Dionex UHPLC coupled to an ultra-high resolution Qq-time of flight (UHR-QqTOF) mass spectrometer (Impact II, Bruker Daltonics, Germany), according to the method described by (Monforte et al., 2019), with slight modifications. Separation of metabolites was performed using a reversed-phase column ACQUITY UPLC® BEH 130Å C18, 1.7 µm, 2.1×100 mm (Waters, Milford, MA). For polyphenols, mobile phases were as set: A (Water 0.1% formic acid); B (Acetonitrile 0.1% formic acid); Flow: 0.250 mL/min; 0-10 min: 100 to 79% A; 10-14 min: 79 to 73% A; 14-18.3 min: 73 to 42% A; 18.3-24 min: 42 to 0% A; 24-26 min: 0 to 100% A. For carotenoids, mobile phases were as set: A (Water 0.1% formic acid); B (Acetonitrile/Methanol (70/30) 0.1% formic acid); Flow: 0.250 mL/min; 0-2 min: 15 % A; 2-11.6 min: 15 to 0% A; 11.6-14 min: 0 to 15% A; 14-15 min: 15% A (Rivera et al., 2011). For polyphenols,

parameters for MS analysis were set using electrospray ionization (ESI) in negative ionization mode with spectra acquired over a range from m/z 20 to 1000 in an Auto MS scan mode. For carotenoids, parameters for MS analysis were set using electrospray ionization (ESI) in positive ionization mode with spectra acquired over a range from m/z 50 to 2000 in an Auto MS scan mode (Rivera et al., 2011). The selected parameters were as follows: capillary voltage, 2.5 kV (negative mode; polyphenols) and 4.5 kV (positive mode; carotenoids); drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision radio frequency (RF), 300 Vpp; transfer time, 120 µs; and pre-pulse storage, 8 µs. Post-acquisition internal mass calibration used sodium formate clusters, with sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

The carotenoids data are expressed by the recovery index.

9.2.5. In vitro gastrointestinal digestion

Simulated complete digestion of the two SF samples (SFOH and SFCONV) was performed according to the method described by Ribeiro et al. (T. B. Ribeiro, Oliveira, Campos, et al., 2020) with slight modifications. This procedure mainly comprised sequential phases simulating different conditions along the gastrointestinal tract. A schematic representation of the main steps performed, and the fractions obtained along with simulated gastrointestinal digestion are presented in figure 9.1, providing information on the process and its conditions. Briefly, 2 g of each sample were mixed with 20 mL of PBS solution, and α-amylase (100 U/mL) was added to the simulated mouth step, and the mixture was incubated for 2 min at 37 ºC under agitation (200 rpm). Subsequently, a pepsin (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) solution, 25 mg/mL, was added with a ratio of 0.05 mL/mL of a sample at pH 2.0 (simulated gastric solution, stomach) were added following incubation for 2 h, 37 ºC at 130 rpm orbital agitation for the gastric phase. Finally, a solution composed of 2.0 g/L pancreatin (Sigma-Aldrich Chemistry, St. Louis, Missouri, USA) and bile salts, 12 g/L, (Oxoid™, Hampshire, UK) at pH 6.0 was added (0.25 mL/mL) and incubated for 2 h at 37 ºC with 45 rpm on an orbital incubator to simulate the small intestinal phase. Then the samples were placed in a dialysis membrane, closed in vials with a solution of PBS to simulate the colon (non-absorbable sample) and the liquid outside the membrane or blood, basolateral part. Both volumes, inside and outside the membrane, were measured. After the gastrointestinal simulation, the basolateral fraction was freeze-drying for subsequent analysis.

During the gastrointestinal stimulation, samples were collected in each step: mouth; stomach, small intestine, colon, and basolateral fraction to analyze total phenolic compounds, total carotenoids, individual compounds, and metabolites (phenolic, carotenoids, and volatile compounds) by HPLC and UPLC-q-TOF Ms, and antioxidant activity. All analyses were performed in triplicate.

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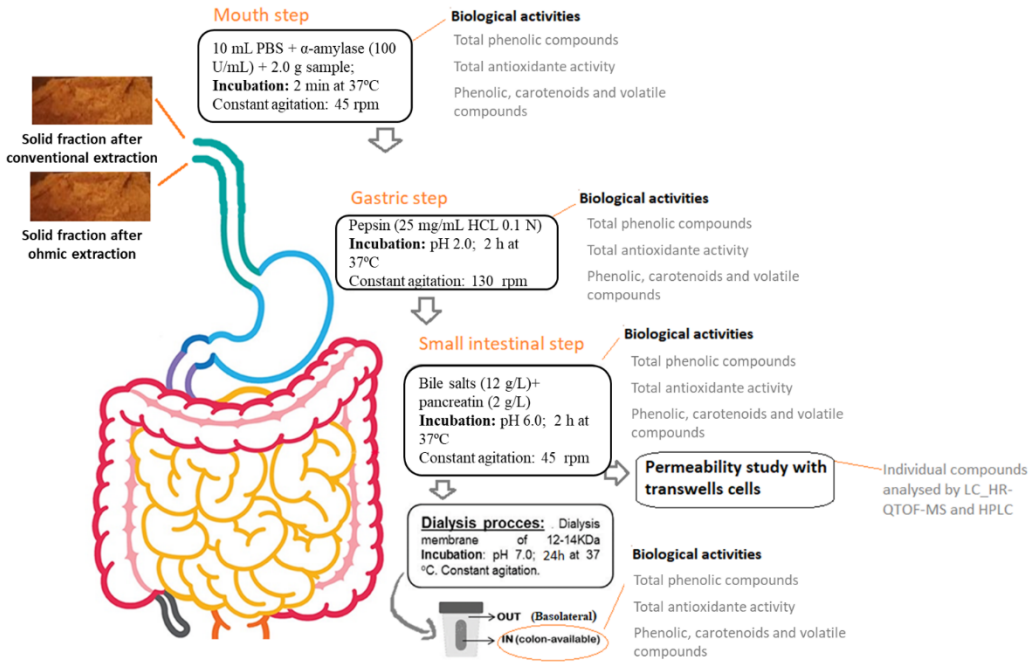


Figure 9.1. Gastrointestinal simulation and biological analysis throughout each step of digestion.

9.2.6. Simulated digestion and transepithelial diffusion across intestinal (Caco-2/HT29-MTX) cell layers

Permeability test was assessed in Corning® Transwell embeds, utilizing well plates. Caco-2/HT29-MTX co-cultures were seeded into the inserts to imitate absorptive epithelia of the human digestive tract, as detailed beforehand (Castro et al., 2019). The human Caco-2 cell line is of a colonic origin, but upon confluence, differentiates to form a well-differentiated polarized monolayer of columnar absorptive cells with a brush border and expressing typical metabolic enzymes and transporters (Costa et al., 2021).

9.2.6.1. Cell culture

Caco-2 (passage 63) and HT29-MTX (passage 55) cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, and non-essential amino acids in a humidified chamber at 37 °C and 5% CO2. The culture media was replaced every 2–3 days until cell seeding.

The cells were seeded on collagen-coated membrane inserts (0.4 µm pore size - Corning, New York, USA) placed in 12-well culture plates. For medium substitution, the medium was removed from the wells and 0.5 and 1.5 mL of new culture medium was added to the apical and basolateral sides, individually. The media was replaced every 2 days for 21 days and the TEER was measured to validate the barrier integrity (TEER > 250 Ω.cm2).

9.2.6.2. Cell layer integrity

Transepithelial electrical resistance (TEER) of Caco-2/HT29 co-cultures is the measurement of electrical resistance across a cellular monolayer and is a very sensitive and reliable method to confirm the integrity and permeability of the monolayer, using a Millicell® ERS-2 Voltohmmeter (Merck, Germany) (P. M. Castro et al., 2019). It was performed during the permeability test and estimated along with cell growth and after each example. During porousness tests, TEER values for Caco-2/HT29-MTX were regularly over 250 Ω. cm2, indicating that cells retained the integrity of their membranes (P. M. Castro et al., 2019).

9.2.6.3. Permeability assay

Upon the arrival of the examination, the culture medium was completely removed. Medium in the basolateral side (receptor part) was supplanted with 1.5 mL of PBS, pH 6.8. After the gastrointestinal simulation, the resultant digested fraction after gut simulation was centrifuge at 2,000 x g, for 5 min, and applied (0.5 mL) into the apical side of Transwells seeded with Caco-2/HT29-MTX co-culture cell layers. The aliquots were pulled back from the basolateral side at 15, 30, 60, 120 and 180 min. The BC released were assessed by LC-ESI-UHR-QqTOF-MS and HPLC, utilizing a similar technique as described above.

9.2.7. Volatile Compounds

Volatile compounds were extracted from SFOH and SFCONV samples using a headspace solid-phase-microextraction technique (SPME), at each sampling point from gastrointestinal compartments.

The used fibre was a DVB/CAR/PDMS (divinylbenzene/carboxy/polydimethylsiloxane) 50/30 mm from Supelco (Bellefonte, PA, USA). A Varian gas chromatograph, Varian CP-450 (Walnut Creek, CA, USA), equipped with a Varian Saturn 240 MS (Walnut Creek, CA, USA) mass spectral detector was used for the identification and quantification of the volatile compounds. The used column for the volatiles separation was a FactorFour VF-WAXms 15 m of length x 0.15 mm of internal diameter x 0.15 µm of film thickness column from Varian (Lake Forest, CA, USA). Briefly, 1 g of SFOH and SFCONV were dissolved in 5 mL of ethanol solution (10%), in a headspace screw vial, and spiked with 20 µL of 3-octanol (50 mg/L). Samples were pre-incubated in a CombiPAL oven at 40 °C and 150 x g for 5 min, and the fibre was exposed after 15 min at 150 x g for extraction. Desorption of the volatile compounds in the injector was performed at 220 ºC for 10 min. All mass spectra were acquired in the electron impact (EI) mode. Compound identification was achieved by comparing retention times and mass spectra obtained from a sample containing pure, authentic standards or by NIST database. Compound quantitation was performed based on standard calibration curves using m/z quantifiers. 1,2-dimethylindole - equivalents of linalool; 2,6-dimethylbenzaldehyde - equivalents of linalool; benzoic acid - pure standard; β-cyclocitral - pure standard; 3,4-diethenyl-1,6-dimethyl - pure standard; camphenol - pure standard; linalyl acetate - pure standard; linalool - pure standard.

9.2.8. Recovery and bioaccessibility indexes of polyphenolic and carotenoids compounds throughout in vitro gastrointestinal digestion

Bioaccessibility is defined as the percentage of the bioactive compound solubilized after intestinal dialysis step; this index defines the proportion of the bioactive compound that could become available for absorption into the blood system:

$$\text{Bioaccessibility index (\%)} = (BC/BCDSI) \times 100 \quad (1)$$

where: BC is the bioactive content (mg) in the digested sample after the dialysis step (OUT) and BCDSI is the bioactive content (mg) in the digested sample after the small intestinal step.
The recovery index determines the main compound concentration during each step of gastrointestinal digestion following the equation:

$$\text{Recovery index (\%)} = (BCDF/BCTM) \times 100 \quad (2)$$

Where BCDF represents the bioactive content (mg) in each digested fraction and BCTM is the bioactive content (mg) quantified in the test matrix.

9.2.9. Mutagenicity

The reference Ames mutagenic test was used to assess the mutagenicity of the tomato flours after the colon simulated digestive process, using Salmonella typhimurium (His-) strains TA-100 and TA-102 following the method described by (M. Coelho et al., 2018). The test was performed with and without a metabolic mix in which 0.5 mL of a liver homogenate (S9 mix) was included in the test tube alongside microorganisms and extract. The positive control used was quercetin (Sigma, St. Louis, MO) without metabolic activation (without rat liver extract, e.g. S9 mix addition) and B[a]P (Sigma, St. Louis, MO) with metabolic activation (with S9 mix). The number of revertants was analyzed and compared with the negative control. All the tests were performed in triplicate.

9.2.10. Statistical analysis

Statistical analysis was done using IBM SPSS Statistics v21.0 (IBM, Chicago, USA). The normality of the data's distribution was evaluated through Shapiro-Wilk's test. As the data proved to follow a normal distribution, a t-student and one-way ANOVA, coupled with Tukey's post-hoc test, was used to determine the differences of the mean values between BC or bioactivities concentrations along digestion. Supervised cluster analysis (Partial Least Squares Discriminant Analysis (PLS-DA)) was applied to evaluate the metabolite patterns of tomato bagasse flour (relative intensity) as a function of time using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) on log-transformed data after autoscaling (mean-centered and divided by the standard deviation of each variable). The differences were considered significant for p-values ≤ 0.05.

9.3. Results and Discussion

9.3.1. Characterization of Solid Fractions obtained after OH and CONV extraction of tomato bagasse

The overall average proximate analysis results for both flours obtained through different extraction methods, SFOH and SFCONV are presented in Table 9.1. In dried samples, ash, protein, fat, and fibre were higher for SFOH than SFCONV samples with significant differences for the last three parameters ($p < 0.05$). The higher nutritional value presented by SFOH than SFCONV samples is mainly due to the heating process that occurs during the OH treatment (Coelho et al., 2019), causing pores formation (electro-permeabilization), and allowing intracellular component diffusion (Ferreira-Santos et al., 2020) that probably remained on the SF after LF extraction.

The significantly higher content of protein observed for SFOH than SFCONV could be related to the interference of electric field, frequency, and consequently, temperature caused by OH in the protein' aggregation, denaturation, and soluble protein content in the matrix (Pereira et al., 2018; D. Xue & Farid, 2015). Moreover, the OH on tomato bagasse causes ions and other charged molecules (e.g. proteins), which move toward the charge electrode opposite (Rodrigues et al., 2015). Pereira et al. (2016) studied the effect of OH on whey protein and showed that fast heating through the joule effect in concomitance with low electric field contributes to obtaining lesser protein aggregates and higher soluble protein content. Regarding the CONV method, this does not use temperature and does not cause denaturation of proteins. Furthermore, this method uses hexane with protein extraction yields similar to ethanol (the latter is used in OH) (Pereira et al., 2016).

Fatty acids were also higher in SFOH than SFCONV sample ($p<0.05$). OH extraction has been associated with higher oil yield, but also with high quantities of phenolic compounds and higher antioxidant activities due to the more intense breakdown of vegetable tissues caused by heating inside the extraction chamber (Al-Hilphy et al., 2020; Moongngarm et al., 2019). Nevertheless, the use of water as an extraction solvent in OH methodology is probably the main reason for the higher fatty acids retention in SFOH than SFCONV.

Another nutritional component of tomato flours positively affected by OH extraction was dietary fibre. The SFOH presented higher total fibre and insoluble fibre than SFCONV samples ($p<0.05$). Ramírez-Jiménez et al. (Ramírez-Jiménez et al., 2019b) presented similar results with corn flours when comparing the extraction effects of OH and CONV method (nixtamalization, whereby corn, is soaked and cooked in an alkaline solution). The results suggest that OH preserve the compounds (e.g., insoluble fibre), not removing the pericarp and aleurone layer during the process, while insoluble fibre losses in the CONV method could be attributed to pericarp removal during this process, which uses organic solvent. The higher amount of insoluble fibre in SFOH makes OH process an important alternative to produce tomato flours with enhanced bioactive profile preserving bound phenolics fraction commonly associated with insoluble dietary fibre (Ramírez-Jiménez et al., 2019b).

Although polyphenols are released towards the liquid extracts, phenolics intimately bound to the fibre (but also to protein and lipids) are not extracted and are part of the flour (Jakobek, 2015). Thus, not

only free phenolic compounds (FPC) were assessed but also bound phenolics compounds (BPC) of SFOH and SFCONV were analyzed to obtain a more complete phenolic characterization from tomato by-products after phenolics recovery (Table 9.1). Significant differences ($p<0.05$) between extracted fractions, SFOH, and SFCONV, were found. SFOH exhibithed a higher TPC in FPC than in BPC. Additionally, FPC of SFOH contained a higher amount of TPC than SFCONV ($p<0.05$). These results could be explained by the extraction solvents used by OH (ethanol:water) and CONV (hexane) extraction. Tomato by-products are a rich source of liposoluble compounds that are more easily extracted by CONV extraction solvent, which justifies the lower free TPC of SFCONV. The higher TPC richness of free phenolics of SFOH were also reflected in a higher antioxidant activity associated with this flour compared to SFCONV, but only in the ORAC method ($p<0.05$). These differences probably arise from different mechanisms to measure the antioxidant capacity of each methodology. ABTS is an electron transfer method based, i.e., measure the ability of a potent antioxidant to transfer 1 electron to reduce radicals and ORAC measure the ability of an antioxidant to quench free radicals by hydrogen donation (hydrogen atom transfer-based method (T. B. Ribeiro, Oliveira, Campos, et al., 2020). Moreover, ORAC assay is a better model of antioxidant reactions with lipophilic bioactive compounds than ABTS, which can explain the higher ORAC value of the free phenolic fraction of SFOH (Schaich et al., 2015).

Regarding BPC fraction, no significant differences were detected for both antioxidant methodologies between SFOH and SFCONV ($p>0.05$), despite the highest TPC value exhibited by BPC of SFCONV compared to SFOH ($p<0.05$). Despite the significantly lower amount of BPC in SFOH, potent antioxidant compounds remained bound to tomato flour macromolecules (dietary fibre, protein, and lipids). Thus, probably this antioxidant activity was linked to other compounds (e.g. bioactive peptides or lycopene) retained in BPC extract, besides the phenolic compounds but not quantified by Folin Ciocalteau methodology.

Despite the tomato bagasse fibre richness, until now the fibre profile of SFOH and SFCONV have been neglected. The results of monosaccharides, lignin composition of IDF and SDF among samples are shown in Table 9.2. There are differences ($p<0.05$) in the monosaccharide from SFOH and SFCONV samples. The SDF profile indicates a higher soluble cellulose and mannose rich character for SFCONV than SFOH ($p<0.05$). Indeed, mannose was not quantifiable in SFOH. The action of OH extraction probably led to the release of polysaccharides rich in glucose and mannose by the cleavage of glycosidic and N-linked bonds, respectively. The release of these neutral sugars from SDF during OH extraction enlightened the lower SDF content of SFOH.

Regarding IDF, SFOH exhibited a higher richness of cellulose (as glucose) than SFCONV. Based on the results, the IDF fraction of tomato flours obtained in this work is essentially a source of cellulose (55.32 - 50.23 g / 100 g fibre) and a less abundant source of hemicellulose (24.72 - 25.2 g / 100 g fibre).

Table 9.1. The proximate composition, total phenolic compounds, and total antioxidant activity of SFOH and SFCONV samples (g / 100g DW).

Chemical Composition (g / 100 g DW)			Sample (g / 100 g)	
			SFOH	**SFCONV**
Proximate composition		*Ash*	3.32 ± 0.21 [a]	2.98 ± 0.16 [a]
		Protein	18.72 ± 0.47 [a]	16.29 ± 0.59 [b]
		Fats	21.12 ± 0.51 [a]	17.82 ± 0.3 [b]
		Dietary Fibre		
		TDF	62.47 ±1.24 [a]	59.06 ± 0.67 [b]
		IDF	50.99 ± 0.16 [a]	46.01 ± 0.13 [b]
		SDF	10.86 ± 0.85 [a]	12.98 ± 0.64 [b]
Phenolic composition	***Free phenolic compounds (FPC)***	*TPC*	1.46 ± 0.05 [a]	0.80 ± 0.01 [b]
		ABTS	0.28 ± 0.01 [b]	0.99 ± 0.15 [a]
		ORAC	2.44 ± 0.34 [a]	1.60 ± 0.09 [b]
	Bound phenolic compounds (BPC)	*TPC*	0.42 ± 0.03 [b]	0.73 ± 0.12 [a]
		ABTS	2.23 ± 0.21 [a]	2.63 ± 0.11 [a]
		ORAC	2.41 ± 0.22 [a]	2.71 ± 0.43 [a]

Values are the mean of three replicates of three independent experiments ± standard deviation. TDF- total dietary fibre; IDF; insoluble dietary fibre; SDF- soluble dietary fibre; TPC- total phenolic compounds (g galllic acid Eq./100g DW); ABTS Antioxidant activity by ABTS method (g ascorbic acid eq. / 100 g DW); ORAC- antioxidant activity (g trolox eq. / 100 g DW). Different letters within the same row are statistically significantly different (determined by ANOVA, $p < 0.05$).

Hemicellulose (as the sum of xylose, galactose, and arabinose) of tomato flours was mainly composed by xylose (13.2 - 15.1 g / 100 g fibre) followed by arabinose (9.31 - 12.81 g / 100 g fibre). The insoluble hemicellulose fraction of tomato flours are essentially a source of arabinoxylans, but SFCONV (9.31 ± 0.51 g / 100 g fibre) presented a lower amount of arabinose than SFOH (12.81 ± 0.28 g / 100 g fibre). This lower content of arabinose in IDF of SFCONV could be the main reason to its smaller IDF content.

Another significant component of IDF was lignin and resistant protein. In tomato flours, the resistant protein was detected as a significant compound of IDF representing between 11-16 % of total IDF. In line with the results obtained above for protein content, SFOH exhibited higher resistant protein content than SFCONV ($p < 0.05$), probably due to the formation of protein aggregates caused by OH (Pereira, Rodrigues, Ramos, et al., 2016; Rodrigues et al., 2015a). The presence of appreciable amounts of resistant protein in IDF has been also previously detected in tomato (≈ 8% of total IDF composition) (Goñi et al., 2009). The appreciable amounts of resistant protein of both tomato flours (more significative in SFOH) had the potential to reach the colon, where it can be used by microbiota as a nitrogen source and also as a secondary source of energy, producing ammonia, branched-chain fatty acids and other metabolites (Goñi et al., 2009). Finally Klason lignin were also present in appreciable amounts in IDF of tomato flours (between 13-14% of total IDF composition), in line with

the previous studies about tomato fibre composition (Silva et al., 2019, 2016). The lignin, as complex macromolecule with linked phenolic compounds (BPC), has the capacity to protect these compounds throughout the gastrointestinal tract up to the colon, where they play a beneficial role in gut health (Ribeiroet al., 2020).

In general, dietary fibre and polyphenols are usually analysed separately, however nowadays bound polyphenols are included as components of dietary fibre due to their well-documented association with significant contribution of bound polyphenols to the health-related properties attributed to dietary fibre (Liu et al., 2019). The industrial purée process eliminates most of the pulp from tomato fruit, resulting in a by-product composed mainly of peels and seeds. Therefore, since phenolic compounds are covalently bound to cell wall structural components, such as hemicellulose, cellulose, lignin, pectin, and structural proteins, these samples are rich in phenolics in insoluble forms (Perea-Domínguez et al., 2018). Also, other studies with fruits and vegetables refer that BPC were largely related to IDF (Goñi et al., 2009; Ribeiro, et al., 2020; Saura-Calixto, 2011). It is important to note that BPC can be released and absorbed in the organism during the digestion process (Perea-Domínguez et al., 2018), furthermore, they also contribute to the antioxidant activity.

As reported before for tomato (Goñi et al., 2009) IDF of tomato flours constitutes a higher source of bound phenolics than SDF ($p < 0.05$). The SFCONV and SFOH exhibited similar amounts of BPC (31.18-34.10 ± 1.21 mg GAE /100g fibre DW). Concerning SDF, SFOH showed a higher BPC value (7.80 ± 0.43 mg GAE /100g fibre DW) than SFCONV (4.99 ± 0.35 g GAE /100g DW), despite the lowest SDF content of SFOH. Comparing the results, OH extraction seemed to be more efficient on the retention of the bound phenolics associated to fibre than CONV extraction. Similar results were found in the literature with corn flours obtained by traditional nixtamalization and OH process (Ramírez-Jiménez et al., 2019b).

Table 9.2 - Constituents (g /1 00 g fibre DW) of SDF and IDF from SFOH and SFCONV samples.

	SFOH		SFCONV	
	SDF	IDF	SDF	IDF
Klason Lignin	n.d.	13.06 ± 0.52 [b]	n.d.	14.09 ± 0.27[a]
Glucose (as cellulose)	24.42 ± 0.43[a]	55.32 ± 1.21[b]	32.1 ± 0.56[c]	50.23 ± 1.76[d]
Hemicellulose	15.12 ± 0.54 [a]	25.2 ± 0.10 [b]	12.71 ± 1.21 [a]	24.72 ± 0.30 [b]
Xylose	*	13.2 ± 0.10 [a]	*	15.1 ± 0.17[b]
Galactose	1.87 ± 0.21	*	*	0.31 ± 0.021
Mannose	*	*	4.10 ± 0.12 [a]	*
Arabinose	13.25 ± 0.86	12.81 ± 0.28	12.71 ± 1.21	9.31 ± 0.51
Uronic acids[**]	67.41 ± 2.15 [a]	81.92 ± 1.98 [b]	58.93 ± 2.56 [c]	78.49 ± 2.32 [b]
Resistant Protein	n.d.	16.03 ± 0.05 [a]	n.d.	11.69 ± 0.03 [b]
Bond Phenolic compounds[***]	7.80 ± 0.43 [a]	31.18 ± 2.31 [b]	4.99 ± 0.35 [c]	34.10 ± 1.21 [b]

* < LOD; n.d. non determinate; IDF – insoluble dietary fibre; SDF – Soluble dietary fibre. ** - mg GUAE / g fibre DW; *** - mg GAE/ 100g fibre DW. Results are the means of three determinations ± standard deviation. Different letters in the same row are significantly different, as determined by ANOVA ($p<0.05$).

9.3.2. Total phenolic compounds, and individual compounds throughout the digestive tract and antioxidant activity

Although polyphenols are released to the liquid extract, phenolics intimately bound to the fibre are not extracted and remain part of the flour in both SFOH and SFCONV. Thus, bound polyphenols-fibre on both fractions were analyzed to evaluate its potential as antioxidant fibre and its bioaccessibility (Figure 9.2 and Table 9.3). The results showed a similar slight increase in total polyphenols content throughout the digestive tract. There is a significant difference between flours (SFOH and SFCONV) ($p<0.05$) resulting in more OH extraction efficiency than with the CONV method to liquid fraction preparation (as confirmed in Chapter 7).

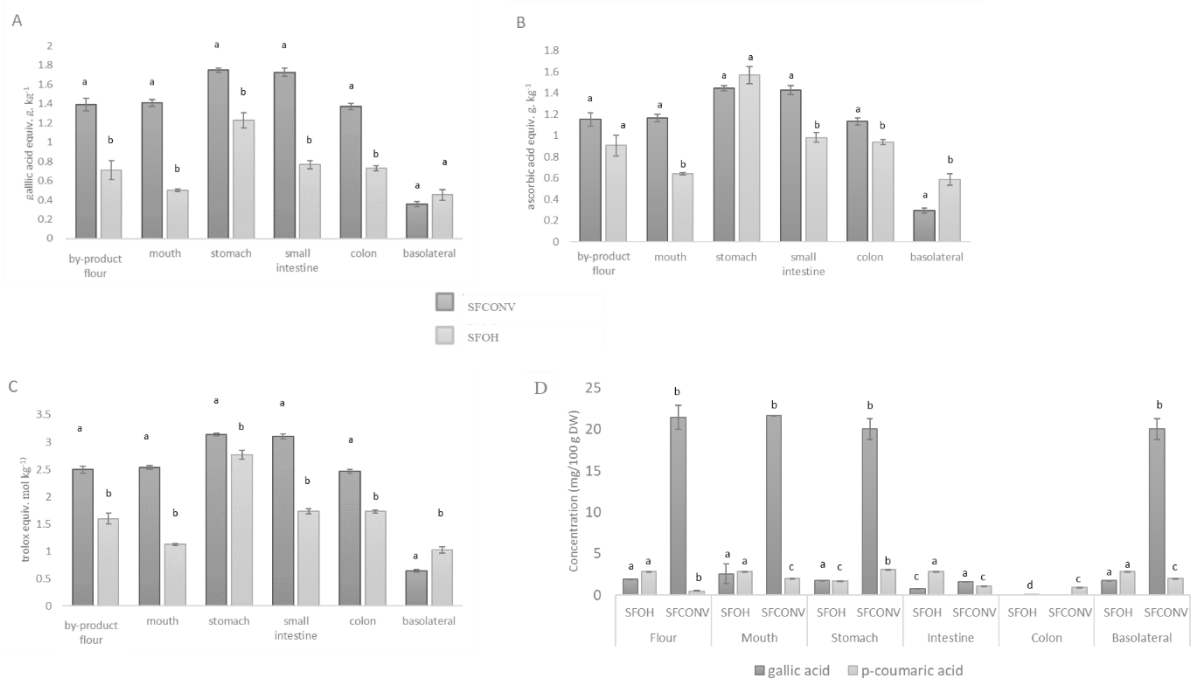


Figure 9.2. Total phenolics content, antioxidant capacity by ABTS, and ORAC method SFOH and SFCONV A -total phenolic compounds; B – total antioxidant activity by ABTS; and C- total antioxidant activity by ORAC method; D the two primary polyphenols linked to fibre. Letters mean the significant difference between methods for each digestive step. $p<0.05$.

The TPC showed a slight increase in the mouth phase in SFCONV samples (RI 101.42%), while the SFOH decreased significantly (RI 70.44%). A significant increase in the stomach for the SFCONV (RI 124.11%) and SFOH (RI 172.186%) was observed ($p<0.05$), followed by a decrease in both samples, SFCONV and SFOH, in the intestine (RI 121.99% and 122.18%, respectively).

Table 9.3. Recovery index and bioaccessibility of total phenolic compounds, antioxidant activity, and individual phenolic compounds from SFCONV and SFOH samples throughout digestion.

		Recovery index (%)					Bioaccessibility (%)
Bioactivities	**Samples**	**Mouth**	**Stomach**	**Small intestine**	**Colon**	**Basolateral**	
Total Phenol	SFCONV	101.42 ± 2.34^{a}	124.11 ± 1.28^{b}	121.99 ± 2.03^{b}	90.78 ± 1.12^{d}	9.22 ± 1.07^{e}	7.03 ± 0.42$^{\alpha}$
	SFOH	70.44 ± 1.56^{a}	172.86 ± 2.34^{b}	122.18 ± 1.24^{c}	103.04 ± 1.28^{d}	15.32 ± 1.25^{e}	11.14 ± 0.76$^{\beta}$
ABTS	SFCONV	103.08 ± 2.23^{a}	134.35 ± 1.78^{b}	124.14 ± 2.02^{c}	98.49 ± 2.45^{a}	18.1 ± 1.96^{d}	14.58 ± 0.13$^{\alpha}$
	SFOH	81.45 ± 1.87^{a}	175.71 ± 2.56^{b}	108.26 ± 1.95^{c}	92.51 ± 1.96	7.49 ± 0.06^{d}	6.47 ± 0.15$^{\beta}$
Orac	SFCONV	109.34 ± 2.25^{a}	121.65 ± 1.25^{b}	119.60 ± 1.21^{b}	90.49 ± 2.54	9.51 ± 0.07^{d}	7.37 ± 0.31$^{\alpha}$
	SFOH	89.19 ± 2.54^{a}	160.36 ± 2.46^{b}	108.26 ± 2.38^{c}	87.50 ± 1.26	12.50 ± 1.01^{d}	10.35 ± 0.12$^{\beta}$
Phenolic compounds							
gallic acid	SFCONV	101.19 ± 1.87^{a}	93.56 ± 1.17^{b}	7.62 ± 0.97^{c}	n.d.	93.56 ± 1.87^{b}	92.46 ± 1.03$^{\alpha}$
	SFOH	134.31 ± 1.12	8.45 ± 0.2 3^{b}	29.76 ± 0.79^{c}	n.d.	100.00 ± 2.05^{d}	77.07 ± 1.44$^{\beta}$
4-hydroxybenzoic acid	SFCONV	100.38 ± 1.56^{a}	87.64 ± 1.68^{b}	12.74 ± 0.26^{c}	n.d.	87.64 ± 1.67^{b}	87.31 ± 1.38$^{\alpha}$
	SFOH	102.54 ± 1.69^{a}	84.92 ± 1.78^{b}	70.98 ± 1.45^{c}	70.98 ± 0.13^{c}	13.94 ± 1.36	16.42 ± 1.22$^{\beta}$
p-coumaric acid	SFCONV	79.35 ± 2.56^{a}	121.14 ± 1.27^{b}	42.49 ± 0.08^{c}	36.15 ± 0.89^{d}	78.65 ± 1.23^{a}	64.92 ± 1.12$^{\alpha}$
	SFOH	89.07 ± 1.98^{a}	59.51 ± 1.25^{b}	100.42 ± 2.76^{c}	0.42 ± 0.03^{d}	99.37 ± 3.01^{c}	49.74 ± 1.12$^{\beta}$
rutin	SFCONV	17.01 ± 0.54^{a}	n.d.	68.83 ± 1.05^{b}	n.d.	58.83 ± 2.16^{c}	46.08 ± 1.56$^{\alpha}$
	SFOH	7.84 ± 0.05^{a}	n.d	n.d.	n.d.	7.56 ± 0.34^{a}	99.99 ± 0.66$^{\beta}$

n.d. not determined. Recovery index and bioaccessibility %. Results are the means of three determinations ± standard deviation. Different letters in the same row are significantly different ($p<0.05$), the greek alphabet means significant differences between methods used in the same column ($p<0.05$). as determined by ANOVA ($p<0.05$).

These increases observed in the stomach could be explained by the compounds presented in both samples, mainly gallic acid, 4-hydroxybenzoic acid, p-coumaric acid, and rutin. Furthermore, during this phase, phenolic compounds hydrolysis occurs by enzymatic action and the acidic condition, which releases the phenolics bound to dietary fibres and proteins either in SFCONV or SFOH samples (Pavez-Guajardo et al., 2020; Ribeiro, et al., 2020). Also, it is crucial phenomena for the protection of polyphenols against degradation (Ribeiro, et al., 2020). The significant decrease of TPC observed in SFOH samples ($p<0.05$) could be explained by the alkaline conditions presented in the small intestine which promotes the degradation of the compounds.

During the dialysis step, mainly to the basolateral fraction, a significant increase ($p<0.05$) of TPC was observed in SFOH comparing to the SFCONV samples, probably due to the electropermeabilization (membrane alterations) during the OH process (Coelho et al., 2019; Coelho et al., 2020; Pavez-Guajardo et al., 2020). Due to this phenomenon, bioactive compounds are better retained and protected within the cell, thus suggesting that they are more available at the time of digestion (Pavez-Guajardo et al., 2020). Thus, these polyphenols could be released by the additional time interaction (24 h) between samples and both intestinal fluids and digestive enzymes present in pancreatin (a pancreas porcine extract constituted mainly by proteolytic, lipolytic, amylolytic, and nucleic acid splitting enzymes) (Ribeiro et al., 2020). Similar results were obtained with enriched apple snacks impregnated with grape juice using the OH method (Pavez-Guajardo et al., 2020).

Taking into account the individual phenolic compounds, the results obtained by HPLC were according to the TPC results. This evidence is clear when compared to the recovery indexes of individual phenolic compounds (Table 3). An example is a *p*-coumaric acid for the SFCONV samples, where a decrease was observed in the mouth (RI 79.35%), and there was an increase in the stomach (RI 121.14%), followed by a decrease in the small intestine (RI 42.49). Another example is rutin, which decreased in the mouth (RI 7.84%) and maintained in the intestine (RI 7.56%). This compound is a water-soluble flavonoid, that was quickly absorbed after mouth simulation, which is in agreement with the literature (Ganeshpurkar & Saluja, 2017; Ou-Yang et al., 2013).

The AA was evaluated by two different methods (ABTS and ORAC). The samples displayed different behaviours. Regarding total AA analyzed by ABTS, no differences were found between SFOH and SFCONV solid fractions ($p>0.05$), however, for the ORAC results, they presented significant differences between both samples ($p<0.05$). The different results could be explained by the chemical reaction used as explained before.

Regarding the AA of SFCONV and SFOH throughout the gastrointestinal tract, different profiles were found for both samples. For SFCONV samples there was a slight increase in the mouth (RI 103.08%), followed by a significant increase in the stomach (RI 134.35%) and a significant decrease in the small intestine (RI 124.14%). While for SFOH, a decrease of AA was observed in the mouth (RI 81.45%), followed by a significant increase in the stomach (RI 175.71%) and a significant decrease in the small intestine (RI 108.26%). The results were in accordance with the results reported for TPC and also with the literature (Pavez-Guajardo et al., 2020; Ribeiro et al., 2020).

Regarding the ORAC method, higher values were found for SFCONV. Nevertheless, the same trend was found for SFOH and SFCONV within the gastrointestinal tract. For the SFCONV sample, a slight increase was observed in the mouth (RI 109.34%), followed by a significant increase in the stomach ($p<0.05$), and decreasing in the small intestine (RI 108.26%).

In the case of AA, a reduction was observed in the small intestine step, manifesting more in the ABTS of SFOH samples (RI 63.45%) than ORAC (RI 52.10%). Nevertheless, after the intestinal absorption simulation, the ORAC recovery index is higher in the IN fraction (colon available fraction) than in OUT fraction (basolateral fraction). Despite the initial AA present in both SFCONV and SFOH samples, only a small proportion of the total AA at the end of digestion (IN +OUT) was detected in the basolateral step. The higher content was observed in the basolateral step for SFOH than the SFCONV samples, indicating that the thermal process, caused by OH, significantly affects this property. Also, better absorption of bioactive compounds from SFOH than from SFCONV was observed.

The digested products' antioxidant properties depend upon the number of polyphenols and other bioactive compounds available for concentration in the upper or lower parts of the digestive tract (bioaccessibility) or on the quantity absorbed. Also, the extraction process can cause some physicochemical alterations in flours: on the one hand, the extraction may not cause cleavage of molecules, on the other hand, in the conventional method, using hexane to extract carotenoids and many polyphenols (polar) is not enough to extract these compounds which remain in the flours probably bound to fibre (Cömert & Gökmen, 2017). During digestion, the molecules present in flours are broken down into smaller ones, and as the bonds between these molecules weaken or break, chemical reactions can occur, and new compounds are produced. These chemical digestion use enzymes through the digestive tract, which break these bonds that hold molecules together, such as polyphenols, carotenoids, fats, proteins, and carbohydrates, that are split into smaller molecules. Polyphenols are commonly bound to the sugar moiety, forming glycones; without the sugar moiety, this simple polyphenol system is the aglycone (Etcheverry et al., 2012).These compounds bound to dietary fibre need to be hydrolyzed by enzymes in the gut's upper region; otherwise, these compounds are not bioaccessible in the intestine but may remain vulnerable to the colonic's degradation microflora at the large intestine (Pérez-Jiménez et al., 2009). Given that dietary fibre acts as the entrapping matrix and limits these enzymes' access to their substrates, most of the polyphenols bound to dietary fibre may pass to the large intestine.

Nevertheless, the smaller molecules pass through the lining of the small intestine and can be absorbed. Thus, they are less bioaccessible than SFOH polyphenols that have polar characteristics and can be incorporated in the polar medium (Barba et al., 2017; Dufour et al., 2018). Dietary fibres may interact with polyphenols and carotenoids at the digestive tract, which might improve compound bioaccessibility in the digestive tract and hence their bioactivities.

This absorption changes the polyphenols extensively: the glycosides are hydrolyzed in the small intestine, or the colon, and release aglycones that can be absorbed. Before entering the bloodstream,

the polyphenols undergo different functional changes because of the conjugation activity, primarily in the liver.

Regarding the carotenoids, see Table 9.4, these are hydrophobic compounds, and their absorption depends upon effective action from the nutrient array and later solubilization by bile acids and digestive enzymes, culminating in their integration into micelles. The main carotenoid observed in both samples presenting a 527 m/z is unknown, with other compounds such as lycopene, phytofluene, and lutein also present. The total carotenoids decreased throughout the gastrointestinal tract (Table 9.2). They are disassociated from their native surroundings in the tomato by-products within the extraction process and digested in the stomach (acid conditions and enzymatic hydrolysis). Also, results showed that OH presents a higher recovery index of lutein and phytofluene than the CONV samples. These significant differences could be explained by the damage that OH does to food structures, which gives access to digestive enzymes and improves bioaccessibility. The results showed that lutein and phytofluene from OH (80.74%, 30.18%) and CONV (14.53 % and 8.66 %) samples presented higher bioaccessibility than lycopene. Nevertheless, the last compound could be cleaved into esters. It appears that these available varieties of fat-soluble vitamins and carotenoids are absorbed by the intestinal mucosa, indicating that these esterified shapes are first hydrolyzed (Nagao, 2014; Shi & Le Maguer, 2000).

Studies on the subject primarily concern retinyl esters. Their hydrolysis may occur in the stomach, where gastric lipase hydrolyzes about 17.5 percent of those triacylglycerols. Nevertheless, the information obtained in healthy studies has indicated that gastric lipase does not significantly hydrolyze retinyl palmitate [64]. The hydrolysis of esters of vitamin A, therefore, occurs basically in the duodenum. On the other hand, the individual carotenoids released into the gut lumen are all highly interactive and appear to impede each other's absorption (Reboul, 2013; Said, 2018; Salter-Venzon et al., 2017).

The results observed are promising regarding the general bioavailability improvement of carotenoids and polyphenols, by the expanded penetrability across absorptive epithelia, by the assurance of the bioactive compounds along the gastrointestinal tract.

Table 9.4. Recovery index and bioaccessibility of carotenoids identified by LC-ESI-UHR-QqTOF-MS from SFCONV and SFOH samples throughout digestion.

Compounds	Mz	Samples	Mouth	Stomach	Intestine	Colon	Basolateral	Bioaccessibility
			(%)					
n.i	525	SFOH	84.85 ± 2.31^{a}	57.97 ± 1.3^{b}	45.52 ± 1.56^{c}	40.33 ± 1.87^{c}	27.17 ± 1.84^{d}	59.67 ± 1.83$^{\beta}$
		SFCONV	86.32 ± 2.05^{a}	60.21 ± 1.31^{b}	49.23 ± 1.55^{c}	41.32 ± 1.98^{d}	31.24 ± 2.13^{e}	63.46 ± 1.76$^{\alpha}$
n.i	527	SFOH	91.55 ± 1.95^{a}	16.86 ± 0.25^{b}	15.68 ± 0.99^{b}	92.48 ± 2.06^{a}	1.18 ± 1.23^{c}	7.52 ± 0.74$^{\alpha}$
		SFCONV	93.41 ± 1.28^{a}	89.31 ± 2.45^{b}	44.66 ± 2.87^{c}	90.23 ± 2.77^{a,b}	3.06 ± 0.22^{d}	6.85 ± 0.32$^{\beta}$
phytofluene	542	SFOH	72.25 ± 1.04^{a}	36.46 ± 0.78^{b}	29.14 ± 1.23^{c}	69.82 ± 1.76^{a}	8.79 ± 0.97^{d}	30.18 ± 0.98$^{\alpha}$
		SFCONV	26.40 ± 0.27^{b}	11.53 ± 0.69^{c}	37.08 ± 1.39^{a}	35.54 ± 1.43^{a}	3.21 ± 0.06^{d}	8.66 ± 0.45$^{\beta}$
lycopene	536	SFOH	n.q	n.q	n.q	n.q	n.q	n.q
		SF	n.q	n.q	n.q	n.q	n.q.	n.q
lutein	569	SFOH	113.9 ± 2.76^{a}	62.52 ± 1.43^{b}	57.65 ± 1.43^{c}	19.26 ± 1.25^{e}	46.55 ± 1.54^{d}	80.74 ± 1.32$^{\alpha}$
		SFCONV	25.53 ± 0.34^{c}	11.15 ± 0.98^{d}	35.85 ± 1.41^{a}	30.53 ± 1.23^{b}	5.21 ± 0.09^{e}	14.53 ± 0.57$^{\beta}$
n.i.	633	SFOH	103.8 ± 2.54^{a}	58.61 ± 1.77^{b}	33.06 ± 1.04^{c}	12.98 ± 0.12^{d}	35.82 ± 2.31^{c}	108.32 ± 2.25$^{\alpha}$
		SFCONV	n.d	n.d	n.d	n.d	n.d	n.q

n.i. non identified; n.d. non determinate; n.q. non quantified. Results are the means of three determinations ± standard deviation. Different letters in the same row are significantly different ($p<0.05$), the greek alphabet means significant differences between methods used in the same column ($p<0.05$). as determined by ANOVA ($p<0.05$).

9.3.3. Carotenoid-derived aroma compounds

Aroma compounds exist in tomato as available volatiles, and they likewise create complexes with non-volatile precursors such as glycosides, carotenoids, and cinnamic acid derivatives. Carotenoids are essential components of foods and their degradation results in the formation of aroma compounds. Their composition and associated physicochemical attributes are related to significant functions and activities (Cardoso et al., 2017). Thus, we analyzed the volatile compounds produced during the digestion process because degradation of carotenoids leads to a significant production of these compounds (see Table 9.5). The main volatile compounds detected by GC-MS were 1,2-dimethylindole; 2,6-dimethylbenzaldehyde; benzoic acid; β-cyclocitral; 3,4-diethenyl-1,6-dimethyl; camphenol; linalyl acetate and linalool. The results presented in Table 9.5 are according to the literature since the main aroma compounds derived from carotenoids are C13-, C11-, C10 -, and C9-derivatives formed via enzymatic reaction of these different carotenoids shown to exist in tomato by-products flour (Dickinson et al., 2019). As examples, we can identify 2,6-dimethylbenzaldehyde, a C9 produced after alpha-amylase action in the mouth, that, in both samples, in the stomach, was transformed into derivatives by acid hydrolysis. The content of α-cyclocitral– formed by β-carotene–derived apocarotenoid enzymatic action - increased after stomach digestion (Dickinson et al., 2019; Ilg et al., 2014). Nevertheless, while this compound was not absorbed through the conventional method, it was absorbed in the SFOH samples. This result following the literature, whereas the type of extraction explains the carotenoids bioaccessibility differences (Moran et al., 2018; Nagarajan et al., 2017; Yonekura & Nagao, 2007). In the conventional method, an organic solvent was used, and many fats were also extracted. In contrast, in ohmic extraction, water:ethanol solution was used and most of the fats remained in the SFOH samples which facilitate the carotenoids' bioaccessibility regarding SFCONV. Results are in agreement with the described literature, in which samples with a higher amount of fatty acids increase the bioavailability of lipophilic micronutrients, facilitating the solubilization mainly of lipophilic micronutrients in the aqueous phase of digestion (F. Liu et al., 2017; Mapelli-Brahm et al., 2018; Nagao, 2014; Reboul, 2019). Different mechanisms that occur during the digestion process are responsible for these results. The pancreatic enzymes of gastrointestinal digestion hydrolise on the oil-water interface, making the fatty acids of SFOH more bioavailable. Also, through small intestine digestion, the carotenoids are incorporated with other lipids, such as cholesterol, phospholipids (present in bile salts), and lipid resultants of digestion products (i.e., free fatty acids, monoacylglycerols, lysophospholipids) into mixed micelles by bile salts. These amphipathic compounds are mainly constituted by water, electrolytes, and organic molecules, including bile acids, cholesterol, phospholipids, and had two essential properties, 1) they are emulsifying agents of lipid aggregates and 2) they solubilize and transport lipids in an aqueous environment (Reboul, 2019). Since biles aggregated into micelles they can solubilize other lipids in their interstices. The absorption of fat-soluble compounds increases the carotenoids' absorption capacity due to bile salts. This way, since SFOH has more fatty acids, together with the bile salts they will form more aggregates, mixed micelles in which they will allow greater integration of the carotenes and, therefore, passively pass to the basolateral membrane.

Table 9.5. Volatile compounds analysis by GCMS.

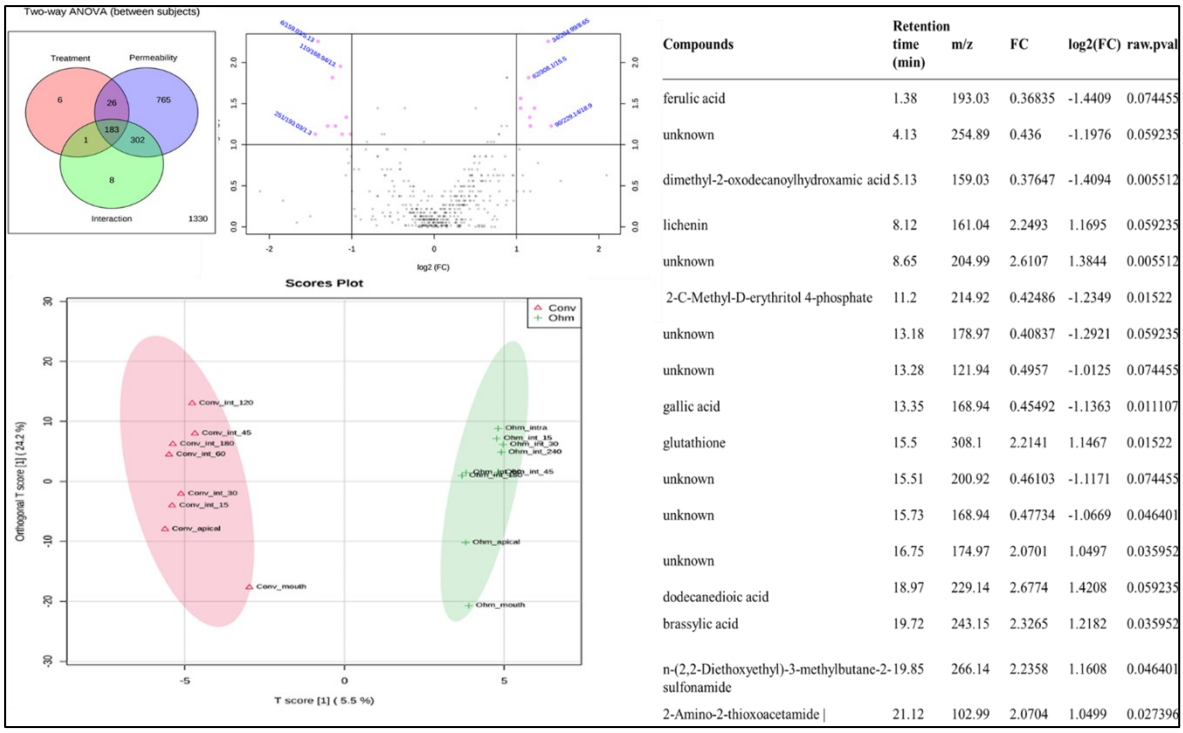
Samples	Compounds (Name)	1,2-Dimethylindole	2,6-Dimethylbenzaldehyde	Benzoic acid	β-cyclocitral	3,4-diethenyl-1,6-dimethyl-	Campheno l	Linalyl acetate and linalool
	m/z quantifiers	133	105	118	119	93	93	93
	Molecular formula	$C_{10}H_{11}N$	$C_9H_{10}O$	$C_7H_6O_2$	$C_{10}H_{16}O$	$C_{12}H_{18}$	$C_{10}H_{16}O$	$C_{12}H_{20}O_2$/ $C_{10}H_{18}O$
	MW	145	134	122	152	162	152	196/154
Conventional method	CBF	226.6 ± 13.4	0.4 ± 0.1	14.4 ± 4.5	3.3 ± 0.5	0.4 ± 0.1	14.1 ± 0.3	0.0 ± 0.0
	Mouth	190.9 ± 5.4	2.7 ± 0.1	14.0 ± 0.9	2.0 ± 0.1	0.4 ± 0.0	9.2 ± 2.5	0.8 ± 0.3
	Stomach	189.0 ± 3.9	2.2 ± 0.4	4.1 ± 0.8	3.2 ± 0.4	0.9 ±0.1	10.4 ± 1.1	0.3 ± 0.1
	Small Intestine	113.1 ± 4.5	0.0 ± 0.0	1.6 ± 0.6	1.3 ±0.1	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0
	Colon	1.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	1.10 ± 0.3	0.0 ± 0.0
	Basolateral	4.6 ±0.6	0.0 ± 0.0	20.8 ± 3.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ohmic conventional	CBF	232.1 ± 21.7	0.0 ±0.0	8.6 ± 1.4	2.8 ± 0.2	0.1 ± 0.0	2.8 ± 0.3	0.0 ± 0.0
	Mouth	155.5 ± 12.43	1.5 ± 0.3	6.6 ± 1.8	2.6 ± 0.3	0.1 ± 0.0	3.1 ± 0.4	0.3 ± 0.1
	Stomach	208.5 ± 15.84	1.3 ± 0.2	4.6 ± 0.7	4.8 ± 0.5	0.1 ± 0.0	3.7 ± 0.7	0.2 ± 0.0
	Small Intestine	79.5 ± 7.49	0.0 ± 0.0	5.5 ± 0.8	1.3 ± 0.1	0.0 ± 0.0	0.5 ± 0.1	0.0 ± 0.0
	Colon	19.0 ± 4.61	0.0 ± 0.0	2.0 ±0.3	0.0 ± 0.0	0.3 ± 0.1	3.5 ± 0.7	0.0 ± 0.0
	Basolateral	17.3 ± 3.95	0.0 ± 0.0	39.4 ± 4.9	0.6 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0

Results are given in µg per liter and are a result of the means of three determinations ± standard deviation.

9.3.4. Metabolomics Analysis – LC-ESI-UHR-QqTOF-MS

From the UPLC-QTOF-MS analysis, an untargeted analysis was applied based on an established pipeline. In the Venn diagram, 765 features were associated with permeability and only 6 features related to the treatment (Figure 9.3).

From the 3D view of the PCA score plot, it was possible to see a clear separation between the mouth samples and the apical zone. Nevertheless, intracellular and intestine are well clustered together. Besides, with fold changes analysis, a clear separation was observed between flour by-products after ohmic extraction and conventional extraction, in agreement with reported data. In each sample, differences were also observed throughout the digestion process, which indicates that the flours were metabolized. Furthermore, the orthoPLSDA graphical analysis showed that the mouth fraction was more similar to the absorbed fraction in both treatments than with the intestine fraction. According to fold-changes analysis, brassylic acid, didecaniodic acid and dimethyl-2-oxodecanoylhydroxamic presented significant differences between samples. Thus, the results suggest that the compounds were bioaccessible due to their capacity to be absorbed in the intestine.



Compounds	Retention time (min)	m/z	FC	log2(FC)	raw.pval
ferulic acid	1.38	193.03	0.36835	-1.4409	0.074455
unknown	4.13	254.89	0.436	-1.1976	0.059235
dimethyl-2-oxodecanoylhydroxamic acid	5.13	159.03	0.37647	-1.4094	0.005512
lichenin	8.12	161.04	2.2493	1.1695	0.059235
unknown	8.65	204.99	2.6107	1.3844	0.005512
2-C-Methyl-D-erythritol 4-phosphate	11.2	214.92	0.42486	-1.2349	0.01522
unknown	13.18	178.97	0.40837	-1.2921	0.059235
unknown	13.28	121.94	0.4957	-1.0125	0.074455
gallic acid	13.35	168.94	0.45492	-1.1363	0.011107
glutathione	15.5	308.1	2.2141	1.1467	0.01522
unknown	15.51	200.92	0.46103	-1.1171	0.074455
unknown	15.73	168.94	0.47734	-1.0669	0.046401
unknown	16.75	174.97	2.0701	1.0497	0.035952
dodecanedioic acid	18.97	229.14	2.6774	1.4208	0.059235
brassylic acid	19.72	243.15	2.3265	1.2182	0.035952
n-(2,2-Diethoxyethyl)-3-methylbutane-2-sulfonamide	19.85	266.14	2.2358	1.1608	0.046401
2-Amino-2-thioxoacetamide |	21.12	102.99	2.0704	1.0499	0.027396

Figure 9.3. Metabolomic analysis, two-way ANOVA, OrthoPLSDA, and fold-change analysis.

9.3.5. Mutagenicity

The mutagenicity of the SFOH and SFCONV samples after the simulated digestion was assessed as some chemical compounds may be harmful or break down into mutagenic compounds within the digestive system (Figure 9.4B).

The results showed an increase in mutagenicity of CONV samples. In contrast, the OH samples were not mutagenic, with no increase in the number of revertant with increasing extract concentrations. Moreover, the mutagenicity also increased when extracts were exposed to metabolic activation (in contact with S9 mix), suggesting that conventional extract yields compounds with a more significant potential to induce DNA damage when undergoing metabolism. The differences between both extracts may be attributed to less soluble components in the ethanolic extract due to their non-polar nature. These results are following previous studies (Sharif et al., 2017). Thus, OH heating is a safer process than the CONV method, which uses organic solvents and could be retained in the flours after extraction, with mutagenic activity.

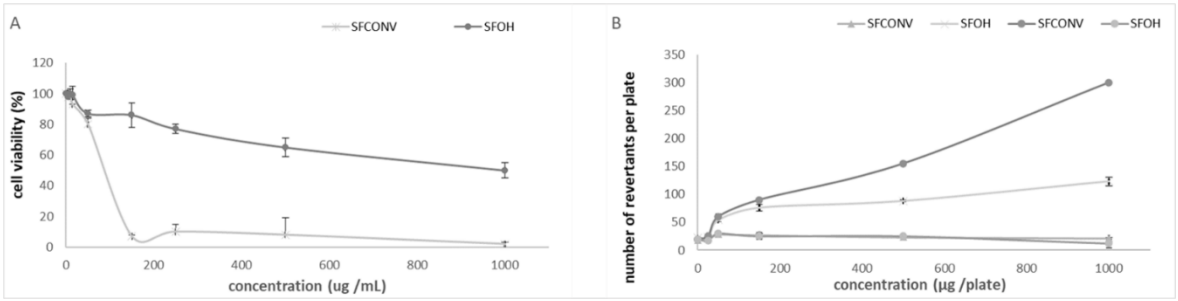


Figure 9.4. Cytotoxicity (A) and mutagenicity (B) of SFOH and SFCONV samples. In Figure B SFCONV SFOH represents without metabolization, and SFCONV SFOH with metabolization. Results are the means of three determinations ± standard deviation.

Regarding the cytotoxicity assessed by the XTT assay, the SFOH extraction presents lower cytotoxicity than SFCONV extracts (Figure 9.5 A). The CONV extraction showed an IC50 of 60 µg/mL. These IC50 values are similar to those measured for medicinal plants (Guil-Guerrero et al., 2011). Sharif et al. (2017) also obtained mutagenicity and cytotoxicity results of Kalanchoe laciniate n-hexane extracts (corresponding to our conventional method) showing that digested conventional bagasse flour seems to be a source of different toxic compounds against cells. After the digestion procedure, less polar components, such as carotenoids and sterols, are bioavailable as dynamic species. The mechanism of cell toxicity could be due to an arrest of cell cycle at the G0/G1 and G2/M stage and by apoptosis, which has been recently reported for a β-carotene enhanced tomato variety (Palozza et al., 2001). Some studies have reported an impacts of carotenoids, such as neoxanthin and fucoxanthin, on DNA fragmentation in human prostate cancer cells (Guil-Guerrero et al., 2011; Palozza et al., 2001, 2009).

9.3.6. Permeability

The Caco-2/HT29-MTX co-culture is an alternative method to mimic the human intestinal epithelium. Few studies simulate the absorption of the carotenoids in the epithelia which could be a good tool to understand the impact of mucins on the bioavailability of bioactive compounds with potential health benefits (Grootaert et al., 2015). Both samples were put in contact with co-culture, and the cumulative permeability was studied throughout the simulated digestion to understand the bioavailability of cells and the experienced continuity (Castro et al., 2019). In this case, both samples were non-toxic. For both analyses, cell integrity was suitable for analysis (Figure 9.6). These following the results present before whereas the SFOH samples present more permeability than SFCONV samples after 60 min. These compounds' bioavailability depends on the ingredient matrix and the water-soluble metabolites which allows increase permeability (Grootaert et al., 2015). Possibly, the compounds can passively pass the formation of mixed micelles being more bioavailable.

Observed results are auspicious regarding the overall bioavailability enhancement of carried both polyphenols and carotenoids, either due to increased permeability across absorptive epithelia and regarding the protection of the bioactive compounds along the gastrointestinal tract.

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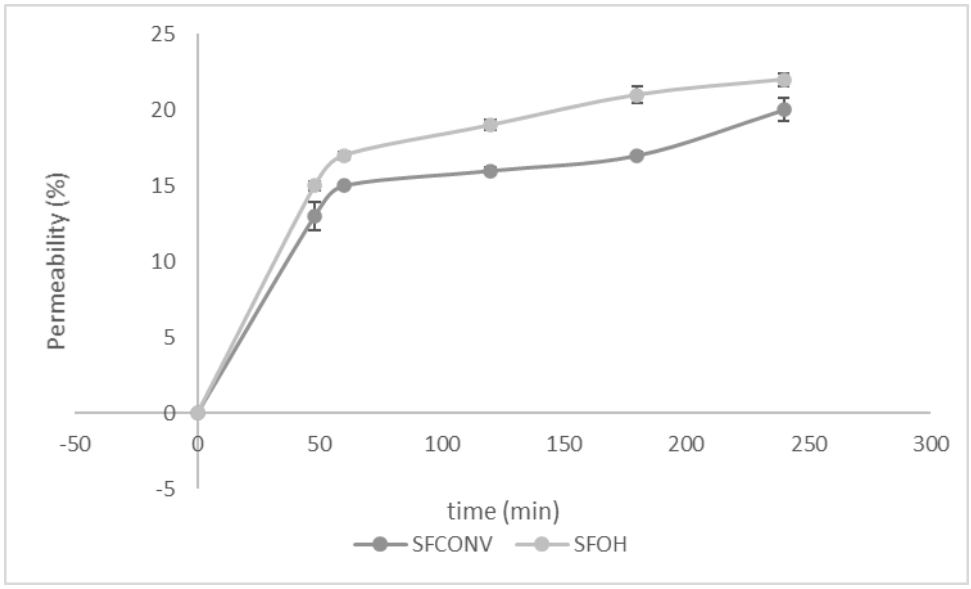

Figure 9.5. Cumulative permeability of cells to SFOH and SFCONV samples.

9.4. Conclusions

In the present work, the chemical composition of two flours obtained by different extraction methods were evaluated. The SFOH presented higher values of IDF fibre and higher protein resistant values than the SFCONV. The study of SFCONV and SFOH. throughout a simulated gastrointestinal tract demonstrated that OH increased the bioaccessibility of phenolic compounds and carotenoids. During this process, carotenoids, such as lycopene, lutein, and phytofluene, were identified. Both volatile compounds and metabolic analysis was performed to understand the metabolic pathways of both carotenoids and polyphenolic compounds during the digestion process. The results confirmed that volatile compounds are precursors of carotenoids, and they are absorbed in the bloodstream. Furthermore, the SFOH is safer and contains more bioaccessible bioactive compounds than SFCONV. The information provided in this work gives insight into the impact of alternative methodologies on the bioaccessibility of bioactive compounds from tomato, which allow us to better understand the derived health benefits of tomato bioactive compounds and their potential as a functional ingredient. The introduction of OH technology to recover the bioactive could be an opportunity of adding value to the greater by-product stream from the tomato paste production.

Chapter 10.
Potential prebiotic impact of tomato flours obtained after conventional and ohmic heating extraction

Abstract

Several studies have supported the positive functional health effects of both prebiotics and probiotics on gut microbiota. Among these, the selective growth of beneficial bacteria due to prebiotics and bioactive compounds (BC) as an energy and carbon source is critical to promoting a healthy microbiota within the human gut. The present work aimed to assess the fermentability of tomato flour obtained after ohmic (SFOH) and conventional (SFCONV) extraction of phenolic compounds and carotenoids and their potential impact upon specific microbiota groups. Thus, the attained bagasse flour was submitted to an *in vitro* simulation of gastrointestinal digestion (GID) followed by studying its potential fermentability and impact upon gut microbiota (using an *in vitro* faecal fermentation model). Different impacts on pure probiotic strains studied were for SFCONV that promoted mainly the *B. animalis* growth, while SFOH promoted more *B. longum,* probably based on the different carbohydrate's profiles of these flours.

The study using a faecal fermentation model showed that these flours could function as a direct substrate to support the growth of the potential probiotic, mainly for *Bifidobacterium*. The results showed the highest Bacteroidetes levels with SFOH and the highest values of *Bacteroides* with SFCONV. A correlation between microorganisms' growth and short-chain fatty acids (SCFA) production was also found for each of the studied tomato flour. Overall, both flours demonstrated prebiotic activity, however, it is evident that the technology influenced the final biological activity.

These new ingredients produced from tomato by-product seems to promote beneficial effects on microbiota flora and could be a potential prebiotic ingredient, although extra *in vivo* trials would be necessary to confirm this initial evidence.

Keywords: gut microbiota; short-chain fatty acids; prebiotic;

10.1. Introduction

The gut microbioma depends on individual intrinsic factors (e.g., age, ethnicity, genetic markers) and on environmental factors (e.g., geographic area, lifestyle, diet, and drugs) (Bell et al. 2018; Carvalho et al. 2019). The gut microbiota modulates various physical functions (e. g., nutrient processing and digestion, immune cell growth and immune response, immunity towards pathogens, among others), representing a mutualistic relationship. At the same time, the host intestine provides the necessary environmental conditions for the bacteria therein to survive and reproduce.

As mentioned before, numerous endogenous or exogenous components can impact the microbiota structure and therefore impact the host's health and well-being. For instance, an infant's microbiota has a low assorted variety, picking up multifaceted nature as the kid ages until it reaches a more constant and unmistakable microbial profile - similar to an adult - after ca. 2.5 years. Moreover, the gut microbiota can be effectively regulated by diet, which is impacted by the geographic area, explicitly concerning fibres, proteins, sugar, and fat admission. The practical relation between diet and intestinal microbiota is easily demonstrated (Filippis et al., 2016). Therefore, new trends of nutritional therapy advantage from diet modifications to improve human well-being have been reported, namely, through the target optimization of gut microbiota performance (Cho and Blaser 2012). Still, even though diet components have been demonstrated to have a modulating effect on gut microbial communities, knowledge on the effect exerted by particular foods in driving gut microbial variety is limited, hence hampering its optimal use.

Most bacterial fermentation occurs in the proximal colon, where there is higher substrate accessibility. Toward the distal colon, the convenience of substrate falls, and the recovery of available food reduces both substrates and microbial products' distribution. Thus, the proximal portion of the colon is the central area of fermentation. The fermentation results in short-chain fatty acids (SCFAs) and gas (CO_2 and H_2). The SCFAs represent saturated aliphatic essential doses with the bonding of one to six carbons and are one of the most thoroughly studied intestinal microbiome metabolites (Cait et al., 2018; Feng and Peng, 2018). These molecules are mainly generated in the large intestine by gut microbiota fermentation of carbohydrates that had escaped digestion and absorption in the small intestine, although non-digested proteins or peptides are also essential upstream compounds for its production (Sivaprakasam, Prasad, and Singh 2016; Fan et al. 2015). These compounds sources are found in the plant, such as tomato fruit. While also used for fresh consumption, tomato is primarily used for processing into juice, pulp and sauces, hence originating many by-products whose potential valorization is still scarce.

Tomato by-products include seeds, peels, and pulp, all of which are rich in nutrients and bioactives, including carbohydrates, organic acids, pigments, fibre, proteins, oils, and vitamins giving beneficial effects to health (Coman et al. 2019).

Different extraction techniques have been tested to valorise these by-products. Bioactive extracts were firstly obtained with ohmic (OH) processing, showing significant differences from extracts

obtained by conventional extraction (Coelho et al. 2019). Applying a complete and integrated recovery from tomato by-products with zero waste, in a circular economy context, the final solid extraction by-product can be dried under controlled conditions, resulting in a flour with a high fibre content combined with bond phytochemicals (Ramírez-Jiménez et al., 2019b). As such, the resulting material could be used with as an ingredient that, given its characteristics, could have an attractive biological potential, particularly in the modulation of the gut microbiota (Campos et al., 2020; Chen et al., 2017; Tomás-Barberán et al., 2016).

Therefore, the present work aimed to characterize the prebiotic potential of two tomato flours obtained after ohmic (SFOH) and conventional (SFCONV) extraction of phytochemicals from tomato bagasse. Both flours were subjected to an *in vitro* stimulation of the gastrointestinal tract. After, the impact of this processing upon each sample, submitted to digestion, was evaluated upon pure probiotic cultures and on fresh human faecal samples to assess the prebiotic potential and the effect on gut microflora's metabolic and population dynamics.

10.2. Materials and Methods

10.2.1. Tomato bagasse flours

Two different tomato bagasse (peel and seeds) flours were used in the present work. The first (OH) was obtained as the solid by-product after ohmic extraction (70 ºC,15 min,70% ethanol) tomato bagasse subjected to ohmic extraction (peel and seeds) as described elsewhere Coelho et al. (2019). The second (CONV) was obtained as the solid by-product after a conventional solid-liquid extraction described in the literature using hexane as solvent (Oliveira et al., 2016). In both cases, after extraction, the leftover solid byproduct fraction (SF) was dried at 55 ºC overnight and stored in a desiccator at room temperature until use. The SF for OH tomato by-products is shortened as SFOH to simplify the nomenclature, while the SF for CONV samples is SFCONV.

10.2.2. *In vitro* digestion simulation (GID)

10.2.2.1. Sample preparation

The tomato SF were suspended in water (10%) and homogenized using an Ultra-Turrax (IKA Ultra-turrax T18, Wilmington, USA) at 13000 x g for 1 min. The tomato bagasse solution was set up at 10% (w/v), as composition showed that the dried sample contained ca. 60% fibre and, as per the European Food Safety Agency (EFSA), 6 g of fibre for each 100 g of the item (Vilella and Vaqué 2018; European Commission 2016).

10.2.3.2. *In vitro* digestion simulation

Before executing faecal fermentation assays, samples were subjected to an *in vitro* simulation of the GI tract (including dialysis) to better mimic *in vivo* conditions. The tomato bagasse mixture's pH value was adjusted to 5.6 – 6.9, utilizing 1M HCl. Mouth digestion was simulated by adding α-amylase from human saliva (100 U/mL in 1 mM aqueous CaCl2), homogenizing the mixture for 2 min and incubating at 37 °C and 200 rpm. Afterwards, to simulate stomach conditions, the mixture's pH value was lowered to 2.0 (utilizing 1 M HCl) and pepsin from gastric juice was added

(12.5 mg/mL in HCl 1 M) at a ratio of 0.05 mL/mL of sample. The mixture was then incubated in a water bath for 2 h at 37 °C and 130 rpm. A pH' mixture was adjusted to 6.0 utilizing 1 M NaHCO3 to stimulate small intestine conditions. Pancreatin and bile salts (0.4 g de pancreatin and 1.2 g bile salts in 200 mL of NaHCO3 1M) were added to the mixture at a ratio of 0.25 mL/mL of sample. Finally, the obtained solution was maintained, for 2 h, at 37 °C and 45 rpm. Then dialysis was performed with membranes 12 kDa for 24 h, with known volume (to simulate the blood circulation). At the end of the dialysis process, the dialysis tubing (OUT) solution represented the non-absorbable sample (colon- available). This fraction was then freeze-dried and stored in a desiccator for later use in the faecal fermentation.

10.2.3. Preliminary evaluation of the prebiotic potential of tomato SF

10.2.3.1. Microorganisms

Probiotic bacteria species were selected for the present work, namely *Lactobacillus casei* 01, and *Bifidobacterium animalis subsp lacties* BO.

10.2.3.2. Selection of the best tomato flour concentration

To evaluate the tomato flours effect on microorganisms growth, tomato SF (before and after digestion) at 2, 4 and 6% (w/v) were suspended in basal media, inoculated, using a 24 h inoculum, at 10% (v/v) and incubated for 24 h at 37 °C, in an anaerobic environment. After this period, the viable cell numbers were determined by plating, using the spread plate method, in de Mann, Rogosa, and Sharpe agar (MRS) enhanced with 0.5 g/L of L-cysteine hydrochloride. After 48 h incubation at 37 ºC, under anaerobiosis, the bifidobacteria and lactobacilli colonies were enumerated, and the outcomes were plotted as log CFU/mL. All inoculations were performed in triplicated, and plain inoculated basal media was utilized as a control.

10.2.3.3. Evaluation of the effect of tomato flours as potential prebiotic

The tomato SF were tested at 2% (w/v), the effect of the SF on the targeted probiotic's enhancement and organic acid generation was tested. The digested bagasse, prepared as described in section 2.3, was inoculated at 2% (v/v) with every microorganism (using a 24 h inoculum). Samples were taken after 0, 12 and 24 h to determine the viable cells, pH value and organic acid generation. Total viable cells were determined using decimal dilutions and plating through spread plate technique in MRS agar enhanced with cysteine and bromophenol blue. In addition, pH values were measured using a 52-02 Crison electrode, and the organic acids production was assessed through HPLC-IR, as described elsewhere by Sousa et al. (2015).

10.2.4. In vitro faecal fermentations

10.2.4.1. Collection and preparation of faecal inocula

Fresh faecal samples were provided by five healthy donors (A-E, three men and two women, ages between 23 to 39 years old), whose selection was based on established criteria regarding health status and dietary habits, namely the existence of chronic diseases, allergies, probiotics ingestion, among others. Moreover, an informed consent form was distributed among donors to

provide the participant's information about the study and the consent certificate assigned for each. Donors were healthy un-related anonymous volunteers, ≥18 and <50 years of age, who had not received antibiotics in the preceding six months or consumed any prebiotic supplement. The faecal samples were maintained under anaerobic conditions for a maximum of 2 h before being used. The *faecal inoculum* (FI) was then prepared by diluting the faecal matter in Reduced Physiological Salt solution (RPS) (constituted by 0.5 g/L cysteine-HCl (Merck, Darmstadt, Germany) and 8.5 g/L NaCl (LabChem, Zelienople, USA), with a final pH value of 6.8, at 100 g/L in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK) (10% CO_2, 5% H_2 and 85% N_2).

10.2.4.2. Nutrient Base Medium preparation

Faecal fermentations were performed with Nutrient Base Medium. The medium comprised 5.0 g/L trypticase soy broth without dextrose (Fluka Analytical, St. Louis, Missouri, EUA), 5.0 g/L bactopeptone (Becton Dickinson Biosciences, New Jersey, USA), 0.5 g/L cysteine-HCl (Merck, Darmstadt, Germany), 1.0% (v/v) of salt solution A [100.0 g/L NH4Cl (Merck, Darmstadt, Germany), 10.0 g/L MgCl2·6H2O (Merck, Darmstadt, Germany), 10.0 g/L $CaCl_2 \cdot 2H_2O$ (Carlo Erba, Chaussée du Vexin, France)], 1.0% (v/v) of trace mineral solution (ATCC, Virginia, USA), 0.2% (v/v) of salt solution B [200.0 g/L $K2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany)] and 0.2% (v/v) of a 0.5 g/L resazurin solution (Sigma-Aldrich Chemistry, St. Louis, USA). The medium final pH value was adjusted to 6.8 and was then bubbled with N_2 until it presented a translucent/yellowish colour. Following this, 50 mL parts were then distributed into several containers. Fructooligosaccharides (FOS) (Nutripar, Matosinhos, Portugal) as positive control and the freeze-dried digested tomato flours were added to the respective vessels at a final concentration of 2%. The bottles were capped and autoclaved. Following sterilization, and before adding the faecal inocula, the atmosphere of each flask was refluxed with a sterile gas mixture (10% CO2, 5% H2 and 85% N2).

10.2.4.3. Faecal fermentations

The flasks prepared before (2.4.2) were inoculated at 2% (v/v) with FI (section 10.2.4.1) and incubated for 48 h at 37 ºC under an anaerobic atmosphere (10% CO2, 5% H2 and 85% N2). Samples were collected after 0, 12, 24 and 48 h of incubation and the pH values were measured using a MicropH 2002 pH meter (Crison, Barcelona, Spain), equipped with a 52-07 pH electrode (Crison, Barcelona, Spain). The positive and negative controls were, respectively, designated as C+ (FOS) and C- (plain media), while the tomato flours digested biomass were named OH and CONV, which are in accordance with flours. Afterwards, the samples were stored at -30 ºC until analysis. All the steps considered in this section were carried out inside an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

10.2.4.4. Faecal fermentation sample's processing

Aliquots of each sample were collected in section 2.7.3. were centrifuged for 6 min at 4000 g. The resulting supernatants were used to evaluate sugars and short-chain fatty acid (SCFA), according to section 2.4.4.1, and the pellet was used to extract the genomic DNA.

10.2.5. Sugars and SCFA analysis

Sugar consumption and organic acid production during fecal fermentation were analyzed using an HPLC system comprised of a Knauer K-1001 pump (Berlin, Germany), an ion exchange Aminex HPX87H (300 x 7.8 mm) (Bio-Rad, Hercules, USA) column and two detectors assembled in series, namely a UV-vis detector (220 nm) and a refractive index detector, both from Knauer (Berlin, Germany) at a temperature of 65 ºC. An isocratic gradient was used (13 mM H_2SO_4 Merck, Darmstadt, Germany) at a 0.6 mL/min flow rate. The injection volume was 40 µL and the running time was 30 min. Fermentation supernatants were filtered through a 0.22 µm syringe filter and each sample was injected in duplicate.

10.2.6. Bacterial population analysis

10.2.6.1. DNA extraction

A NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) was used to extract DNA with slight modifications. Briefly, pellets were washed with TE (pH 8.0; Tris EDTA buffer), vortexed and centrifuged at 4000 g for 10 min. Then, 180 µL of a freshly prepared lysozyme solution (10 mg/mL lysozyme in a NaCl-EDTA (30 mM:10 mM) solution) was added and incubated for a period of 1 h, at 37 ºC, with periodic shaking. Afterwards, 350 µL of NT1- buffer was added to samples, then vortexed and incubated at 95 ºC. After 10 min, samples were centrifuged (11000 x g, 10 min, 4 ºC), supernatants (200 µL) were mixed with 25 µL of proteinase K and incubated at 70 ºC for 10 min. The remaining steps were performed accordingly to the manufacturer's instructions. After extraction, DNA's purity and concentration were assessed using a Thermo Scientific™ µDrop™ Plate coupled with a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, USA).

10.2.6.2. Real-Time Quantitative Polymerase Chain Reaction

Real-time PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA) under the conditions described in the table below (Table ST1).

The PCR reaction mixture comprised of 5 µL of 2x iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA), 2 µL of sterile ultrapure water, 1 µL of sample DNA (equilibrated to 20 ng/µL) and 1 µL of forward and reverse primers (100 nM) targeting the 16S rRNA gene. The primers used were obtained from STABvida (Lisbon, Portugal) and are listed in table ST2. Standard curves were constructed using tenfold dilutions (from 2 log to 6 log of number of copies of 16S rRNA gene/µL) of bacterial genomic DNA standards (DSMZ, Braunschweig, Germany) (Table ST2). Melting curve analysis was performed for each PCR to evaluate the

specificity of the amplification, considering a temperature interval from 60 to 97 ºC. All assays were performed in quadruplicate.

10.2.7. Statistical analysis

Data's statistical analysis was done using IBM SPSS Statistics v21.0 (IBM, Chicago, USA). The normality of the data's distribution was evaluated through Shapiro-Wilk's test. As the data proved to follow a normal distribution, One-way ANOVA, coupled with Tukey's post hoc test, was used to determine the significance of Tomato bagasse biomass' effect on bacterial populations at each time point. Repeated Measures ANOVA was used to evaluate the effect of Tomato bagasse biomass on the bacterial population over time. Differences were considered significant for p-values ≤ 0.05.

10.3. Results and Discussion

10.3.1. Probiotic effect

The most used probiotic microorganisms belong to the *Lactobacillus* and *Bifidobacterium* genera. So, at the first stage, these microorganisms were used to understand the potential prebiotic effects of tomato bagasse flours after extractions. For this, the bacteria were inoculated into a growth medium with different concentrations of tomato flours (0%, 1%, 2%, 4%) to select the minimum concentration exerting a prebiotic effect. The results (Figure 10.1) showed that the flours had little impact, with differences observed between the SFCONV and SFOH samples to 2 and 4% by-product's concentration. The viable cells number of *Lactobacillus* was ca. 10^8 CFU/mL for the various percentages of tomato samples, while for *Bifidobacterium*, the observed values were lower.

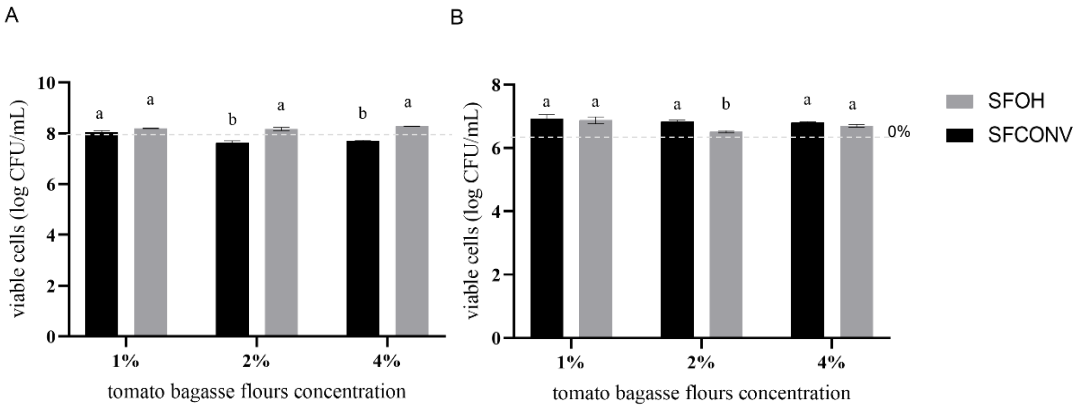

Figure 10.1. Impact of different concentrations of digested tomato by-products (after carotene extraction, OH and CONV) upon the growth of *Lactobacillus* (A) and *Bifidobacterium* (B) after 24 h anaerobic incubation.

For *Lactobacillus casei* when comparing the viable cells of the positive control and the sample at 2%, the by-product appeared to allow for some growth of this bacteria, i.e. the total viable counts were above those registered for the control and allowed for more prolonged survival of the bacterial cells, indicating that tomato biomass may be used as a source of nutrients by this

microorganism. The SFOH extraction had a significant impact when compared with SFCONV ($p<0.05$). The results suggest better bacteria accessibility to nutrients, such as carbohydrates and promoting its growth (Sousa et al. 2015; Gullón et al. 2015; Carvalho et al. 2019; Drakoularakou, Rastall, and Gibson 2011; Costa et al. 2019). Also, according to the chemical flours profile, the SFOH has a greater amount of galactose, arabinose, and uronic acids than CONV, which could promote the growth of these bacteria as described in the literature (McLaughlin et al., 2015; Watson et al., 2013). The results are according the literature given the recognized metabolic diversity of *Lactobacillus*, as previous results also reported strain-specific effects of tomato flours (Carvalho et al. 2019; Gaglio et al. 2018). Thus, these flours can also be used as a medium for probiotic growth.

It was reported that XOS is not fermented by most of the lactobacilli tested, whereas arabinoxylan was not used by any of the strains examined (Crittenden et al., 2002). However, McLaughlin et al. (2015) verified a *L. casei* growth in arabinoxylan. The authors also demonstrated that another strain of *Lactobacillus*, *L. brevis* DSM 20054, was genetically equipped with functional arabinoxylan-oligosaccharide degrading hydrolases which could explain the use of arabinose to growth.

In addition, tomato by-products could promote *L.casei* growth, suggesting that this sample may be used as a source of essential nutrients by bacteria, as has been the case of some studies that utilized tomato juice as the material for the manufacture of a probiotic drink. A recent study showed that the tomato juice enriched with *Lactobacillus plantarym ST III* strain positively affected fermented skimmed milk's taste and health-promoting activity. Liu et al. (2018) also used a fermented tomato juice with *L. casei* and *L. plantarum* to create a high-bioactivity probiotic drink. They observed a significant increase in antioxidant capacity by ABTS assay and increased compounds, such as phenolic compounds, lycopene, and other carotenoids.

Relatively to *B. animalis,* significant differences were observed at 24 h between CONV and OH extracts with 2% of tomato by-products. The CONV seemed to promote the growth of this microorganism as viable cell numbers after 24 h incubation. Nevertheless, OH-extracted flour led to a slight decrease in bacteria growth over time. These differences between results may be originated from the fact that different *Bifidobacterium* strains may have distinctive carbohydrate metabolic abilities, as has been observed in several previous studies (Pokusaeva, Fitzgerald, and Van Sinderen 2011; Mazé et al. 2007; Schell et al. 2002; Viborg et al. 2014; Parche et al. 2006; Degnan and Macfarlane 1993). Studies revealed that the *B. longum* encodes ABC transporters, PEP-PTS systems, and secondary transporters required to carry mono- and disaccharides. In comparison, *B. animalis* has a significantly smaller genome than *B. longum*, with a lower number of metabolic pathways to take advantage of carbon sources, does not encode PEP-PTS frameworks and contains just two qualities determining sugar specific ATP-binding proteins characteristic of ABC transporter (Pokusaeva, Fitzgerald, and Van Sinderen 2011; Barrangou et al. 2009). Therefore, since the previous studies (Coelho et al., 2021) showed that CONV has more disaccharides and monosaccharides (glucose, fructose, and mannose) than OH (which contains more polysaccharides, such as cellulose, hemicellulose, and pectins), so it is plausible

that more metabolically limited bacteria, such as *B. animalis,* cannot use it as a carbon and energy source to grow (Coelho et al. 2019).

Since there were no marginal gains in growth or the death of the target microorganism provided by the different concentrations of tomato pomace, subsequent experiments used the 2% concentration as there was limited sample availability, and in future, it will be easy to justify as a commercial ingredient to minimize interference in final food products.

10.3.1.1. Impact of the digested tomato SF on pH values

The pH values of the media are frequently considered an indicator of fermentation occurring or not, as this process leads to the formation of organic acids that cause a drop in the environmental pH values (Slavin J, 2006). When considering the variation of the pH values, it can be seen that at time 0 h, the initial control pH value was higher than that observed in the sample of tomato by-products for *Bifidobacterium* (Figure 10.2), and even though these values dropped slightly in control over time, in the presence of tomato by-products the same did not happen. As the initial pH was already low, it is possible to observe some inhibition of bacterial growth.

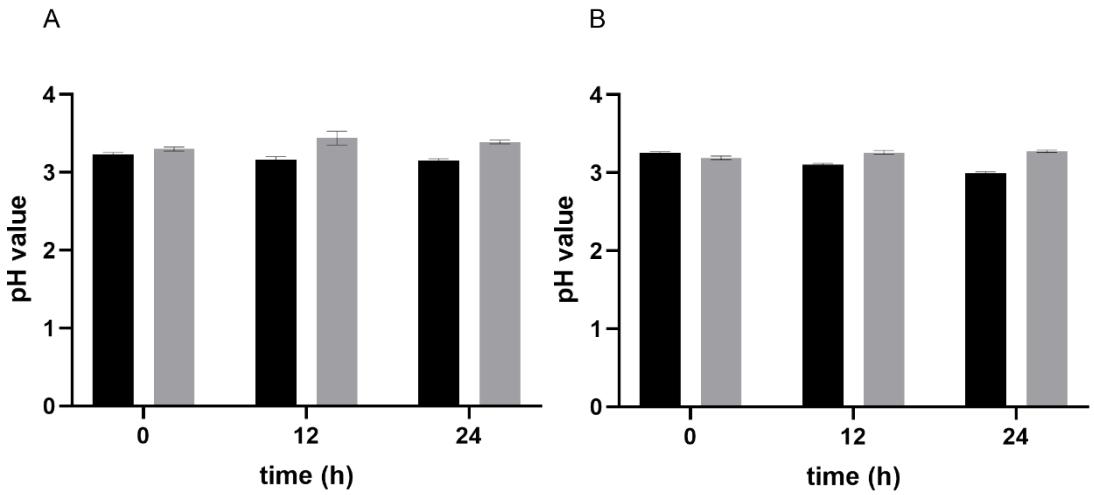

Figure 10.2. pH values variation of the individual bacteria incubated in the presence (light grey) and in the absence (dark grey) of tomato by-products 2%, after simulation on the gastrointestinal tract; *A -L. casei; B – B. longum*

10.3.1.2. Impact of the digested tomato SF on organic acid production

Probiotic bacteria can produce a variety of organic acids. The primary fermentation product from the breakdown of complex dietary carbohydrates is lactic acid, specially synthesized by *Lactobacillus* and *Bifidobacterium*. In Figures 10.3 and 10.4, it can be seen that at time 0 h, lactic acid is detected in the tomato SF. Greater concentrations of lactic acid concentrations were obtained, both in the SFOH and in the SFCONV at 2%, after 24 h. Moreover, the tomato by-product's presence promoted an overall increase in the production/accumulation of lactic acid.

Notwithstanding, there's a contradictory result in the samples at 2% of *L. casei* (after 24 h incubation) where a decrease in lactic acid was observed – it is not statistically significant ($p<0.05$) the existing standard deviation must be taken into account. Likewise, the low pH variation (from T12 to T24 h) previously observed can be attributed to this low production of lactic acid. Else, a

slight increase in the production of lactic acid in *Bifidobacterium* species can be noted. This result corroborates the literature: '*Bifidobacterium* produce lactic and acetic acids in large amounts, that is, larger than the amounts secreted by *Lactobacillus,* even though the latter is known to be very acid-tolerant (Sinderen & Mayo, 2010).

Bifidobacterium and *Lactobacillus* fermentations also result in the production of acetic acid. Acetic acid was not identified in the tomato byproduct control at time 0 h. Plus, during the graphical execution of the acetic acid concentrations in the function of time, it would be expected that the concentration of this product would increase over time. However, this condition wasn't verified for *L. casei,* in the presence of tomato by-products, at 24 h, which presents a high standard deviation and for the mixture of *Lactobacillus* and *Bifidobacterium* in the absence of tomato, at 12 h. Amakiri (2016) reported that bifidobacteria produced acetic and lactic acids at proportions of 3:2, which was not comprised by analyzing the fermentation end products: the concentration of acetic acid was approximately three times inferior to the concentration of lactic acid obtained.

Lactobacillus and *Bifidobacterium* can break down and metabolize a variety of substrates. The glucose concentration is initially high due to large amounts of this monosaccharide (Figure 10.3 and 10.4). After 12 h, there is a decrease in its concentration since the bacterial strains consume this substrate. At 24 h, the monosaccharide concentration is even lower, but only small amounts of sugars were consumed by the end. The metabolic capacity to consume sugars did not differ significantly between the various probiotic bacteria, except for *L. casei*, in the presence of tomato by-products, which degraded glucose more sharply after 24 h through the production of lactic acid as well as of acetic acid did not increase.

Besides glucose, the disaccharide maltose was also found in the SF but at a lower concentration. During the incubation and in the SFCONV, the overall amount of maltose decreased. As bacterial enzymes hydrolyze maltose in two glucose molecules, the bacteria are likely consuming it. However, in the SFOH, consumption of maltose by the microorganisms was, overall, significantly lower, with the amount of maltose in the media increasing after 12 h (possibly released from the matrix), with subsequent reduction of the concentration after 24 h (Figure 10.3 and 10.4 In the SFOH only *B, animalis* was capable of consuming present maltose.

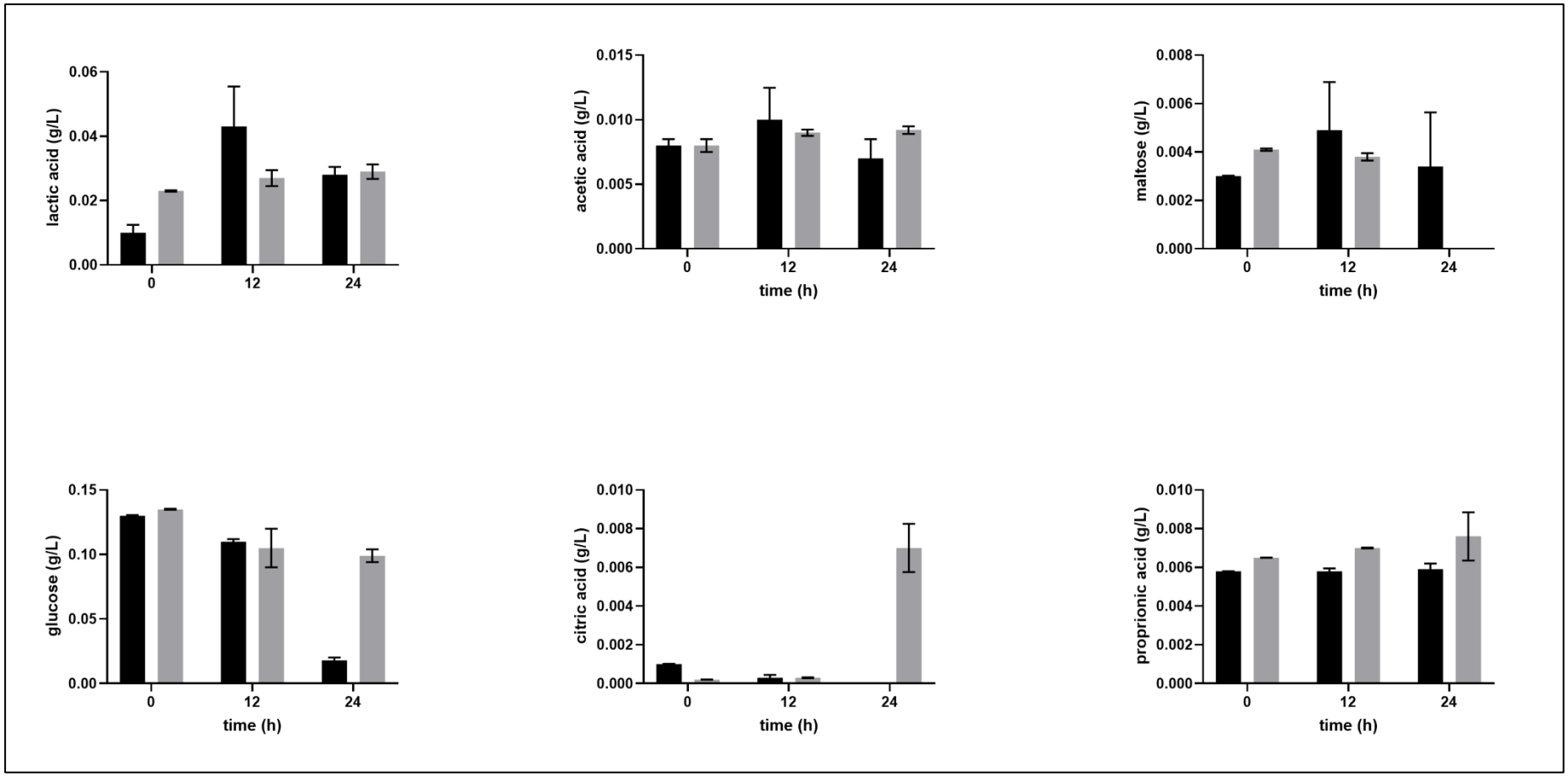

Figure 10.3. Concentrations of organic acids and sugars during the 24 h of growth of prebiotic bacteria *Lactobacillus*, incubated in the presence of SFOH (black) and, in the SFCONV (grey) of tomato SF 2%, after simulation of the gastrointestinal tract.

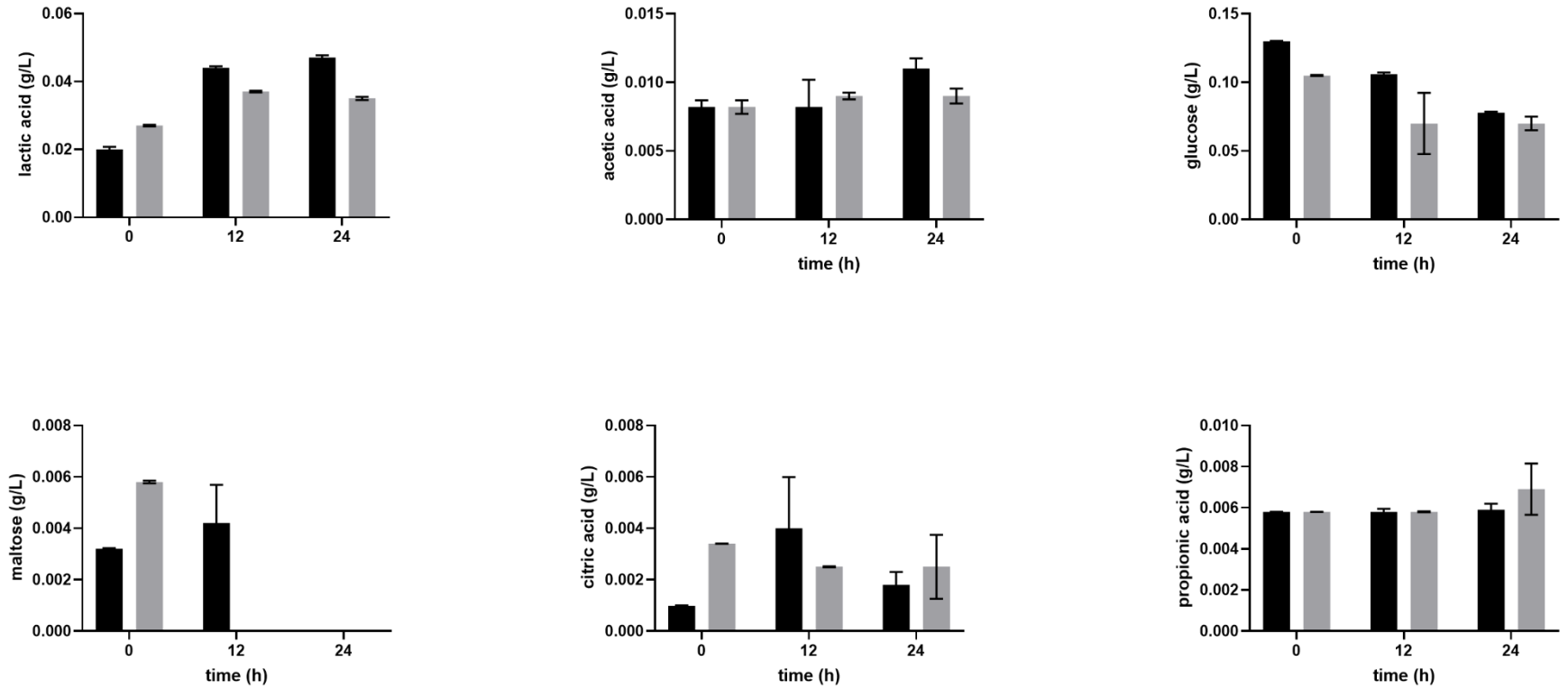

Figure 10.4. Concentrations of organic acids and sugars during the 24 h of growth of prebiotic bacteria *Bifidobacterium*, incubated in the presence of SFOH (black) and in the SFCONV (grey) of tomato SF 2%, after simulation of the gastrointestinal tract.

Citric acid, in the control, increased throughout the incubation time for all probiotic bacteria as well as in the mixture of prebiotics. In addition, a significant concentration of citric acid was produced by *L. casei*. Towards the presence of tomato by-products, an increase in citric acid is visible in the first 12 h, however after this time the concentration decreases substantially, with *L. casei* production reaching null values. It's possible to conclude that *B.animalis* and *B.longum* achieved the highest concentrations in the presence of these flours.

Propionic acid is a major fermentation end product in the human gut with health benefits that extend beyond the gut epithelium. It is thought to 'lower lipogenesis, serum cholesterol levels and exerts immunosuppressive actions against pathogens'. Therefore, microbial production of propionic acid through the diet could be a potential strategy to increase health effects from carbohydrate fermentation (Hosseini E, 2014).

The initial propionic acid concentration was slightly higher, but despite that, during the 24 h, all concentrations increased in the SFCONV and in SFOH, except *L. casei*. No significant differences were observed between *Lactobacillus* and *Bifidobacterium* samples concerning propionic acid concentrations.

10.3.2. Impact of tomato flour after extraction on gut microbiota

10.3.2.1. Microbial population modulation

The gut microbiota assay mimics our organism's complexity, which goes far beyond *Lactobacillus* and *Bifidobacterium*. There is a set of microorganisms that interact with each other and with different preferences for substrates. Moreover, the colonic microbiota is constituted by a complex microbial community that contributes to intestinal homeostasis and impacts food digestion, either culture-dependent or culture-independent (metagenomics). Thus, it is crucial to consider the two-tomato flour (obtained as the second residue from two carotenoids extractions) to understand the intestinal microbiota influences.

The potential impact of the digested tomato flours upon the gut microbiota was evaluated using a batch faecal fermentation system in which fluctuations in target microbial groups and SCFA were measured throughout time.

A simulated gut microbiota fermentation was made through an *in vitro* model to evaluate the potential prebiotic impact of tomato flours (promotion of positive microorganisms' growth and metabolite production) obtained after CONV and OH extraction.

The phyla Bacteroidetes, a Gram-negative and Firmicutes, a Gram-positive, are the most plentiful in the human gut. Relatively to Bacteroidetes and Bacteroides presented significant differences between the SFCONV and the controls. For the Bacteroidetes cluster, there was an increase in the presence of SFCONV at 12 h, with significant differences compared with C^-. Also, according to Figure 10.3, there is a greater dispersion of the number of gene copies for SFOH than for SFCONV. The latter population is less spread, concentrating in the 5 log of number of copies of 16S rRNA / ng DNA). It is also possible to verify that for SFOH about 25% of the population presented a 6 log 16S rRNA gene copies/ng of DNA, similar behaviour to C+. The results are in

agreement with the literature, as Bacteroidetes may metabolize complex nutrient polymers, many of which are molecules in the plant cell wall (e. g. cellulose, pectin and xylan), which through the of action human digestive enzymes' cleavage activity are released and may reach the colon intact (Xue et al., 2016). Studies reveal that dietary habits and lifestyle turn into determinants and play a critical part in gut microbiota variations. The high-fibre and animal protein foods increase Bacteroidetes, whereas the presentation of high-fibre and carbohydrates foods increases Firmicutes and Prevotella (Rinninella et al., 2019; Simpson & Campbell, 2015). This information reinforces our results since the SFOH presents more protein than SFCONV, consequently, more Bacteroidetes than SFOH ($p<0.05$).

Also, SFCONV samples contain more bound phenolics than SFOH. Xue and colleagues (2016) showed that phenolic compounds, namely quercetin and catechin, inhibit the Bacteroidetes and Firmicutes growth. Nevertheless, other microorganisms maintain the ability of carbohydrate and energy metabolism in each group. It is yet unknown how other bacteria use FOS and their metabolites (Xue et al., 2016). The presence of multiple FOS transport systems with different specificities in each strain may also explain the selective metabolism of particular oligosaccharide components observed here. Also, bound phenolic compounds may be responsible for altering the metabolism pathway inhibiting the Bacteroidetes growth. The increase of Bacteroidetes leads to an increase of acidic compounds like pyruvic, citric, fumaric, and malic acids, indicators of higher energy metabolism, and thus contributes to the healthy metabolome (Baümler & Sperandio, 2016; Jandhyala et al., 2015; Kamada et al., 2013). For Bacteroides, results also showed the same tendency as to Bacteroidetes genera. The SFOH presented a more heterogeneous distribution for Bacteroides than SFCONV, which presents similar behaviour for different donors (Figure 10.3).

Furthermore, the results showed that 25% of SFOH and SFCONV have gene copies number higher than C+. Also, there is a significant increase in the Bacteroides population caused by SFOH and SFCONV at 12 h when compared to controls ($p<0.05$) (Figure 10.3). The SFCONV have more rutin than SFOH, a polyphenol compound as described in chapter 9. Rastmanesh et al. (2011) claimed that polyphenols might modify microbiota balance through the biased effects on Bacteroides (Rastmanesh, 2011).

Regarding the Firmicutes results, a slight increase of 16S rRNA at 24 h for SFCONV samples exposure was observed compared to control samples, with significant differences ($p<0.05$). According to Figure 10.3, while the SFOH samples presented similar results to C+, the bacterial population is more dispersed than the observed SFCONV bacterial population. The SFOH contain more fatty acids and fibre than the SFCONV (table 9.1). Studies have demonstrated that a diet rich in fibre and low fat promotes Firmicutes growth that metabolises dietary plant-derived polysaccharides to SCFAs. (Di Paola et al., 2011; Simpson & Campbell, 2015).

The ratio of Firmicutes to Bacteroidetes (F/B) was also analysed (data not shown) being 1:1 for all samples during the experience. Commonly, healthy individuals display a nearly 1:1 ratio of F/B (Parkar et al., 2013; Xue et al., 2016), and the ratio's increase (e.g., to 20:1, F/B) or decrease has

been associated with obesity and weight loss, respectively (Koliada et al., 2017). Also, dietary intake, such as fibres, and phytochemicals have a higher impact on microbiota. An example is a diet rich in fibres which increases Bacteroidetes, and diets rich in calories increase Firmicutes. The maintenance of the F/B ratio during all experiences corroborates with higher amounts of fatty acids and dietary fibre present in samples, contributing to ratios equilibrium. Thus, this is a good indicator to use SFOH in diet and contribute to healthy health individuals. Other studies with obese individuals or malnourished could be interesting to understand the alterations caused by these samples.

In general, the *Bifidobacterium* showed a slight increase over time for both tomato SF tested. According to Figure 10.3, about 50% of the initial population of *Bifidobacterium* is between 1 and 4 log of copies of number of 16S rRNA / ng DNA). At 6 h fermentation, a significant increase was observed in the number of copies with time ($p<0.05$) for SFOH. In addition, CONV fermentations showed higher levels of gene copies at 12 h, compared to the controls. Also, at 24 h 25% of this bacteria presented more copies than the C+. Given the results showed in chapter 9, it appears that SFCONV has more soluble fibres than SFOH and C+, being a good carbon source and promoting a greater growth of this bacteria. Studies have shown that, depending on the strains, they use different substrates for growth. While not all strains are capable of using (most of the components) of the galactooligosaccharides, the capacity of intestinal communities for the metabolization of galactooligosaccharides would not be excluded. This is reinforced by the study of Xu et al. (2007), who demonstrated that the combined activity of multiple bacteria is responsible for the fermentation of complex carbohydrates in the gut. Another study indicated that certain prebiotics encourages, but not others, the growth of some strains/species (Watson et al., 2013). Authors investigated several strains and revealed that 11 of the bifidobacterial strains were significantly growing on polydextrose (soluble fibre), final OD_{600}>05, whereas 34 of the bifidobacterial strains exhibited positive growth FOS. This is an essential result to deduce the prebiotic potential of different carbohydrates to increase the diversity of the gut microbiota. McLaughlin et al. (2015) verified a superior growth with inulin to *B. longum* subsp. CCUG 18157, when compared to the other strains tested. Possibly this strain produces a specific enzyme, such as β-fructofuranosidase with specificity for FOS or inulin.

One intestinal bacterium naturally existing in gut microbiota of healthy people is *Akkermansia.* When present in the intestinal flora, this group also produces propionate and acetate; however, the faecal samples contained lower gene copies of *Akkermansia.* The fermentation of both samples induced a significant reduction in *Akkermansia* levels from 0 to 12 h. Nonetheless, no differences were found between samples and controls ($p>0.05$). The lower concentrations of this bacteria, when compared with the other groups of bacteria, could be associated with its human intestinal colonization at a very young age (it is found in breast milk and infant formula) (Lukovac et al. 2014) and the donators age media was 40 years old. Also, recent studies have shown that diets rich in fibres and proteins decrease the *Akkermansia* population (Dao et al., 2016; Everard et al., 2013). Our results agree with previous reports, where SFOH presents more fat and soluble

fibres than SFCONV, resulting in population distribution with less 16 rRNA gene copies of Akkermansia.

The *C. leptum* belongs to the group of anaerobic bacteria that mainly produce propionate and butyrate in gut microbiota and use amino acids as the primary energy source. No differences were observed in cell numbers ($p>0.05$); nevertheless, interesting results were observed in Figure 10.3. The first quartile population (25%) of SFOH presented a lower number of gene copies than other samples, while the second and third quartile, 50% of *Clostridium leptum* population, presented a similar 16 rRNA gene copies with C+. Regarding SFCONV, this seems to promote the growth of this bacteria better. As seen for *Akkermansia*, a diet rich in fermentable fibres can promote the growth of clostridium. However, the presence of lipids can also inhibit them, thus verifying the discrepancies in the growth of this microorganism (Simpson & Campbell, 2015; Zhou, 2017).

The differences obtained for microorganisms agree with recent research, suggesting that food changes may drastically modify endogenous microbial communities' total composition and organization in the gut.

10.3.2.2. SCFA analysis

As referred, some of the welfare benefits attributed to the fibre fermentation by the colonic bacteria are related to the metabolites generated. Carbohydrates are fermented to organic acids that allow energy for different microorganisms, the gut epithelium and peripheral tissues. SCFA represent the top end products of carbohydrates fermentation. These weak acids (pKa ~4.8) help decrease the colon's pH, thereby inhibiting the development and action of pathogenic microorganisms. Other small organic acids created include lactate, succinate and formate.

Relatively to butyrate, this SCFA is the primary energy source for normal, healthy colon cells. In addition, it safeguards against colon cancer and inflammation due to its capacity to help defend the gene-expression structure that discourages the development and proliferation of cancer cells. Results suggest a significant increase of n- butyrate at 6 h in samples fermented with SFOH- compared with control samples. At 24 h the samples of SFCONV fermented also showed an increase of n-butyrate higher than SFOH samples, but no statistical differences were found ($p>0.05$).

Acetate developed at microbial fermentation in the intestine mostly escapes the first-pass metabolism in the human. Some acetate is converted to butyrate by lumenal bacteria (Chambers et al., 2018). It may bring acetyl units to lipogenesis at the cytosol of hepatocytes and adipocytes but its central place of reaction in peripheral tissues. It also restricts adipose tissue lipolysis and maybe on central appetite control. Results showed a significant higher acetate concentration for tomato flour CONV than OH ($p< 0.05$). In addition, there was an increase in acetate concentration over time. The results align with previously reported observations since this SCFA is, for the most part, created by enteric microorganisms as Bacteroidetes and *Bifidobacterium* because of carbohydrate fermentation through the hydrolysis of acetyl-CoA. A little part is synthesized by acetogenic microorganisms that use hydrogen, carbon dioxide or formic acid through the Wood-

Ljungdahl pathway (Schuchmann and Müller 2016; Ríos-Covián et al. 2016). The results are aligned with the literature, since the observed formic acid concentration decreases as acetate concentration increases.

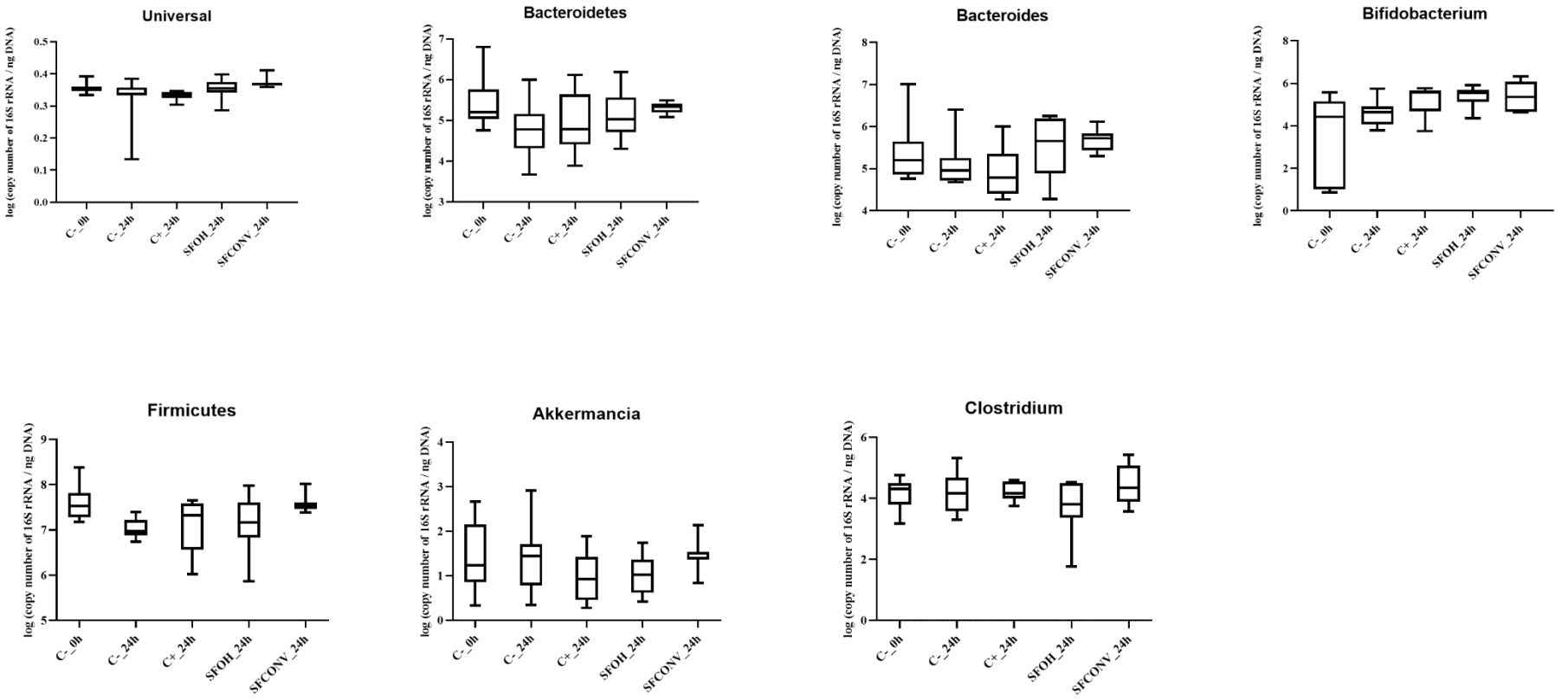

Figure 10.5 Distribution of gut bacterial populations (log 16S rRNA gene copies/ng of DNA), means ± SD) detected by PCR in Fecal samples. The used probes: *Clostridium leptum* (A*), Bacteroidetes* (B), *Bacteroides* (C), *Firmicutes* (D), *Bifidobacterium* (E), and *Akkermansia* (F) Different letters mark statistically significant ($p<0.05$) differences between samples at each sampling point.

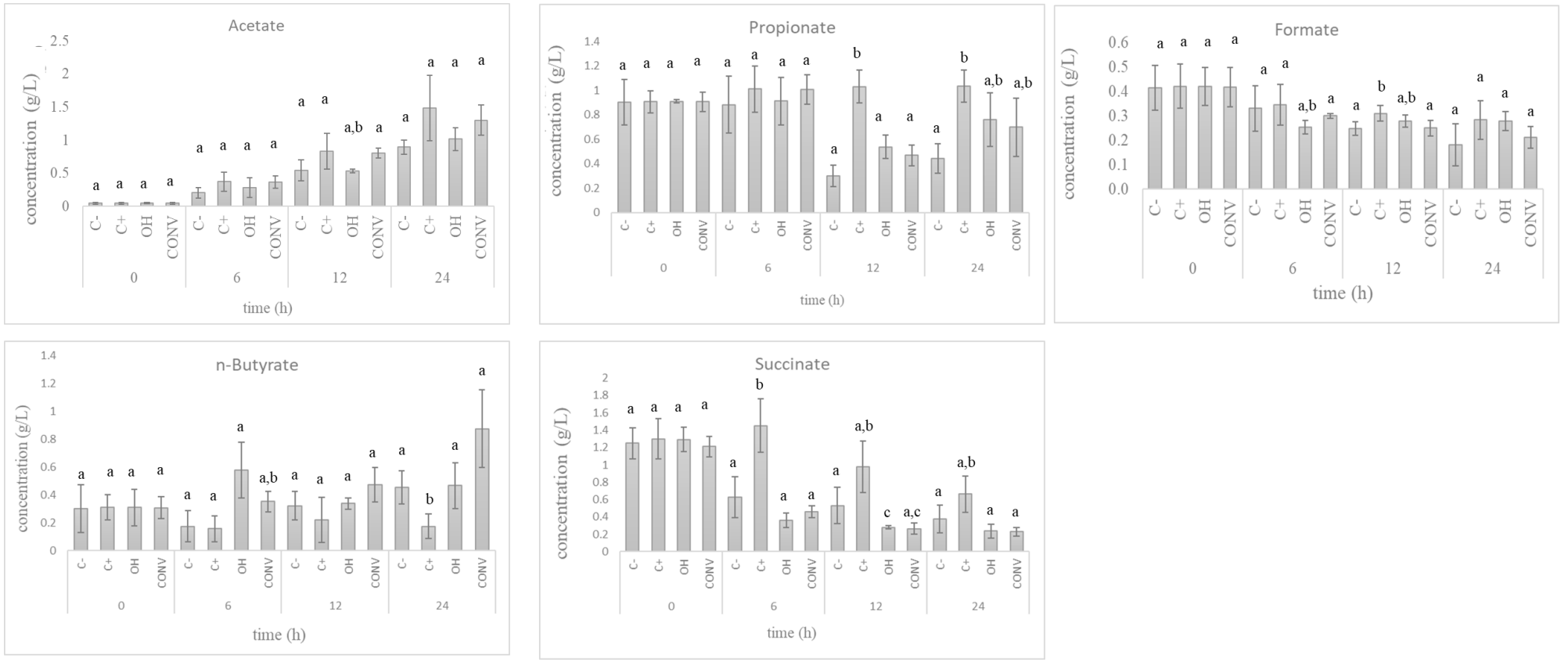

Figure 10.6. Concentration (mg / mL ± SD) of the SCFA produced along fermentation time in fecal samples. (negative control (C-), positive control (C+), tomato residue flour after OH extraction (OH) and tomato residue flour after conventional extractio (CONV). Different letters mark statistically significant ($p < 0.05$) differences

Propionate behaves locally in the intestine on enteroendocrine L-cells to induce the activity of anorexigenic gut hormones PYY and GLP-1. It is mainly absorbed across the gut and sequestrated mainly in the organs where it may be oxidized or applied in glucogenesis. There was a slight decrease of propionate concentration at 12 h on the SFOH sample compared with the positive control, with a significant difference ($p<0.05$). Nevertheless, increased propionate concentration was found at 24 h for both positive control and tomato bagasse flours (SFOH, SFCONV), with significant differences compared to the negative control. The different propionate's pathways may explain these results: succinate, acrylate, and propanediol. The succinate pathway is related to the Firmicutes and Bacteroidetes (Ríos-Covián et al. 2016; Morrison and Preston 2016). The results are illustrated in Figure 10.6, where succinate concentration decreases over time, suggesting a production of propionate based on the succinate pathway.

Butyrate is mainly oxidised in the gut epithelium, where it plays a central part in orchestrating the hard junction protein complexes to ensure gut barrier use. Results showed higher production at 24 h for SFOH and SFCONV samples than C-, which indicates a tomato bagasse flour fermentation stimulating the production of this acid.

Although acetate and propionate production for SFOH and SFCONV were almost the same, the CONV sample had higher butyrate production than the OH sample. Also, the acetate and propionate are related to the advancement of satiety, thus taking into account the phytochemical profile of these tomato bagasse flour and the results obtained for propionate and acetate production; they could be applied as a substitute for animal-derived proteins and fibre in foods.

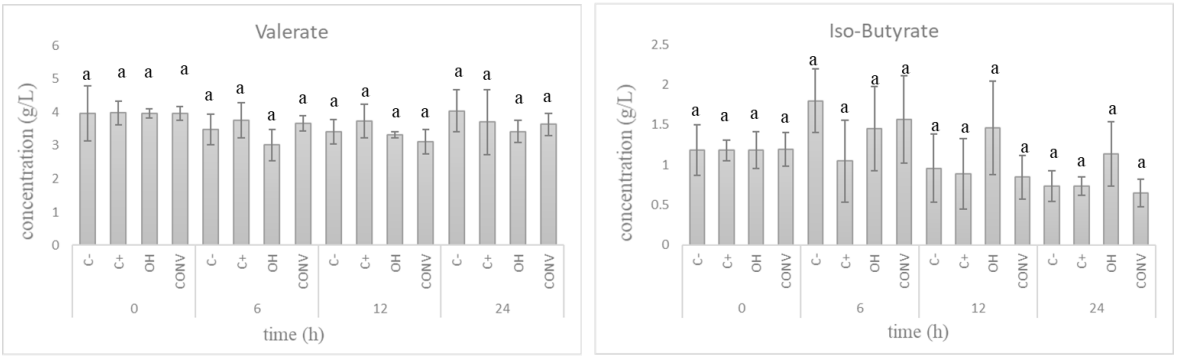

Figure 10.7. Concentration mg/mL ± SD) of iso-butyrate and valerate produced along fermentation time in Fecal samples. (negative control (C-), positive control (C+), tomato residue flour after OH extraction (OH) and tomato residue flor after CONV extraction(CONV).Different letters mark statistically significant ($p <0.05$) differences samples at each sampling point.

Regarding BCFA (branched-chain fatty acids), these results were at lower concentrations than SCFAs and agreed with the literature (Carvalho et al., 2019; Ríos-Covián et al., 2016). For valerate, the concentrations were primarily constant over time. This BCFA is an absorption marker for undigested proteins. Also, these compounds are mainly created from protein degradation, especially from branched amino acid fermentation (Carvalho et al., 2019).

According to the Pearson correlation (Figure 10.8), a significant impact is observed of some SCFAs on some microorganism's expression, which is considered in the evaluation of the previously described results. Propionate is correlated with the growth of Clostridium and Firmicutes, and formate and succinate production is correlated with Akkermancia. The last one also influences the Firmicutes.

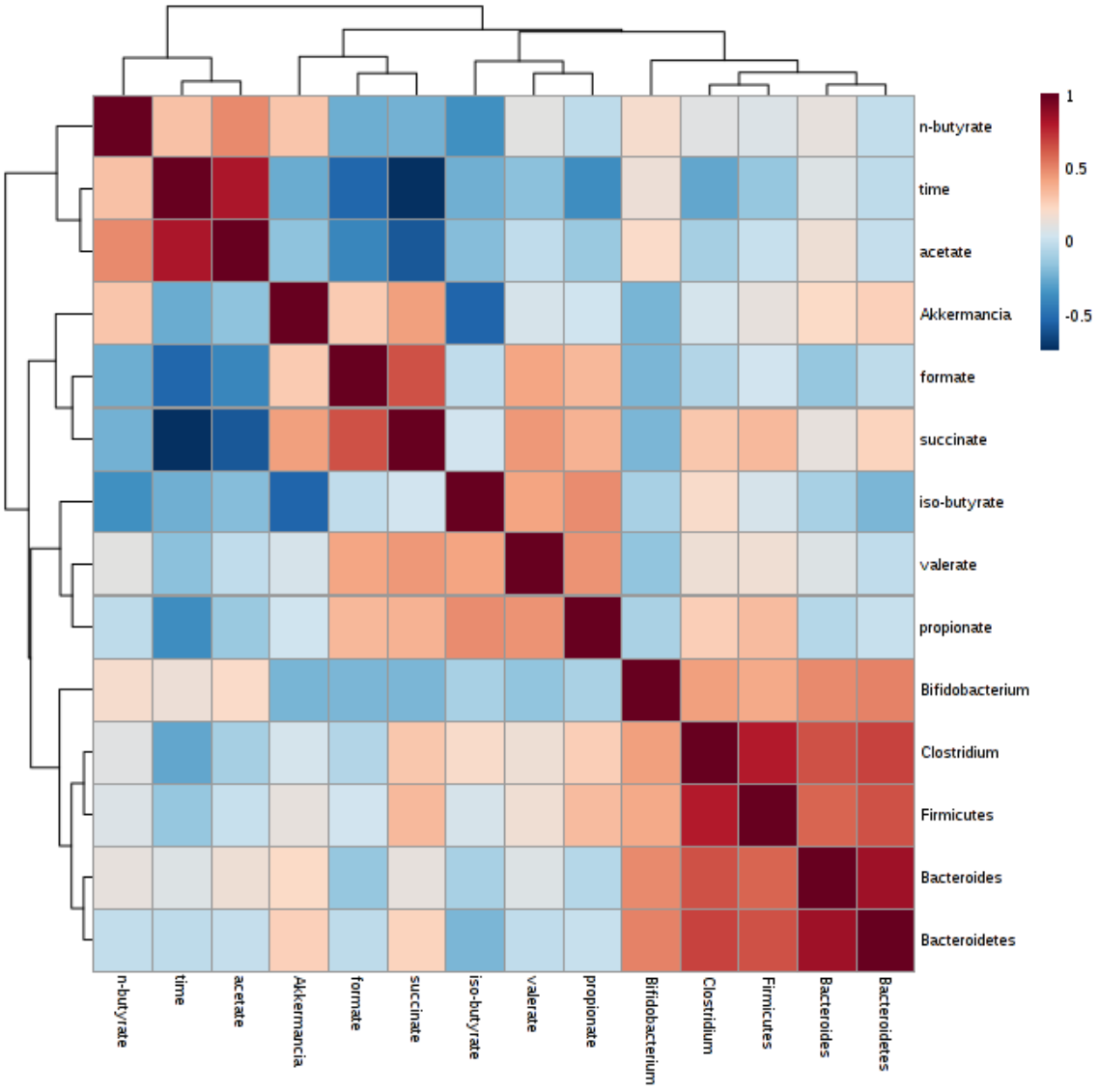

Figure 10.8. Pearson correlation between microorganisms and SCFAS produced during fermentations.

10.4. Conclusions

The screening of the prebiotic properties of SF obtained after OH, and CONV extraction from tomato by-products were assessed using *Lactobacillus* and *Bifidobacterium* as probiotics. Differences in bacterial carbohydrate utilization patterns between species were identified, with the best results being obtained for *Bifidobacterium animalis BO*. The impact of SFOH and SFCONV on these probiotics was small, with differences observed for *Lactobacillus* and *Bifidobacteria* strains. The SFOH at 2% and 4% contribute to *L. casei* growth comparatively with SFCONV. While for *Bifidobacterium* SFCONV at 2% promotes it growth.

Regarding the faecal fermentation based on volunteers faeces, both flours' main groups are the *Bifidobacterium* and *Akkermansia*. Also, 25% of SFOH and SFCONV samples presented gene copies higher than the positive control to Bacteroides. Regarding SFOH, this sample enhanced the Bacteroidetes growth, and also, 50% of population testes presented similar results with C+ to *C. leptum*. While SFCONV higher number of genes copies than the positive control to *Bifidobacterium*.

Concerning SCFA results, both flours increased the propionate, butyrate and acetate concentration compared to the negative control, which indicates the production capacity of these acids by SFOH and SFCONV during fermentation. Also, SFCONV produces more butyrate than the OH samples.

Yet, a relation was observed between certain bacterial groups and SCFA concentration. For the *Bifidobacterium* both acetate and n-butyrate influence its growth. While for *Clostridium*, iso-butyrate and propionate.

The outcomes propose that both tomato flours favour a potential modulatory impact upon the gut microbiota, thus giving a counteractive action of different diets. Also, SFOH comes from a cleaner extraction than SFCONV, making it a potential sustainable ingredient with a prebiotic impact through the growth enhancement of *Bifidobacterium animalis* and improving the generation of SCFA.

Nonetheless, initial and promising evidence of their potential prebiotic effect was demonstrated, raising the need for more extensive testing *in vivo* as part of future work.

PART V

Final Remarks

Chapter 11.
Final conclusions

The actual industrial goal of the European Union is to transit to a circular economy, to benefit the planet, people, sustainability, and prosperity. Both winemaking and tomato processing industries produce large quantities of byproducts, especially seeds and skins, which are wasted and can exacerbate the degradation of the environment. Thus, the work presented in this PhD thesis is an effort to find a scientific basis for valorising the byproducts with the most significant impact in the Portuguese agro-industry, winemaking, and tomato paste industries throughout an emergent technology. We expect to contribute towards an integral and sustainable valorisation based on the circular economy process of winemaking and tomato byproducts by applying an emergent technology, the ohmic heating (OH). Also, the advantages of its application for bioactive compounds (BC) recover from both byproducts were compared with conventional methods (CONV) application which uses organic solvents (pollutants, toxic, with needs of an additional cleaning process, and expensive).

Initially, the characterisation of the proximal composition and phytochemical profile from winemaking byproducts - stems and bagasse (peels and seeds) - from white and red grape cultivars; and tomato pomace (peels and seeds) allow identifying the key actions necessary to enable efficient recovery of critical key molecules, taking into account its potential benefits and guarantee the maximum value from the agro-industrial waste and minimisation of final waste.

The results showed that the stems presented higher mineral content in grape byproducts than grape bagasse (~4 and ~2 g/100 g, respectively), with significant differences between the cultivars used. At the same time, the bagasse contains more protein and carbohydrates than stems.

White and red pomaces present an excellent source of BC, such as total dietary fibre (almost 60%), proteins (5 to 12%), and polyphenols (6 to 15%) (mainly flavonols: quercetin, kaempferol, and myricetin; flavan-3-ols: catechin and epicatechin; and anthocyanins, mainly delphinidin – 3-glucoside; petunidin- glucoside, peonidin-3-glucoside). Experimental results demonstrated that exploiting the white and red bagasse as a functional ingredient contributing to a high economic value and supporting the circular economy would be advantageous. Thus, the study continued with applying ohmic in white and red bagasse to recover the bioactive compounds. Only the moisture of the samples was used to increase the conductivity of the sample and to use a simple method without other solvents added and reduce operational costs.

Results showed that OH technology allows recovering approximately 30% more phenolic compounds quantities such as anthocyanins than the CONV method. This approach may thus be employed directly in bagasse with advances to improve the extraction of beneficial phytochemicals and generate a new product's residual solid fraction. In addition, this study reveals that these solid fractions are a rich source of fibre binding to polyphenols and carotenoids, and they constitute ingredients with potential health benefits. Furthermore, this technology may provide integral recovery

with two functional ingredients: one liquid fraction directly obtained from tomato byproduct and the corresponding solid fraction obtained after extraction.

Regarding tomato byproducts, they presented higher amounts of protein ranging from 16.29 to 19.4 g /100 g DW, it is also rich in fibre close to 60.0 g /100 g DW, and it is also abundant in fatty acids, 17.0 g /100 g DW, including polyunsaturated fatty acids, such as linoleic acid, monounsaturated fatty acid such as oleic acid and saturated fatty acids, palmitic acid. Valuable by-products are disposed of annually, and from a functional point of view, byproducts may generate substantial quantities of fibres, fatty acids, lycopene, polyphenols, and sustainable protein as a suitable source for future foodstuffs and cosmetics.

According to these results, these byproducts contain many BC with health benefits which could also be more sustainable. There are no studies of the effects of alternative methodologies, like OH, on tomato bagasse. Thus, we also applied a green methodology strategy, like OH with food-grade solvents on tomato bagasse, to allow higher recovered yields of BC such as proteins and phenolic compounds, namely kaempferol and rutin. After OH based on solid-liquid extraction, two fractions were obtained, a liquid fraction (LF) and a solid fraction (SF). About tomato bagasse, the results for the extraction of bioactive compounds in the first instance were not as promising as in CONV methods nor as seen previously for grape bagasse. The experimental results revealed that tomatoes presented more lipophilic compounds than grapes, and therefore the extraction would need to be optimised to obtain extractions with yields similar to those obtained conventionally. The OH might also be used in the tomato industry process, allowing valuable compounds to be extracted and approaches across entire value chains, helping achieve a circular economy. The economic potential of OH on tomato byproducts and their BC as either an ingredient or an integrated product was for the first time demonstrated.

After validating the potential byproducts from grape and tomato bagasse as a source of new food ingredients, the next step was to optimise the recovery BC processes, obtaining LF and SF with added value. Considering the lowest pigmented compounds extracted yields observed from tomato bagasse, the red grape bagasse potential compared with white grape bagasse, and high volume of samples, the experimental procedure progressed only with these two byproducts (tomato and red grape bagasse).

The extracts obtained with OH and characterisation have been scarce and exhibited a good potential as multifunctional ingredients. Also, as mentioned above, the sample moisture was used to promote conductivity and OH technology. However, to preserve the samples and use them throughout the study, they were dehydrated, making it necessary to optimise the method to obtain high extraction yields. Thus, water was maintained as the main solvent to BC recovery, with the addition of acidification as a variation, as in the conventional methods, the extraction of anthocyanins is done with acidified MeOH to promote its extraction and its stability. The extracts were chemically characterised, and their bioactivity (antioxidant activity, antimicrobial, and anti-hypertensive) and biological safety were assessed). Also, the combination of OH with water acidified with citric acid (without cytotoxicity verified) presented higher AA than the CONV method, which uses acidified

methanol, a cytotoxic solvent (inhibition of cell viability of 80%). The main anthocyanins recovery was of malvidin-3-O-glucoside, delphinidin-3-O-glucoside, and petunidin-3-O-glucoside. Additionally, OH with citric acid at 1 mg/mL exhibited antimicrobial properties against pathogens, namely *P. aeruginosa, Y. enterocolitica, S. Enteritidis, MSSA, MRSA*, and *B. cereus*.

The results showed that OH coupled with food-grade solvents (water and citric acid) enables stable anthocyanins to be recovered in compliance with Directive 2009/32/EC on the European Union. The results showed a pertinent opportunity in a circular economy to valorise red grape byproducts. Also, the implementation of this technology in the agro-industrial process, either in the winemaking process or in the tomato process industry, and the possibility to reuse water, allow extract BC and at the same time to tackle water scarcity across the EU. At the same time, our results demonstrated that this process is non-toxic and exhibit health properties. Although LF and SF obtained from grape bagasse demonstrated bioactive properties supporting these ingredient's potential application as a functional ingredient, the digestion impact on phenolics compounds, fatty acids and sugars needs to be evaluated to guarantee their health benefits. The bioavailability and bioaccessibility of BC from LF and SF were accessed. Also, the SF impact on gut microbiota was assessed. Nevertheless, this thesis did not show the data due to colleagues' extensive work presented and similar work published before.

Regarding tomato bagasse, we also intended to guarantee the maximum recovery yield of BC, a factorial experimental design 3^3 was carried out to OH, and some procedures were taken, including 1) the use of a food-grade solvent, ethanol to extract lipophilic compounds, such as carotenoids; 2) use a control (CT) with the same solvent used in OH and at the same temperature to verify the effects of OH and non-temperature effects; 3) different electric fields were tested. These extracts were characterised chemically (phenolic compounds and carotenoids) and their bioactivity (antioxidant capacity). The results showed 58% higher recovery with OH when compared with CT; the same solvent was utilised in OH extraction at the same temperature conditions. Nevertheless, compared to CONV methods, the tomato bagasse obtained better results in LF for CONV samples, and the OH presents better SF results. Thus, the SF can also be used with a higher potential functional ingredient.

Additionally, we observed a significant recovery of polyphenols and carotenoids compounds when 6 and 11 V.cm-1 electric fields were applied by the Joule effect and fast heating in association with high electric field intensities. We conclude that OH could work as a selective extraction method and may be an efficient method to extract BC from these byproducts. It allows the same yields to be achieved as in CONV processes but at a higher extraction rate (less time). The LF, both OH and CONV, are rich in carotenoids, mainly lutein, lycopene, and β-carotene, as well as polyphenols, mainly hydroxycinnamic acids, and benzoic acids. The high antioxidant, antimicrobial, and prebiotic effect potential exhibited by LF from tomato bagasse could be explored as a source of potential health benefits and as a potential food preservative. On the other hand, the richness of SF in antioxidants mainly carried by fibre and its simultaneous richness in polyunsaturated fatty might give rise to additive or synergic effects as gut health benefits and lower lipid peroxidation. Biological safety

was confirmed to tomato bagasse fractions, and they demonstrated good functional properties for food applications.

The BC extracted and solid fraction from tomato bagasse was studied regarding their stability throughout the GID tract, demonstrating the predicted bioaccessibility and bioavailability of the main compounds present – carotenoids and polyphenols. The type of extraction showed a substantial impact on the bioaccessibility and bioavailability of polyphenols and carotenoids of tomato bagasse extracts and related biological properties. The OH extraction improved polyphenols bioaccessibility in comparison with CONV and improved the bioavailability of antioxidants. In addition, OH extract showed a possible prebiotic effect and anti-hypertensive activity and anti-inflammatory activity in general with better results than CONV. Differences in the use pattern of bacterial carbohydrates were discovered across species, and *Bifidobacterium animalis* BO yielded the best results. Regarding the faecal fermentation simulation, a relation between certain bacterial groups and SCFA concentration was observed. For the *Bifidobacterium*, both acetate and n-butyrate influenced its growth, while iso-butyrate and propionate influenced clostridium growth.

The outcomes propose that SFOH extracts exhibit a prebiotic impact through the growth of *Bifidobacterium animalis* and improving the generation of SCFA, favouring a potential modulatory impact upon the gut microbiota and thus giving a counteractive action of different diets.

In conclusion, OH combined with food-grade solvent (water, citric acid, or/and ethanol) allows the recovery of stable BC, such as anthocyanins or other phenolic compounds, carotenoids, proteins with health benefits. Therefore, it is necessary to devise strategies for using byproducts directly, considering their properties as a whole. These results demonstrate a relevant opportunity to valorise grape and tomato byproducts, with a simple eco-friendly technique, OH, safety, economical and selectively in a circular economy context. This study also suggests that the OH application on tomato and grape byproducts can produce valuable powder fractions with improved antioxidant properties, antimicrobial, anti-hypertensive without cytotoxicity and genotoxicity effect.

Considering the high nutritional value and potential health benefits identified in this work for grape and tomato byproducts, the valorisation approach proposed in this work could be a good key for winemaking and the tomato processing industry, increasing its environmental, economic sustainability towards a circular economy.

New activities are necessary to develop, manufacture and effectively empower customers concerning circular products and services and embrace more sustainable lives. Also, transit to a circular economy in the agro-industry is necessary to ensure transparency, traceability, and security. An example is the blockchain technology application, which improves the record of information such as byproducts source, quality of products and food safety, monitoring the agro-industries chain while satisfying consumer demands and contributing to sustainability assessments in a Circular Economy.

Chapter 12.
Future prospects

The studies proposed in this PhD thesis encompass a novelty on their own, concerning the valorization of by-products, such as grape and tomato bagasse, through the use of OH as a green and sustainable method to extract BC with health benefits. Also, several scientific, technological, and regulatory issues should be comprehensively addressed to understand the feasibility of this technique to be applied directly to the tomato production process to obtain BC's rich LF and SF achieving a "zero waste food system" towards a circular economy concept and leading to new research lines.

Regarding Chapters 4 and 5, an OH was used in a lab-scale experiment. A possible future optimisation at a pilot-scale further modification to use during the grape pomace separation to wine production, and tomato processing being an affordable and feasible process. However, simulation and optimisation studies in conjunction with life cycle analysis (LCA) should be performed to improve the BC recovery sustainability and economics proposed as an integrated biorefinery scheme and analyse its environmental impacts.

Concerning Chapters 6 and 7, it could be interesting to explore the use of other food-grade solvents, like NADES, which could allow better recovery of lipophilic compounds, like fatty acids and carotenoids. Also, the evaluation of enzymes to recover BC with OH could enhance the recovery yields and make these industries more sustainable. Thus, with only an investment of the OH equipment, using the water present in the tomato industry and winemaking process, and the enzymes present in both tomato and grape, it could answer to an economy more sustainable with zero waste.

Regarding data presented in Chapters 8, 9 and 10, more studies are necessary to overview component behaviour, absorption rate and metabolism, and bioavailability and dose levels. To confirm tomato's gut health benefits, other GID with pH control; could be used, and also, *in vitro* faecal model pH-controlled using human or pig faecal inoculum must be carried out before *in vivo* experiments are held. Other studies regarding antimicrobial, antimutagenic, and anti-inflammatory activity in gut cells should also be performed to explore additional tomato bagasse health benefits.

Also, it is vital to enhance its cost-benefit efficiency for various applications, such as functional ingredients, food and feed, cosmetics and natural pesticides. The result is that the acquisition of value-added health care benefits placed on the market to implement a circular economy in the agricultural sector will alleviate a particularly negative impact of the accumulation of by-products in landfills.

Finally, these ingredients must be tested in terms of microbiological quality and storage conditions defined. Besides the sensory performance of these ingredients must be demonstrated as direct ingredients, but specially to be applied in final products as proof of concept. In this sense, although many different applications could be envisaged, in particular the LF could be tested as presevetives

in a meat products demonstrating the antioxidant and antimicrobial properties and the SF could be tested as a functional flour to produce new bread, cookies or snacks.

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