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Evaluation of the influence of technological treatments on bioactive compounds in vegetable foods

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A mia mamma Libera e mia nonna Angela,
fondamenta dei miei giorini e mio cuore.
A loro dedico il frutto dei silenzi, delle paure e
delle emozioni che mi hanno condotto fin qui.

Con immensa gratitudine.

During the development of this PhD Thesis, the following articles derived from the work carried out have been published, copies of which are included as annexes in the present memory:

[1] A. Fratianni, **A. D’Agostino**, S. Niro, F. Lucarelli and G. Panfili. **Wild edible species of the Mediterranean area: contents of bioactive compounds and effects of domestic cooking**. 35th EFFoST **2021** – International Conference, 1- 4 Novembre, Losanna, Svizzera.

[2] D’Agostino. **Evaluation of domestic cooking on the content of bioactive compounds in green leafy vegetables**. In: Proceedings book of the First Virtual Workshopon the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology. Palermo, **14-15 September 2021.**

[3] S**.** Niro, A. Fratianni, **A**. **D'Agostino**, I. Notardonato and G. Panfili. **Chemical characterization of 'Pecorino Di Farindola' cheese during ripening**. *Italian Journal of Food Science* **2021**, 33(1), pp. 46-51.

[4] A. Fratianni, **A. D’Agostino**, S. Niro, A. Bufano, B. Paura and G. Panfili. **Loss or Gain of Lipophilic Bioactive Compounds in Vegetables after Domestic Cooking? Effect of Steaming and Boiling**. *Foods* **2021**, 10, 960; https://doi.org/10.3390/foods10050960.

[5] G. Panfili, S. Niro, A. Bufano, **A. D’Agostino**, A. Fratianni, B. Paura, L. Falasca and L. Cinquanta. **Bioactive Compounds in Wild Asteraceae Edible Plants Consumed in the Mediterranean Diet.** Plant Foods for Human Nutrition **2020**, volume 75, pages 540–546; https://doi.org/10.1007/s11130-020-00842-y.

[6] **A. D’Agostino**. **Evaluation of the influence of technological treatments on bioactive compounds in vegetable foods**. In: Proceedings book XXIV Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology. Florence, **11-13 September 2019**.

[7] S. Niro, **A. D’Agostino**, A. Fratianni, L. Cinquanta and G. Panfili. **Gluten - Free Alternative Grains: Nutritional Evaluation and Bioactive Compounds.** *Foods* **2019**, 8, 208; doi:10.3390/foods8060208.

Articles in process:

[8] S. Niro, A. Fratianni, R Ievoli, **A. D'Agostino**, P. Avino, I. Notardonato and G. Panfili. **Tocols and fatty acid as markers of different bakery fats in biscuits.** Submitted to *Food Chemistry.*

Index

Abstract 1

Riassunto 5

CHAPTER 1
Bioactive compounds in human health

Introduction 11

1.1 The world of vegetable products 13

1.2 Bioactive compounds of vegetable origin 14

1.2.1 Carotenoids in vegetable products 18

1.2.2 Vitamin E in vegetable products 22

1.2.3 Phenolic compounds 24

1.2.4 Dietary fiber 27

1.2.5 Hydrosoluble vitamins 28

1.3 Antioxidants 30

References 31

CHAPTER 2
Functional products

Introduction 38

2.1 Functional foods 39

2.2 Health benefits and vegetable products in functional foods 41

References 44

CHAPTER 3
Technological treatments on bioactive compounds

Introduction 47

3.1 Overview of food processing 49

3.2 Heat processing 50

3.3 Traditional culinary treatments 51

References 53

CHAPTER 4
Methods of analysis

4.1 Chemical analysis 57

4.1.1 Moisture, ashes and proteins 57

4.1.2 Fats 57

4.1.3 Determination of total fibre 57

4.1.4 Carbohydrates 57

4.2 Extraction of soluble and hydrosoluble vitamins 58

4.2.1 Extraction of carotenoids and tocols 58

4.2.2 Determination of hydrosoluble vitamins 60

4.2.3 Chemicals and Reagents 61

4.3 Polyphenol, antioxidant activity and flavonoid extraction and determination 61

4.3.1 Total polyphenol content 62

4.3.2 ABTS assay 62

4.3.3 Total flavonoid content 63

4.5 Fatty acid analysis 63

4.6 Sensory analysis of pasta 63

4.7 Statistical analysis 64

4.7.1 Statistical analysis 64

4.7.2 Statistical analysis for bakery products 64

References 66

CHAPTER 5
Gluten-Free Alternative Grains

Abstract 69

Introduction 70

Material and Methods 71

Sample Collection and Preparation 71

Chemical analysis 72

Results and Discussion 75

Nutritional composition 75

Carotenoids 76

Tocols 77

Thiamine and riboflavin 79

Conclusion 81

References 82

CHAPTER 6
Tocols as products and process markers

Introduction 87

6.1 Bakery products 89

6.2 Method to identify oils and fats in bakery products 90

6.3 Materials and methods 91

6.3.1. Preparation of biscuits 91

6.3.2. Chemical analysis, tocol analysis, fatty acid analysis 93

6.4 Results and discussion 93

6.4.1. Tocol and fatty acid composition 93

6.4.2. PCA analysis 95

6.5 Impact of cooking on the content of tocols in final bakery products 107

6.6 Conclusions 110

References 111

CHAPTER 7
Evaluation of the influence of domestic cooking on bioactive compounds in green leafy vegetables

Introduction 116

7.1 Wild edible plants “WEPs”: a challenge for future diet and health 117

7.2 Description of WEPs under study 118

7.2.1. Crepis vesicaria 119

7.2.2. Sonchus asper 120

7.2.3. Sonchus oleraceus L. 121

7.2.4. Beta Vulgaris L. 122

7.3 Cooking conditions 122

Bioactive compound content of wild edible Asteraceae species present in the Mediterranean diet 125

Abstract 126

Introduction 127

Materials and Methods 128

Plant Material 128

Chemicals and Reagents 129

Proximate Analysis 129

Carotenoid and Tocol Analysis 129

Thiamine and Riboflavin Analysis 130

Statistical Analysis 131

Results and Discussion 131

Nutritional Composition 131

Conclusion 139

References 140

7.4 Results and discussion of *Spinacea oleracea, Cicoria intybus, Beta vulgaris* 144

7.4.1 Nutritional composition 144

Loss or Gain of Lipophilic Bioactive Compounds in Vegetables after Domestic Cooking? Effect of Steaming and Boiling 147

Abstract 148

Introduction 149

Materials and Methods 150

Plant Material 150

Cooking Conditions 151

Chemicals and Reagents 151

Tocols and Carotenoids Extraction and Quantification 152

Results and Discussion 154

Conclusions 161

References 162

7.5 Effect of domestic cooking on *Beta vulgaris* 165

7.6 Hydrosoluble compounds 167

7.7 Antioxidant activity 168

References 169

CHAPTER 8
Pasta enriched with green leafy vegetables

Introduction 173

8.1 Pasta in the world 174

8.2 Enriched pasta-like products 175

8.3 Materials and methods 176

8.3.1 Ingredients 176

8.3.2 Proximate Composition, tocols and carotenoids analysis 176

8.3.3 Pasta making 176

8.4 Results and discussion 178

8.4.1 Chemical composition 178

8.4.2 Nutritional properties 179

8.4.3 Cooking quality 181

8.5 Conclusions 185

References 186

CHAPTER 9
General conclusion

9.1 General conclusion and future perspective 190

INDEX OF FIGURES

Figure 1. Chemical structure of selected carotenoids and their main dietary sources 21

Figure 2. Chemical structures of tocopherols and tocotrienols and the main dietary sources of vitamin E (edible oils, nuts, seeds, spinach and cereals). 24

Figure 3. Classification and structure of polyphenols. 25

Figure 4. Structures of phenolic acids. 26

Figure 5. Chemical structure of B1 and B2 vitamins. 29

Figure 6. Shortbread biscuit production. 93

Figure 7. HPLC chromatograms of tocols 94

Figure 8. Score plot of the first three principal components (A) and plot of the principal component analysis (B) with fatty acids as variables, in the made biscuits. For fats/oils identification, see Table 9 and Table 10. 103

Figure 9. Score plot of the first three principal components (A) and plot of the principal component analysis (B) with tocols as variables, in the made biscuits. For fats/oils identification, see Table 9 and Table 10. 104

Figure 10. Score plot of the first three principal components (A) and plot of the principal component analysis (B) with fatty acids and tocols as variables, in the made biscuits. For fats/oils identification, see Table 9 and Table 10. 105

Figure 11. Score plot of the first three principal components (A) and plot of the principal component analysis (B), with fatty acids and tocols as variables, in the fat/oil ingredients. For fats/oils identification, see Table 9 and Table 10. 106

Figure 12. Percentage decrease of tocols after cooking in bakery products. 109

Figure 13. *Crepis vesicaria.* 119

Figure 14. *Sonchus asper L.* 120

Figure 15. *Sonchus oleraceus L.* 121

Figure 16. *Beta vulgaris* L. 122

Figure 17. Steps from plant collection to analysis 124

Figure 18. Typical chromatogram of carotenoids of *Crepis vesicaria* (a) and a standard mix (b). 135

Figure 19. Typical chromatogram of tocols of *Crepis vesicaria* (a) and a standard mix (b) 135

Figure 20. TPC contents (nmoles of catechin/g f.w.) in fresh and cooked plants. Different letters indicate a significant difference. 167

Figure 21. TFC contents (nmoles of catechin/g f.w.) in fresh and cooked plants. Different letters indicate a significant difference. 167

Figure 22. Antioxidant activity (nmoles of trolox/g f.w.) in fresh and cooked plants. Different letters indicate a significant difference. 168

Figure 23. Formation of gluten in the dough. 166

Figure 24. Preparation of pasta samples 169

INDEX OF TABLES

Table 1.	Bioactive compounds and health benefits of selected vegetables.	14
Table 2.	Content of bioactive compounds in vegetable products.	15
Table 3.	Important phytochemicals in functional foods and their health benefits.	42
Table 4.	Rating scale for pasta sensory analysis (ISO 7304-1).	64
Table 5.	List of analysed gluten-free grains.	72
Table 6.	Nutritional composition of gluten free grains (g/100 g).	75
Table 7.	Carotenoid composition in gluten free grains (μg/100g d.w.).	76
Table 8.	Tocol composition in gluten-free grains (mg/100g d.w.).	78
Table 9.	Thamine and riboflavin content in gluten-free grains (mg/100g d.w.).	79
Table 10.	Oils, fats and their blends used in the twelve different biscuit samples	92
Table 11.	Formulation of recipes	92
Table 12.	Contents of tocols (mg/100 g d.w.) in the fat/oils and other used ingredients and made biscuits.	99
Table 13.	Fatty acids (%) in the fats/oils and other used ingredients and in the made biscuits.	101
Table 14.	Theoretical content of tocols in bakery products (mg/100 g d.w.).	108
Table 15.	Synthesis of sampling operations.	119
Table 16.	Proximate composition of WEPs (g/100 g WB).	133
Table 17.	Content of carotenoids in WEPs (mg/100 g WB) in the two harvest years.	134
Table 18.	Content of tocols in WEPs (mg/100 g WB) in the two harvest years.	136
Table 19.	Content of thiamine and riboflavin in WEPs (mg/100 g WB) in the two harvest years.	137
Table 20.	Proximate composition of green leafy vegetables (g/100 g W.B.).	144
Table 21.	Content of carotenoids in the analyzed green leafy vegetables (mg/100 g W.B.).	145
Table 22.	Content of main tocols in green leafy vegetables (mg/100 g W.B.).	146
Table 23.	Content of thiamine and riboflavin in WEPs (mg/100 g W.B.)	146
Table 24.	Contents of the main carotenoids and tocols in the investigated leafy vegetables before and after cooking (mg/100 g d.m.).	154

Table 25. Soluble solid losses (g) in cooking water from 100 g of dry matter of the cooked samples 157

Table 26. Contents of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *S. oleraceus.* 158

Table 27. Contents of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *S. asper*. 158

Table 28. Content of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *Sp. oleracea.* 159

Table 29. Content of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mgz/g d.m.) and in cooking water (mg/g d.m.) of *C. intybus.* 159

Table 30. Content of the main carotenoids and tocols in fresh and in cooked *Beta vulgaris* (d.w.) 165

Table 31. Soluble solid losses (g) in cooking water from 100 g of dry matter of the cooked samples. 166

Table 32. Contents of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *B. vulgaris.* 166

Table 33. Control purchased samples. 176

Table 34. Pasta formulations. 177

Table 35. Proximate composition of dried pasta (g/100 g f.w.). 178

Table 36. Tocol composition in the investigated pasta samples (mg/100 g f.w.). 180

Table 37. Carotenoid composition in investigated pasta samples (mg/100 g f.w.). 181

Table 38. Cooking quality of experimental pastas. 183

ABBREVATIONS

BCs	Bioactive Compounds
BC	Bioactive Component
WHO	World Health Organization
FAO	Food and Agriculture Organization
USDA	United States Department of Agriculture
EFSA	European Food Safety Authority
NSP	Non-Starch Polysaccharides
RDA	Recommended Daily Allowance
T	Tocopherol
T_3	Tocotrienol
α-, β-, γ-, δ-T	α-, β-, γ- and δ-Tocopherols
α-, β-, γ-, δ-T_3	α-, β-, γ- and δ-Tocotrienols
IU	International Unitis
TE	Tocopherol Equivalent
B1	Thiamin
B2	Riboflavin
TPP	Thiamin Pyrophosphate
FOSHU	Food for Specified Health Uses
ILSI	International Life Science Institute
FDA	Food and Drug Administration
FMN	Flavin Mononucleotide
FAD	Flavin Adenine Dinucleotide
SDF	Soluble Dietary Fibre
IDF	Insoluble Dietary Fibre
TDF	Total Dietary Fibre
T.E.	Tocopherol Equivalent
GC	Gas chromatography
FA	*Fatty acids*
BF	Butter
LF	Lard
OO	Olive oil
EVO	Extra virgin olive oil
SO	Sunflower

HSO	High oleic sunflower oil
PO	Palm oil
CO	Corn oil
SOO	Sunflower oil/olive oil
POO	Palm oil/olive oil
SPO	Sunflower oil/palm oil
HSPO	High oleic sunflower oil/palm oil
WEPs	Wild edible plants
W.B.	Wet basis
f.w.	Fresh weight
d.w.	Dry weight
AMD	Age-related macular degeneration
d.m.	Dry matter
TPC	Total Polyphenol Content
TFC	Total Flavonoid Content

Abstract

Nowadays, consumers all over the world are ever more concerned about having a healthy and balanced nutrition and, in this sense, the consumption of vegetables and vegetable-based products could play a fundamental role. In fact, the beneficial effects on human health deriving from the intake of vegetable products are now widely recognized. Many epidemiological studies report the positive correlation between the vegetables and vegetable-based products consumption and the reduced risk of several diseases such as cardiovascular disease, type 2 diabetes, obesity, Alzheimer's disease, cataracts and several forms of cancer. These positive effects are the result of the synergic action of the several bioactive compounds and micronutrients of which vegetable products (vegetables, fruits, herbs, legumes, cereals or even oils) are characterized, for example, carotenoids, tocols, dietary fiber, flavonoids and other phenolics, vitamins, minerals and many other phytonutrients. This convincing evidence suggests that a change in dietary behavior such as an increasing consumption of fruit, vegetables, and grains is a practical strategy for significantly reduce the incidence of chronic diseases. Prevention is a more effective strategy than treatment of chronic diseases. The level of bioactive compounds in vegetable products could depend on botanical family, growing conditions, storage and food processing carried out. Nevertheless, it should be considered that there might be bioactive compounds losses in the final product during processing, cooking and storage, due to the influence of several factors such as temperature, light, air, pH, moisture content, water activity. All this causes alter their structure and induce significant changes in chemical composition, nutritional value and bioavailability of bioactive compounds. The study of these products could help in predicting losses during processing and in identifying the best technological conditions for process optimization and preservation of their content and their bioavailability in food.

In the light of these considerations, in this doctoral thesis, the aim of the research was the qualitative and quantitative evaluation of bioactive compounds of different vegetable products, the influence of the technological treatments on their content, the identification of product and process markers and, finally, the development of healthy foods enriched with the identified bioactive compounds. The reserch was developed on three main activities:

1. Gluten-free minor cereals and pseudocereals were characterized for their nutritional value, with a particular focus on some bioactive compounds (carotenoids and tocols) and hydrosoluble vitamins (thiamine and riboflavin), in order to increase the awareness of their nutritional profile.
2. The determination of tocols and fatty acids of commercial and laboratory made bakery products, together with chemiometric analysis, was used to discriminate among different oils and fats in the investigated products. On realized products, the effect of technological treatments of the tocol amount was studied.
3. Investigation of local wild plants, comparing them with those marketed, through their nutritional characterization and the analysis of bioactive compounds. Since the leafy vegetables under study are also consumed after cooking, the nutritional evaluation could not be made without considering changes of bioactive compounds due to principal thermal treatments, such as boiling and steaming. To have a more complete picture, in this last part, the research activities focused on the development of a protocol for the production of traditional innovative healthy foods with the investigated leafy vegetables, considering the significant quantities of found bioactive compounds. The nutritional and sensorial characteristics of the made final products were evaluated.

The results obtained in the first part of the experimentation can provide more information on bioactive compounds in gluten-free minor cereals and also pseudocereals, in order to increase the awareness of their nutritional profile, to date limited in literature. All analyzed samples showed a high content of bioactive compounds; in particular, amaranth, cañihua and quinoa are good sources of vitamin E, while millet, sorghum and teff are good sources of thiamine. Moreover, millet provides a fair amount of carotenoids, and in particular of lutein. In view of these results, it is possible to use the combined mix of these flours in order to improve the nutritional value of cereal-based gluten-free products and avoid the monotony of the celiac diet.

The second research area aims at verifying the origin of vegetable oils and fats in commercial and laboratory made bakery products. The combined use of the profile of fatty acids and tocols present in biscuits was used to demonstrate the correspondence between the profile of these indices and the vegetable oils, fats or their blends, used as ingredients. The reported results showed significant differences in the tocol and fatty acids profiles, which reflected the tocol and fatty acids composition of the specific fat/oil used as ingredient, providing the possibility to use these compounds as markers to identify

the origin of the oils/fats used. Moreover, the obtained data can provide more information on the effects of technological treatments on tocols in biscuits.

Finally, the third research area dealt with the study of different green leafy vegetables, chosen because of their wide diffusion in several traditional recipes of the Mediterranean diet. They were four wild edible plants, *Sonchus asper* L. *Hill*, *Sonchus oleraceus* L. (Asteraceae), *Crepis vesicaria* and chard (*Beta vulgaris*), and two more commercial ones, spinach (*Spinacia oleracea* L.) and chicory (*Cichorium intybus* L.). All the samples were examined for their nutritional composition and their content of bioactive compounds, such as carotenoids, tocols, polyphenol, flavonoid, hydrosoluble vitamins and antioxidant activity. Analysis of data shows that all species were found to be sources of carotenes (α-carotene, β-carotene, 9-cis-βcarotene and 13-cis-β-carotene) and xanthophylls (violaxanthin, neoxanthin, lutein, zeaxanthin and β-cryptoxanthin). Furthermore, they possessed good amounts of tocols, in particular α-tocopherol and γ-tocopherol. Therefore, the analyzed plants can be declared as a source of fiber, vitamin A and E, considering the recommended daily allowance (RDA) established by the EU Regulation. All plants showed a good amount of thiamin. Most of the green leafy vegetables are consumed in-house after removing no edible parts, washing, cutting and different thermal treatments that can cause, at different degrees, damage in color, taste and the nutritional value. Therefore, some of these different domestic cooking treatments such as boiling and steaming were compared, in order to investigate their effect on the bioactive contents, so that to define the optimal process conditions able to reduce structural damage, preserving the nutritional and the organoleptic properties of the products.

The obtained results showed that all lipophilic compounds were not affected by boiling; on the contrary, steaming slightly significantly decreased the contents of lutein and β-carotene (on average 20% and 15%, respectively). For phenolic compounds it was found that, after the boiling treatment, there was a decrease of the phenolic and flavonoid content (from 40% to 80%), due to their loss in the cooking water.

In this context, in the last activity of this present PhD research study, after a careful quantitative analysis of bioactive compounds in vegetable products, focused on the enrichment of food matrices with the analyzed vegetables. In particular, a cereal product, such as pasta, was used as a vehicle of beneficial substance from vegetables, being a staple food within human diet, consumed worldwide. Pasta was enriched with freeze-dried leaves of chicory, chard and spinach. Each case study addressed proved that

vegetable products could be used as high value food ingredients, allowing to better satisfying consumer demand for healthy food products in a more sustainable perspective.

Riassunto

Al giorno d’oggi, i consumatori di tutto il mondo sono sempre più indirizzati verso un'alimentazione sana ed equilibrata e, in questo senso, il consumo di ortaggi e prodotti a base vegetale potrebbe svolgere un ruolo fondamentale. Infatti, gli effetti benefici sulla salute umana derivanti dall'assunzione di prodotti vegetali sono ormai ampiamente riconosciuti. Molti studi epidemiologici riportano la correlazione positiva tra il consumo di prodotti vegetali e il ridotto rischio di malattie non trasmissibili, come malattie cardiovascolari, diabete di tipo 2, obesità, morbo di Alzheimer, cataratta e diverse forme di cancro. Questi effetti positivi sono il risultato dell'azione sinergica dei numerosi composti bioattivi e micronutrienti di cui i prodotti vegetali (ortaggi, frutta, erbe aromatiche, legumi, cereali o anche oli) sono caratterizzati, quali carotenoidi, tocoli, fibre alimentari, flavonoidi e altri composti fenolici, vitamine, minerali e molti altri fitonutrienti. Questa prova convincente suggerisce che un cambiamento nel comportamento alimentare, come un aumento del consumo di frutta, verdura e cereali è una strategia pratica per ridurre significativamente l'incidenza delle malattie croniche. La prevenzione è la strategia più efficace del trattamento delle malattie croniche. Il livello di composti bioattivi negli alimenti vegetali dipende da molteplici fattori: dalla famiglia botanica, dalle condizioni di crescita, dalle condizioni in post-raccolta, ma anche dalla conservazione e dalla lavorazione degli alimenti praticata.

Infatti, si deve considerare che possono verificarsi perdite di composti bioattivi nel prodotto finale durante la lavorazione, la cottura e la conservazione a causa dell'influenza di diversi fattori quali temperatura, luce, aria, pH, umidità e attività dell'acqua. Tutto ciò può alterare la struttura e indurre a cambiamenti significativi nella composizione chimica, nel valore nutritivo e nella biodisponibilità dei composti bioattivi. Lo studio di questi prodotti potrebbe aiutare a prevenire le perdite durante la lavorazione e ad identificare le migliori condizioni tecnologiche per l'ottimizzazione dei processi e la conservazione degli alimenti al fine di prevenire il contenuto e la biodisponibilità dei composti bioattivi. Alla luce di queste considerazioni, in questa tesi di dottorato, la ricerca è stata indirizzata alla valutazione qualitativa e quantitativa dei composti bioattivi presenti in diversi prodotti vegetali, sull'influenza che possono avere i trattamenti tecnologici sul loro contenuto, sulla possibile identificazione di marcatori di prodotto e di processo, ed infine sullo sviluppo di alimenti salutistici arricchiti con i composti bioattivi identificati. La ricerca si è sviluppata in tre attività principali:

1. Caratterizzazione dei cereali minori e pseudocereali senza glutine per il loro valore nutrizionale, con particolare attenzione ad alcuni composti bioattivi (carotenoidi e tocoli) e vitamine idrosolubili (tiamina e riboflavina), al fine di aumentare la consapevolezza del loro profilo nutrizionale.

2. Determinazione dei tocoli e degli acidi grassi presenti nei prodotti da forno commerciali e realizzati in laboratorio. Il profilo di tocoli e acidi grassi, unitamente all'analisi chemiometrica, è stato utilizzato per discriminare i diversi oli e grassi impiegati nei prodotti esaminati. Inoltre, sui prodotti realizzati è stato valutato l'effetto dei trattamenti tecnologici sulla quantità dei tocoli presenti.

3. Indagine sulle piante spontanee locali confrontandole con quelle commerciali, attraverso la loro caratterizzazione nutrizionale e l'analisi dei composti bioattivi. Dal momento che i vegetali oggetto di studio vengono consumati anche dopo la cottura, la valutazione nutrizionale non potrebbe essere fatta senza considerare le variazioni dei composti bioattivi dovute ai trattamenti termici domestici, quali cottura tradizionale e cottura a vapore. Per avere un quadro più completo, in quest'ultima parte, le attività di ricerca hanno portato allo sviluppo di un protocollo per la produzione di cibi tradizionali salutistici e innovativi con i vegetali studiati, considerando le loro quantità significative di composti bioattivi. Sono state valutate le caratteristiche nutrizionali e sensoriali dei prodotti realizzati.

I risultati ottenuti nella prima parte della sperimentazione, possono fornire maggiori informazioni sui composti bioattivi presenti nei cereali minori e peseudo cereali senza glutine, al fine di aumentare la consapevolezza del loro profilo nutrizionale, ad oggi molto carente in letteratura. Tutti i campioni analizzati hanno mostrato un elevato contenuto di composti bioattivi; in particolare, l'amaranto, la cañihua e la quinoa sono risultate buone fonti di vitamina E, mentre miglio, sorgo e teff sono buone fonti di tiamina. Il miglio, inoltre, fornisce una discreta quantità di carotenoidi, nello specifico di luteina. Alla luce dei dati ottenuti, è possibile utilizzare la miscela combinata di queste farine per migliorare il valore nutritivo dei prodotti senza glutine a base di cereali ed evitare così la monotonia della dieta per i celiaci.
La seconda area di ricerca trattata riguarda lo studio di un metodo, finalizzato alla verifica dell'origine di oli e grassi vegetali nei prodotti da forno commerciali e realizzati in laboratorio. Lo sviluppo del metodo ha previsto l'uso combinato del profilo in acidi grassi e tocoli presenti nei biscotti per dimostrare la corrispondenza tra il profilo di questi indici e gli ingredienti utilizzati come oli vegetali, grassi o loro miscele. I risultati ottenuti mostrano differenze

significative del profilo in acidi grassi e tocoli, che riflettono la composizione dei tocoli e degli acidi grassi del grasso specifico utilizzato come ingrediente, offrendo la possibilità di utilizzare questi composti come marker per identificare l'origine degli oli/grassi utilizzati come ingrediente. Inoltre, i dati ottenuti contribuiscono a fornire maggiori informazioni sugli effetti dei trattamenti tecnologici sui tocoli nei biscotti.

Infine, la terza area di ricerca indagata riguarda lo studio di specie diverse di vegetali a foglia verde, scelti in base alla loro ampia diffusione in diverse ricette tradizionali della dieta Mediterranea. Sono state analizzate quattro piante selvatiche commestibili, quali *Sonchus asper* L., *Sonchus oleraceus* L., *Crepis vesicaria* L., (Asteraceae), e bieta (*Beta vulgaris*), ed altre due piante commerciali, ossia spinaci (*Spinacia oleracea* L.) e cicoria (*Cichorium intybus* L.). Tutti i campioni sono stati esaminati per la loro composizione nutrizionale e il loro contenuto di composti bioattivi, quali carotenoidi, tocoli, polifenoli, flavonoidi, vitamine idrosolubili, fibra alimentare ed infine, è stata determinata l'attività antiossidante. Dall'analisi dei dati si può che tutte le specie sono risultate essere fonti di caroteni (α-carotene, β-carotene, 9-cis-β-carotene e 13-cis-β-carotene) e xantofille (violaxantina, neoxantina, luteina, zeaxantina e β-criptoxantina). Inoltre, possiedono buone quantità di tocoli, dove i maggiori composti presenti sono stati l'α-tocoferolo e il γ-tocoferolo. Per tali ragioni, le piante analizzate possono essere dichiarate come fonte di vitamina A ed E, considerando la dose giornaliera raccomandata (RDA) stabilita dal Regolamento UE. Tutte le piante hanno mostrato di possedere una buona quantità di tiamina. La maggior parte delle verdure a foglia verde, viene consumata in casa dopo aver rimosso le parti edibili, lavaggio, tagli e diversi trattamenti termici che possono causare, a diversi gradi, danni al colore, al gusto e al valore nutrizionale. Pertanto, due diversi trattamenti di cottura domestica (bollitura e vapore) sono stati confrontati, al fine di indagarne l'effetto sul contenuto dei composti bioattivi, in modo da definire le condizioni di processo ottimali in grado di ridurre i danni strutturali, preservando così le proprietà nutritive e organolettiche di partenza dei prodotti. I risultati ottenuti hanno mostrato che tutti i composti lipofili non sono influenzati dalla cottura tradizionale, al contrario, la cottura a vapore ha ridotto in modo leggermente significativo il contenuto di luteina e β-carotene (rispettivamente, in media, del 20% e del 15%). Per i composti fenolici è stato riscontrato che, dopo il trattamento di cottura tradizionale, si è verificata una diminuzione del contenuto fenolico e di flavonoidi (dal 40% all'80%), a causa della loro perdita nell'acqua di cottura. L'ultima attività del presente studio di ricerca di dottorato, dopo un'attenta analisi quantitativa dei composti bioattivi nei prodotti vegetali a foglia verde, si è conclusa con l'arricchimento di matrici alimentari con i vegetali analizzati. In particolare, prodotti a base di cereali, come la pasta, sono stati utilizzati come veicolo di

sostanze benefiche, essendo alimento base nella dieta umana consumata in tutto il mondo. La pasta è stata arricchita con liofilizzati di foglie di cicoria, bieta e spinaci. Ogni caso di studio affrontato ha dimostrato che i prodotti vegetali a foglia verde possono essere utilizzati come ingredienti alimentari ad alto valore bioattivo, consentendo di soddisfare al meglio la domanda dei consumatori di prodotti alimentari salutistici, in una prospettiva più sostenibile.

CHAPTER 1

Bioactive compounds in human health

Introduction

Mom’s conventional wisdom of *"eating fruits and vegetables to lead a healthy life”* has evolved with scientific, fact-finding research due to advances in science and of the “Foods for Health”. Vegetable products play a vital role in our diet and lives, and hence the claims for such important food commodities have exponentially increased because of the growing world population and the changes in eating habits. Different literature study suggests that vegetables products may have an important role in the maintenance of a healthy lifestyle (Joshipura et al., 1999; Arts et al., 2005). Renewed interest in the health-benefiting properties of plant foods is driven, in part, by the need for alternatives to conventional strategies of disease management, as well as the potential to prevent onset of certain diseases and reduce health care costs. This observation also led to a million dollar question: “*which components of vegetable products may be responsible for the protective effects?*”

In addition to their nutritive value, vegetable products contain a variety of phytochemicals with biological activities, which act in different ways and interact with different metabolic processes. These compounds called “bioactive compounds (BCs) or phytochemicals” are nonessential biomolecules that are present in foods and exhibit the capacity to modulate one or more metabolic processes, which results in the promotion of better health. We have several important factors influencing bioactive content (BC) in vegetable-based products, such us genetics and growing conditions (that is, fertilization, moisture, pest, and disease burden, and so on) handling, storage conditions, processing, particularly cooking. All these factors can be beneficial or detrimental to the total content of bioactive compounds in vegetable products. The intake of these compounds is strongly linked with the high consumption of fruits, vegetables, and unrefined cereals. A whole-diet approach to these food constituents is intended to render the current definition of Mediterranean diet based on food consumption more comprehensive.

The great progress in technological processes (advanced and innovative technologies), in agricultural practices (food production, transport and processing) and changes in lifestyle have focused attention on local productions and functional foods as main elements for improving food quality. In particular, the study of traditional, local and

seasonal foods becomes important: food products, grown with techniques based on a historical and cultural tradition of a specific territory and which is needed only in a specific place. In addition, functional foods are developing; these foods, compared to conventional foods, could have beneficial properties and effects on human health. Functional foods are “foods that can affect one or more target functions in the body, foods in which a bioactive component has been incorporated, or foods in which a component has been eliminated, or foods where the bioavailability of more components has been changed”.

Information on food preparation methods and the processes to which foods are subjected could help in understanding the differences in the nutritional picture in relation to cooking or the different treatments undergone. The gaps in the composition and consumption data must be filled. As for the nutritional composition, the starting point must be the formulation of "new and complete" databases, which take into account all the specific characteristics of the food and its relationship with the environment. Real data on the nutritional composition of individual foods are becoming essential for the formulation of appropriate therapeutic diets, and there is an increasing number of foods made "interesting" by their nutritional and therapeutic properties. The nutritional value of plant matrices can be assessed through the study of the macronutrient composition, but the aspect of greatest interest currently is the large number of natural compounds present in fruit and vegetables. The research activity carried out aimed at better understanding and describing the factors that influence food quality. The interest aroused by the beneficial effects of phytochemical compounds has focused the attention of researchers towards the identification of techniques and protocols for the improvement of food quality through the achievement of an optimal content of bioactive molecules, increasing and maximizing their levels in foods and consequently towards more nutritious and healthier food productions.

1.1 The world of vegetable products

From ancient times, vegetables and vegetable-based products have always had a crucial role in our diet and human life due to their valuable chemical composition. Pivotal links have been established between dietary components and human health (Schwager et al. 2008; Ares et al. 2009). In fact, growing evidence suggests that vegetable products rich diet is well established for its efficiency to promote human health for disease prevention and treatment (Tiwari & Cummins, 2013; Mozaffarian, 2016). This is more and more boosted by the increased need for natural products by the consumers, who require sustainable solutions for improvement of quality of life focused on personalized nutrition. For this reason, consumption of fruits and vegetables is considered by many organizations (World Health Organization —**WHO**, Food and Agriculture Organization — **FAO**, United States Department of Agriculture —**USDA** and European Food Safety Authority —**EFSA**) as a major public health issue and is the subject of nutritional recommendations worldwide (Su et al., 2006). Given the importance of vegetables and vegetable-based products for health, many health policies insist on increasing consumption of fruits and vegetables due to the resulting health benefits (Djuric, 2011). A high amount of plant- based food consumption, at least 400 g of vegetable products, is recommended in dietary guidelines (Agudo 2005). It is well known that they are able to reduce risk of several chronic diseases, such as obesity, diabetes, cancer, cardiovascular and neuro degenerative diseases, because they contain significant amounts of functional compounds such as carotenoids, dietary fiber, polyphenols, tocopherols, vitamins and other substances (Trichopoulou et al. 2003; Yahia, 2009). For example, dietary fiber is able to reduce cholesterol, diabetes, coronary heart disease and ease constipation. Antioxidants are other great interest compounds for food sector because they are able to inhibit or delay the oxidation reactions and furthermore these allow substituting synthetic antioxidants. Depending on plant species, variety and tissue, high levels of health protecting antioxidants, such as vitamin C and E, phenolic compounds including flavonoids, and carotenoids can be found. Phenols can serve as not only antioxidant agents but also as potential natural antimicrobial substances. Conversely, deficiency of carotenoids results in clinical signs of conjunctiva and corneal aberrations including, xerophthalmia, night blindness, keratomalacia, corneal ulceration, scarring, and resultant irreversible blindness. Other than the above, deficiency of provitamin A carotenoids leads to vision-disability in human and increased mortality due to a weakened innate immunity and adaptive immunity.

It is quite apparent from the scientific literature that the concentrations of bioactive compounds in vegetable products are certainly not constant, but widely varies within botanical families and it is also influenced by growing conditions, moisture, and other factors. In addition to the effects on human health, when ingested in the diet, bioactive compounds are believed to affect may also extend shelf life and increase stress tolerance, leading to lower postharvest losses of produce.

The list of major bioactive components of fruits and vegetables that have beneficial effects in the human body is summarized in **Table 1**.

Table 1: Bioactive compounds and health benefits of selected vegetables.

Name	Phytochemicals	Health benefit
Asparagus	Dietary fibe , vitamin, and fl vonoid	Antibiotic, antispasmodic, demulcent, diaphoretic, diuretic, laxative
Broccoli	Sulphoraphane, indoles, β-carotene, lutein, and quercetins	Antioxidant status, anticancer potential, reduce LDL oxidation
Brussels sprouts	Sulphoraphane and indoles	Protects from DNA damage, lowers risk of heart attack, anticancer potential, improves gastrointestinal tract
Cabbage	Sulphoraphane and indoles	Antioxidant, anticancer, improves digestion and skin tone
Carrots	β-Carotene	Effective in diabetes mellitus, lowers cholesterol, reduces colon cancer, improves skin tone
Caulifl wer	Sulphoraphane and indoles	Improves antioxidant capacities and detoxificatio mechanisms
Cucumber	Antioxidants, carotenoids, and vitamins	Antioxidant potential, improves the skin tone
Garlic	Sulfur-containing compound	Antioxidant potential, cardiovascular disorders, and anticancer activities
Lettuce	Quercetins and carotenoids	Antioxidant, anticancer properties, and cardiovascular disorders
Okra	Antioxidants	Prevents constipation, improves heart health
Peas	Proteins and fiber	Lowers cholesterol and improves bone health
Potato	Antioxidants	Antioxidant, effective against diabetes mellitus
Pumpkin	β-Carotene	Antioxidant, anti-inflammator perspectives, inhibits arthritis and prostate cancer, improves skin tone
Radish	Anthocyanins	Reduces weight, respiratory problems, and diabetes mellitus
Spinach	β-Carotene, lutein, and zeaxanthin	Prevents lung, colon, and breast cancer; protects from blindness and memory loss
Squash	α- and β-Carotenes, zea-xanthin	Prevents atherosclerosis, lowers the cholesterol
Sweet potato	β-Carotene	Antioxidant and anti-inflammator properties, lowers cholesterol and LDL, effective in diabetes mellitus
Tomato	Lycopene	Potent antioxidants, lowers cholesterol/glucose, anticancer potential
Turnips	Indoles and sulphoraphane	Cardiovascular disorders, high blood glucose

Source: Butt et al. (2009), Gorbach and Goldin (1992), Howard and Kritchevsky (1999), and Hsing et al. (2002).

1.2 Bioactive compounds of vegetable origin

Bioactive compounds are defined as non-nutritional substances that are found at very low concentrations in foods, having some biological activity. They determine the color of vegetables, protect plants against herbivores and microorganisms, attract pollinators and seed dispersing animals, and act as signal molecules under stress conditions (Crozier et al. 2006). As the name suggests (Greek ‘bios’ means life and Latin ‘activus’ means

dynamic or full of energy), a bioactive compound (or substance) has its direct physiological or cellular effects on a living organism. Such effects may be positive or negative depending on the nature of the substance, its dose, and its bioavailability. The bioactive compound biosynthesis is encouraged by light; hence, they assemble in the skin and leaves of the fruits and vegetables (Bernhoft 2010). They are found in very low concentrations in foods and that intervene in the secondary metabolism of vegetables and may have a significant effect on human health in addition to the basic nutritional value of the product they provide. These compounds vary widely in chemical structure and function. Some examples of bioactive compounds are carotenoids, flavonoids, tocols, polyphenols and dietary fiber. Since vitamins and minerals elicit pharmacological effects, they can be categorized as bioactive compounds as well. Although bioactive compounds are naturally present in various foods, such as grains, legumes, vegetables and fruits they are can also be added to foods or food products for the enhancement of their health promoting properties, but also as an additives to give color and a processing aid as well. The content of selected bioactive compounds in vegetables is presented in **Table 2**.

The commonly used methods for their extraction are the conventional liquid–liquid or solid–liquid extraction.

Table 2. Content of bioactive compounds in vegetable products.

Compounds	Vegetables	Concentrations	References
Carotenoids			
α-Carotene	Broccoli	1 mg/100 g FW	USDA (2005)
	Carrot	4.6	
	Pea	19	
	Sweet pepper	59	
	Tomato	112	
β-Carotene	Broccoli	779 mg/100 g FW	
	Brussels sprout	450	
	Carrot	8.8	
	Pea	485	
	Tomato	393	
β-Cryptoxanthin	Sweet pepper	2.205 mg/100 g FW	
Zeaxanthin	Carrot	23 mg/100 g FW	
	Celery	3	
	Kale	173	
	Lettuce	187	
	Spinach	331	
Lycopene	Tomato	3.025 mg/100 g FW	
Quinones			

Phylloquinone	Broccoli	102 µg/100 g FW	Damon et al. (2005)
	Carrot	8.3	
	Celery	29	
	Cucumber	16.4	
	Lettuce	24.1–127	
	Onion	0.2	
	Sweet pepper	4.9–21.4	
Tocopherols and tocotrienols			
α-Tocopherol	Broccoli	1.44 mg/100 g FW	Chun et al. (2006)
	Cabbage	0.21	
	Carrot	0.86	
	Celery	0.26	
	Onion	0.04	
	Spinach	1.96	
	Tomato	0.53	
β-Tocopherol	Carrot	0.01 mg/100 g FW	
	Cucumber	0.01	
	Lettuce	0.01	
β-Tocopherol	Broccoli	0.31 mg/100 g FW	
	Cauliflo wer	0.20	
	Lettuce	0.11–0.74	
	Spinach	0.21	
	Tomato	0.14	
α-T3	Cabbage	0.04 mg/100 g FW	
	Cauliflo wer	0.06	
	Onion	0.12	
γ-T3	Corn	0.21 mg/100 g FW	
	Pea	0.05	
Flavonoids			
Quercetin	Broccoli	3.12 mg/100 g FW	USDA (2005)
	Cabbage	0.01	
	Endive	7.71	
	Lettuce	1.95	
	Onion	13.27	
	Tomato	0.57	
Apigenin	Cabbage	0.01 mg/100 g FW	USDA (2005)
	Celery	4.61	
	Lettuce	0.01	
Luteolin	Cauliflower	0.08 mg/100 g FW	USDA (2005)
	Celery	1.31	
	Spinach	1.11	
	Sweet pepper	0.63	
Myricetin	Lettuce	0.02 mg/100 g FW	USDA (2005)
	Spinach	0.01	
Cyanidin	Green lettuce	0.3 mg/100 g FW	Harnly et al. (2006)
	Red lettuce	13.7	
Lignans			
Lariciresinol	Broccoli	972 mg/100 g FW	
	Cauliflo wer	124	
	Kale	599	
	Lettuce	5	
	Onion	19	
	Sweet pepper	164	
Pinoresinol	Broccoli	315 mg/100 g FW	Milder et al. (2005)
	Cabbage	568	
	Kale	1,691	

	Endive	9	
	Leek	3	
	Sweet pepper	1	
Secoisolariciresinol	Broccoli	38 mg/100 g FW	
	Brussels sprout	34	
	Leek	38	
	Carrot	93	
	Lettuce	8	
	Tomato	2	
Matairesinol	Kale	12 mg/100 g FW	
Phenolic acids			
Chlorogenic acid	Bean	0.29 mg/100 g FW	Mattila and Hellstro¨m (2007)
	Carrot	10	
	Cauliflower	0.14	
	Lettuce	0.42–23	
	Soya bean	2.0	
	Tomato	0.86	
Caffeic acid	Carrot	0.1 mg/100 g FW	
Ferulic acid	Turnip	0.42 mg/100 g FW	
p-Coumaric acid	Cabbage	0.21 mg/100 g FW	
	Cauliflower	0.31	
Vanillic acid	Soya bean	0.5 mg/100 g FW	
Sinapic acid	Cauliflower	0.15 mg/100 g FW	
	Turnip	1.4	
Protocatechuic acid	Bean	0.26 mg/100 g FW	
	Carrot	0.46	
Tannins			
Proanthocyanidin monomers	Avocado	0.96 mg/100 g FW	USDA (2005)
	Kidney bean	16.25	
	Lentil	0.53	
	Pea	0.02	
	Squash	1.63	
Proanthocyanidin dimers	Avocado	1.46 mg/100 g FW	
	Kidney bean	22.90	
	Lentil	1.20	
	Squash	1.98	
Proanthocyanidin trimers	Avocado	1.36 mg/100 g FW	
	Kidney bean	23.60	
	Lentil	0.11	
	Squash	1.49	
Proanthocyanidin polymers	Kidney bean	258 mg/100 g FW	
	Squash	3.50	
Alkaloids			
α-Tomatine	Tomato	521–795 μg/g FW	Kozukue et al. (2004)
Dehydrotomatine	Tomato	41.6–68.0 μg/g FW	
α-Solanine	Potato	0.01–0.43 mg/kg FW	Sengul et al. (2004)
α-Chaconine	Potato	0.7–1.93 mg/kg FW	
Lactucin	Chicory	178–245 mg/kg FW	Peters et al. (1997)
Lactucopicrin	Chicory	112–143 mg/kg FW	
Vitamin B complex			
Thiamine	Broccoli	0.15 mg/100 g FW	FAO (1981)
	Cucumber	0.04	
	Garlic	0.32	
	Leek	1.46	
	Spinach	0.16	

Riboflavin	Brussels sprout	0.12 mg/100 g FW	FAO (1981)
	Lettuce	0.15	
	Onion	0.17	
	Spinach	0.19	
	Tomato	0.05	
Total dietary fiber	Cabbage	23.24 g/100 g DW	Anderson & Bridges (1988)
	Carrot	23.76	
	Lettuce	21.02	
	Potato	9.48	
	Tomato	13.13	

FW, fresh weight; FAO, Food and Agriculture Organization of the United Nations Database; USDA, United States Department of Agriculture National Nutrient Database.

1.2.1 Carotenoids in vegetable products

Carotenoids are biosynthesized by photosynthetic organisms (cyanobacteria, algae, plants) as well as by some fungi and bacteria; which are responsible for the yellow, orange, and red colors in various fruits and vegetables (Namitha & Negi, 2010). More than 600 carotenoids have so far been identified in nature. However, in a typical human diet, only about 40 are present. Carotenoids can be classified into two groups based on their function:

- *xanthophylls*, including violaxanthin, neoxanthin, lutein, zeaxanthin and β-cryptoxanthin;
- *carotenes*, such as α-carotene, β-carotene, 9-cis-β-carotene and 13-cis-β-carotene and lycopene.

They cannot be synthesized *in vivo* by humans or animals and are consumed only through the diet. Carotenoids present in human diets typically contain C40 skeleton (tetraterpenoids), although there are some examples with a lower carbon number, such as apocarotenoids. Carotenoids in plants can be found in free form or esterified to fatty acids; although esterification does not alter the chromophore properties of the carotenoid, it modifies the chemical and biological properties by changing its immediate environment (Pérez-Gálvez et al., 2005). These properties also depend on the kind of fatty acid bound to the carotenoid molecule. Esterification facilitates carotenoid storage, aidiing the integration of these highly lipophilic molecules within lipid rich plasto globules. Esterification is the natural biological mechanism to protect triacylglycerols, unsaturated lipids, and other light sensitive compounds from photooxidation. They are insoluble in water, but dissolve very well in non-polar or slightly polar solvents (Kączkowski, 2009). Determination of the carotenoid contents in foods has been the main objective of many studies, and the resulting data have been compiled in databases

and other food carotenoid compilations. There is an increasing interest in searching new natural sources of carotenoids (e.g., underutilized wild fruits and vegetables), as well as in the selection, breeding, and enhancement of traditional cultivars of well-known staple food (potato, maize, wheat, etc.) (Brown 2008; Murillo et al., 2010). The majority of dietary carotenoids consumed by humans are obtained from plant-derived foods; in fact, vegetables and based-vegetable products are considered the most important sources for carotenoids in the human diet (Britton et al., 2009). Content and types of carotenoids in plants depend on several pre- and pro-harvesting factors, genotype, ripening time, cultivation method and climatic conditions, and processing. The availability of carotenoids in fruits and vegetable can be predicted by their color, such as yellow-orange vegetables and fruits are generally rich in β-Carotene and the α-Carotene. Alfa-Cryptoxanthin and zeinoxanthin can be found in orange fruits, such as mandarin, orange, and papaya. Similarly, lycopene pigment (responsible for bright red color) is the major constituent of tomatoes and tomato products. Lutein (nearly 45%) and β-carotene (25–30%) followed by violaxanthin (10–15%), and neoxanthin (10–15%) are the predominant forms of carotenoids in green leafy vegetables (Lakshminarayana et al., 2005), although the absolute concentration of each carotenoid varies considerably among different vegetables. Different parts of the same plant may also contain different types and amount of carotenoids. For instance, peel of the fruits is generally richer in carotenoids compared to pulp. α-Carotene, β-Cryptoxanthin, zeaxanthin, antheraxanthin, and lutein 5,6-epoxide (luteoxanthin) are also recorded in green leafy vegetables in minor concentrations. In most of the fruits and vegetables, β-Carotene is generally dominating compared to its geometric isomer α-carotene. Significant high contents of α-carotene can be found in a limited number of fruits and vegetables, such as sweet potato, carrots, pumpkin, and dark green vegetables, such as green beans, spinach and broccoli (Khoo et al., 2011). Knowledge on carotenoid composition in different edible parts and cultivars will be useful to nutritional experts for the selection of nutrient-rich plants for food fortification and proper diet recommendation. Carotenoids play an important role in diet due to vitamin A activity (Haskell, 2013). Carotenoids with pro-vitamin A activity are essential components of the human diet. Vitamin A is involved in hormone synthesis, regulation of cell growth and differentiation, and immune responses (Combs, 1998). Lack of carotenoids in the human diet can lead to xerophthalmia (night blindness) and fetal death. Carotenoid-rich diets are correlated with a significant reduction in the risk for certain forms of cancer,

coronary heart disease, and some degenerative diseases, such as cataract (Johnson, 2002). Apart from this, carotenoids are also important for antioxidant activity, intercellular communication and immunosystem activity. The general procedure for the determination of carotenoids in different matrices can be divided into the following steps: sample preparation, extraction and saponification followed by separation, identification and quantification of the carotenoids. Among other factors, carotenoids are very sensitive to heat, light, oxygen, and acids resulting in some degree of degradation and/or isomerization. Consequently, precaution must be taken throughout the analysis to minimize the possible loss of carotenoids and thereby achieve reliable data.

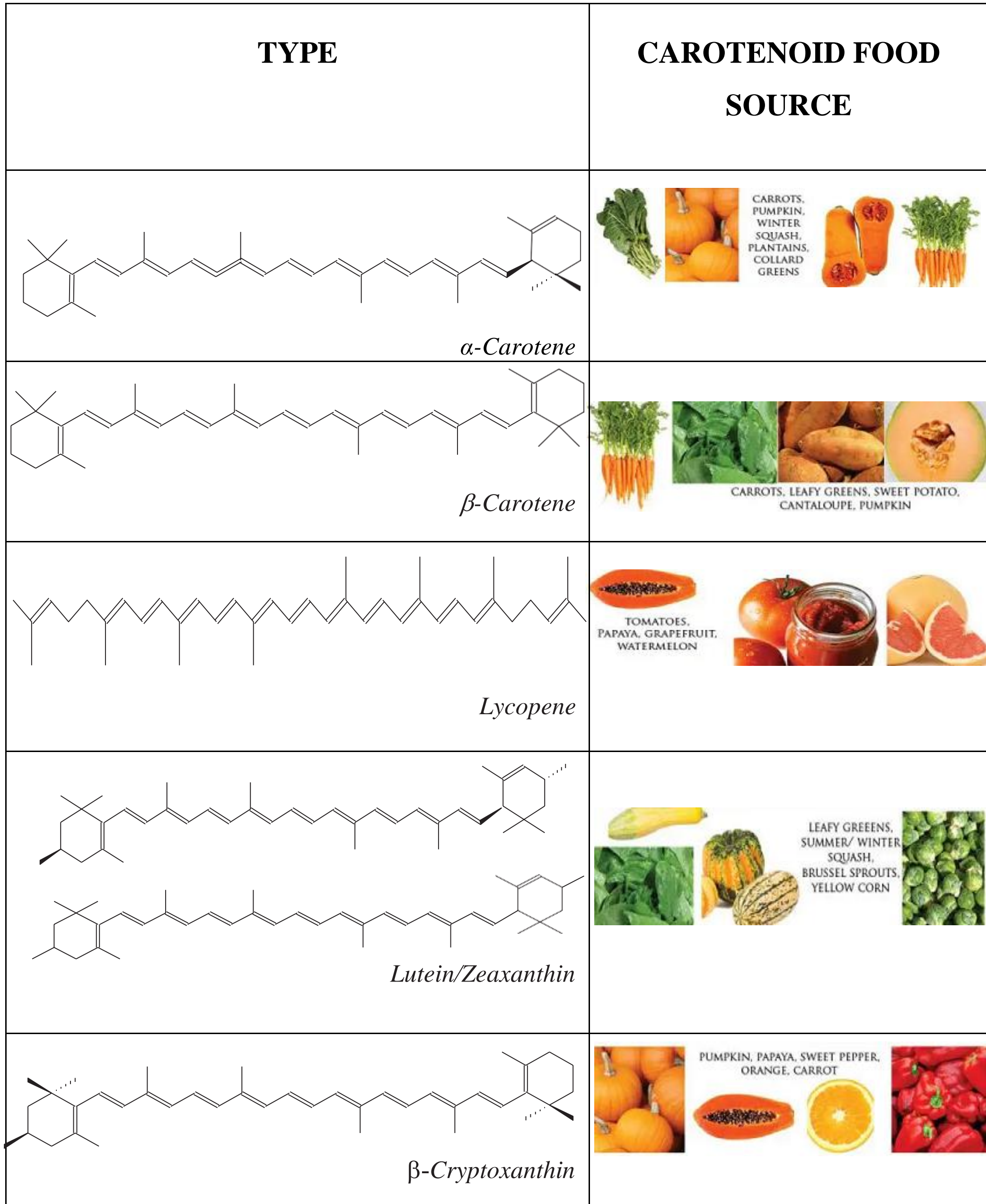


Figure 1. Chemical structure of selected carotenoids and their main dietary sources.
Source: **USDA**, United States Department of Agriculture National Nutrient Database.

Vitamin A activity

Vitamin A is a fat soluble vitamin obtained from the diet as retinol and retinyl in animal foods or as carotenoids in plant foods (a limited number have provitamin A activity such as β-carotene, α-carotene and β-cryptoxanthin).

Although carotenes have been acknowledged as beneficial for human health, they are sadly not defined as essential nutrients as such, which is why they are not assigned a value for daily reference intakes (Rao et al., 2007). Vitamin A is an essential nutrient, as

it is a bioactive compound involved in the visual cycle of the retina, in the continued growth and integrity of the cells in body tissues. Unfortunately, the human body is unable to synthesize compounds with vitamin A activity. The biological value of substances with vitamin A activity is expressed as retinol equivalent (RE) (EFSA, 2015). In the assessment of this value reference is made to the conversion factors suggested by EFSA, namely 1 µg RE corresponds to 1 µg retinol, 6 µg β-carotene and 12 µg other provitamin A activity carotenoids, such as α-carotene, β–cryptoxanthin and cis isomers of β-carotene with lower vitamin activity (EFSA, 2015). The demand for Vitamin A can be satisfied with any mixture supplying a quantity of vitamin A equivalent to the reference value expressed in µg RE per day, corresponding to 800 µg according to EU Reg n. 1169/2011.

1.2.2 Vitamin E in vegetable products

Vitamin E comprises tocopherols (α-, β-, γ- and δ–T) and tocotrienols (α-, β-, γ- and δ–T3), a group of vitamers known collectively as "tocols." Their structure is characterized by a chromanol ring with a 13-carbon chain at the C2 position. These lipophilic compounds occur naturally in plants as four homologues of α, β, γ, and δ according to the number and position of methyl groups attached to the chromanol ring. Tocopherols have a saturated side chain and three chiral carbons resulting in eight stereoisomers, whereas tocotrienols have an unsaturated side chain containing three double bonds and one chiral carbon forming two stereoisomers. **Figure 2** illustrates the chemical structures of tocols. One feature worth considering concerns tocopherols, which are always present in plants, while tocotrienols appear only in seeds and fruits (Falk et al., 2010). In detail, are found in asparagus, broccoli, Brussels sprout, cabbage, carrot, cauliflower, kale, lettuce, spinach, sweet potato, tomato, and turnip (Eitenmiller et al., 2004). Moreover, in foods, the main sources of vitamin E are also cereals, meat and by-products. In plants, tocopherols protect chloroplast membranes from photooxidation to provide an optimal environment for the photosynthetic machinery (Munne´-Bosch et al., 2002). In humans, vitamin E is present in all cell membranes and plasma lipoproteins (especially in red blood cells). As the major lipid soluble chainbreaking antioxidants in humans, tocopherols and tocotrienols protect DNA, low-density lipoproteins, and polyunsaturated fatty acids from free radical-induced

oxidation. They also play a role in stabilizing the structure of membranes, haemoglobin biosynthesis, and modulation of immune response (Brigelius-Flohe et al., 1999).

In the past, the scientific world focused mainly on α-tocopherol, considered as being the constituent with the highest vitamin activity (100%), but recent studies have shown that other homologues carry out important functions for human health. In fact, a mixture of α-, γ- and δ-tocopherol has proved to have a higher antioxidant and anti-inflammatory power on biological systems than α-tocopherol alone.

Moreover, tocotrienols, which are plentiful in vegetable oils (eg. Palm oil), despite a lower vitamin activity than α-tocopherol, have a significantly higher antioxidant capacity compared to tocopherols. Tocotrienols show further promising healthy effects, compared to tocopherols, such as lowering plasma cholesterol levels and triggering immune system response. Several *in vitro* studies point out that tocotrienols feature anticancer, cardioprotective and neuroprotective effects (Aggarwal et al., 2010).

Consequently, an adequate supply of all eight homologues of vitamin E can contribute to counteract oxidative stress within cell membranes (D'Evoli et al., 2013).

In the past, the biological activity of Vitamin E was defined in terms of International Units (IU), while nowadays it is defined in terms of Tocopherol Equivalent (TE), where 1 TE corresponds to 1 mg of α-tocopherol, the biologically most active form of vitamin E. The different tocols feature a wide variety of bioactivity, which lessens dramatically as follows: $\alpha\text{-T} > \beta\text{-T} > \alpha\text{-T}_3 > \gamma\text{-T} > \beta\text{-T}_3 > \delta\text{-T}$ (Panfili et al., 2008).

According to EU Regulation n° 1169/2011 dealing with the nutritional labelling of food products, the recommended daily allowance (RDA) of vitamin E is 12 mg.

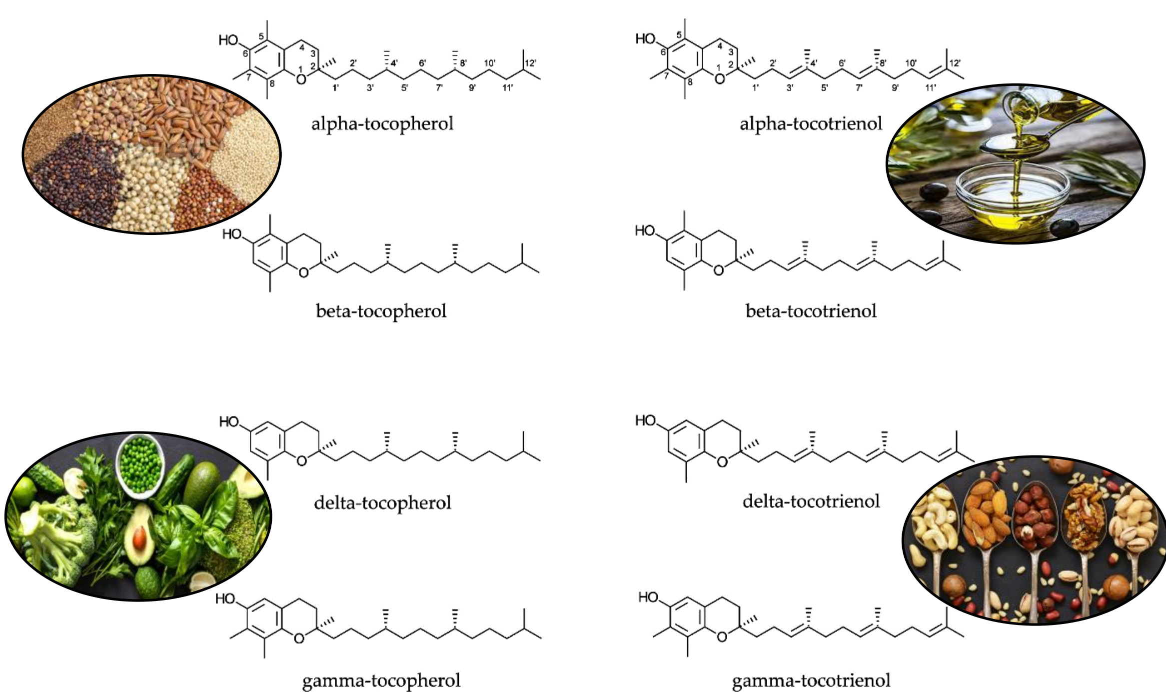


Figure 2. Chemical structures of tocopherols and tocotrienols and the main dietary sources of vitamin E (edible oils, nuts, seeds, spinach and cereals).

1.2.3 Phenolic compounds

Phenolic compounds are one of the main class of plant secondary metabolites and among the most abundant natural antioxidants in the diet, which are important determinants in the sensory and nutritional quality of fruits, vegetables and other plants. Phenolic potential of fruits depends on many external factors (climatic conditions, technical itineraries, origins) and internal factors (physiological state of the fruit, position of the fruit on the tree, genotype) (Dragovic-Uzelac et al., 2007). Plant phenolic compounds include around 8,000 metabolites (Goldberg 2003), in according to their structure, in particular based on the number of carbon atoms in their skeleton, polyphenols can be classified into 5 major families: phenolic acids (C6–C1), flavonoids (C6–C3–C6), lignans ([C6–C3]n), stilbenes (C6–C2–C6) and curcuminoids (C6-C7-C6), represented in **Figure 3** (Panickar et al., 2011). Phenolic compounds occur most widely in plants as simple phenolics, phenolic acids, flavonoids, coumarins, stilbenes, tannins, lignans, and lignins. Phenolics may act as phytoalexins, anti feedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and

protective agents against UV light, amongst others (Naczk, et al., 2006). These bioactive properties made these compounds to play an important role in plant growth and reproduction, providing an efficient protection against pathogens and predators, besides contributing to the colour and sensory characteristics of fruits and vegetables (Alasalvar et al., 2001). In particular, natural phenols have been reported to have excellent properties as food preservatives as well as having an important role in the protection against a number of pathological disturbances, such as atherosclerosis, brain dysfunction and cancer. Moreover, polyphenols have many industrial applications, for example, they may be used as natural colourants and preservatives for foods, or in the production of paints, paper, and cosmetic. For these reasons, great effort has been made to characterize the phenols occurring in different plant tissues (Pinelo et al., 2005). The main food sources of polyphenols are fruits, vegetables, derived beverages (wine, tea, coffee, and fruit juices), cereals, oilseeds and pulses. Fruits and vegetables contribute to about one-half of the total nutritional intake of polyphenols, the other half being provided by derived beverages (Brat et al., 2006). In most cases, foods contain complex mixtures of polyphenols. Besides beneficial properties for human health, phytochemicals are responsible for color, flavor and odor (Miglio et al., 2008). Their content is influenced by crop type, variety, environmental conditions, location, germination, maturity, processing and storage (Björkman et al., 2011).

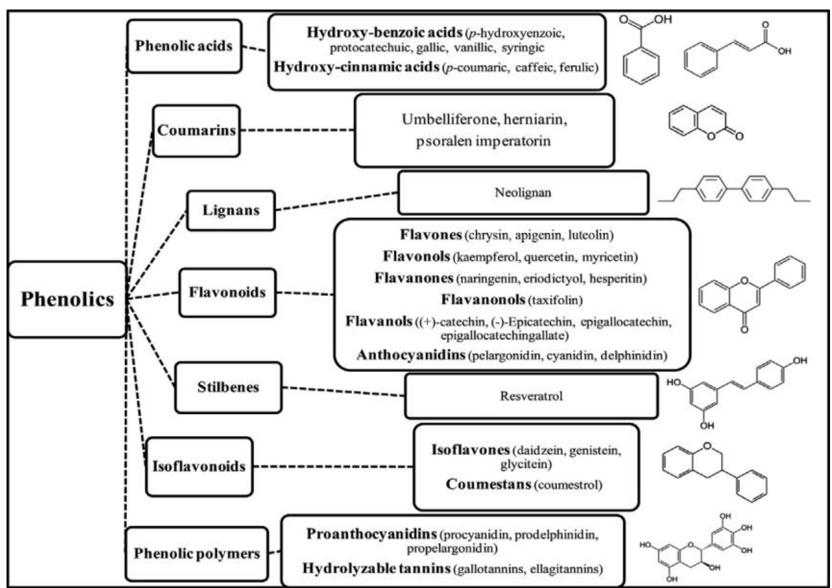
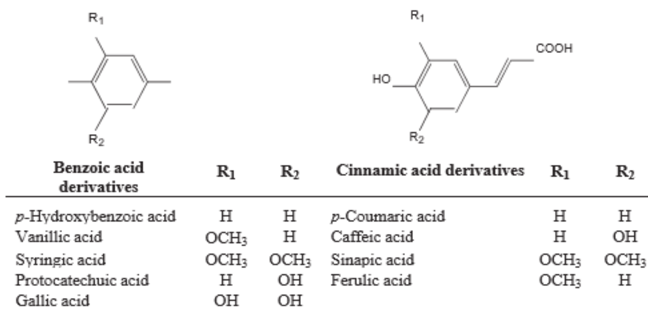


Figure 3: Classification and structure of polyphenols.

Phenolic acids are chemical compounds with at least one aromatic ring bearing one or more hydroxyl groups. These compounds include phenolic acids (e.g., hydroxybenzoic, gallic, and vanillic), hydroxycinnamic acids (e.g., ferulic, coumaric, and caffeic), and phenylacetic acids (e.g., phenylacetic and hydroxyphenylacetic) **Figure 4.** Chlorogenic acid has been found in bean, carrot, cauliflower, and lettuce; coumaric in cabbage and cauliflower; protocatechuic in bean and carrot; and sinapic in cauliflower and turnip (Mattila et al., 2007). In plants, these compounds fulfil antipathogen, antiherbivore, and allelopathic roles (Nicholson et al., 1992). Salicylic acid plays an important role in cell signalling under stress conditions (Klessig et al., 1994). Dietary phenolic acids, such as benzoic, hydroxybenzoic, vanillic, and caffeic, were reported to have antimicrobial and antifungal action, probably due to enzyme inhibition by the oxidized compounds (Cowan, 1999). Hydroxycinnamic acid derivatives, such as caffeic, chlorogenic, sinapic, ferulic, and p-coumaric acid, possess strong antioxidant activity due to the inhibition of lipid oxidation and scavenging reactive oxygen species (Cheng et al., 2007). Some phenolics such as syringic acid may contribute to the bitter and astringent taste of vegetables (Drewnowski et al., 2000).



Benzoic acid derivatives	R_1	R_2	Cinnamic acid derivatives	R_1	R_2
p-Hydroxybenzoic acid	H	H	*p*-Coumaric acid	H	H
Vanillic acid	OCH_3	H	Caffeic acid	H	OH
Syringic acid	OCH_3	OCH_3	Sinapic acid	OCH_3	OCH_3
Protocatechuic acid	H	OH	Ferulic acid	OCH_3	H
Gallic acid	OH	OH			

Figure 4: Structures of phenolic acids.

Flavonoids represent the most numerous (4,000 compounds) group of plant phenols (Harborne, 1993). Flavonoids are classified as:

- flavones, e.g., apigenin, luteolin, and chrysoeriol;
- flavonols, e.g., quercetin, kaempferol, and isorhamnetin;
- flavanones, e.g., naringenin and hesperetin;
- catechins, e.g., catechin and epigallocatechin;
- anthocyanidins, as pelargonidin, cyanidin, and malvidin;
- isoflavones, e.g., genistein and daidzein;
- chalcones, as butein and phloretin.

Flavonol quercetin, and isorhamnetin have been found in bean, broccoli, endive, leek, onion, and tomato; flavones apigenin and luteolin in bean, red peppers, parsley, and thyme; thocyanidins in onion, radish, red cabbage, and red lettuce; and isoflavones in soy (Horbowicz et al. 2008). Most of the flavonoids present in plants are attached to sugars (glycosides) (Ross et al., 2002). Many flavonoids, such as anthocyanidins, chalcones, and flavones, are plant pigments, which determine the color of vegetables. Dietary flavonoids possess antiviral, antinflammatory, antihistamine, and antioxidant properties. They have been reported to inhibit lipid peroxidation, to scavenge free radicals, to chelate iron and copper ions (which can catalyze production of free radicals), and to modulate cell signalling pathways (Rice-Evans et al., 2003). Flavonoids protect low-density lipoprotein cholesterol from being oxidized, preventing the formation of atherosclerotic plaques in the arterial wall. They stimulate enzymes involved in detoxification of cancerogenic substances and inhibit inflammations associated with local production of free radicals (Hollman et al., 1999). Most flavonoids have a bitter or astringent taste, or a bitter taste with sweet aftertaste (Drewnowski et al., 2000).

1.2.4 Dietary fiber

Dietary fibre is not a simple and well defined chemical compound, but a combination of chemical substances in composition and structure, such as cellulose, hemicelluloses, and lignin, being defined as *"edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine"* (Mongeau and Brooks 2003). Thus, dietary fibre comprises food material, particularly plant material that is not hydrolysed by enzymes secreted by the human digestive tract but that may be digested by microflora in the gut. Plant components that fall within this definition include non-starch polysaccharides (NSP) such as celluloses, some hemicelluloses, gums and pectins, as well as lignin, resistant dextrins and resistant starches (IFST 2007).

The amount of dietary fiber varies due to the type of vegetables, maturity, growing conditions, etc. The composition of dietary fiber determines the physiological effect of a specific vegetable (Marlett et al. 2002). The components of dietary fiber are water-soluble and insoluble fractions. Dietary fiber can be partly digestible or completely indigestible by the bacteria in the colon. The components that are partly digestible include pectin, hemicelluloses, and cellulose; however, lignin is not digestible by the

bacterial enzymes. Lignin also lowers the digestibility of the other fiber components. Dietary fiber has the potential to lower blood cholesterol level, thus reducing the risk of cardiovascular disorders; it is also effective in gastrointestinal problems and weight management. In consequence, vegetables are effective on physiological parameters such as satiety, gastrointestinal tract physiology, metabolic parameters (post-prandial lipemic response, long term basal lipemia) and microorganism local population through prebiotic effects (Eswaran et al., 2013). Vegetables, especially green leafy types, contain dietary fibers. artichoke, sweet potato. Brussels sprouts and turnip are among the rich sources. Generally, dietary fiber concentrates in the peel portion; therefore, many researchers recommend consuming vegetables with their edible peel or skin portion. In addition, consumption of vegetables products helps covering fiber requirements with contained energy intake when compared to refined foods.

1.2.5 Hydrosoluble vitamins

The vitamin B complex of vegetables is a group of eight compounds all acting as enzyme cofactors essential in metabolism. Includes the following hydrosoluble vitamins: *thiamin* (vitamin B1) and *riboflavin* (vitamin B3), and others not examined in this Doctoral thesis (B3, B5, B6, B8, B9 and B12). Apart from vitamin B 12, plants can synthesize all group B vitamins (Fitzpatrick et al., 2012). The chemical structure of B vitamins is shown in **Figure 5**. In plants, these compounds are vital cofactors for enzymes involved in photosynthesis (riboflavin), respiration (thiamine, riboflavin), synthesis of organic and amino acids (thiamine). Vitamin B compounds are produced by different metabolic pathways, including the pentose phosphate pathway, glycolysis, and amino acid metabolism (Roje, 2007). In humans, B vitamins are involved in tissue respiration and carbohydrate, fatty acid, and amino acid metabolism. Deficiency of vitamin B1 can cause polyneuritis; deficiency of vitamin B2 can lead to cheilosis, angular stomatitis, and dermatitis (Combs, 1998). Green leafy vegetables, such as asparagus, Brussels sprout, cauliflower, lettuce, spinach, and turnip, are good sources of B vitamins (USDA 2005). Vitamins B1, B2 have been found in cabbage, carrot, cauliflower, lettuce, potato, spinach, and tomato (Hanif et al. 2006);

Thiamine

Riboflavin

Figure 5. Chemical structure of B1 and B2 vitamins.

Thiamine (B1): it acts as a coenzyme in energy-yielding reactions from carbohydrate, fat, and protein. It is the key component of coenzyme thiamin pyrophosphate (TPP) that participates in decarboxylation of glucose, essential for nucleic acids synthesis. Is made of a pyrimidine ring and a thiazole ring linked together by a methylene bridge (EFSA, 2016). Thiamine is widespread both in vegetable foods (as free thiamine) and in animal foods (in its phosphorylated form). Food processing (alkaline pH, high temperatures, sulphite exposure) entails a significant loss of thiamine (Damodaran et al., 2007).
According to UE Regulation n°1169/2011 about the nutritional labelling of food products, the recommended daily allowance (RDA) for vitamin B1 is 11 mg daily.

Riboflavin (B2): is part of the functional group of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors.
A large number of enzymes require these flavin cofactors, including those involved in electron transport at mitochondrial level, in photosynthesis and in fatty acid oxidation. Man cannot synthesise it but can produce FMN and FAD from riboflavin. Closely associated with thiamin, riboflavin is lacking when thiamin deficiency prevails and vice versa; however, its deficiency symptoms are not obvious as thiamin. Leafy vegetables are a rich source of vitamin B2. In fact, in vegetables, the leafy parts contain plenty of riboflavin, which decreases as the vegetable ripens, whereas cereals, for example contain little riboflavin (National Academy of Sciences, 1989). For example broccoli, turnip greens, asparagus and spinach are a good source of B2. Recommended intake levels naturally vary depending on age, sex and physiological condition. In this respect, according to UE Regulation n°1169/2011 about the nutritional labelling of food products, the recommended daily allowance (RDA) for vitamin B1 is 1.4 mg/daily.

1.3 Antioxidants

Vegetable products are a rich source of dietary antioxidants, which are defined as “food compounds that significantl decrease the adverse effects of reactive oxygen species,

reactive nitrogen species, or both, on the normal physiological function in humans" (SEDRI 1998). Reactive oxygen species (e.g., oxygen ions, free radicals, and peroxides) and reactive nitrogen species (e.g., nitrous anhydride, peroxynitrite, and nitrogen dioxide radical) cause oxidation, nitration, halogenation, and deamination of biomolecules of all types, including lipids, proteins, carbohydrates, and nucleic acids, with the formation of toxic and mutagenic products (Castro et al., 2001). Antioxidants delay the start or slow the rate of free radical formation. These compounds are characterized by the ability to donate the hydrogen atom (or electron/proton), or to chelate metal ions involved in formation of reactive oxygen species. In most cases antioxidant action involves a combination of different mechanisms; therefore, antioxidant properties cannot be attributed to a certain class of chemical compounds or to certain functional groups in these compounds. Antioxidants have been found among many classes of primary and secondary plant metabolites, such as amino acids, amines and polyamines, organic acids, terpenoids, phenolics, alkaloids, and organosulfur compounds. A number of studies have demonstrated synergistic interactions among different antioxidant compounds. It has been shown that mixtures of antioxidants have higher antioxidant activity than the sum of individual compounds. Mixtures of carotenoids were more effective against oxidative damage compared to single carotenoids (Stahl et al., 1998). Synergistic interactions have been demonstrated between phenolic acid, β-carotene, and ascorbic acid, as well as between flavonoids and tocopherols (Marinova et al., 2008).

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CHAPTER 2

Functional products

Introduction

Nowadays, society's attitude to food, as a natural and inevitable necessity, has altered in line with changes in social conditions and development of technology. Current consumers are interested in the composition, properties, safety, and health effects of food products. The desire to consume foods with high biological value from natural origins poses a huge challenge for modern food science and industry. Thus, in the response to the market needs industry and researchers are involved in optimizing production technology to improve the quality, taste, functionality and bioavailability of food matrices. Recently, there has been a global trend in developing novel products supplemented with natural antioxidants, which are derived from vegetable world (Hao and Beta, 2012). Obviously, the incorporation of vegetable material into food eaten daily is a great challenge because it improves the nutritional composition, increasing antioxidant activity level, the phenol compounds amount, etc. and on the other hand it affects the technological and the sensory properties. It followed that, in the last years, a new category of "enhanced" foods, known as "functional foods", with healthy-pharmaceutical characteristics, have appeared on supermarket shelves and health food stores (Karelakis et al., 2019). These foods, besides satisfying hunger and providing nutrients that are necessary for the organism, also prevent nutrition related diseases and improve the body on both a physical and mental level (Menrad, 2003; Roberfroid, 2000).

Therefore, functional foods are regarded as innovative and promising products, which can provide additional health benefits beyond the basic nutrition. Although the functional foods have no formal definition, some groups define the primary category of functional foods as modified foods that claim to have been fortified with nutrients or enhanced with phytochemicals or botanicals to provide specific health benefits, when they are consumed at efficacious levels as part of a varied diet on a regular basis (Hao and Beta, 2012; Hasler, 2002). In this way, is it possible to satisfy consumer demands for food products with functional properties.

2.1 Functional foods

Functional food cannot be a single well-defined/well-characterised entity. Indeed, a wide variety of food products are or will, in the future, be characterised as functional food with a variety of components, some of them classified as nutrients, affecting a variety of body functions relevant to either a state of well-being and health and/or to the reduction in risk of a disease. There are several definitions for "functional foods"; however, a universally accepted definition is missing, functional food has thus to be understood as a concept, and the unique features that must be respected are:

- to be beneficial for human health beyond its basic nutritional value, enhancing wellbeing or reducing the risk of disease;
- its effects must be recognized/ensured by the scientific community;
- being a food or food-ingredient which is conventional or consumed as part of the normal/usual diet;
- the food should be natural and not synthetic, it is not a pill, drug or dietary supplement.

The term functional foods was first coined in Japan in 1984 (Kubomara, 1998); according to the Japanese Ministry of Health and Welfare, FOSHU (Food for Specified Health Uses) they are:

- foods that are expected to have a specific health effect due to relevant constituents, or foods from which allergens have been removed;
- foods where the effect of such an addition or removal has been scientifically evaluated, and permission has been granted to make claims regarding the specific beneficial effects on health expected from their consumption.

In that context, the European Commission's Concerted Action on FuFoSE, coordinated by the International Life Science Institute (ILSI) Europe described functional foods as follows:

"*A food can be regarded as 'functional' if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease.Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern"* (Diplock et al., 1999).

The main aspects of this working definition are:

- the food nature of functional food that is not a pill, a capsule or any form of dietary supplement;

- the demonstration of the effects to the satisfaction of the scientific community;
- the beneficial effects on body functions, beyond adequate nutritional effects, that are relevant to improved state of health and well-being and/or reduction of risk (not prevention) of disease;
- the consumption as part of a normal food pattern.

The definition encompasses all main features of functional foods identified above; it is aimed at stimulating research and development in the field of nutrition so as to contribute adequately to the scientific knowledge that will be required to define optimum (optimised) nutrition by elaborating new dietary guidelines. However, it should be emphasised that a functional food will not necessarily be functional for all members of the population, and that matching individual biochemical needs with selected food component intakes may become a key task as we progress in our understanding of the interactions between genes and diet (Milner, 2000).

From a practical point of view, a functional food can be:

- a natural food;
- a food to which a component has been added;
- a food from which a component has been removed;
- a food where the nature of one or more components has been modified;
- a food in which the bioavailability of one or more components has been modified, or any combination of these possibilities.

However, in recent years, due to the rapid spread of these products, there is an atmosphere of confusion beetween consumers, health experts and foods companies, so what followed was the need to regulate production and marketing of functional foods. through the definition of standards and guidelines (Benassi et al., 2017). In order to harmonize regulation on foods throughout the EU, Regulation (EC) No. 1924/2006 and Regulation (EU) No. 432/2012 were drafted to regulate the use of health and nutritional claims on labels, approved, upon scientific evidences, by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA). EFSA is asked to express its opinion in the authorization of health claims for the different bioactive molecules, the opinions of EFSA are contained in the European Register. In May 2012 EFSA published online a register is constantly evolving, and it is updated whenever the Authority expresses new favourable or contrary opinions (Benassi et al., 2017) al the following link:

- http://ec.europa.eu/food/safety/labelling_nutrition/claims/register/public/?event=search.

2.2 Health benefits and vegetable products in functional foods

Plant foods are rich sources of nutrients and phytochemicals that are increasingly being shown to play a major role in the health benefits associated with a plant-rich diet. According to several studies, the great number of potentially beneficial compounds and their multifunctional properties makes plant products perfect candidates for the production of health-promoting food. The World Health Organisation recommends that the intakes of fruits and vegetables, legumes, whole grains and nuts are increased in order to reduce the risk of disease (WHO, 2004). This particular recommendation clearly emphasises the importance of plant-derived foods in meeting dietary goals. In order to achieve these goals, the idea of enriching food with plant products is increasingly gaining ground rich in bioactive compounds. In the table (**Table 3.**) below some important phytochemicals found in functional foods are shown.

Table 3. Important phytochemicals in functional foods and their health benefits.

Bioactive compounds	Functional Food Sources	Health Benefits
Anthocyanins	Red, purple, & blue veggies: Red cabbage, straw/black/blueberries, radishes, plums, & eggplant; elderberry; blackrice; bananas	Reduction of cholesterol (Wallace et al. 2016); Prevention of Cardiovascular diseases (Mink et al. 2007; Qin et al. 2009; Cassidy et al. 2011; Hassellund et al. 2012; Jennings et al. 2012; McCullough et al. 2012; Hassellund et al., 2013); Antiobesity or Weight loss (CAB Reviews 2010; Bertoia et al. 2016; Azzini et al. 2017); Cancer prevention (Wang and Stoner 2008; Lin et al. 2017); Enhances cognitive function (Whyte and Williams 2012; Wallace and Giusti 2013)
Beta-carotene	Sweet potato; carrots; spinach; butternut squash; cantaloupe; lettuce; bell pepper; apricot; broccoli; pea; other yellow and orange colored vegetables and fruits	Precursor of Vit. A (Grune et al. 2010; Gul et al. 2015); Inhibits the peroxidation of lipids induced by free radicals (Antioxidant) (Grune et al. 2010; Gul et al. 2015); Prevention of sunburns and photodamage (Krutmann 2008; Stahl and Sies 2012), porphyria (Sassa 2006; Mir 2018) and psoriasis (Rollman and Vahlquist 1985)
Catechins	Green tea, apples (peel on), apricots, cherries, peaches, blackberries, black grapes, strawberries, blueberries and raspberries	Antioxidant (Cabrera et al. 2006); Lowers blood pressure and low density lipoprotein (Kim et al. 2011; Khalesi et al. 2014)
Flavonoids	Citrus fruits, berries, purple grapes, onions, black and green tea, cocoa, legumes, soy, & whole grains	Antioxidant (Brunetti et al. 2013; Banjarnahor and Artnti 2015), Decreases inflammation, may protect against heart disease, stroke & cancer (Serafini et al. 2010; Perez-Cano and Castell 2016)
Lutein	Green leafy vegetables: kale, collards, spinach, romaine & broccoli; yellowcorn	Protection from eye disease by absorbing damaging blue light that enters the eye (Johnson 2002); May reduce risk of age-related macular degeneration (Ugusman et al. 2014)
Lycopene	Tomatoes, watermelon & pink grapefruit, pink guava, papaya, seabuckthorn and rosehip	Decreases risk for prostate cancer (Ilic et al. 2011; Ilic and Misso 2012; Wang et al. 2015; Chen et al. 2015; Rowles et al. 2017) and Cardiovascular diseases (Cheng et al. 2017); Cholesterol lowering action (Ried and Fakler 2011); Protection against Metabolic Syndrome (Senkus et al. 2018); Strong antioxidant effect (Chen et al. 2013)

Quercetin	Yellow fruits & veggies: Apples/pears, citrus fruits, onions, some berries, black/green tea	Blood pressure reduction (Serban et al. 2016); Antioxidants, decreases inflammation, & may help protect from heart disease, stroke & cancer(Shah et al. 2016)
Resveratrol	Red/purple grapes, red wine, purple grape juice, cocoa/dark chocolate & peanuts	Promotes cardiovascular health; decreases blood pressure and body mass index (Fogacciet al. 2018)
Rutin (vitamin Por Rotuside)	Buckwheat; Asparagus; elderflower tea; unpeeled apples; figs	Anti-inflammatory (Guardia et al. 2001; Gunawardena and Munch 2014; Horcajada et al. 2014; Ganeshpurkara and Salujaa 2017); Neuroprotection against brain ischemia (Pu et al. 2007; Khan et al. 2009; Javed et al. 2012); Improves endothelial function by enhancing nitric oxide production (Ugusman et al. 2014)
Zeaxanthin	Green leafy vegetables: kale, collards, spinach, romaine & broccoli; yellowcorn	Protection from eye disease by absorbing damaging blue light that enters the eye (Johnson 2002); May reduce risk of age-related macular degeneration (Ugusman et al. 2014)

Source: Dable-Tupas at al., 2020

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CHAPTER 3

Technological treatments on bioactive compounds

Introduction

The increasing request of fruit and vegetables is bringing the food industry, together with the academia, to improve the processes targeted to prolong crops shelf life. Since ancient times, foods have undergone processing as a way to protect and improve their nutritional and organoleptic properties. In addition, because of the varied growing and harvesting seasons of different vegetables at different locations, the availability of fresh vegetables that differs greatly in different parts of the world. Therefore, processing can transform vegetables from perishable products into stable foods with long shelf life and thereby aids in the global transportation and distribution of many varieties of vegetables. Generally, the traditional techniques are based on the use of temperature as process variable. Beside the positive effects, we have to consider that these processes can also result with some undesired consequences, such as losses of nutrients and formation of toxic compounds with negative effects on flavor, texture, or color. Like all transformation treatments, even hot processes can determine, at different degrees, tissue softening, colour change, formation of aromas and inactivation of compounds considered to be anti-nutrient, but they can be responsible for a damage to color, taste and nutritional value. In addition to industrial treatments, vegetables often undergo home heat treatments to increase their edibility and palatability. Domestic cooking processes in different ways, can significantly affect natural phytochemical profile and biological properties of vegetable products (Zhao et al., 2019). In some cases, there has been a decrease in their content. The extent of degradation depends on temperature, light, the presence of oxygen, pH, water activity and interactions with other antioxidants (Fratianni et al., 2020). Different cultures and countries have different cooking processes: boiling, steaming, frying, microwaving and baking are the most used techniques for vegetables cooking at home. Several studies have been carried out to understand which effects technological treatments have on overall nutritional quality or on beneficial factors and results are different. In fact, some studies have concluded the reduction of the bioactive contents of leafy vegetables after thermal treatment (Ismail et al., 2004), while others have shown no such effects (Turkmen et al., 2005; Delchier et al., 2012). Ismail et al. (2004) observed a decrease in the total phenols by 14% in spinach, 20% in cabbage, and 12% in kale by boiling treatment. The cooking process has shown a considerable effect on bioactive contents of leafy vegetables, and their health-promoting compounds (Moreno et al., 2007; Zhao et al., 2019). To achieve the balance between food quality and safety, there is a need to identify the best technological conditions for process optimization and food

preservation of the content of bioactives and their bioavailability. Among the vegetable thermal treatments, in this Doctoral thesis, the influence of different thermal treatments on bioactive compounds will be discussed in biscuits, in leafy vegetables, taken fresh, and after domestic cooking.

3.1 Overview of food processing

Since ancient times, foods have undergone processing processes as a way to protect and improve their nutritional and organoleptic properties. In fact, according to archaeological and ethnographic evidence, the hunter-gatherer societies utilized the first food processing methods to prevent famine, improve their diet and increase eating quality (Fellows, 2000). They used open fire heat and boiling water to prepare their meals, as well as increase palatability. Due to their nomadic life style, they did not need to preserve their food; however, after the invention of agriculture, societies started to store and preserve their food. By 3000-1500 BC, the Egyptians started using the sun to dry/preserve fish and poultry, fermentation to produce alcohol, and cereal grinding to make leavened bread. As societies progressed, they started specializing in different food processing techniques, such as milling, baking, brewing, and cheese making. In those early days, the food processing techniques use were simple craft skills that passed from one generation to another, and little to no effort was done to understand the science behind the processes (Fellows 2000). Most food processing techniques, nowadays, are still used during the handling of food from raw ingredients to finished or ready-to-eat products. These procedures influence the shelf life, safety, sensory, physical, and chemical properties of foods. In addition to the eating quality, current food industries aim to provide nutritional foods. Food products are being enriched with vitamins, minerals and prebiotic cultures, resulting in functional foods. Some of changes induced by processing in foods are desired; such as a increased shelf life through microbial inactivation, increased digestibility and improved texture, flavor, and edibility. However, processing may also trigger side reactions that cause unwanted changes in the physicochemical, sensory, and nutritional characteristics of foods (Boekel et al. 2010). For instance, size reduction (via milling) results in desirable textural and rheological properties, but can also result in unwanted aroma and flavor in some foods (Fellows, 2000). By milling, one can improve mixing and heat transfer, but such processing can disrupt cells and increase surface area, which in turn facilitates oxidative deterioration, and microbiological and enzymatic activities (Fellows, 2000). While, as regards thermal and non-thermal processes, they are usually carried out to ensure safety of processed juice, and heat-assisted processing is generally given to ensure microbial food safety. Conventional heat treatment is routinely used to extend shelf life and preserve fruits and vegetables and their products (Chipurura et al., 2010). For instance, contents of antioxidant compounds, such as phenolics and their biological activities in foods, may be affected by certain thermal techniques.

Conventional cooking of vegetables generally reduces total phenolic content and antioxidant activity. Fellows (2000) reported that, in some spices and nuts, increased temperature during milling could result in loss of volatile compounds.

3.2 Heat processing

Cooking is a very common food processing technique that improves the nutritional and organoleptic properties of foods. Like all treatments, even hot processes can determine, at different degrees, tissue softening, colour change, formation of aromas and inactivation of compounds considered to be anti-nutrient; they can be responsible for damage to color, taste and nutritional value. It is known that cooking can change the content of bioactive compounds. Several authors have studied variations in vegetable nutrients and non-nutrients after cooking and great variability in the data has been reported. Changes in bioactive compounds after cooking may result in two opposite phenomena: thermal degradation, which reduces their concentration, but also matrix softening effect, which increases the extractability of bioactive compounds, resulting in a higher concentration with respect to the raw material. However, the final effect of cooking on bioactive compounds concentration depends on the processing parameters, the structure of food matrix and the chemical nature of the specific compound (Palermo et al., 2014). The cooking methods at the industrial scale, include a very wide variety of processes, including boiling, heating, steaming, frying, blanching, sterilizzation or pasteurization (Palermo et al., 2014). The extent of degradation that thermal treatments can cause depends on temperature, light, presence of oxygen, pH, water activity and interactions with other antioxidants (Fratianni et al., 2020,). It is important to understand what effects these processes have on the overall nutritional quality or on beneficial factors. Some of the bioactive compounds are remarkably stable to heat. Others are severely depleted following harvesting, long periods of storage and cooking (Howard, 2008). The cellular localisation and the structure of the plant often influence the retention of bioactive compounds during processing. For instance, water soluble compounds, such as phenolics and flavonoids, are leached into water during blanching or canning, as a result of membrane destruction, whilst cell wall bound phenolics resist leaching and may be more easily extracted or bioavailable after processing, due to tissue softening. Lipid-soluble compounds, such as carotenoids, are retained after blanching, although losses are possible through thermal degradation and oxidation. Many processed vegetables have higher

levels of carotenoids than the raw material or in a more bioavailable form (Dewanto et al., 2002). Mechanical destruction of the food matrix and heating enhance the release of carotenoids from foods. Some studies have noted that high temperature short time processing favours the retention of anthocyanins (Jackman et al., 1987) and carotenoids (Lin et al., 2005).

3.3 Traditional culinary treatments

Most dietary vegetables are eaten at home, as final step "home-cooking", to improves their sensory acceptability of consumers. Vegetables can be cooked in different ways, according to individual taste, the recipes and the culinary traditions of the various countries. For example, in the habits of the South U.S., the boiling of vegetables is usually protracted, with a soft consistency and a blend of flavors in combining ingredients considered desirable. In the *nouvelle cuisine* of France, on the other hand, Chinese influence dictates minimal boiling or steaming to preserve fresh color, texture, and flavor. In general, it is possible to say that dense vegetables require longer cooking times (e.g. carrots, potatoes; vegetables with higher water content), while others a faster cooking (e.g. capsicums, leafy vegetables). Domestic cooking is based on common techniques, which include boiling, frying, steaming, baking and roasting, in traditional and microwave ovens (Van Boekel M., et al. 2003). It is known that also domestic cooking can induces significant changes in chemical composition, influencing the concentration and bioavailability of bioactive compounds in vegetables. However, both positive and negative effects have been reported, depending on differences in process conditions and morphological and nutritional characteristics of the vegetable species (Podsêdek, A. 2007). First of all, cooking increases food safety as a result of the destruction of microorganisms and the inactivation of anti-nutritional factors. A second beneficial effect of cooking is the enhancement of the digestibility of food and the bioavailability of nutrients; for example, the denatured proteins are generally more digestible than native proteins and the gelatinisation of starch improves its hydrolysis by amylases. Cooking is also involved in the formation of desired compounds, such as flavour compounds, antioxidants and colouring agents. On the other hand, cooking can damage food quality, leading to undesired consequences, such as losses of certain nutrients, due to chemical reactions, formation of undesired compounds (e.g. acrylamide or molecules with negative effect on flavour perception), loss of texture and discolouration (Van Boekel M., et al.

2003). Domestic processing and cooking methods are presumably considered to be one of the most important factors influencing the daily intake of carotenoids and tocols (Van den Berg et al., 2000). On the content of bioactive compounds in vegetables, and on those that promote health, the cooking process has shown a considerable effect (Moreno et al., 2007; Shonte et al., 2020). Therefore, the most recent knowledge on the effects of two specific domestic cooking conditions (conventional boiling and steam) on the bioactive content of vegetables is presented in this Doctoral thesis. The boiling treatment, in particular, can produce changes in cell structure and cell composition, the breaking of the food matrix (formed mainly by dietary fiber), which can cause the release of low molecular weight compounds into water and solid losses (Southon et al., 2002). Studies on processed foods have shown that processed foods have a better bioavailability of carotenoids than their raw materials (Gärtner et al., 1997; Hedren et al., 2002). In fact, carotenoids and tocols, being fat soluble, are not significantly lost in water-soluble media during processing. In any case, domestic cooking processes, in different ways, can significantly influence their content in vegetables (Zhao et al., 2019, Ruiz-Rodriguez et al.,2008). In some cases, there has been a decrease due to their thermal lability and their sensitivity to oxidation. In a study conducted by Chang et al. (2013), a significant increase of β-carotene was observed in selected vegetables during boiling for 8 min. Nevertheless, they observed retention of β-carotene in Chinese cabbage and spinach after boiling for 4 minutes, while lutein showed a good stability during boiling. Studies conducted on steaming have shown that this treatment improved the digestibility of food, the extraction and bioavailability of nutrients, softening the food matrix (Palermo et al., 2014). However, the final effect of heat treatment depends mainly on the processing parameters and the structure of the food matrix (Palermo et al., 2014). This thermal process is considered a safer method in terms of retention and of bioavailability of vegetable bioactives. For example, in the study conducted by Wachtel-Galor et al. (2008) it has been found that antioxidant content of the investigated Brassica species was high during the steaming process, compared to microwave or boiling methods. In fact, during steaming, the investigated species maintain a better consistency quality. In addition, also the total antioxidant capacity remains stable after steaming (Mazzeo et al., 2011). The content of polyphenols after steaming was significantly increased, and it was also noticed that this treatment preserved in a better way the content of carotenoids (Pellegrini et al., 2010).

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CHAPTER 4

Methods of analysis

4.1 Chemical analysis

The compostion in moisture, ashes, proteins and fats was determined according to the AOAC methods.

4.1.1 Moisture, Ashes and Proteins

Moisture, ash and protein content were determined according to ICC methods 110/1, 104/1 and 105/2 respectively (ICC, 1995).

Moisture is the measure of the water content of a food product. It moisture was determined using an oven set at 105 °C for 4 hours;

A*sh* content of a sample represents the amount of minerals that remain after the carbonization and calcination of a food matrix. Ash was quantified using a muffle furnace set at 525 °C until a white residue was obtained.

Protein content was determined though the Kjeldhal method (N x 6.25). This method involves three stages of analysis: sample mineralization in the presence of H_2SO_4, steam distillation in the presence of NaOH and subsequent titration with H_2SO_4 at a known concentration.

4.1.2 Fats

Total fat was determined by acidic hydrolysis (AACC, 2000) for leafy vegetables, and with Soxhlet Method for minor and pseudo-cereals and backery products.

4.1.3 Determination of total fibre

Dietary fibre was assessed with the AOAC method 991.43 (1995), and AACC method 32-07 (1995). Soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) were estimated. Total dietary fibre (TDF) content corresponds to the sum of SDF and IDF.

4.1.4 Carbohydrates

Carbohydrates were calculated as the difference to 100 of all other nutrients.

4.2 Extraction of soluble and hydrosoluble vitamins

4.2.1 Extraction of carotenoids and tocols

Carotenoid and tocol extraction was carried out using the saponification method reported by Panfili et al. (2003; 2004) suitably modified in based of analyzed matrix, whether cereals, vegetables, fresh or freeze-dried. This method requires a first stage of hot saponification of the sample followed by solvent extraction and chromatographic determination by HPLC. Milled sample was weighed and placed in a screw-capped tube. The weight differs according to the analyzed sample, 1 g in triplicate was weighed for minor *cereals, pseudocereals* and *bakery products*; while, for *green leafy vegetables* 0.2 g freeze-dried were weighed in triplicate. Then, on the basis of the sample, whether cereal or vegetable, in succession were added:

	CEREALS	**VEGETABLES**
Ethanolic pyrogallol (60 g/l)	5 mL	5 mL
Ethanol (96%)	2 mL	3 mL
Sodium chloride (10 g/l)	2 mL	1 mL
Potassium hydroxide (600 g/l)	2 mL	2 mL
Glass beads	~10	~10

After bubbling nitrogen in the tubes to remove oxygen, the tubes were placed in a 70°C water bath and mixed every 5 – 10 min during saponification. After alkaline digestion at 70°C for 45 min, the tubes were cooled in an ice bath, and 15 mL of sodium chloride (10 g/L) were added. The suspension was then extracted with 15 mL portions of n-hexane/ethyl acetate (9:1, v/v) replicating the steps until it was colorless. Subsequently, the organic layers, containing carotenoids or tocols, were evaporated until dryness, with a rotating evaporator, in a water bath at the set temperature of 40°C.

The dry residue was dissolved in isopropyl alcohol (10% for carotenoid and 1% for tocols) in n-, stored at -20 °C and analyzed trought HPLC.

Determination of carotenoids through High Performance Liquid Chromatography (HPLC)

The analytical determinations of carotenoids have been carried out with a HPLC Dionex (Sunnyvale, CA) analytical system, consisting of a U6000 pump system and a 50 uL injector loop (Rheodyne, Cotati) was used. The chromatographic separation of the compounds was achieved by means of a 250 mm x 4.6 mm i.d., 5 μm particle size, Kromasil Phenomenex Si column (Torrance, CA, USA). The mobile phase has been described in detail below in each carried out activity.

For a more complete evaluation of carotenoids in some vegetable samples, the analytical procedure was carried out using a gradient and the separation of carotenoids was made through the combination of a normal- phase and reversed-phase HPLC methodology.

- *Normal-phase*: the mobile phase was n-hexane:isopropyl alcohol in a multilinear gradient elution from 10% (A) to 20% (B) of isopropyl alcohol with a flow rate of 1,5 mL/min. The chromatographic separation of the compounds was achieved by means of a 250 mm x 4.6 mm i.d., 5 μm particle size, 100A Luna Phenomenex Si column (Torrance, CA).
- *Reverse phase*: separation was performed, at a flow rate of 1 mL/min, by using a 5 μm C30 YMC (Hampsted, NC, USA) stainless steel column (250×4.6 mm i.d.). The mobile phase was methanol: Methyl tert-butyl ether: water. The gradient profile is given in Mouly et al., 1999.

Through reversed phase the elution order of the different compounds was: violaxanthin, neoxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, 9-*cis*-β-carotene, β-carotene, 13-*cis* β-carotene. Through normal-phase carotenes were eluted as it follows: lutein, zeaxanthin, violaxanthin, neoxanthin.

Spectrophotometric detection was achieved by means of a diode array detector set in the range of 350–500 nm. Peaks were detected at 450 nm. Carotenoids were identified through their spectral characteristic, and comparison of their retention times with known standard solutions. Data were stored and processed by a Dionex Chromeleon Version 6.6 chromatography system (Sunnyvale, CA, USA). The quantitative analysis of carotenoids was carried out with the external standard method, building up a calibration curve for each compound, by using known concentration of standard solutions.

Determination of tocols through High Performance Liquid Chromatography (HPLC)

The determination of tocols was carried out with an HPLC Dionex system equipped with an ULTIMATE 300 Pump, under normal phase conditions.
The chromatographic separation of the compounds was achieved by means of a 250 mm x 4.6 mm i.d., 5 µm particle size, 100A Luna Phenomenex Si column (Torrance, CA). Fluorometric detection of all compounds was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm by means of an RF 2000 spectrofluorimeter (Dionex, Sunnyvale, CA, USA). The mobile phase was n-hexane/ethylacetate/acetic acid (97.3:1.8:0.9 v/v/v), at a flow rate of 1.6 mL/min (Panfili et al., 2003). The chromatographic run lasted 28 minutes during which the different vitamin E vitamers were eluted in the following order: α-tocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol, β-tocotrienol, γ-tocotrienol, δ-tocopherol, δ-tocotrienol. Compounds were identified by a comparison of their retention times with those of known available standard solutions, and quantified through the calibration curves of the standard solutions. The concentration range was 5–25 ug/mL for every tocol standard. Vitamin E activity was expressed as Tocopherol Equivalent (T.E.) (mg/100 g of fresh weight- f.w.), calculated as reported by Sheppard et al., 1993.

4.2.2 Determination of hydrosoluble vitamins

Thiamine (B1) and *riboflavin* (B2) were extracted applying the INRAN method taken from the original one of Hasselmann et al., (1989), with appropriate modifications. Briefly, samples were placed in 100 mL volumetric flasks containing 20 mL of 0.1 N HCl and heated in a water bath at 100 °C for 30 min. After cooling at room temperature, the pH of the samples was adjusted to 4.5 with 2.5 M Ammoniunm acetate (NaOAc). Following the addition of 0.2 mL of aqueous Clara-Diastase (50 mg/mL). Samples were incubated for 3 h at 37 °C. After cooling, samples were brought up to 25 mL with distilled water. Then samples were centrifuged and filtered through a 0.45 µm filter. Thiamine was converted to thiochrome by adding 1.25 mL of 1% potassium ferricyanide (in 15% aqueous NaOH) to 2.5 mL of filtered extract. After 1 min of oxidation, 0.25 mL of 85% H_3PO_4 was added. The extract was purified on a Sep-Pak C18 cartridge. The cartridge was washed with 5 mL MeOH, followed by 5 mL of 0.05 M NH_4OAc (adjusted to pH 5.0 with Acetic acid). Samples (5 mL) were loaded into the Sep-Pak C18 cartridge, and then the cartridge was washed with 0.05 M NH_4OAc (5 mL), the eluated was

discarded. Finally, vitamins were eluted with 5 mL mobile phase Methanol:NaOAc (40:60 v/v). Extracts were separated by a HPLC Dionex (Sunnyvale, CA, USA), with a U3000 pump and an injector loop (Rheodyne, Cotati). Separation was made at a flow rate of 0.8 mL/min with Methanol:NaOAc (40:60 v/v) as a mobile phase, by using a 5 um C18 Luna, Phenomenex (Torrance, CA, USA) stainless steel column (250 x 4.6 mm i.d.). Fluorometric detection was performed at an excitation wavelength of 366 nm and an emission wavelength of 453 nm for thiamine, and an excitation wavelength of 453 nm and an emission wavelength of 580 nm for riboflavin, by means of an RF 2000 spectrofluorimeter (Dionex, Sunnyvale, CA, USA). Data were processed by a Dionex Chromeleon Version 6.6 chromatography system (Sunnyvale, CA, USA). Thiamine and riboflavin were compared with known available standards, and identified considering their retention times and relative elution order.

4.2.3 Chemicals and Reagents

Solvents were commercially obtained (Sigma Aldrich) at the highest quality. All other used reagents were of analytical grade. Violaxanthin, neoxanthin α-carotene, 9-cis-β-carotene and 13-cis-β-carotene standards were obtained from CaroteNature (Lupsingen, Switzerland); lutein, zeaxanthin, and β-cryptoxanthin were from Extrasynthese (Z.I. Lyon-Nord, Genay, France). All-trans-β-carotene, thiamine and riboflavin standards were from Sigma Chemicals (St. Luis, MO, USA). α-, β-, γ- and δ-tocopherol standards were from Merck (Darmstadt, Germany); α-, β-, γ- and δ-tocotrienol standards were purified as reported in Panfili et al. 2003.

4.3 Polyphenol, antioxidant activity and flavonoid determination

The total content of polyphenols, of flavonoids, and antioxidant activity was determinated only on fresh and cooked freeze-dried leafy vegetables. The leafy vegetables were extracted in MeOH:H_20 (80:20 v/v) for 30 min at 4°C and in the dark and subsequently centrifuged for 10 min at 1500 rpm. The analyses were carried out on the obtained supernatants.

4.3.1 Total polyphenol content

Total polyphenol compounds (TPC), expressed as nmoles catechin/g fresh weight (f.w.), were determined by VIS spectrophotometry according to the Folin-Ciocalteu method. This method, is based on the oxidability of phenols at basic pH, while Folin-Ciocalteu reagent works as an oxidant agent. The total phenolic compounds were determined as described by George at al., (2005), with some modifications. Breifly, 0.5 mL of properly diluited extract and 2,5 mL of Folin-Ciocalteu reagent (0.5 N) were mixed and left to rest at room temperature for 2 min. An amount of 2.5 mL of 7.5% sodium carbonate was then added and the mixture was allowed to rest again for 15 min at 50° C. The absorbance was then read, by using a spectrophotometer UV/VIS (Perkin Elmer) at 760 nm. Total phenolic compounds were quantified by a calibration curve. For each sample, the analysis was carried out in triplicate.

4.3.2 ABTS assay

The antioxidant activity was measured using the ABTS assay, according to the method of George at al., (2005). Before the analysis, the calibration curve was built, and the antioxidant activity was expressed as mg Trolox equivalents (TEs) for gram of fresh weight (f.w.). This test is based on the ability of antioxidants to interact with the radical cation $ABTS^{\bullet+}$ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) inhibiting its absorption at 734 nm. The ABTS radical cation ($ABTS^{\bullet+}$) was obtained by dissolving ABTS in distilled water and 44 uL potassium persulphate was mixed allowing the mixsture to stay in the cold and in the dark at for 12-16 h before use. The resulting blue-green ABTS radical solution was diluted 70 times with ethanol to obtain an absorbance of 0.700 ± 0.050 at 734 nm. Then, 1mL of $ABTS^{\bullet+}$ was added to different dilutions of each extract (usually 1/10 or 1/20) in microcuvettes. The microcuvettes are left to rest in the darkness for 20 minutes to reach the stability of the reaction plateau and then the solutions are read through a UV-Vis spectrophotometer at 734 nm.

4.3.3 Total flavonoid content

The total flavonoid content (TFC) was evaluated by the aluminum chloride colorimetric method, according to Gunathilake et al. (2018) with slight modifications. Extracts (0.5 mL), prepared as previously described, were mixed with 3 mL of distilled water and 0.3 mL of a 5% sodium nitrite ($NaNO_2$) solution. After 5 minutes, 0,3 mL of a 10% aluminum chloride ($AlCl_3$) solution was added and the mixture was allowed to stand for 6 minutes. Finally, 2 mL of 1M sodium hydroxide (NaOH) was added, and

the solution was made up to 10 mL with distilled water and was mixed. For each sample the absorbance was read against an appropriate blank at 510 nm using a UV/VIS spectrophotometer. The total amount of flavonoids was expressed as nmoles catechin per g fresh weight (f.w.), after a proper calibration curve. The analysis was carried out in triplicate for each sample.

4.5 Fatty acid analysis

Fats were extracted with the Soxhlet method (AACC, 1995). Each extract was treated according to the following analytical protocol: approximately 0.1 g of each sample was dissolved in 2 mL of *n*-heptane. The solution was vortexed for 30 seconds; 0.2 mL of KOH in MeOH (2 M) were added and the solution was debated for few seconds. One μL of supernatant was directly injected into the GC-FID. The gas chromatography used was a Dany MasterGC, equipped with a Flame Ionization Detector (FID, 280 °C) fed with hydrogen (30 mL min^{-1}), air (300 mL min^{-1}) and helium (20 mL min^{-1}). A Programmed Temperature Vaporizer (PTV) was used, starting at 110 °C (1 min) and rising up to 280 °C (5 min) at 800 °C min^{-1}. A ThecnoKroma capillary column model MetaX5 (30 m × 0.25 mm × 0.25 μm) was used for the determination. The programmed oven started at 100 °C (1 min) and rose up to 280 °C (5 min) at 10 °C min^{-1} (Campanella, et al., 1999). The chromatograms were processed by means of a specific Clarity software.

4.6 Sensory analysis of pasta

Optimum cooking time (measured every 30 sec, by observing time of disappearance of the central core strand during cooking), firmness (by chewing), liveliness (by manual handling) and starch release (by manual handling) were determined according to International Standard ISO 7304 -1. A rating scale ranging from 10 to 100 was used (from absence to maximum), showed in **Table 4**. Pasta with a total score of ≤ 40 was classified as of poor or mediocre quality; >40 to ≤50 was not completely satisfactory; >50 to ≤70 was fair; >70 to 80 was good; and >80 was excellent. Each cooking test was carried out in triplicate under controlled temperature A panel of 10 trained judges was used to assess pasta characteristics, and each sample was evaluated for three times. The total score was calculated by adding the single ratings obtained for firmness, liveliness and starch release and then dividing the sum by 3.

Table 4. Rating scale for pasta sensory analysis (ISO 7304-1).

Firmness	Liveliness	Starch Release
100 - very high (very firm)	100—very high (not at all sticky)	100 - very low (no starch)
80 – high	80 - high	80 - low
50 – medium	50 - medium	50 - medium
30 – low	30 - low	30 - high
10 - very low (very tender)	10—very low (very sticky)	10—very high (large quantity of starch)

4.7 Statistical analysis

4.7.1 Statistical analysis

All experimental data were subjected to one-way analysis of variance (ANOVA), by using a Statistical Software Package for Windows (SPSS Inc. Version 13, Chicago, IL, USA), except for bakery products, where statistical analysis was carried out using R, Version 3.5.0.

The data reported for all parameters are the average values of three results obtained from analysis of three different aliquots of each sample. Analysis of variance was performed to determine significant differences (Tukey's HSD test $^{*}P \leq 0.05$) between means.

4.7.2 Statistical analysis for bakery products

Three different biscuit preparations were made for every typology of fat/oil. Three analytical determinations on each preparation were made. Results were reported as average of the three different batch samples. Principal Component Analysis (PCA) (Husson, Lê, & Pagès, 2011) was performed using tocol and fatty acids as active variables, while the classification of fat/oil is used as supplementary variable. The analysis was carried out through R Studio Software, using the package *FactoMineR* (Lê, Josse, & Husson, 2008).

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CHAPTER 5

Gluten-Free Alternative Grains

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Gluten-Free Alternative Grains: Nutritional Evaluation and Bioactive Compounds

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Abstract

The interest in gluten-free grains is increasing together with the major incidence of celiac disease in the last years. Since to date the knowledge of nutritional and bioactive compounds profile of alternative gluten-free grains is limited we evaluated the content of water-soluble (thiamine and riboflavin) and liposoluble vitamins, such as carotenoids and tocols (tocopherols and tocotrienols), of gluten-free minor cereals and pseudocereals. The analysed samples showed a high content of bioactive compounds; in particular, amaranth, cañihua and quinoa are a good source of vitamin E, while millet, sorghum and tef are a good source of thiamine. Moreover, millet provides a fair amount of carotenoids, in particular of lutein. These data can provide more information on bioactive compounds in gluten free grains. The use of these grains can improve the nutritional quality of gluten free cereal-based products and could avoid the monotony of the celiac diet.

Keywords: minor cereal, pseudocereal, bioactive compound, gluten-free grain, tocols, carotenoids.

Introduction

Celiac disease is a chronic systemic, autoimmune disorder in genetically predisposed individuals triggered by exposure to dietary gluten and resulting in mucosal inflammation, villous atrophy and crypt hyperplasia [1]. It is characterized by an abnormal immune reaction consisting in an excessive response of the immune system to a group of cereal proteins, called prolamines (gliadin, hordein, sekalina, avenin) found in wheat, barley, rye and oats. Celiac disease affects approximately 1% of the world population and it has significantly increased due to an underestimation, since it is often left undiagnosed [2]. The only treatment for people with the celiac problem is the adherence to gluten-free foods for their whole lifetime.

Several studies demonstrated that sticking to a gluten-free diet for a lifetime can lead to nutritional imbalance in celiac subjects, such as malabsorption of nutrients and deficiencies of several vitamins and minerals. These deficiencies are due both to the phenomena of malabsorption at the intestinal level and to the monotony of the diet based mainly on rice and maize [3-6].

Recently, more attention has been given to gluten free minor cereals and pseudocereals as alternatives to those conventionally used for celiacs. Many of them have been defined as “orphan crops” o “underutilised crops”; they are indigenous crops scarcely documented and rarely used by food industries [7]. Many underutilized crops are relatively more drought-tolerant than most major cereals; they play a significant role in many developing countries, providing food security and income to resource-poor farmers [8]. Gluten-free alternative sources studied in this work include minor cereals (sorghum, tef, millet and wild rice), and pseudocereals (quinoa, cañihua, chia, and amaranth). These grains are mainly consumed as flours and seeds, which can be added to preparations such as soups, yogurt, cakes, breads and others cereal-based products; nevertheless, commercialization of these products is still quite limited in the Italian market. Some of these are a source of nutrients and bioactive compounds that could improve the nutritional quality of gluten free products.

Carotenoids are a significant group of bioactive compounds with health promoting properties [9, 10] and responsible for the colour of a wide variety of grains [11]. Some carotenoids are precursors of retinol (vitamin A) and are very strong natural antioxidants. Carotenoids are known to be efficient physical and chemical quenchers of singlet oxygen, as well as potent scavengers of other reactive oxygen species [9].

Vitamin E is a natural antioxidant comprising two groups of vitamers, tocopherols and tocotrienols, occurring in eight forms: α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T) and α-tocotrienol (α-T3), β-tocotrienol (β-T3), γ-tocotrienol (γ-T3), and δ-tocotrienol (δ-T3). Vegetable oils are the main tocol sources, however, substantial amounts of these compounds are also reported in most cereal grains [12-14]. Potential health benefits of tocols include the prevention of certain types of cancer, heart diseases and other chronic diseases [15, 16]. Thiamine (B1) is one of the major water-soluble vitamins, it plays an important role as a co-factor of several key enzymes involved in the carbohydrate metabolism and defence mechanism [17]. It can be found in moderate amounts in all foods: nuts and seeds, legumes, wholegrain/enriched cereals and breads, pork [18]. Thiamine deficiency is rare in healthy individuals in food-secure settings, where access to thiamine-rich foods ensures adequate intakes [19]. Riboflavin (B2) is a precursor of the co-enzymes flavin mononucleotide (FMN; riboflavin phosphate) and flavin adenine dinucleotide (FAD), which are components of oxidases and dehydrogenases. It is also important for skin health and normal vision and can be found in whole cereals, breads, leafy green vegetables, milk products [18].

To date, the evaluation of nutritional and bioactive compound profiles of alternative gluten-free grains is limited, if not lacking [20-23]. These researches are of a great importance in order to formulate gluten free cereal-based products with a higher nutritional value. Thus, in this work, samples of minor cereals and pseudocereals commercialized in Italy have been characterized for their nutritional value, with a particular focus on some bioactive compounds, such as carotenoids, tocols, thiamine and riboflavin, in order to increase the awareness of their nutritional profile. Moreover, data coming from this study may be included in food nutrient databases.

Material and Methods

Sample Collection and Preparation

Thirty one different minor cereals and pseudocereals were bought in Italian specialized shops (**Table 5**.). Different brands were considered for each grains. Grains were grounded with a refrigerated IKA A10 laboratory mill (Staufen, Germany), carefully mixed and stored at -20°C until analysis. Each sample was analysed in triplicate.

Table 5. List of analysed gluten-free grains.

Minor cereals	**Samples (n)**
Millet (*Panicum miliaceum* L.)	6
White Sorghum (*Sorghum bicolor* L.)	3
Tef (*Eragrostis tef* (Zucc.) Trotter)	4
Wild rice (*Zizania aquatica* L.)	2
Pseudocereals	
White quinoa *(Chenopodium quinoa* Willd.)	3
Pigmented quinoas (red and black) *(Chenopodium quinoa* Willd.*)*	4
Cañihua *(Chenopodium pallidicaule)*	3
Amaranth *(Amaranthus spp.)*	3
Chia *(Salvia hispanica* L.*)*	3

Chemical analysis

Proximate analysis

Moisture, ash, fat, and protein contents were determined using an ICC standard procedure [24]. Briefly, moisture was determined using an oven set at 130 °C and ash was quantified using a muffle furnace set at 525 °C. Protein content was determined though the Kjeldhal method (N×6.25) and lipids were determined by the Soxhlet method. Carbohydrates plus fiber were calculated as difference, using the following equation: [100 - (% moisture + % lipids + % proteins + % ash)].

Carotenoid analysis

Carotenoid extraction was carried out using the saponification method reported by Panfili et al. [14]. About 0.2 g of milled sample was weighed and placed in a screw-capped tube. Then, 5 ml of ethanolic pyrogallol (60 g/L) was added as an antioxidant, followed by 2 ml of absolute ethanol, 2 mL of sodium chloride (10 g/L) and 2 mL of potassium hydroxide (600 g/L). The tubes were placed in a 70 °C water bath and mixed every 5–10 min during saponification. After alkaline digestion at 70 °C for 45 min, the tubes were cooled in an ice bath and 15 mL of sodium chloride (10 g/L) were added. The suspension was then extracted twice with 15 mL portions of n-hexane/ethyl acetate (9:1, v/v). The organic layers, containing carotenoids, were collected and evaporated to dryness; the dry residue was dissolved in 2 mL of isopropyl alcohol (10%) in n-hexane.

A HPLC Dionex (Sunnyvale, CA) analytical system, consisting of a U6000 pump system and a 50 µl injector loop (Rheodyne, Cotati) was used. The chromatographic separation of the compounds was achieved by means of a 250 mm x 4.6 mm i.d., 5 µm particle size, Kromasil Phenomenex Si column (Torrance, CA). The mobile phase was n-hexane/isopropyl alcohol (5%) at a flow rate of 1.5 mL/min. Spectrophotometric detection was achieved by means of a diode array detector set in the range of 350-500 nm. Peaks were detected at 450 nm. Carotenoids were identified through their spectral characteristic and comparison of their retention times with known standard solutions. Data were stored and processed by a Dionex Chromeleon Version 6.6 chromatography system (Sunnyvale, CA). All-trans-β-carotene and lutein were obtained from Sigma Chemicals (St. Luis, MO, USA); zeaxanthin and β-cryptoxanthin were obtained from Extrasynthese (Z.I. Lyon-Nord, Genay, France).

Tocol analysis

Tocols were determined after the same saponification method described for carotenoids. An aliquot of the carotenoid extract was collected and evaporated to dryness and the dry residue was dissolved in 2 ml of isopropyl alcohol (1 %) in n-hexane and was analysed by HPLC, under normal phase conditions, using a 250 x 4.6 mm i.d., 5 mm particle size Kromasil Phenomenex Si column (Torrance, CA, USA) [14]. Fluorometric detection of all compounds was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm by means of an RF 2000 spectrofluorimeter (Dionex, Sunnyvale, USA). The mobile phase was n-hexane/ethylacetate/acetic acid (97.3:1.8:0.9 v/v/v), at a flow rate of 1.6 mL/min [14, 25]. Compounds were identified by comparison of their retention times with those of known available standard solutions and quantified through the calibration curves of the standard solutions. The concentration range was 5-25 µg/ml for every tocol standard. Vitamin E activity was expressed as Tocopherol Equivalent (T.E.) (mg/100 g of fresh weight f.w.), calculated as reported by Sheppard et al. [26].

Thiamine and riboflavin analysis

Thiamine and riboflavin were extracted as in Hasselman et al. [27]. Briefly, samples were placed in 100 ml volumetric flasks containing 20 ml of 0.1 N HCl and heated in a water bath at 100 °C for 30 min. After cooling at room temperature, the pH of the samples was adjusted to 4.5 with 2.5 M NaOAc. Following the addition of 0.2 ml of aqueous Clara-Diastase (50 mg/ml), samples were incubated for 3 h at 37 °C. After

cooling, samples were brought up to 25 ml with distilled water. Then samples were centrifuged and filtered through a 0.45 μm filter. Thiamine was converted to thiochrome by adding 1.25 ml of 1% potassium ferricyanide in 15% aqueous NaOH to 2.5 ml filtered extract. After 1 min for oxidation, 0.25 ml of 85% H_3PO4 was added. The extract was purified on a Sep-Pak C18 cartridge. The cartridge was washed with 5 ml MeOH, followed by 5 ml of 0.005 M NH_4OAc (adjusted to pH 5.0 with HOAc). The sample (5 ml) was loaded into a Sep-Pak C18 cartridge, and then the cartridge was washed with 0.005 M NH_4OAc and, finally, the vitamins were eluted with 5 mL mobile phase. Extracts were separated by a HPLC Dionex (Sunnyvale, CA), with a U3000 pump and an injector loop (Rheodyne, Cotati). Separation was made at a flow rate of 0.8 mL/min with Methanol:NaOAc (40:60 v/v) as mobile phase, by using a 5 μm C18 Luna, Phenomenex (Torrance, CA, USA) stainless steel column (250×4.6 mm i.d.). Fluorometric detection was performed at an excitation wavelength of 366 nm and an emission wavelength of 453 nm for thiamine, and an excitation wavelength of 453 nm and an emission wavelength of 580 nm for riboflavin, by means of a RF 2000 spectrofluorimeter (Dionex, Sunnyvale, USA). Data were processed by a Dionex Chromeleon Version 6.6 chromatography system (Sunnyvale, CA). Thiamine and riboflavin were compared with known available standards and identified considering their retention times and relative elution order. Thiamine and riboflavin standards were obtained from Sigma Chemicals (St. Luis, MO, USA).

Results and Discussion

Nutritional composition

The nutritional composition of analysed minor cereals and pseudocereals is shown in **Table 6**.

Table 6. Nutritional composition of gluten free grains (g/100 g).

	Minor cereals				Pseudocereals			
	Millet	***Sorghum***	***Tef***	***Wild rice***	***Quinoa (white and pigmented)***	***Cañihua***	***Amaranth***	***Chia***
Moisture	12.7	12.5	11.5	10.5	11.5	8.6	11.0	8.4
	(2.0)[a]	(6.9)	(1.4)	(0.4)	(9.8)	(5.7)	(1.0)	(6.7)
Ash	1.0	1.4	2.3	1.8	2.2	2.4	2.3	4.5
	(63.0)	(8.6)	(5.6)	(7.2)	(3.0)	(6.6)	(8.7)	(2.7)
Protein	11.7	9.0	11.7	12.4	12.9	14.1	13.8	21.5
	(3.3)	(0.1)	(1.7)	(6.1)	(1.5)	(2.6)	(3.4)	(7.6)
Fat	4.4	2.6	2.4	1.2	5.8	8.4	6.1	35.4
	(0.4)	(26.9)	(4.1)	(4.5)	(12.0)	(1.1)	(5.7)	(2.1)
Carbohydrate +Fiber*	70.2	74.5	72.1	74.1	67.6	66.8	66.8	30.2

* Calculated by difference; [a]: coefficient of variability.

The composition of the chia seeds notably differs from all the other cereal and pseudocereal samples, showing high concentrations of fats (35.4 g/100g), proteins (21.5 g/100g) and ash (4.5 g/100g). These values are similar to those observed by other authors for chia seeds [28]. In general, wild rice and pseudocereals are a good source of proteins. Taking into account the European law [29], wild rice, all quinoa seeds, cañihua and amaranth can be declared in label with the claim “source of protein”, since they contain at least 12 g of protein per 100 g. Chia seeds can be declared with “high protein content”, since they contain at least 20 g of protein per 100 g. The fat content was

significantly higher for pseudocereals, if compared to minor cereals. Wild rice shows the lower fat content (1.2 g/100g).

Carotenoids

Table 7 shows the carotenoid amounts of analysed samples. Carotenoids content (μg/100 g dry weight d.w.) varied significantly from 22 μg/100 g in amaranth to 763 μg/100 g in millet. In all gluten-free grains the main compounds are lutein and zeaxanthin. A comparison with literature related to the HPLC analysis of carotenoids is very difficult since the available few data are obtained by different methods and these pigments may vary depending on genotype and location. The total carotenoid content of millet, wild rice, quinoas and cañihua is comparable with that of wheat (about 305 μg/100 g for durum and about 150 μg/100 g for soft wheat) [30, 31] and of pigmented rice (460-50 μg/100 g) [32], but it is significantly lower than that of maize (about 1110 μg/100 g) [30, 33]. Among minor cereals, literature data are reported only for sorghum [34], where the authors found an average amount of 20 μg/100 g as sum of lutein and zeaxanthin, with a high variability among the different genotypes.

Table 7. Carotenoid composition in gluten free grains (µg/100g d.w.).

	Minor cereals				Pseudocereals				
Carotenoids	***Millet***	***Sorghum***	***Tef***	***Wild rice***	***White quinoa***	***Pigmented quinoas***	***Cañihua***	***Amaranth***	***Chia***
β-Carotene	19.8 (15.0)[a]	9.86 (10.0)	7.8 (20.0)	6.23 (10.0)	12.3 (10.0)	23.6 (23.0)	20.2 (28.0)	tr	12.4 (10.0)
β-Criptoxanthin	20.0 (30.0)	nd	tr	tr	tr	tr	tr	nd	nd
Lutein	535.5 (3.4)	11.2 (64.0)	36.45 (30.0)	196.2 (36.6)	85.6 (1.3)	265.2 (33.0)	325.3 (0.1)	19.8 (5.0)	tr
Zeaxanthin	188.3 (10.0)	28.9 (10.0)	18.4 (40.0)	9.7 (10.0)	11.2 (11.0)	13.2 (30.0)	40.2 (4.2)	2.2 (11.3)	33.5 (10.0)
Total Carotenoid	763.1 (4.0)	50.46 (8.0)	62.6 (28.0)	212.3 (8.0)	109.1 (11.0)	302.0 (26.0)	385.7 (10.0)	22.0 (10.0)	45.9 (8.0)

[a]: coefficient of variability; nd: not detectable; tr: traces.

In the present study, the variability of the total carotenoid content within the same cereal (expressed by the coefficient of variability, CV%), is from 4% in millet to 33% in pigmented quinoa. This variability may be due to genetic, pedoclimatic and varietal factors [35]. Regarding pseudocereals, results for chia are similar to those obtained in the work of da Silva et al. [28]. Significant differences between white and pigmented quinoas were found for total carotenoids, due to the different lutein amounts, as also observed by Tang et al. [36] who indicate a direct correlation between the higher total carotenoid content and the darkness of the seed coat.

Tocols

The characterization of tocols in minor cereals and pseudocereals is reported in **Table 8**. Except for wild rice, that shows a minor content of total tocols (TC) (about 0.4 mg/100 g), TC of minor cereals and amaranth are comparable with that of wheat, maize and rice (about 3.5-7.0, 6.0-7.0 and 2.3-2.7 mg/100g, respectively) [12, 14, 37] while, for the remaining pseudocereals, values are significantly higher. Among minor cereals, tef shows the highest amount of total tocols (6 mg/100g d.w.), followed by millet and sorghum with about 4 and 3 mg/100g respectively.

Table 8. Tocol composition in gluten-free grains (mg/100g d.w.).

Tocols	Minor cereals				Pseudocereals			
	Millet	*Sorghum*	*Tef*	*Wild rice*	*Quinoa (white and pigmented)*	*Cañihua*	*Amaranth*	*Chia*
α-T	0.16 (6.2) [a]	0.60 (83.0)	0.11 (18.2)	0.13 (11.5)	2.86 (9.58)	4.2 (35.7)	1.28 (44.5)	0.33 (33.3)
β-T	0.06 (16.6)	0.08 (62.5)	0.06 (20.0)	0.10 (13.0)	0.11 (23.0)	0.28 (21.0)	3.43 (46.0)	nd
γ-T	2.73 (47.2)	2.32 (41.4)	5.52 (8.3)	0.10 (1.0)	5.9 (8.3)	12.50 (4.3)	0.30 (36.7)	13.59 (21.5)
δ-T	0.45 (29.0)	0.03 (33.0)	0.14 (14.0)	nd	0.22 (1.0)	0.40 (5.0)	1.28 (35.0)	0.38 (34.0)
α-T3	nd	nd	nd	nd	nd	nd	nd	nd
β-T3	0.12 (50.0)	nd	nd	0.03 (16.6)	tr	nd	nd	nd
γ-T3	0.04 (25.0)	nd	0.15 (73.0)	nd	tr	nd	nd	0.13 (23.0)
δ-T3	0.24 (45.8)	nd	nd	nd	nd	nd	nd	nd
Total tocols	3.80 (45.0)	3.09 (51.0)	5.99 (5.0)	0.36 (1.0)	9.10 (8.0)	18.06 (3.9)	6.31 (42.0)	14.43 (22.0)
T.E.	0.43 (28.0)	0.78 (82.0)	0.56 (21.0)	0.17 (5.9)	3.62 (1.0)	4.5 (2.0)	2.7 (33.0)	1.6 (24.0)

[a]: coefficient of variability; nd: Not detectable; tr: traces; T.E.: Tocopherol equivalent (mg/100g f.w.).

Except for wild rice, where α-tocopherol is the prevalent isomer, the main tocopherol isomer is γ-tocopherol, which represents the 92%, 72% and 75% of the total content in tef, millet and sorghum, respectively. For pseudocereals, the highest content of total tocols was found in cañihua (about 18 mg/100g), followed by chia seeds (about 14 mg/100 g d.w.) and quinoas, with an average of 9.1 mg/100 g d.w. Contrarily to carotenoids, among all analysed quinoa seeds, all found vitamers did not shown significant qualitative and quantitative differences. Amaranth is the pseudocereal with the lowest total tocol amounts (about 6mg/100g). For chia, cañihua and quinoa the predominant isomer is γ-tocopherol (94%, 69% and 64% of total tocols), while for amaranth the prevalent isomer is β-tocopherol, which represents the 54% of total tocols. γ-Tocopherol has been found as the main vitamer in quinoa and chia also in other works [28, 36, 38]. References for tocols are not available for all analysed gluten-free grains and, where present, they show similar results in millet and sorghum [23, 34]. Moreover, a comparison with literature data related to tocol analysis is very difficult, for the same reasons already explained for carotenoids.

Table 8 also reports values of vitamin E activity provided by 100 g of product, expressed as Tocopherol Equivalent (T.E.) (mg/100 g product) [26]. Taking into account the Recommended Daily Allowance (RDA) for vitamin E, which is of 12 mg/day [39], 100 g of amaranth contribute to 22 % of the RDA, while quinoas and cañihua approximately to 35% of the RDA, so as to be declared in label as a "source of vitamin E". A portion of these pseudocereals (70 g) contributes approximately to 15 % of the RDA for amaranth and to 25% of the RDA for quinoas and cañihua.

Thiamine and riboflavin

Table 9 reports values of thiamine and riboflavin of analysed grains. The concentrations of thiamine are different between minor cereals and pseudocereals, except for wild rice. In whole wheat grains about 0.40 mg/100g are found in literature [40, 41]. Low values of riboflavin were found for all samples, except for wild rice with values comparable to those of whole wheat grains and maize (0.15 and 0.20 mg/100g, respectively) [40, 41].

Table 9. Thiamine and riboflavin content in gluten-free grains (mg/100g d.w.).

Minor cereals	**Thiamine (mg/100g d.w.)**	**CV %**	**%RDA (1.1 mg/100g f.w.)**	**Riboflavin (mg/100g d.w.)**	**CV %**	**%RDA (1.4mg/ 100g f.w.)**
Millet	0.28	49	23	0.02	25	1
Sorghum	0.28	61	23	tr	75	-
Tef	0.22	35	17	0.02	15	1
Wild rice	0.08	28	6	0.17	3	11
Pseudocereals						
Quinoa (white and pigmented)	0.13	50	9	0.02	32	1
Cañihua	0.04	6	3	0.09	8	6
Amaranth	0.03	23	3	0.01	20	1
Chia	0.06	2	5	0.02	20	1

tr: traces

Taking into account the Recommended Daily Allowance (RDA) for thiamine, which is of 1.1 mg/day [39], 100 g of tef contribute approximately to 17 % of the RDA, while

100 g of millet and sorghum to 23 % of the RDA, so as to be declared in label as a "source of thiamine". A portion of 80 g contributes approximately to 16 % of the RDA for tef and to 20% of the RDA for millet and sorghum.

Conclusion

Naturally gluten-free products are corn, rice, potatoes, soybean, millet, buckwheat, tapioca, amaranth, cassava, lentils, beans, sago, sorghum, nuts, as well as meat, fruit and vegetables. Among these, cereals and pseudocereals are becoming increasingly important. This work confirms that minor cereals and pseudocereals are an important source of bioactive compounds. In particular, wild rice and all analysed pseudocereals are good sources of protein. Taking into account the Recommended Daily Allowance (RDA) for vitamins established by the Commission of the European Communities, amaranth, cañihua and quinoa can be declared on the label as a source of vitamin E, the main antioxidant found in cells involved in the prevention of several diseases. Moreover, millet, sorghum and tef can be declared on the label as a potential source of thiamine. Millet also provides a fair amount of lutein. In the light of these results, it is possible to use the combined mix of these flours in order to improve the nutritional value of cereal-based gluten-free products.

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CHAPTER 6

Tocols as product and process markers

Introduction

Bakery products, like biscuits, snacks and croissant loaves, are very popular among people, in particular kids. They contain several ingredients, sugars, cereals and/or pseudocereals, eggs, cocoa, vegetable and animal fats. The quality of baked products is affected by lipids in many ways, including air incorporation, lubrication, heat transfer, tenderness and texture, moisture, mouthfeel, flavor and shelf life (Ghotra et al., 2002). Moreover, these products are a rich source of bioactive compounds, with nutritional and health effects, like vitamin E. Traditionally, in the production of bakery foods, animal fats, like butter and lard, are often employed, but also different vegetable oils and fats, such as corn, sunflower oil, olive oil, palm oil or hydrogenated fats or their blends are used. With the introduction of the Regulation EU, from December 2014, it is mandatory to specify on the label the type of fat used in food, in particular the origin of vegetable oils. The profile of fatty acids (FA) is a fundamental characteristic of every vegetable oil and has traditionally been used as an indicator of vegetable oil authenticity, by using different techniques (gas chromatography, mass spectroscopy, HPLC); however, the sole application of fatty acids profile for the determination of the botanic origin of a mixture of vegetable oils is not sufficient to distinguish one oil from the others (Osorio et al., 2014). Analysis of minor constituents, such as tocols and sterols, has been shown to aid authenticity in vegetable oils, therefore, it is possible to resort to the analysis of tocols grouped under the generic term of vitamin E (Aparicio et al., 2000, Cerretani et al., 2010, Dionisi et al., 1995).

On the basis of this evidence, in a previous work by Mignogna et al. (2015), tocol profile (tocopherols and tocotrienols) was evaluated in different commercial bakery products, to verify the veracity of the information in the label about the vegetable oils used in samples of commercial bakery products.

In this Doctoral thesis, the same type of investigation was carried out, but on shortbread biscuits made *ex-novo* in laboratory, in which the composition and type of vegetable oil or fat used is perfectly known. The development of the method involved the combined use of the profile in fatty acids and tocols of biscuits to demonstrate the correspondence between the profile of these indices and the ingredients used as vegetable oils, fats or their blends. The reported results showed significant differences in the tocol and fatty acids profiles, which reflect the tocol and fatty acids composition of the specific fat used as an ingredient, providing the possibility to use these compounds as markers to identify

the origin of the oils/fats used as an ingredient. Moreover, the obtained data can provide more information on the effects of technological treatments on tocols in biscuits.

6.1 Bakery products

Bakery products are one of the most consumed products in the world (Matsakidou et al., 2010). They play very important roles in the diet of the modern and are widely accessible. In fact, a wide variety of bakery products can be found on supermarket shelves, such as breads, sweetened rolls, doughnuts, croissant, dessert pies, quiche, crackers, cookies and other products. They provide a high quantity of carbohydrates, proteins, minerals and also bioactive compounds, as dietary fibres and different vitamins. The nutritional value of bakery products largely depends on the type and grade of flour, recipe components, as well as the humidity of the products.

The basis of bakery products is wheat flour, which contains a significant amount of carbohydrates, as well as vegetable proteins, but other ingredients can also be added for a better final product.

The main indicator of the nutritional value of the product is the content of nutrients, such as proteins, amino acids, carbohydrates, vitamins and minerals (Minevich et al., 2008).

To improve taste, nutritional and biological value of bakery products, oil and fat can introduce into wheat flour.

Fats play a key role in bakery products, providing desired rheological properties and specific sensory properties (aroma, flavor, softness, volume, palatability, bright appearance), as well as stabilizing the products toward oxidation reactions, stale and moisture migration (Pagani et al., 2010); for these reasons they have been important bakery ingredients for centuries.

In its loosest sense, the term "fat" is often used interchangeably with the term *"shortening"*. Shortening is applied in the broader sense to edible fats used to shorten or tenderize baked products, with many functions in bakery foods (McClements et al., 2010; O‘Brien, 2009; Ghotra et al., 2002):

- tenderness and texture;
- mouthfeel;
- structural integrity;
- lubrication;
- air incorporation;
- heat transfer;
- shelf life extention.

Quantitatively, fats are the second most important ingredients used in bakery products. Therefore, their properties affect quality of the final products, influencing some shelf life and sensory characteristics. Is necessary to know the type and content of oils and fats used. In the production of bakery foods, the bakery industry often uses animal fats, like butter and lard, together with different vegetable oils. The most frequently used ones are margarine, palm oil, manufactured shortenings and butter, all of which contain high levels of saturated fatty acids and, in some cases, trans-fatty acids (Ghotra at al., 2002).

Tocols as product markers

6.2 Method to identify oils and fats in bakery products

Several methods can be used to identify and quantify additions of other fats in olive oil and the origin of other fats/oils in bakery products. Analysis of minor constituents, such as tocols, sterols, has been show to aid authenticity in vegetable oils (Mignogna at al., 2015) in conjunction with fatty acids profile.

Sterols are commonly analyzed using gas chromatography (GC) and are used in olive oil as indicators for the detection of seed oils; however, in all-refined oil mixtures, due to the low and extremely variable sterol level, it is a less useful approach (Osorio et al., 2014).

The *profile of fatty acids* (FA) is a fundamental characteristic of every vegetable oil and has traditionally been used as an indicator of vegetable oil authenticity, by using different techniques (gas chromatography, mass spectroscopy, HPLC); however, the sole application of FA profile for the determination of the botanic origin of a mixture of vegetable oils is not sufficient to distinguish one oil from the other (Osorio et al., 2014);

Tocols (tocopherols and tocotrienols) are well represented in vegetable products, such as cereals (Fratianni et al., 2013; Mignogna at al., 2015; Niro et al., 2019), nuts and oils, showing a qualitative and quantitative variability. In particular, in fats and oils they exhibit differences due to natural variation, different refining processes and their complex profile in vegetable oil blends.

Although tocol profile was shown to be able in discriminating animal fats from vegetable oils and among different vegetable oils, it fails in differentiating those fats whose tocol profile is very similar, such as butter and lard. On the basis of this evidence, in this Doctoral thesis, the possibility to use tocol profile, in conjunction with fatty acids profile, as a methodology to verify the origin of vegetable oils and fats in baked products was investigated. A chemiometric approach, combining the use of tocols and the profile of fatty acids, was used to demonstrate the correspondence between these indices and the fat ingredients used, such as vegetable oils, fats or their blends, so to verify the information given in the label.

6.3 Materials and methods

6.3.1. Preparation of biscuits

Twelve different types of shortbread biscuits were produced at “Parco Scientifico e Tecnologico” of the Molise Region (Campobasso, Italy). Eight samples contained different oils/fats: butter (BF), lard (LF), olive oil (OO), extra virgin olive oil (EVO), sunflower oil (SO), high oleic sunflower oil (HSO), palm oil (PO) and corn oil (CO). Four samples were made with a 50:50 blend of two different oils: sunflower oil/olive oil (SOO), palm oil/olive oil (POO), sunflower oil/palm oil (SPO) and high oleic sunflower oil/palm oil (HSPO) (**Table 5**).

TABLE 10. Oils, fats and their blends used in the twelve different biscuit samples

1.	Butter
2.	Lard
3.	Olive oil
4.	Extra virgin olive oil
5.	Sunflower oil
6.	High oleic sunflower oil
7.	Palm oil
8.	Corn oil
9.	Palm oil / olive oil
10.	Palm oil / sunflower oil
11.	Palm oil / high oleic sunflower oil
12.	Sunflower oil / olive oil

All the ingredients, consisting of 210 g refined soft wheat flour (00), 70 g granulated sugar, 60 g UHT milk, 1 teaspoon chemical yeast, fat/oil (100 g for BF, 80 g for all other fats/oils, 40 g for each fat/oil of the blend), were mixed together. Water was added as a complement to the final weight of the dough (440 g) (**Table 11.**).

Table 11. Formulation of recipes

Butter	*Vegetable oils/lard*	*Oil blends*
210 g plain flour	210 g plain flour	210 g plain flour
70 g sugar	70 g sugar	70 g sugar
60 ml UHT milk	60 ml UHT milk	60 ml UHT milk
1 teaspoon of baking powder	1 teaspoon of baking powder	1 teaspoon of baking powder
100 g butter	80 g oil/lard + 20 g water	40 g oil 1 + 40 g oil 2
440 g Total of dough		

A planetary mixer with a K whisk was used. After a 30 min rest, the dough was fed into the cookie drop machine and round shaped biscuits were collected on a baking tray. Biscuits were then baked at 180 °C for 20 min in a ventilated rotating oven, followed

by cooling at room temperature for 30 min. Samples were grounded with a laboratory mill (IKA A10, Staufen, Germany) and stored at -20 °C until analysis **(Figure 6).**

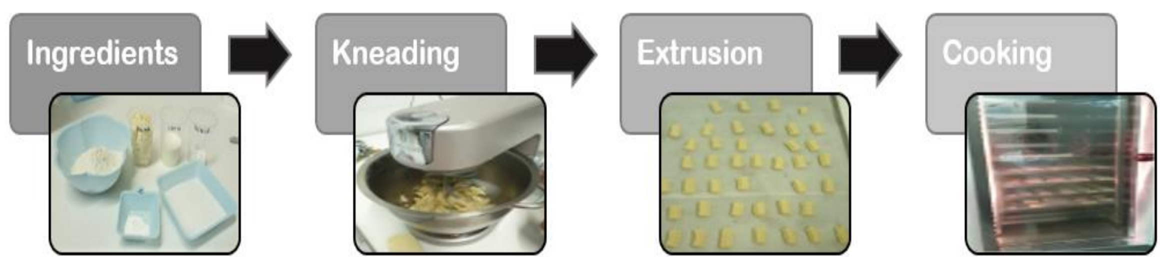


Figure 6. Shortbread biscuit production.

6.3.2. Chemical analysis, tocol analysis, fatty acids analysis

Methods of analysis have been reported in chapter 4.

6.4 Results and discussion

6.4.1. Tocol and fatty acid composition

Both used ingredients and made biscuits were analysed for tocol and fatty acid profiles. **Table 12** reports the mean values of tocols, expressed as mg/100 on dry weight (d.w.), in the used ingredients and in the made biscuits. Results obtained from tocols are given as single forms, as total tocols and as ratio tocotrienols to tocopherols (T3/T). It is worth noticing that, in the analysed bakery products, although fat/oil tocols represent the predominant compounds found, the tocol profile also reflects that of cereal tocols, which are differently distributed in cereals and cereal products (Fratianni, Di Criscio, Mignogna, Panfili, 2012; Fratianni et al., 2013; Mignogna et al., 2015). Products with the highest content of tocols were vegetable oils, while animal fats had the lowest levels. In all analysed animal fats, either butter or lard, the only tocol was α-T. In sunflower oil, olive and extra virgin olive oil α-T was the most representative tocol, followed by low amounts of β-T and γ-T. In corn oil, the tocol at the highest amounts was γ-T3 followed by α-T, δ-T and γ-T3. In all the respective realized biscuits, also α-T3 and β-T3, and β-T in those made with corn oil, were detected. These compounds were also

provided by the flour. Palm oil was characterized by the presence of γ-T3, α-T3, α-T, γ-T and δ-T3. Also in this case, in the realized biscuits, the presence of β-T3 was found. In palm oil and in the respective products the presence of another peak, tentatively identified as α-tocomonoenol (α-T1), was detected, accounting for 5% and 4% of total tocols respectively. The different tocol profile in the analysed oils/fats was confirmed by several papers (Bonvehi, Coll, & Rius, 2000; Ng, Choo, Ma, Chuah, & Ali, 2004; Puah, Choo, Ma, & Chuah, 2007, De Leonardis et al., 2016; Mignogna et al., 2015, Müller, Hammann, & Vetter, 2018).

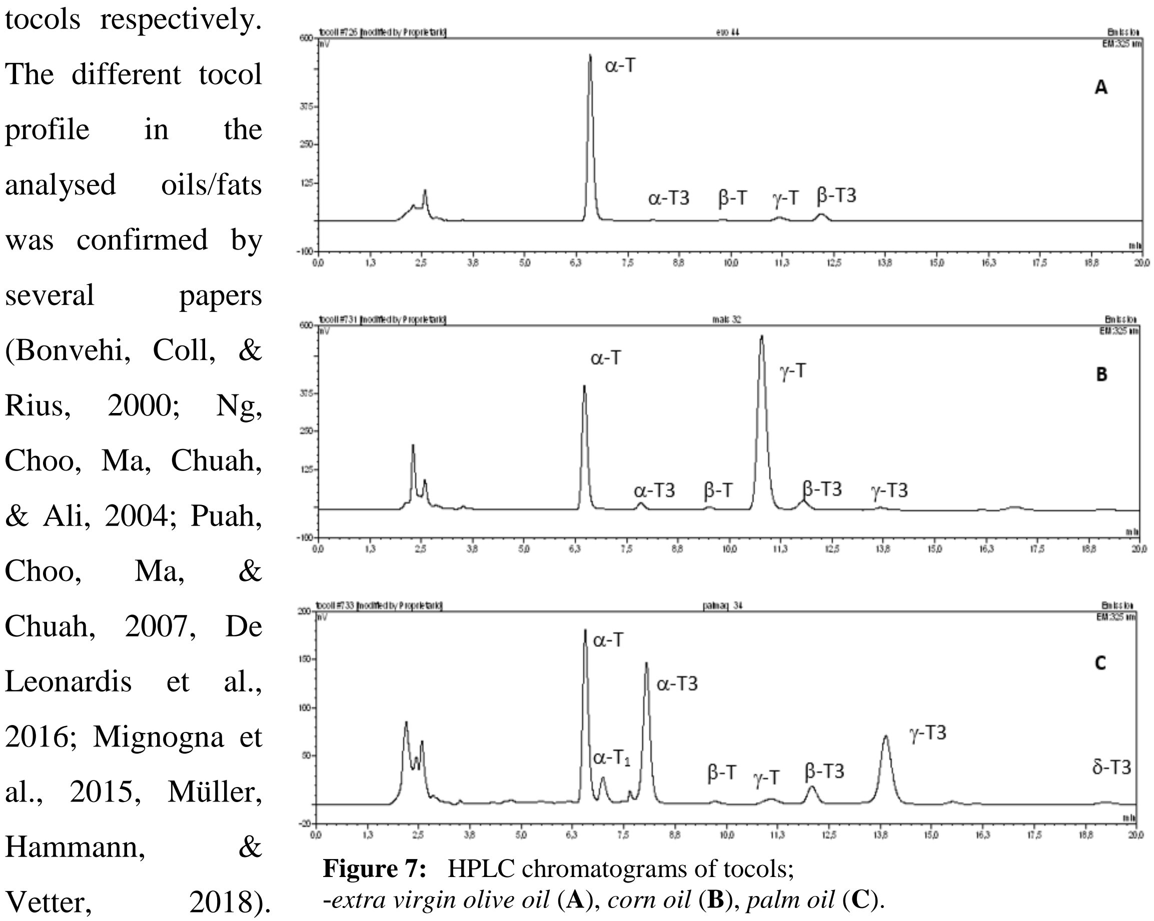


Figure 7: HPLC chromatograms of tocols; -*extra virgin olive oil* (**A**), *corn oil* (**B**), *palm oil* (**C**).

Figure 7 shows the HPLC chromatograms of tocols in bakery products made with extra virgin olive oil (A), corn oil (B) and palm oil (C).

Results of fatty acids in the used ingredients and produced biscuits, expressed as percentages, are reported in **Table 13.** As expected, in butter and butter made products the following acids were detected: miristic (C14:0), capric (C10:0) and lauric acid (C12:0). In both butter and lard samples the most representative fats were oleic (C18:1) and palmitic (C16:0). Among vegetable oils and derived products, corn and sunflower samples were characterized by the predominance of linoleic (C18:2), oleic and palmitic acid. In sunflower and high oleic sunflower oils, low percentages of lauric acid were found. Both in high oleic sunflower, oleic and extra virgin olive oil and the respective biscuits the most representative fatty acids were oleic, linoleic, palmitic and oleic acid (C18:1). In palm oil and its products, palmitic and oleic acid were present at the highest percentages. Except where already specified, in all samples capric and lauric acid were

absent and long chain fatty acids (C20:0, C20:1, C22:0) were present at low amounts or not detected (lard). The reported data on fatty acids are in accordance with those of literature for the same oils and fats (Tsimidou et al., 1987, Dubois, Breton, Linder, Fanni,, & Parmentier, 2007, Devi & Khatkar, 2018). Either for tocols or for fatty acids the 50:50 blends and the respective products reflected the composition of the starting ingredients.

6.4.2. PCA analysis

To validate the suitability of tocols and fatty acids for classifying the different made biscuits, PCA was applied for the categorization of samples. PCA was used to derive the first principal components from the data and to examine the grouping of samples, to visualise the relative distribution of the made biscuits according to their fat/oil contents. For the interpretation of the results, the following parameters were discussed: a) *squared cosines*, measuring the quality of representation on the PCA dimension of both variables and individuals, b) the *coordinates* of individuals on the factor map, c) the linear correlation the variables with the axis (dimensions) and d) the *contribution* in percentage, computed from squared cosines. The score plot of the first three principal components (PC1, PC2 and PC3) for the classification of the made biscuits according to their fatty acid content is shown in **Figure 8a**. The first two PCs accounted for about 62% of the variation in the analysed samples, where PC1 explains 46% and PC2 16% of the variation related to the different fatty acids coming from the different fat/oil ingredients. Moreover, the third component (PC3) explained also the 15% of the variability (Fig 1A). According to the squared cosines, that directly measured the quality of the representation on the PCA map, fatty acids C10:0, C12:0, C14:0 and C18:0 were well represented on the PC1, presenting squared cosines between 0.71 (C18:0) and 0.76 (C14:0). These four fatty acids also shared high and positive correlation with the first dimension (PC1), with values exceeding 0.8, also giving the highest contribution (in percentage) to PC1. Therefore, also C16:1 was positively correlated with PC1 (0.75), with a quite satisfying representation on this axis. Conversely, C20:0 and C20:1 were also moderately well represented (squared cosines equal to 0.50 and 0.54), but they were negatively correlated with the PC1 (-0.71 and -0.73). Considering the second dimension, only fatty acids C16:0, C18:2 and C22:0 presented a squared cosine (goodness of fit) greater than 0.4. The first of them were negatively correlated with PC2, while the latter

two presented a positive correlation with this axis. Apart from those three fatty acids, also C20:0 gave a not negligible contribution (in terms of percentage) to PC1. For what concerns the observations, **Figure 8b** shows that samples with butter (BF) and lard (LF) were the only ones presenting positive coordinates on PC1. Contributes of the three BF samples were essential for PC1 (27%, 22% and 21% respectively), as well as the quality of their representation that was very high (squared cosines between 0.89 and 0.96). In addition, the samples of SOO were well represented in PC1 (squared cosines between 0.61 and 0.77). Considering PC2, POO samples presented the highest squared cosines (between 0.79 and 0.82), while also PO and SO appeared quite well represented on the axis. According to percentages, SO, PO, POO and HSO were the samples that highly contributed to PC2. To summarize these results, in PC2 the contrast between SO and SOO and oils containing palm was evidenced.

Figures 9a and **9b** show the PCA performed using tocols as variables (α-, β-, γ-, δ-tocopherol, α-tocomonoenol, α-, β-, γ-, δ-tocotrienol, the ratio T3/T). The first two PCA dimensions explained the 65% of the variability (40% and 24%). A third dimension was also quite relevant (22% of the variability) (**Figure 8a**). Among tocols, α-T3, γ-T3, δ-T3 and α-T1 were well represented on the PC1, with squared cosines between 0.67 (α-T1) and 0.81 (α-T3), also giving the highest contributions to PC1 in terms of percentages.

Their correlations with PC1 were positive and overall greater than 0.8. Other tocols exhibited a negative correlation with PC1 (**Figure 9a**). Regarding the second dimension (PC2), four tocols were quite well represented (squared cosines around or greater than 0.5): α-T, β-T, δ-T, and γ-T. The correlation with PC2 was negative for the first two tocols and positive in the case of the last two. As expected, the contribution of these variables to PC2 were very high in percentages (between 20% of α-T and the 27% of β-T). By looking at the individuals samples containing palm oil, they were well or quite well represented by the PC1 and positioned in the right part of the individual map (Figure 8B). CO samples mainly contributed to explain the second dimension (PC2), followed by most of the sunflower-based product (SO, SOO and SPO). CO products were positioned at the top-left of the map, while sunflower products without palm oil can be found in the third quadrant. To summarize, if the first axis helps to understand the separation between palm-based products and the others, PC2 opposed CO samples to SO and SOO samples.

In order to verify if tocols and fatty acid profiles taken together provided a better mapping of samples, the PCA was performed using all the previous variables (tocols and fatty acids) (**Figure 10a** and **10b**). The same analysis was also performed on the oil/fat ingredients (**Figure 11a** and **11b**). The first three PCs accounted for about 71% of the variation in the analyzed biscuits, where PC1 explains 32%, PC2 25%, PC3 14% (Figure 3A). In the first dimension (PC1), some variables were almost quite well represented (squared cosines greater than 0.45) and most of them are in the class of fatty acids (C18:.0, C14:0, C20:1, C10:0, C12:0, C16:1). Apart from α-T and C20:1, all these variables were negatively correlated with PC1. Therefore, the most well represented variables for PC2 are four tocols (α-T3, γ-T3, δ-T3 and α-T1) and C16:0. These five variables resulted highly positively correlated with PC2. In Figure 3B, BF and LF samples (in the third quadrant) are well or quite satisfyingly represented by PC1, together with SOO products (fourth quadrant). In PC2, many products with palm oil appeared well represented and positioned at the top of the individual map (with positive coordinates). While the first dimension was mainly driven by BF and LF against other fats/oils, PC2 helped in separating palm-based biscuits from those made with other oils. As far as it concerns fat/oil ingredients (Figure 4A), the first three principal components explained 78% of the overall variance (36%, 27% and 13%, for PC1, PC2 and PC3, respectively). The most contributing variables for PC1 were α-T1, α-T3, γ-T3, δ-T3 and T3/T, C20:0 and C18:3. These variables were positively correlated with PC1 (all values were greater than 0.86) and were well represented on this dimension. The tocols α-T and β-T and the fatty/acids C14:0, C16:0 and C10:0 were the most well represented variables in PC2. Tocols were negatively correlated with PC2, while fatty acids are positively correlated. Observing the individual factor map (**Figure 11b**) it shows a separation between BF and LF (in the second quadrant of the PCA map), palm-based products (depicted in the first and fourth quadrant) and other samples (third quadrant). PO and POO samples were well represented by the first dimension, while BF shared high values of squared cosines for the second dimension.

The combination of fatty acid and tocol profiles allowed a better identification of fats/oils, separating palm oil (PO) and its blended (POO, SPO and HOSPO), poorly misclassified in **Figure 8b**, and improving the separation of close groups in **Figure 9b** (BF and LF).

Table 12. Contents of tocols (mg/100 g d.w.) in the fat/oils and other used ingredients and made biscuits.

Fat/oil	α –T	β –T	γ –T	δ –T	α –T3	β –T3	γ –T3	δ–T3	α –T1	T3/T	Totals
					Ingredients						
OO	14.1 (0.1)*	0.6 (0.0)	1.1 (0.0)	-	-	-	-	-	-	-	15.8 (0.1)
CO	20.1 (0.0)	-	72.6	2.1 (0.1)	-	-	1.4 (0.2)	-	-	0.01 (0.00)	96.2 (2.1)
PO	11.2 (0.7)	0.2 (0.0)	0.9 (0.0)	-	12.6	-	12.6	0.5	2.2 (0.2)	2.08 (0.02)	40.2 (2.5)
LF	0.3 (0.1)	-	-	-	-	-	-	-	-	-	0.3 (0.0)
BF	0.6 (0.1)	-	-	-	-	-	-	-	-	-	0.6 (0.1)
SO	62.2 (1.3)	1.9 (0.5)	0.6 (0.0)	-	-	-	-	-	-	-	64.8 (1.9)
HSO	58.4 (0.9)	2.8 (0.8)	0.3 (0.0)	-	-	-	-	-	-	-	61.5 (1.3)
EVO	16.9 (0.1)	0.9 (0.1)	1.4 (0.1)	-	-	-	-	-	-	-	19.3 (0.1)
SOO	38.2 (0.6)	1.2 (0.2)	0.8 (0.0)	-	-	-	-	-	-	-	40.3 (0.9)
POO	12.7 (0.4)	0.4 (0.0)	1.0 (0.0)	-	6.3 (0.4)	-	6.3 (0.5)	0.2	1.1 (0.1)	0.91 (0.04)	28.0 (1.3)
SPO	36.7 (0.9)	1.1 (0.2)	0.8 (0.0)	-	6.3 (0.4)	-	6.3 (0.4)	0.2	1.1 (0.0)	0.33 (0.02)	52.5 (1.7)
HSPO	34.8 (0.7)	1.5 (0.2)	0.6 (0.0)	-	6.3 (0.4)	-	6.3 (0.5)	0.2	1.1 (0.0)	0.35 (0.01)	50.9 (1.6)
Flour	0.4 (0.0)	0.2 (0.0)	-	-	0.2 (0.0)	1.6	tr	-	-	3.00 (0.02)	2.4 (0.1)
Milk	0.1 (0.0)	-	-	-	-	-	-	-	-	-	0.1 (0.0)
					Biscuits						
OO	2.9 (0.1)	0.1 (0.0)	0.3 (0.0)	-	0.1 (0.0)	0.7		-	-	0.25 (0.01)	4.1 (0.1)
CO	4.2 (0.2)	0.1 (0.0)	14.5	0.4 (0.0)	0.2 (0.0)	0.9	0.2 (0.0)	-	-	0.07 (0.00)	20.4 (0.1)
PO	2.3 (0.2)	0.1 (0.0)	0.3 (0.0)	-	2.2 (0.1)	0.7	2.5 (0.2)	0.4	0.3 (0.0)	2.23 (0.02)	8.8 (0.6)
LF	0.1 (0.0)	0.1 (0.0)	-	-	0.1 (0.0)	0.8	-	-	-	3.70 (0.18)	1.0 (0.1)
BF	0.3 (0.0)	0.1 (0.0)	-	-	0.1 (0.0)	0.7	-	-		2.13 (0.06)	1.2 (0.0)

SO	12.8 (1.3)	0.6 (0.0)	0.2 (0.0)	-	0.1 (0.0)	0.8	-	-	-	0.07 (0.00)	14.4 (1.4)
HSO	10.3 (0.7)	0.5 (0.0)	0.3 (0.0)	-	0.2 0.0)	0.8	-	-	-	0.08 (0.00)	12.3 (0.8)
EVO	5.9 (0.0)	0.1 (0.0)	0.4 0.0)	-	0.1 (0.0)	0.8	-	-	-	0.13 (0.01)	7.3 (0.2)
SOO	9.4 (1.6)	0.3 (0.0)	0.3 (0.0)	-	0.2 (0.0)	0.8	-	-	-	0.10 (0.00)	10.9 (1.7)
POO	3.9 (0.1)	0.2 (0.0)	0.4 (0.1)	-	2.3 (0.1)	0.9	2.4 (0.1)	0.1	0.3 (0.0)	1.27 (0.07)	10.5 (0.7)
SPO	7.5 (0.1)	0.3 (0.0)	0.3 (0.0)	-	1.7 (0.0)	0.8	1.8 (0.2)	0.1	0.5 (0.0)	0.55 (0.04)	13.0 (0.8)
HSPO	5.5 (0.8)	0.2 (0.0)	0.2 (0.0)	-	1.4 (0.2)	0.8	2.2 (0.0)	0.1	0.3 (0.0)	0.78 (0.08)	10.7 (1.1)

* In brackets the standard deviation is reported; tr, traces.

OO: olive oil; **CO**: corn oil; **PO**: palm oil; **LF**: lard; BF: butter; **SO**: sunflower oil; **HSO**: high oleic sunflower oil; **EVO**: extra-virgin olive oil; **SOO**: sunflower/olive oil; **POO**: palm/olive oil; **SPO**: sunflower/palm oil; **HSPO**: high oleic sunflower/palm oil.

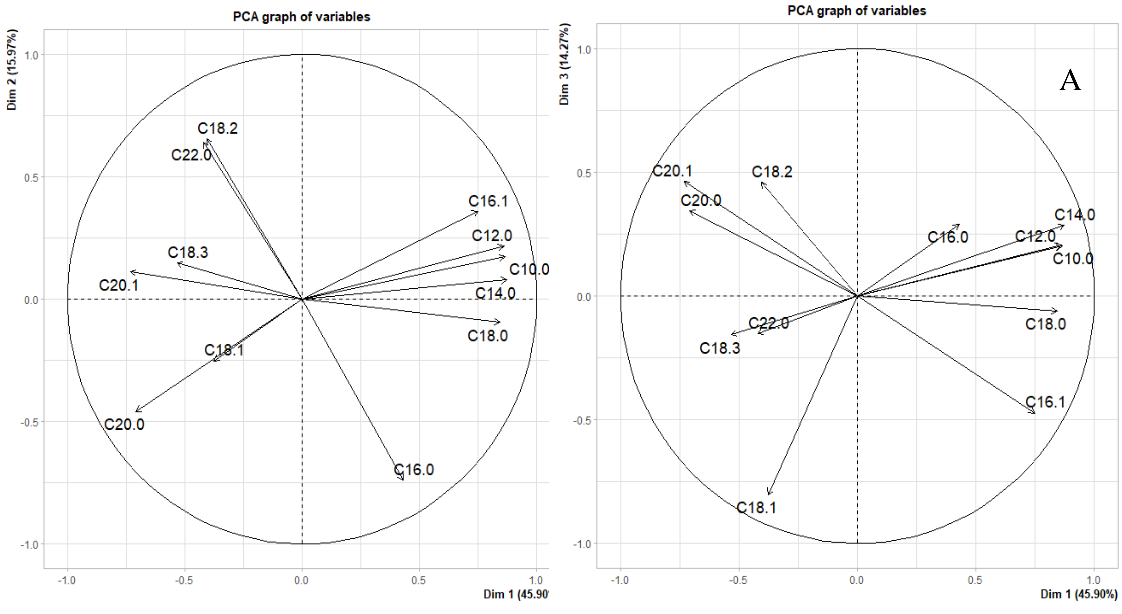
Table 13. Fatty acids (%) in the fats/oils and other used ingredients and in the made biscuits.

Fat/oil	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:0	C18:3	C20:1	C22:0
						Ingredients						
OO	-	-	0.2	18.0 (0.2)	-	4.3	60.1	13.6	1.2 (0.1)	1.3 (0.1)	0.8 (0.1)	0.4 (0.0)
CO	-	-	0.4	13.6 (0.1)	-	8.7	28.3	44.7	1.3 (0.1)	1.1 (0.1)	1.7 (0.1)	0.3 (0.0)
PO	-	-	2.8	38.2 (0.4)	-	5.7	37.5	10.7	1.6 (0.1)	1.7 (0.1)	1.3 (0.0)	0.2 (0.0)
LF	-	-	0.5	27.1 (0.4)	2.4 (0.1)	12.6	40.4	16.0	0.4 (0.1)	0.2 (0.0)	-	0.1 (0.0)
BF	2.9	4.8	16.7	27.6 (0.1)	2.1 (0.1)	10.8	25.6	8.1	0.3 (0.0)	0.3 (0.0)	0.6 (0.1)	0.3 (0.0)
SO	-	0.2	0.4	10.8 (0.1)	-	5.2	23.3	56.2	0.6 (0.0)	0.6 (0.1)	0.9 (0.0)	2.0 (0.1)
HSO	-	0.2	0.4	9.5 (0.2)	0.9 (0.1)	4.7	64.6	15.1	0.6 (0.0)	0.5 (0.1)	0.9 (0.0)	2.7 (0.5)
EVO	-	-	0.2	17.9 (0.4)	-	3.7	60.5	16.2	0.6 (0.1)	0.2 (0.0)	0.4 (0.0)	0.2 (0.0)
SOO	-	0.1	0.3	14.4 (0.2)	-	4.8	41.7	34.9	0.9 (0.0)	0.9 (0.0)	0.8 (0.0)	1.2 (0.3)
POO	-	-	1.5 (0.0)	28.1 (0.1)	-	5.0	48.7	12.1	1.4 (0.1)	1.5 (0.0)	1.0 (0.0)	0.4 (0.0)
SPO	-	0.1	1.6 (0.0)	24.5 (0.1)	-	5.5	30.4	33.4	1.1 (0.0)	1.1 (0.1)	1.1 (0.1)	1.3 (0.3)
HSPO	-	0.1	1.6 (0.0)	23.8 (0.3)	0.4 (0.0)	5.2	51.0	12.9	1.1 (0.0)	1.1 (0.0)	1.1 (0.1)	1.5 (0.3)
Flour	-	-	tr	0.2 (0.1)	tr	tr	0.1 (0.0)	0.7 (0.1)	-	0.1 (0.0)	-	-
Milk	0.1 (0.0)	0.1	0.4 (0.0)	1.0 (0.2)	0.1 (0.0)	0.4 (0.0)	0.9 (0.1)	0.1 (0.0)	-	0.1 (0.0)	-	-
						Biscuits						
OO	-	-	0.5 (0.1)	18.1 (2.0)	0.9 (0.1)	5.3	59.6	12.3	1.3 (0.1)	1.3 (0.2)	1.1 (0.0)	0.4 (0.1)
CO	-	-	0.6 (0.1)	9.8 (0.3)	0.9 (0.0)	6.6	30.5	48.6	1.1 (0.1)	1.3 (0.3)	1.4 (0.3)	0.3 (0.1)
PO	-	-	2.1 (0.1)	41.3 (2.4)	0.1 (0.0)	5.8	36.3	11.5	1.4 (0.4)	0.8 (0.3)	1.3 (0.3)	0.2 (0.0)
LF	-	-	0.8 (0.1)	28.0 (0.7)	2.1 (0.1)	12.2	40.1	16.6	0.2 (0.1)	-	-	0.6 (0.1)
BF	2.6	4.6	15.3	31.1 (3.2)	2.0 (0.2)	10.4	24.7	7.5 (0.4)	0.2 (0.0)	0.3 (0.0)	0.3 (0.1)	0.1 (0.0)

SO	-	0.3	0.6 (0.1)	11.9 (0.3)	0.9 (0.1)	4.2	24.1	53.5	0.9 (0.2)	0.3 (0.1)	1.3 (0.2)	2.7 (0.3)
HSO	-	0.2	0.4 (0.1)	8.4 (0.4)	1.1 (0.0)	3.9	65.4	14.2	0.7 (0.1)	1.8 (0.3)	1.1 (0.1)	3.0 (0.2)
EVO	-	-	0.1 (0.0)	18.5 (0.5)	0.9 (0.1)	4.1	59.7	15.1	0.7 (0.1)	0.5 (0.1)	0.7 (0.1)	0.2 (0.1)
SOO	-	0.1	0.3 (0.0)	13.4 (0.3)	0.4 (0.1)	3.4	48.1	30.3	1.2 (0.3)	1.0 (0.1)	1.1 (0.1)	1.3 (0.2)
POO	-	-	1.3 (0.1)	30.3 (0.9)	0.2 (0.0)	6.7	47.8	11.5	1.3 (0.1)	0.6 (0.2)	0.6 (0.1)	0.2 (0.1)
SPO	-	tr	1.6 (0.1)	30.5 (0.9)	0.1 (0.0)	4.1	28.1	31.1	1.0 (0.0)	1.3 (0.2)	1.0 (0.1)	1.3 (0.1)
HSPO	-	tr	1.5 (0.1)	32.0 (0.6)	0.2 (0.0)	5.8	45.6	10.6	1.3 (0.2)	0.3 (0.1)	1.3 (0.1)	1.5 (0.1)

* In brackets the standard deviation is reported; tr, traces.

OO: olive oil; **CO**: corn oil; **PO**: palm oil; **LF**: lard; BF: butter; **SO**: sunflower oil; **HSO**: high oleic sunflower oil; **EVO**: extra-virgin olive oil; **SOO**: sunflower/olive oil; **POO**: palm/olive oil; **SPO**: sunflower/palm oil; **HSPO**: high oleic sunflower/palm oil.


PCA graph of variables
Dim 2 (15.97%)
Dim 1 (45.90
C18.2
C22.0
C16.1
C12.0
C10.0
C18.3
C20.1
C14.0
C18.0
C18.1
C20.0
C16.0
PCA graph of variables
Dim 3 (14.27%)
A
C20.1
C18.2
C20.0
C14.0
C16.0
C12.0
C10.0
C22.0
C18.0
C18.3
C16.1
C18.1
Dim 1 (45.90%)

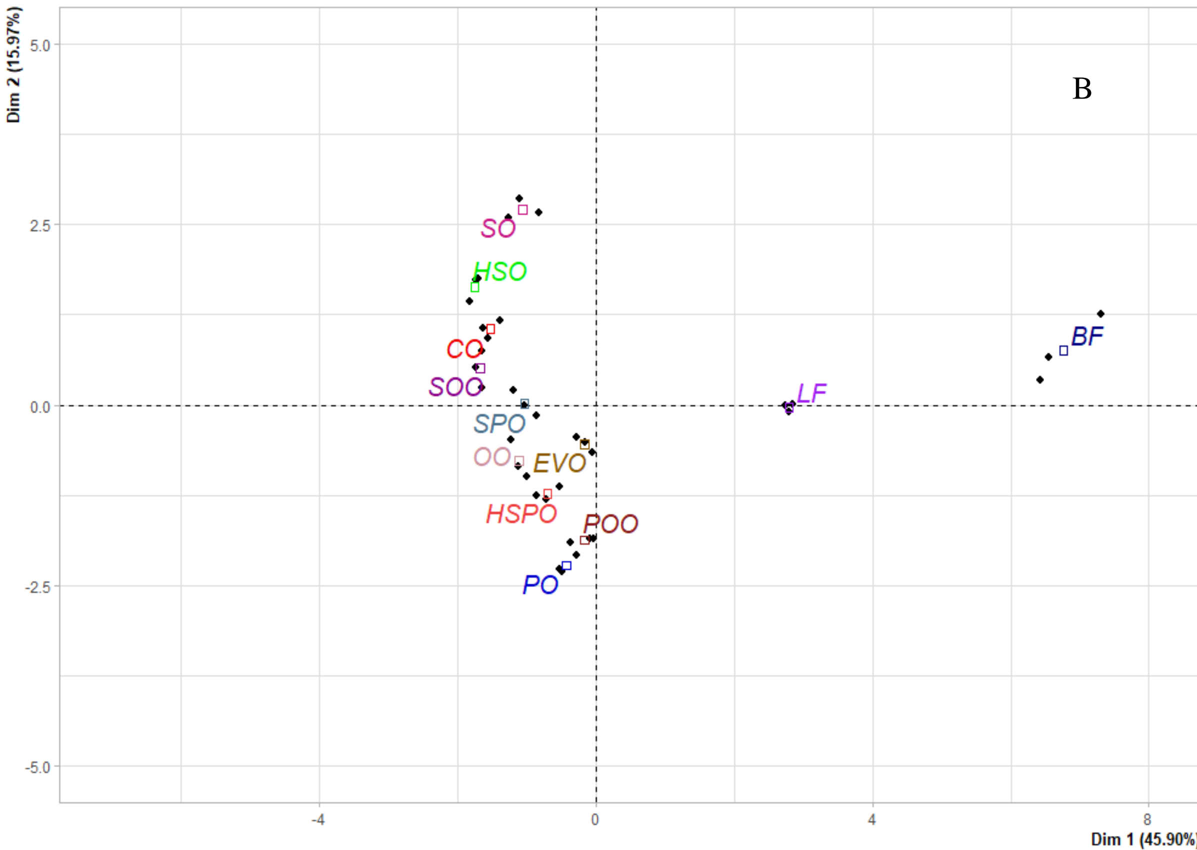
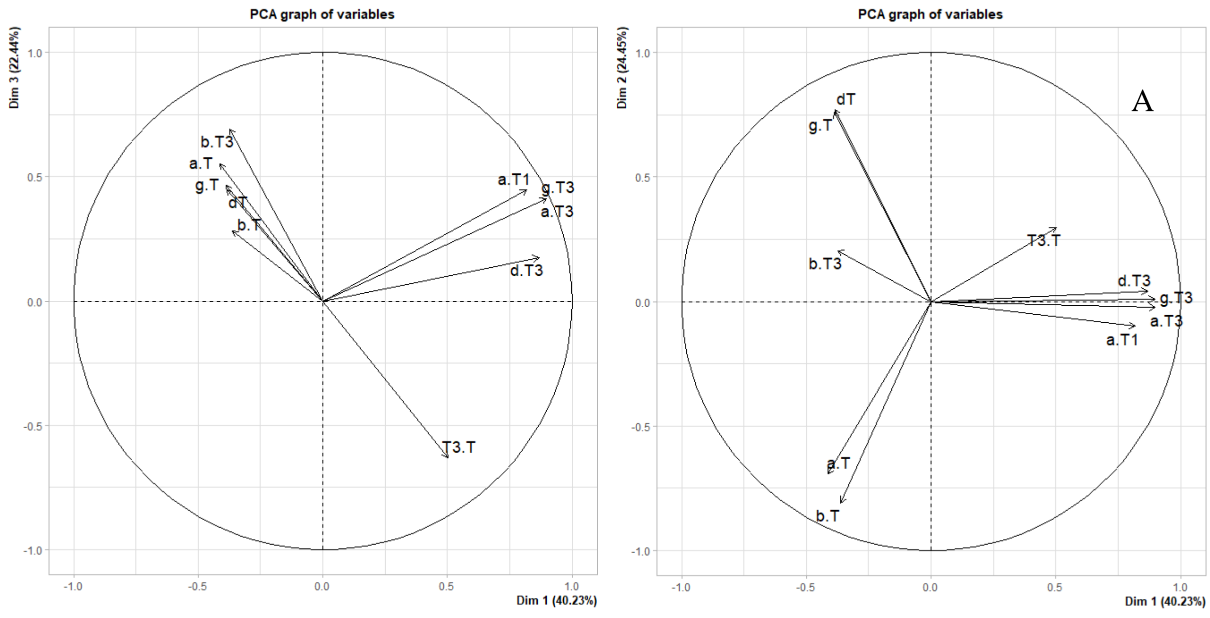

Dim 2 (15.97%)
B
SO
HSO
CO
SOO
SPO
OO
EVO
HSPO
POO
PO
LF
BF
Dim 1 (45.90%)

Figure 8. Score plot of the first three principal components (A) and plot of the principal component analysis (B) with fatty acids as variables, in the made biscuits. For fats/oils identification see Table 7 and Table 8.



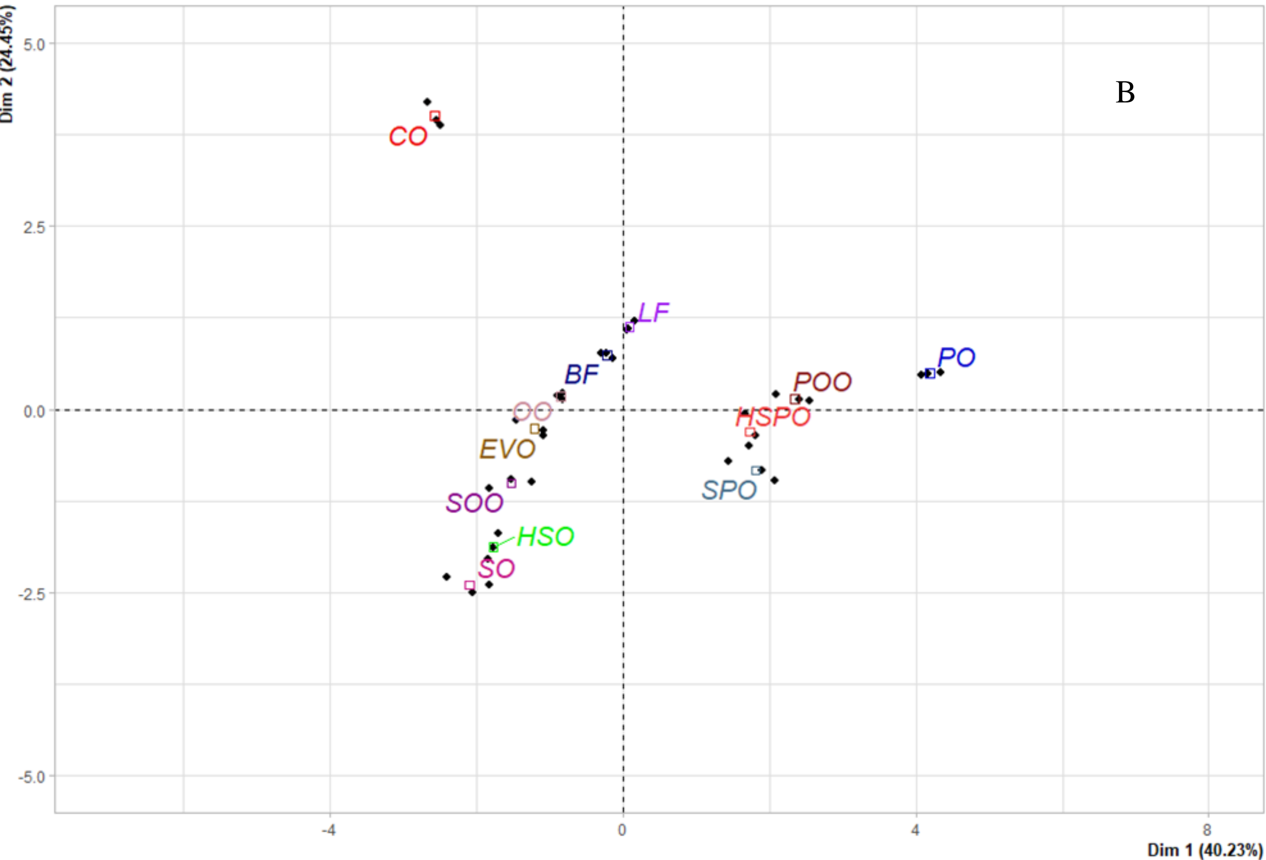


Figure 9. Score plot of the first three principal components (A) and plot of the principal component analysis (B) with tocols as variables, in the made biscuits. For fats/oils identification see Table 7 and Table 8.

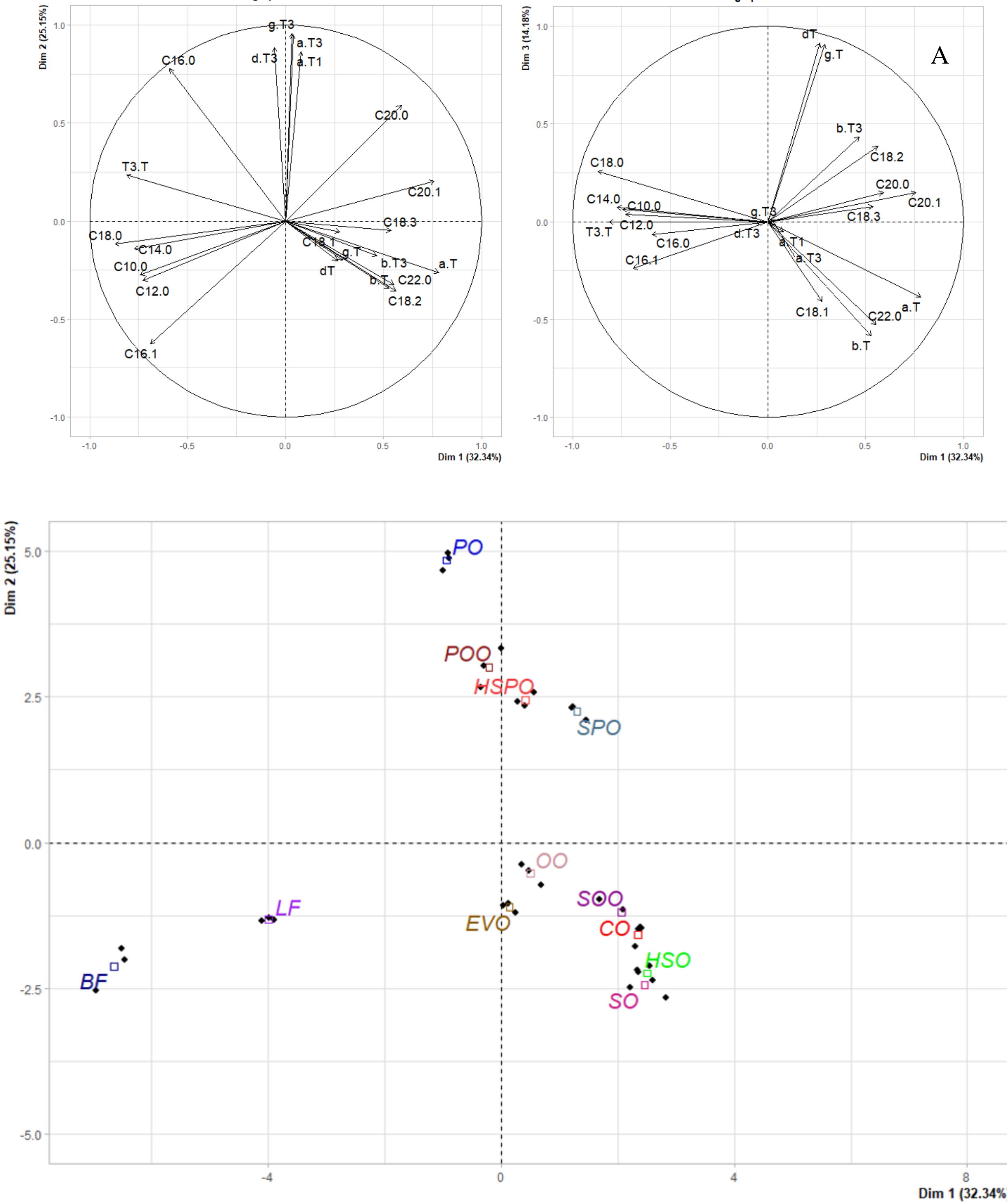
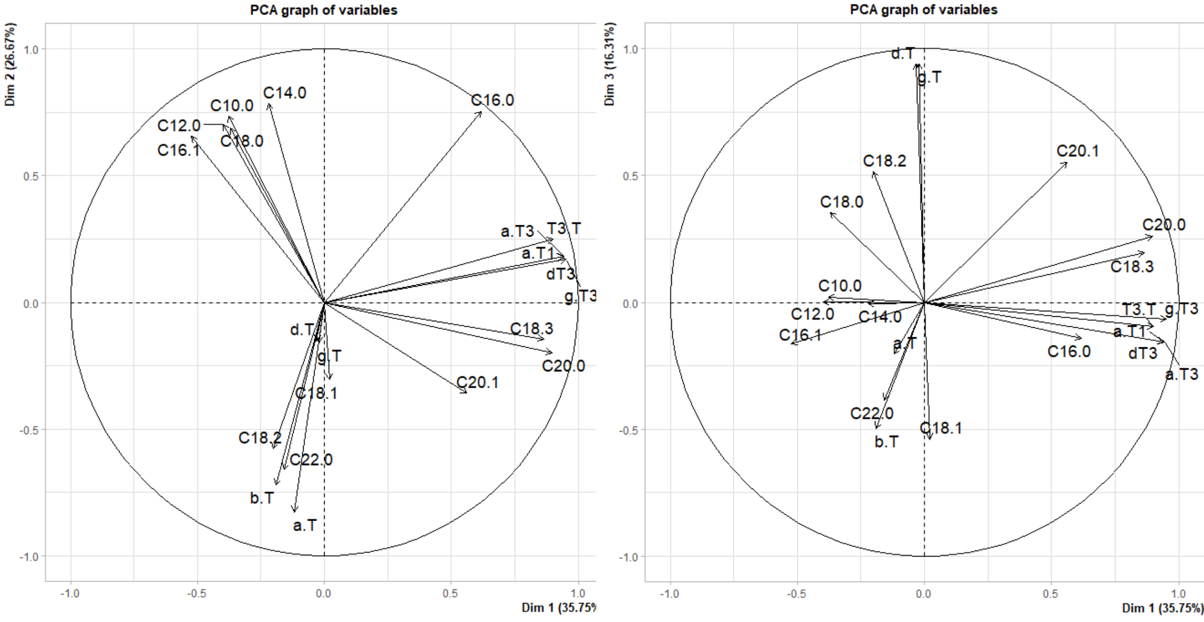


Figure 10. Score plot of the first three principal components (A) and plot of the principal component analysis (B) with fatty acids and tocols as variables, in the made biscuits. For fats/oils identification see Table 7 and Table 8.



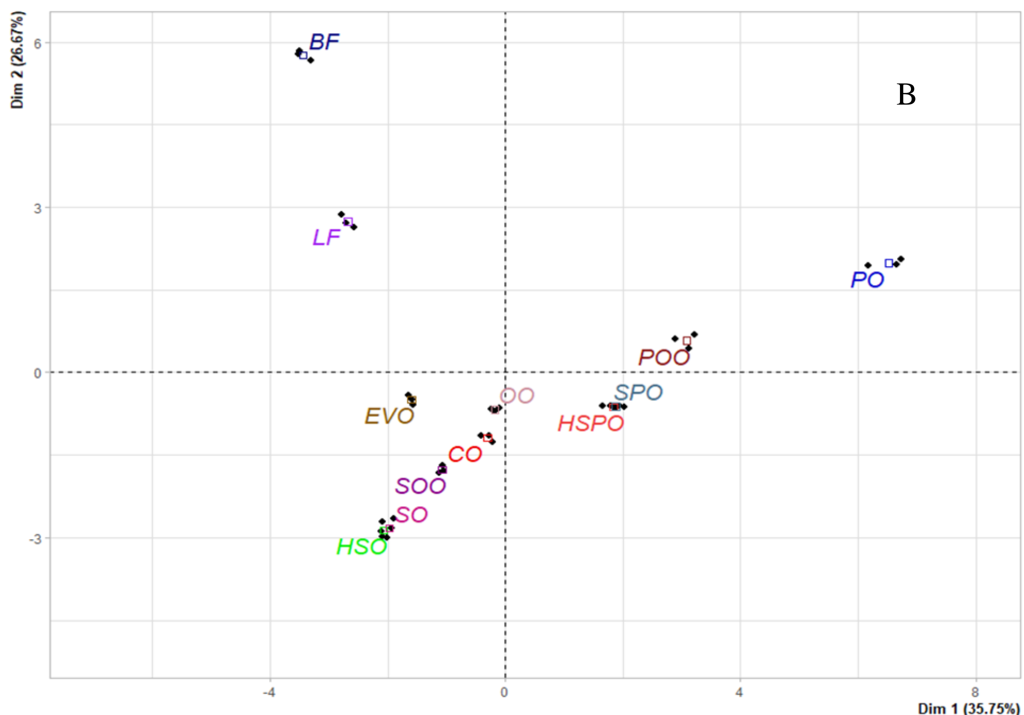


Figure 11. Score plot of the first three principal components (A) and plot of the principal component analysis (B), with fatty acids and tocols as variables, in the fat/oil ingredients. For fats/oils identification see Table 7 and Table 8.

6.5 Impact of cooking on the content of tocols in final bakery products

Tables 14 and **Table 12 s**hows respectively the calculated (from the contents of raw ingredients of **Table 14**) and found tocol contents in the made biscuits. Values are reported as single forms of tocopherols (T) and tocotrienols (T3), and as total tocols (T + T3). The figure (**Figure 12.**) shows the effect of the cooking process on tocol amount, expressed as percentage as to the calculated ones. Tocols losses after cooking varied widely according to Fratianni et al. (2015). The percentage of reduction ranged from a minimum of 11% in biscuits with butter to a maximum of 40% in the palm biscuits. The individual tocols showed a different variation for each type of biscuit. The most sensitive tocotrienol was δ-T3 in palm oil biscuits, specifically, it showed losses of 56% in PO and 75% in POO, SPO, HSPO. α-T1, a typical compound of palm oil, also reported a loss of 67%. The percentage of reduction of β−T3 showed a maximum of 16% in PO and OO.

For tocopherols, losses of β−T were 50% in OO, PO, HSPO and on average of 26% in HSO and EVO. Losses of α-T reached a maximum of 50% in LF. The percentage loss of the γ-T ranges from 19% in CO to 68% in HSPO.

Table 14. Theoretical content of tocols in bakery products (mg/100 g d.w.).

	α–T	α–T3	β–T	γ–T	β–T3	γ–T3	δ–T	δ–T3	α–T1	Totals
OO	3,7	0.1	0.2	0.3	0.8	-	-	-	-	**5.0**
CO	5,1	0.1	0.1	17.8	0.8	0.3	0.5	-	-	**24.8**
PO	3.5	4.2	0.2	0.2	0.8	3.9	-	0.9	0.9	**14.7**
LF	0.2	0.1	0.1	-	0.8	-	-	-	-	**1.2**
BF	0.3	0.1	0.1	-	0.8	-	-	-	-	**1.3**
SO	15.3	0.1	0.5	0.1	0.8	-	-	-	-	**17.1**
HSO	14.6	0.1	0.7	0.1	0.8	-	-	-	-	**16.3**
EVO	4.3	0.1	0.3	0.3	0.8	-	-	-	-	**5.8**
SOO	7.7	0.1	0.3	0.4	0.8	-	-	-	-	**9.3**
POO	2.9	1.7	0.2	0.6	0.8	1.6	-	0.4	0.3	**8.6**
SPO	7.6	1,8	0.3	0.3	0.8	1.6	-	0.4	0.3	**13.1**
HSPO	7.2	1.9	0.4	0.3	0.8	1.6	-	0.4	0.3	**12.9**

OO: olive oil; **CO:** corn oil; **PO:** palm oil; **LF:** lard; **BF:** butter; **SO:** sunflower oil; **HSO:** high oleic sunflower oil; **EVO:** extra-virgin olive oil; **SOO:** sunflower/olive oil; **POO:** palm/olive oil; **SPO:** sunflower/palm oil; **HSPO:** high oleic sunflower/palm oil.

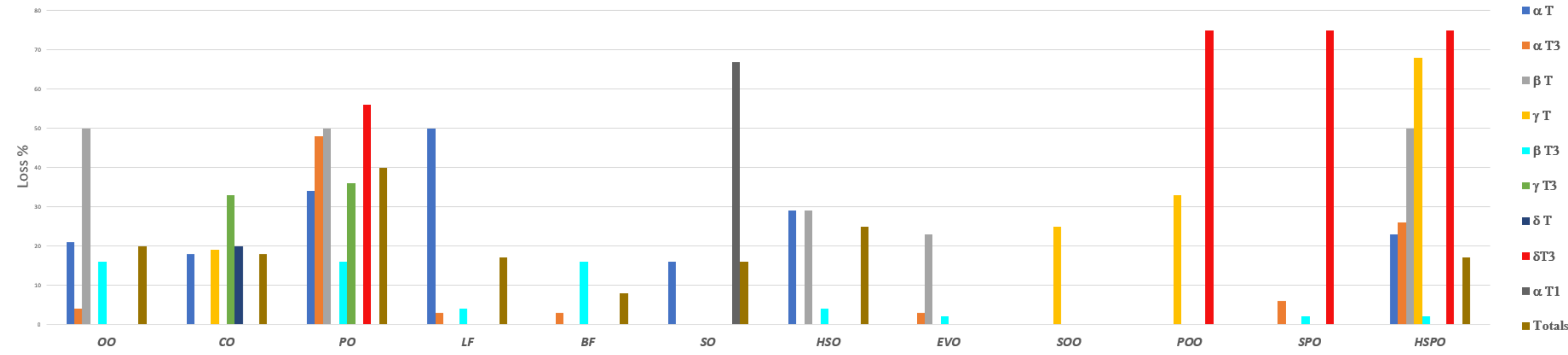


Figure 12. Percentage decrease of tocols after cooking in bakery products.

OO: olive oil; **CO:** corn oil; **PO:** palm oil; **LF:** lard; **BF:** butter; **SO:** sunflower oil; **HSO:** high oleic sunflower oil; **EVO:** extra-virgin olive oil; **SOO:** sunflower/olive oil; **POO:** palm/olive oil; **SPO:** sunflower/palm oil; **HSPO:** high oleic sunflower/palm oil.

6.6 Conclusions

The results of this study demonstrated that the use of tocol profile in conjunction with that of fatty acids, by using a chemiometric approach, such as a PCA analysis, could offer an advantage in the identification of the origin of vegetable oils/fats used as ingredients in bakery products.

A careful validation of the obtained results should be made on commercial samples so to verify the information given in the food label.

Regarding the cooking processes, cooking influences the levels of tocols in the final products to a different extent, depending on the type of oil/fat used.

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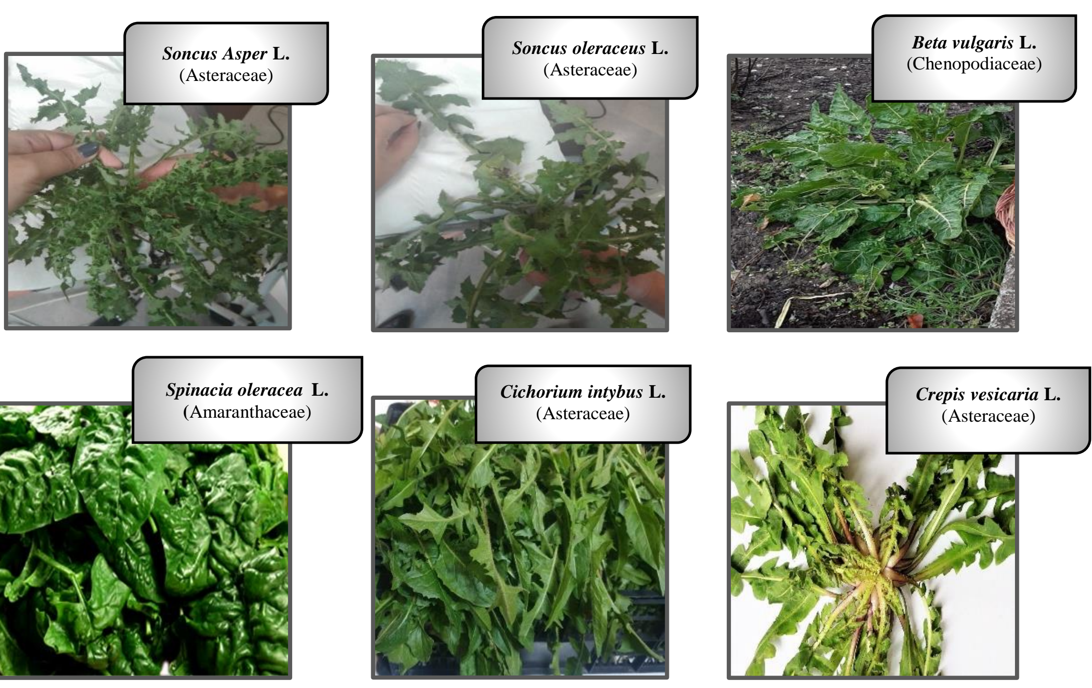
CHAPTER 7

Evaluation of the influence of domestic cooking on bioactive compounds in green leafy vegetables

Introduction

In this section, six different green leafy vegetables, chosen because of their wide diffusion in several traditional recipes of the Mediterranean diet, were investigated: *Sonchus asper* L. *Hill, Sonchus oleraceus L. Crepis vesicaria* L. (Asteraceae), and chard (*Beta vulgaris,* Chenopodiaceae), and two more commercial ones, spinach (*Spinacia oleracea* L.) and chicory (*Cichorium intybus* L.). Plants of *Sonchus* species and *Crepis* were collected during the years 2019–2020, *while Beta vulgaris* in the years 2020-2021. *Sp. oleracea* and *C. intybus* were purchased from local markets. These plants have been characterized by their centesimal composition and bioactive content, with particular reference to the content of carotenoids, tocols, B vitamins (thiamine and riboflavin), poliphenols, flavonoids and antioxidant activity. The results of the discussion and conclusion related to the nutritional composition of *Sonchus asper*, *Sonchus oleraceus* and *Crepis vesicaria*, are reported in the paper attached to this thesis: "*Bioactive Compounds in Wild Asteraceae Edible Plants Consumed in the Mediterranean Diet"* (Panfili et al., 2020). Subsequently, the influence of domestic cooking on bioactive compounds is reported in the second paper: *"Loss or Gain of Lipophilic Bioactive Compounds in Vegetables after Domestic Cooking? Effect of Steaming and Boiling"* (Fratianni et al., 2021). For chard and chicory, the data will be separately reported. Finally, given the positive feedback obtained, functional products were produced.



7.1 Wild edible plants “WEPs”: a challenge for future diet and health

Wild edible plants (WEPs) can be defined as “native species that grow and reproduce naturally in their natural habitat without being cultivated”. Humans have gathered WEPs since ancient times and nowdays they have become part of the human diet and traditional food systems, with numerous studies reported on their therapeutic and nutritional properties (Pieroni A., 1999; Pardo de Santayana et al., 2007).

In some cases, WEPs still play an important role where food crops are scarce, ensuring food sovereignty in some regions of the world, where people still experience hunger or do not have access to a nutritious diet, but they are spreading more and more as an essential component of people diets, providing greater dietary diversity for those who rely on them. Due to their clearly positive influence on health, these foods recognition has a significant contributor to the human, thanks to their high contents of vitamins, phenols, flavonoids, antioxidants, microelements, and fiber. Moreover, WEPs are also perceived as a healthy alternative to cultivated vegetables that might be rich in pesticides and other chemicals. Therefore, WEPs may have great potential as sources of unusual colors and flavors, bioactive compounds and of dietary supplements (Nwafor I. et al., 2017; Gamba M et al., 2020).

Recently, many studies have documented that the Mediterranean Diet meets several important criteria for a healthy diet. Since 2013, the Mediterranean Diet has been recognized as Intangible Cultural Heritage of Humanity. At the base of this diet there are vegetable products, wild fruits and wild vegetables in addition to cultivated ones (Leonti et al., 2006; Rivera et al., 2006). Finally, wild plants are an integral part of local cultural heritage and are often consumed for their socio-economic and environmental sustainability. In other words, their consumption and gathering can provide cultural ecosystem services.

These scenarios and the rediscovery of popular culture has become a topic of scientific investigation, but also a preservation and an enhancement of the local traditions and of the "popular knowledge" as protection of territories. In fact, the increased knowledge of WEPs could also have a useful impact on the agriculture of marginal areas. It should be emphasized that, in light of the above, the promotion of the use of WEPs could play a key role in the future of the Sustainable Development.

The bioactive components and nutritional profiles of the analysed species can contribute, with other ingredients, to improve the nutritional and/or sensory quality of food.

In conclusion, wild plants represent a crucial section of the human diet. It is hoped that an increasing amount of scientific research will focus on plant diversity, traditional knowledge and agricultural studies and will foster bio-conservation strategies and sustainable food production.

7.2 Description of WEPs under study

The study of WEPs focuses on four species*: Crepis vesicaria* L. (s.l*.), Sonchus asper (*L.) Hill s.l., *and Sonchus oleraceus* L. (Asteraceae), and *Beta Vulgaris* L. (Chenopodiaceae). The first three species used in the experimental testing were collected from two different sites in the Molise region, Italy. In particular, samples of *C. vesicaria* were collected in spring, in two consecutive years, namely 2018 and 2019, in the fields of Ripalimosani (altitude 600 m a.s.l., coordinates: Lat. 41° 36′41.25 ″ N, Long. 14° 39′59.58 ″ E) while *S. asper, S. oleraceus* were sampled in the pastures of San Massimo, Matese Massif (altitude 709 m a.s.l., coordinates Lat. 41° 29′19,09 ″ N; Long. 14° 23′29,28 E) in their stage of basal rosette. *Sonchus spp*. samples were first gathered in autumn 2018 and the second harvest occurred in spring 2019. The best season to gather wild plants is spring, when up to 56% species are available, followed by summer (29%), autumn (9%) and winter (6%) (Gonzalez et al., 2011). Only samples of *Beta vulgaris* were collected in spring 2020 and spring and authumn 2021, in the fields of Circello, site in the Campania region, Italy (altitude 650 m a.s.l., coordinates: Lat. 41° 21'21"60 N, Long. 14° 48'35"28 E).

Table 15 reports a synthesis of the data concerning the studied species, harvest sites, years and seasons when samples were collected. With specific reference to these last two criteria it is important to point out that the decision of analysing samples gathered in different years arises from the necessity to assess possible variations of data, depending on the seasonal component.

Table 15. Synthesis of sampling operations.

SPECIES	FAMILIES	PLACE OF COLLECTION	YEAR OF HARVEST
Crepis vesicaria L.	Asteraceae	Fields near Ripalimosani Pastures near S. Massimo - Matese massif	Spring 2018 and 2019
Sonchus asper L. Hill	Asteraceae	Region Molise Fields near Ripalimosani Pastures near S. Massimo - Matese massif	Autumn 2018; Spring 2019
Sonchus oleraceus L.	Asteraceae	Fields near Ripalimosani Pastures near S. Massimo - Matese massif	Autumn 2018; Spring 2019
Beta vulgaris L.	Chenopodiaceae	Region Campania Countryard near Circello	Spring 2020 and 2021; Autumn 2021

7.2.1 Crepis vesicaria

Family: Asteraceae

Botanical description: It is an annual or biannual herbaceous plant (rarely perennial). The stalk appears more or less lignified, reddish-purple at the basis, upright, ramose and bristly. The leaves divide into basal and caulis: the basal leaves are always present, called basal rosette, are rather pressed down on the ground; while, the caulis leaves are smaller and smaller, sessile, auriculate and amplexicaul. The young plant features a series of self-enclosed buds. The flowers are 2 cm capitula with yellow ligulate corollas often featuring numerous reddish streaks; they tend to form a terminal umbel or gather as a corymbiform raceme. The inflorescence has a cylindrical casing with paper-like outer bracts and bristly scales.

Figure 13. *Crepis vesicaria*

Nutritional qualities: This species possesses the same properties as many bitter herbs; therefore, it is detoxifying and purifies the blood, it is diuretic and hypoglycemic The phenolic substances contained in wild plants act as antioxidants of free radicals and help prevent cardiovascular diseases and tumor diseases.

Culinary uses: The tender leaves of *Crepis vesicaria,* commonly known as knot grass, are used boiled and sauteed, to prepare soups, omelets, risotto or as filling for ravioli and savory pies. The tender shoots are kept in salt. In Sardinia ground toasted roots were used as a coffee substitute. (Paura et al., 2021).

7.2.2 *Sonchus asper*

Family: Asteraceae

Botanical Description: Annual or biannual herbaceous plant, growing 3 to 10 dm; its stalks are strong, hollow, streaked, sometimes reddish and poorly ramified. The basal leaves are gathered into a rosette in the first months of growth, from whole to pinnatifid, and later appear rigid, ± leathery, glabrous, bright green on the upper side; the stem leaves are sparse, amplexicaul with snail like winding auricles; with small thorns on the margins. Capitula inflorescences, 3-4 cm in Ø gathered in irregular umbellifer heads with pear-shaped casings 10-15 mm long. The flowers are all ligulate, yellow, sometimes reddish on the outer surface. The fruit are obovate-elliptic with three longitudinal ribs, smooth, without transversal streaks, with a white sessile pappus.

Figure 14. *Sonchus asper*

Nutritional qualities: It contains a good amouny of mineral salts, iron, calcium and phosphorus, vitamins and fiber; it is purifying, refreshing, diuretic and hepatoprotective.

Culinary uses: The culinary uses are quite like those of wild chicory. The leaves of the basal rosette are used mainly in salads. While tender leaves and even adult leaves (despite their bitter taste) are used to prepare omelets or soups such as "minestra ' ‘Terrana’ or the pistic from Friuli. The shoots are cooked and eaten as vegetables,

often mixed with dandelion to temper their bitter taste, or they can be fried with oil and garlic (Paura et al., 2021).

7.2.3 *Sonchus oleraceus L.*

Family: Asteraceae

Botanical description: An annual or biannual wild herbaceous plant. Its basal leaves are at first gathered as a rosette and are quite limp, dull and varying in shape. The flowers are gathered in capitulum inflorescences, bright yellow in the center; the outer ligulae are paler and open in the early morning but close again after a few hours, as they cannot bear the heat.

Figure 15. *Sonchus oleraceus* L.

Nutritional qualities: The leaves are particularly rich in water, which is actually the main component. It is also rich in mineral salts such as iron, calcium, phosphorus and potassium, vitamins, especially Vitamin C, and fibers. The macromolecules have a higher concentration of carbohydrates, followed by proteins. It is deemed to have purifying properties, especially for the liver, hepatoprotective, diuretic properties (therefore the water used to boil them is commonly drunk) and it also has remineralizing properties.

Culinary uses: *Sonchus oleraceus* has delicate organoleptic features. It is used in the same way as wild chicory. We eat the tender leaves of the basal rosette or the tender leafy stem in mixed salads. These parts can be cooked and used to prepare omelets, risotto, fillings, soups and pasta. It can also be boiled in water and sauteed with smoked ricotta.

7.2.4 Beta Vulgaris L.

Family: Chenopodiaceae

Botanical description: Chard is a biennial plant grown as an annual for its rosette of big crinkly leaves and/or wide crunchy stems. Chard leaves presents prominent, enlarged midribs and are borne on stout petioles, have shiny, green, ribbed leaves, with stems that range from white to yellow to red, depending on the cultivar. Its large leaves can grow to more than 30 cm (1 foot) in length and can be continually harvested throughout the growing season. Chard can be harvested while the leaves are young and tender, or after maturity, when they are larger and have slightly tougher stems.

Figure 16. *Beta vulgaris* L.

Nutritional qualities: The leaves are particularly rich in water, which is the main component. It is also rich in vitamins A, K, and C. It is high in fiber and contains riboflavin and B6. Also having significant content in raw chard are vitamin E and the dietary minerals magnesium, manganese, iron, and potassium. Chards are also high in beta-carotene. Raw chard has low content of carbohydrates, protein, fats.

Culinary uses: Fresh chard can be used raw in salads (have a slightly bitter taste), stirfries, soups or omelets. The raw leaves can be used like a tortilla wrap. Mature chard leaves and stalks are typically boiled or sautéed; their bitterness fades with cooking, leaving a refined flavor which is delicate.

7.3 *Cooking conditions*

All samples were prepared with at least twenty randomly chosen specimens for each species. About 500 g of edible part were collected for each species. After harvest, the vegetable material was taken to the laboratory and was accurately cleaned. For each sample, a part of the vegetable material harvested was cut into small portions to carry out the moisture analysis on fresh sample.

Two different cooking methods were tested: conventional boiling and steaming. Cooking conditions were performed by preliminary experiments carried out for each vegetable, considering the minimum cooking time to achieve softness, palatability and taste, according to the consumption habits or to the recipe. Each plant batch was divided into three parts to have at least three repetitions in the experiments. A total of 100 g of leaves was chopped and boiled in a beaker in 1 L of water (1:10 food:water). For conventional boiling, they were cooked for 10 min. For steaming, the portion of the vegetable was placed on a steaming rack over boiling water in a closed water bath for 10 min. The boiling water was drained off for 5 min. After cooking and draining, the cooked portions, the water samples and the fresh controls were freeze-dried.
This operation was carried out with a Genesis 25ES lyophilizer (Vir Tis Co., Gardiner, New York), consisting in a freezing and sublimation chamber, equipped with 4 cooling/warming plates, a condensation chamber and a vacuum pump. The system is equipped with 4 probes which can be inserted into the product before lyophilization for constant monitoring of the product temperature. The whole system is driven by a Data Center Wizard 2.0. programme. Through the management program, the freeze-drying system was set up with the following recipe:

- Initial vacuum 500 mPa
- Heat treatment step -40 ° C for 120 min (time and temperature to which the chamber plates are brought);
- Freezing temperature -35 ° C (temperature that the product must reach);
- Condenser temperature -40 °C.

Finally, the freeze-dried samples were ground with a refrigerated IKA A10 laboratory mill (IKA®-Werke GmbH & Co. KG, Staufen, Germany), carefully mixed and stored at -20°C until analysis. The dried water residue was weighed in order to determine the soluble loss after cooking **(Table 25)**.

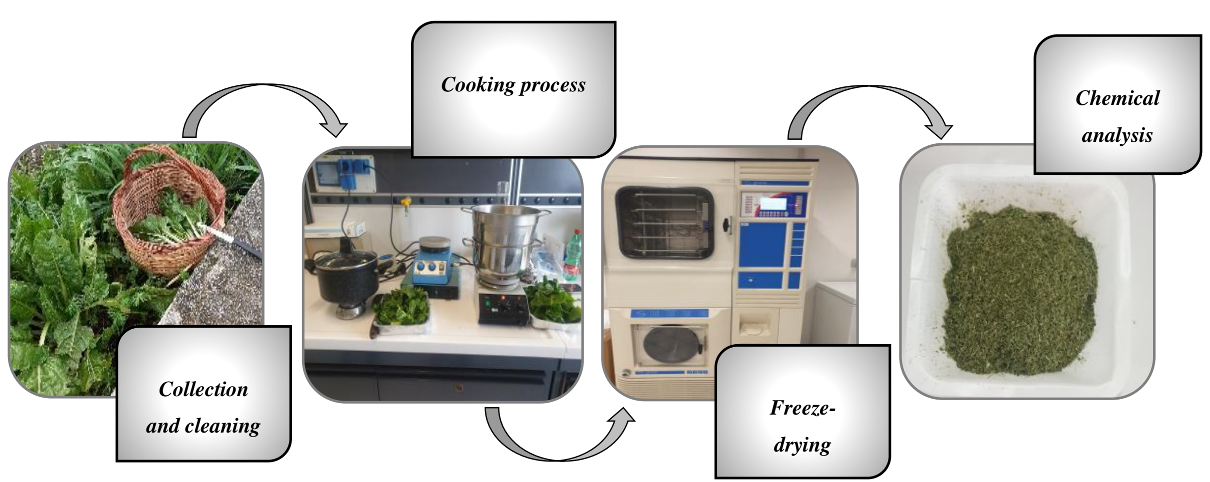


Figure 17. *Steps from plant collection to analysis*

Bioactive compound content of wild edible Asteraceae species present in the Mediterranean diet

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Abstract

Three wild edible species belonging to the Asteraceae family (*Crepis vesicaria L s.l., Sonchus asper L. Hill s.l., and Sonchus oleraceus L.*) were tested for their nutritional composition and carotenoid, tocol, thiamine and riboflavin content. Low amounts of thiamine and riboflavin were found. All species are sources of xanthophylls (violaxanthin, neoxanthin, anteraxanthin, lutein, zeaxanthin and β-cryptoxanthin) and carotenes (α-carotene, β-carotene, 9-cis-β-carotene and 13-cis-β-carotene). Lutein accounts for the highest content (about 4 mg/100 g). They have good tocol amounts, in particular α-tocopherol (about 2-3 mg/100 g). Taking into account the Recommended Daily Allowance (RDA) established by the EU Regulation, the analyzed plants can be declared as a source of fiber, vitamin A and E. These data could be useful for database on the nutritional and bioactive compound profile of studied plants and can contribute in promoting their use in functional foods.

Keywords Wild edible plants · Bioactive compounds · Tocols · Carotenoids · Thiamine · Riboflavin.

Introduction

According to the Food and Agriculture Organization (FAO), over 100 million people in the EU consume wild foods, being a part of people diet around the world and playing an important role in the Mediterranean diet [1]. In the last years, the interest in wild edible plants (WEPs) has quickly grown and a large number of studies on their therapeutic and nutritional properties has been carried out. WEPs have been associated with several health effects against different chronic disorders such as obesity, cancer, cardiovascular diseases, immune deficiency, and brain disorders [1]. Wild plants have high contents of fiber, proteins and different minerals [1–4]; they provide high amounts of bioactive compounds, such as flavonoids, proanthocyanidins, flavonols, vitamin C, tocols (vitamin E), carotenoids (vitamin A) and xanthophylls that can contribute to a healthy condition [1, 2, 5].

Carotenoids and tocols are an important group of bioactive compounds with antioxidant activity and health-promoting properties [6]. Different carotenoids are precursors of vitamin A; moreover, the color of a wide variety of foods depends on their carotenoid content [7]. Tocols, also known as vitamin E, comprise two groups of vitamers, tocopherols and tocotrienols, occurring in eight forms: α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), δ-tocopherol (δ-T) and α-tocotrienol (α-T3), β-tocotrienol (β-T3), -tocotrienol (γ-T3), δ-tocotrienol (δ-T3). Tocols have been demonstrated to prevent certain types of cancer, heart and other chronic diseases [8]. Their main sources are vegetable oils but they are also found in a large amount in different vegetable products [1, 9–13].

Thiamine (vitamin B1) is a water-soluble vitamin involved as a cofactor of several enzymes present in wholegrain, enriched cereals, pork, liver, legumes, nuts and seeds. Riboflavin (vitamin B2) takes part in several flavoprotein enzymes. Milk products, leafy green vegetables, whole cereals provide good amounts of riboflavin [14]. In Italy, the use of alimurgical wild edible species has always been a relevant feature of local cultures; however, WEPs are becoming neglected due to urbanization and the globalization of agriculture. In particular, the Asteraceae family includes numerous native wild edible species usually consumed in the Mediterranean basin, most of which are used as salad vegetables or as vegetable mixtures. By consulting the Italian WEP database (named AlimurgITA) [15], it has been possible to focus on three annual or

biennial species belonging to the Asteraceae family: *Crepis vesicaria* L s.l., *Sonchus asper* L. Hill s.l., and *Sonchus oleraceus* L. They are among the most used plants in Italian and Mediterranean traditional cuisine [1]. In Southern Italy, the WEP mesclun, in fact, represents a base element in the eating habits defined by the term "Mediterranean diet". So far, despite the wide diffusion of the mentioned species, the knowledge of nutritional and bioactive compound profile of alimurgical WEPs is scarce and mostly limited to Sonchus spp. [1, 3, 4, 16, 17]. In this study *Crepis vesicaria* L. (s.l.), *Sonchus asper* (L.) Hill s.l. and *Sonchus oleraceus* L. have been investigated for their nutritional composition with particular regard to carotenoids, tocols, thiamine and riboflavin, in order to highlight their importance in human diet.

Materials and Methods

Plant Material

Plants of *Crepis vesicaria* L. (s.l.), *Sonchus asper* (L.) Hill s.l., and *Sonchus oleraceus* L. (Asteraceae), were collected in two sites of the Molise Region, Italy. *Crepis* samples were harvested in the fields near Ripalimosani (elevation 600 m.a.s.l., coordinates: Lat. 41°36′41.25″N, Long. 14°39′59.58″E) while *Sonchus* samples in the pastures of San Massimo, Matese Massif (elevation 709 m.a.s.l., coordinates Lat. 41°29′19.09″N; Long. 14°23′29.28″E). Plants were gathered during two consecutive years: 2018 (1y) and 2019 (2y), in particular, *Crepis* in spring 2018 and 2019 while *Sonchus* spp. in autumn 2018 and spring 2019. The collected plants have been identified on the basis of their morphological characters, following the nomenclature of Conti et al. [18]. At least 20 specimens of each species were collected, randomly chosen in relation to the basal rosettes status. The basal leaves were harvested and the non edible portion was discarded. From each sample, a minimum of 500 g of edible portion was gathered, cleaned by removing damaged parts and soil particles. Then, the leaves were freeze-dried (Genesis 25SES freeze dryer, VirTis Co., Gardiner, NY) and grounded with a refrigerated IKA A10 laboratory mill (Staufen, Germany), carefully mixed and stored at −20° C. Two bulk samples were prepared by combining the samples of each year and stored in dark at −20° C until analysis. Samples were analyzed in triplicate.

Chemicals and Reagents

Solvents were commercially obtained (Sigma Aldrich) at the highest quality. All other used reagents were of analytical grade. Violaxanthin, neoxanthin α-carotene, 9-cis-β-carotene and 13-cis-β-carotene standards were obtained from CaroteNature (Lupsingen, Switzerland); lutein, zeaxanthin, and β-cryptoxanthin were from Extrasynthese (Z.I. Lyon- Nord, Genay, France). All-trans-β-carotene, thiamine and riboflavin standards were from Sigma Chemicals (St. Luis, MO, USA). α-, β, γ- and δ-tocopherol standards were from Merck (Darmstadt, Germany); α-, β-, γ- and δ-tocotrienol standards were purified as reported in Panfili et al. [12].

Proximate Analysis

Moisture, proteins, fats, ash and fiber were determined according to the AOAC methods [19]. Proteins and fats were determined by the Kjeldhal (N × 6.25) and the Soxhlet method, respectively. Soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) were estimated as in Niro et al. [10]. Total dietaryfiber (TDF) content corresponds to the sum of SDF and IDF.

Carotenoid and Tocol Analysis

The extraction of carotenoids and tocols was carried out according to Panfili et al. [12, 20], with some modifications. About 0.3 g of freeze-dried samples was weighed in a screw-capped tube. Then 5 mL of ethanolic pyrogallol (60 g/L), 3 mL of absolute ethanol, 1 mL of sodium chloride (10 g/L) and 2 mL of potassium hydroxide (600 g/L) for alkaline digestion were added. The tubes were put for 45 min in a 70° C water bath and stirred every 5–10 min. After cooling, 15 mL of sodium chloride (10 g/L) were added. Compounds were extracted with 15 mL of n-hexane/ethyl acetate (9:1, v/v), until the organic layer was colorless. Organic layers were collected and evaporated to dryness. For carotenoid analysis, the dry residues were suspended in 50:50 (v/v) methanol: tert-methyl-butyl-ether (MTBE). Carotenoid extracts were separated, through a HPLC (Dionex, Sunnyvale, CA), as in Mouly et al. [21], by means of a YMC (Hampsted, NC, USA) stainless steel column (250 × 4.6 mmi.d.), packed with 5 μm

silica spheres that were chemically bonded with a C30 material. Methanol: MTBE: water was the mobile phase, at a flow rate of 1 mL/min. Details of the gradient profile are given in the original work. The eluted compounds were detected by a Dionex photodiode array detector set at 430 nm. A Dionex Chromeleon Version 6.6 chromatography system was used to process data. Vitamin A activity was expressed as Retinol Equivalent (R.E.), in µg/100 g of wet basis (WB), as reported by EFSA [22]. For tocol analysis, the dry residues were suspended in 2 mL of isopropyl alcohol (1%) in n-hexane and were analyzed by a Dionex HPLC, using a 250 × 4.6 mm i.d., 5 µm particle size Kromasil Phenomenex Si column (Torrance, CA, USA), as reported in Panfili et al. [12]. Fluorometric detection of all tocols was performed by means of a Dionex RF 2000 spectrofluorimeter, at an excitation wavelength (exc) of 290 nm and an emission wavelength (em) of 330 nm. Compounds were identified by comparing their retention times with standard solutions and through their spectral characteristics, and quantified through the calibration curves of each standard solution. Vitamin E activity was expressed as Tocopherol Equivalent (T.E.) (mg/100 g WB), as in Sheppard et al. [23].

Thiamine and Riboflavin Analysis

The extraction procedure of Hasselmann et al. [24] was applied.0.4 g of sample was weighted in 100 mL volumetricflasks; 20 mL of 0.1 N HCl were added, followed by heatingin a water bath at 100° C for 30 min. Further details are reportedin Niro et al. [10]. A HPLC Dionex (Sunnyvale, CA), with a U3000 pump and an injector loop (Rheodyne, Cotati), was used to separate the extracts, through a 5 µm C18 LunaPhenomenex stainless steel column (250 × 4.6 mm i.d.) (Torrance, CA, USA). The mobi l e phase wasmethanol: sodium acetate (40:60 v/v), at a flow rate of0.8 mL/min. Fluorometric detection was performed at an excof 453 nm and an em of 580 nm, for riboflavin, and at an excof 366 nm and an em of 453 nm, for thiamine, after its derivatizationto thiocrome, by means of a Dionex RF 2000 spectro fluorimeter. A Dionex Chromeleon Version 6.6 chromatographysystem was used to process data. Thiamine and riboflavinwere identified through available standards.

Statistical Analysis

Data were subjected to the analysis of variance (ANOVA). The least significant differences were obtained through an LSD test ($p < 0.05$). A SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results and Discussion

Nutritional Composition

Table 16 reports the nutritional composition of the analyzed WEPs, expressed on wet basis (WB). Fat content was very low (0.3–0.4 g/100 g). No significant differences in fat and ash content were found among samples ($p > 0.05$). *Sonchus oleraceus* contained significant ($p < 0.05$) higher amounts of proteins than the other tested plants (3.0 g/100g as to 1.7 and 1.8 g/100 g of *Crepis vesicaria* and *Sonchus asper*, respectively). Total fiber went from 6.4 g/100 g for *Crepis vesicaria*, to 5.4 g/100 g for *Sonchus asper*, being the insoluble dietary fiber the predominant fraction (from 4.7 g/100 g for *Crepis vesicaria*, to 3.6 g/100 g for *Sonchus asper*). Guil-Guerrero et al. [3] and de Cortes Sánchez-Mata and Tardío [1] report similar results on *Sonchus asper* and *Sonchus oleraceus*. According to the European law [25], *Sonchus asper* and *Sonchus oleraceus* can be declared on the label with the claim "source of fiber", since they contain at least 3 g of fiber per 100 g, while *Crepis vesicaria* can be declared with the claim "high fiber content", since it contains at least 6 g of fiber per 100 g. The reported results are in agreement with different papers, which found green leafy vegetables as rich sources of proteins and fiber [1, 3, 4]. Generally, the chemical composition of plant species could differ, depending on the harvest period and growth conditions (e.g., climate, treatments, rainfall, irrigation, soil quality, etc.). The content of carotenoids (mg/100 g WB) in the investigated plants, in the two harvest years, is reported in **Table 17**. In the present study, 9 carotenoid compounds were detected and identified in all plants: xanthophylls (violaxanthin, neoxanthin, lutein, zeaxanthin and β-cryptoxanthin) and carotenes (α-carotene and β-carotene with its isomers 9-cis-βcarotene and 13-cis-β carotene) (**Fig. 1**). The mean total carotenoid content varied from 10.0 mg/100g in *Crepis vesicaria* to about 15.0 mg/100g in *S. asper*. Lutein was the main carotenoid (range about 3–4

mg/100 g), while β-carotene accounted for about 2–3 mg/100 g. In *Crepis vesicaria* lutein accounted for 35% of total carotenoids, followed by β-carotene (20%), while, in *S. asper* and *S. oleraceus*, lutein and violaxanthin accounted for about 25 and 27% of total carotenoids, respectively, followed by β-carotene (20%). Between the two years of harvesting no difference was found in the qualitative distribution of the different carotenoids while a quantitative variability emerged in both *Sonchus* spp. In these plants appreciable significant ($p < 0.05$) lower amounts of lutein, β-cryptoxanthin, β-carotene, 9-cis-β-carotene and totalcarotenoids were found in the 2019 harvest year.

Table 16. Proximate composition of WEPs (g/100 g WB).

Species	Moisture	Protein	Fat	Ash	Fiber		
					Soluble	*Insoluble*	*Total*
S. asper	89.1 ± 2.68[a]	1.8 ± 0.05[a]	0.3 ± 0.03[a]	1.9 ± 0.02[a]	1.8 ± 0.06[a]	3.6 ± 0.15[a]	5.4 ± 0.51[a]
S. oleraceus	89.3 ± 3.04[a]	3.0 ± 0.13[b]	0.4 ± 0.01[a]	1.5 ± 0.01[a]	1.6 ± 0.03[b]	3.9 ± 0.18[a]	5.5 ± 0.35[a]
C. vesicaria	87.1 ± 3.52[a]	1.7 ± 0.00[a]	0.3 ± 0.02[a]	1.7 ± 0.18[a]	1.7 ± 0.05[a]	4.7 ± 0.20[b]	6.4 ± 0.74[b]

Values are expressed as mean ± standard deviation (n = 3). WB–wet basis. Total fiber–sum of soluble and insoluble fiber. Different letters within the same column indicate a significant difference ($p < 0.05$)

Table 17. Content of carotenoids in WEPs (mg/100 g W.B.) in the two harvest years.

Compound		*C. vesicaria*	% tot	*S. asper*	% tot	*S. oleraceus*	% tot
Violaxanthin	1y	1.1[a]		3.4[a]		3.9[a]	
	2y	1.4[a]		4.4[a]		3.1[a]	
	Mean	1.3	13	3.9	26	3.9	28
Neoxanthin	1y	0.9[a]		1.7[a]		1.7[a]	
	2y	1.1[a]		1.3[a]		1.2[a]	
	Mean	1.0	10	1.5	10	1.5	11
Lutein	1y	3.9[a]		4.9[a]		4.8[a]	
	2y	3.1[a]		3.1[b]		3.1[b]	
	Mean	3.5	35	4.0	27	3.1	22
Zeaxanthin	1y	0.9[a]		0.4[a]		0.2[a]	
	2y	1.7[a]		0.5[a]		0.2[a]	
	Mean	1.3	13	0.5	3	0.2	1
β-Cryptoxanthin	1y	0.1[a]		0.2[a]		0.2[a]	
	2y	0.1[a]		0.1 [b]		0.1[b]	
	Mean	0.1	1	0.1	< 1	0.1	< 1
α-Carotene	1y	0.2[a]		0.7[a]		0.6[a]	
	2y	0.3[a]		0.5[a]		0.4[a]	
	Mean	0.2	2	0.6	4	0.5	3
13-Cis-β-carotene	1y	0.1[a]		0.1[a]		0.1[a]	
	2y	0.1[a]		0.1[a]		0.1[a]	
	Mean	0.1	1	0.1	< 1	0.1	< 1
β-Carotene	1y	2.0[a]		3.8 [a]		3.5[a]	
	2y	2.0[a]		2.3 [b]		2.0[b]	
	Mean	2.0	20	3.0	20	2.8	20
9-Cis-β-Carotene	1y	0.5[a]		1.1 [a]		1.0[a]	
	2y	0.6[a]		1.0[b]		0.7[b]	
	Mean	0.5	5	1.0	7	0.8	6
Total carotenoids	1y	9.5[a]		16.2[a]		16.0[a]	
	2y	10.5[a]		13.3[b]		11.1[b]	
	Mean	10.0		14.7		14.0	
R.E. (μg/100 g WB)		408.0		606.8		644.8	

Values are expressed as mean (n = 3). WB–wet basis. R.E. – Retinol Equivalent [22]1y 2018, 2y 2019. Different letters within the same column indicate a significant difference ($p < 0.05$).

This variability could be due to the harvest period and environmental conditions (temperature, humidity, etc.). For *Crepis vesicaria*, where samples were collected in the same season of the two years, no significant differences ($p > 0.05$) were found, so the results of *Sonchus* WEPs may suggest a major role of seasonality in influencing the carotenoid content of these species that were, instead, collected during different seasons of the two years. Table 2 also reports values of vitamin A, expressed as Retinol Equivalent (R.E.) (µg/100 g WB) [22].

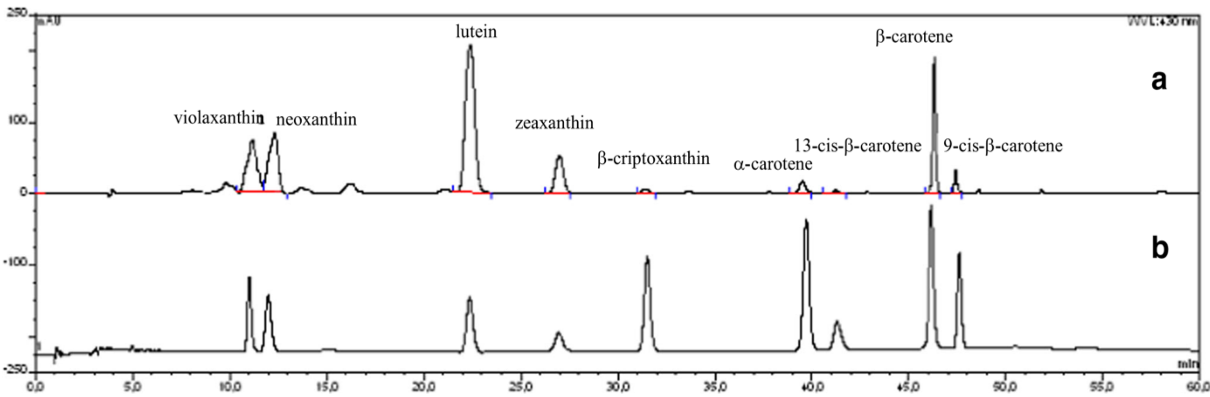

Figure 18. Typical chromatogram of carotenoids of *Crepis vesicaria* (a) and a standard mix (b).

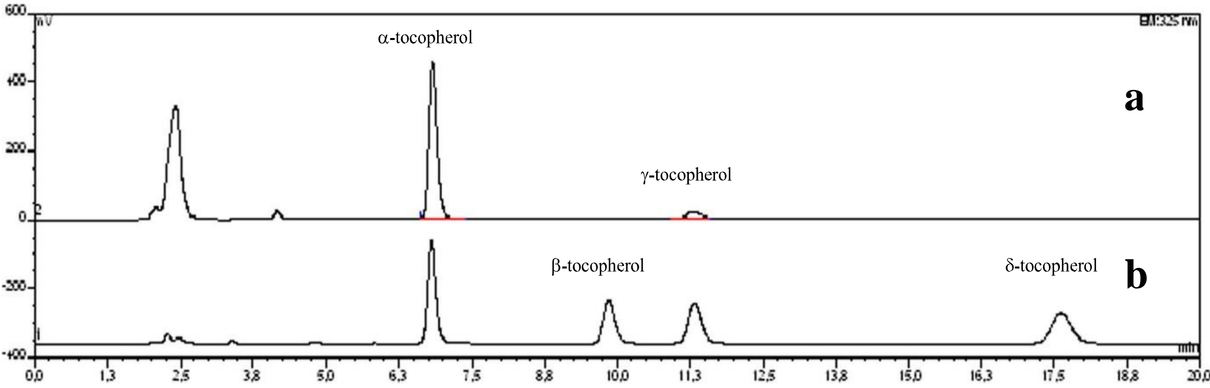

Figure 19. Typical chromatogram of tocols of *Crepis vesicaria* (a) and a standard mix (b).

Taking into account the Recommended Daily Allowance (RDA) for vitamin A, which is 800 µg/day [26], 100 g of leaves contribute from 50% of the RDA in *Crepis vesicaria* to about 80% in *S. oleraceus*, so that all plants can be declared as a "source of vitamin A". Moreover, a mean portion of 200 g provides about 7–8 mg of lutein. According to Alves-Rodrigues and Shao [27], the intake of 6 to 14 mg of lutein per day is associated with more than 50% reduction in risk for age-related macular degeneration (AMD) and

cataract. Our data of *Sonchus* species are confirmed by other literature studies [1, 3, 28]. No data are available for *Crepis vesicaria*. Several authors studied the total carotenoid in species traditionally consumed in the Mediterranean area, with contents going from 2.5 mg/100 g for *Bellis perennis* to 13.3 mg/100 g for *Allaria Petiolata* [1]. Different green leafy vegetables (spinach, chicory, broccoli, lettuce, watercress) were confirmed to be rich sources of lutein [1, 29–31] and good sources of β- carotene, with contents in a range similar to those found in most WEPs [1, 29, 32]. It is difficult to compare the different literature data related to the HPLC analysis of carotenoids, since the available results have been obtained by different analytical methods and these pigments may vary depending on genotype, weather conditions, maturity stage, location, part of the analyzed plant, seasonality [31]. Tocol composition and content (mg/100 g WB) are shown in **Fig. 18** and **Table 18**.

Table 18 also reports values of vitamin E activity provided by 100 g of product, expressed as Tocopherol Equivalent (T.E.) (mg/100 g WB) [23]. The main detected tocopherol was α-tocopherol, which was found in all the studied species and provided about 86% of total tocols; β-tocopherol was detected only in *S. asper*, at less than 0.1%. *Crepis vesicaria* showed the highest value of γ-tocopherol (0.6 mg/100 g), followed by *S. asper* and *S. oleraceus* (0.4and 0.3 mg/100 g, respectively). No tocotrienols were detected. The variability of the tocol content of the investigated samples between the two harvest years showed, in *Sonchus* species, significantly higher values for α-tocopherol and total tocols in plants collected during spring 2019 ($p < 0.05$). Comparison with literature data is very difficult, due to the very few available data and to the same reasons already seen for carotenoids.

Table 18. Content of tocols in WEPs (mg/100 g WB) in the two harvest years

Compound		***C. vesicaria***	**% tot**	***S. asper***	**% tot**	***S. oleraceus***	**% tot**
α-Tocopherol	1y	3.7[a]		2.0[a]		1.7[a]	
	2y	3.3[a]		3.5[b]		2.2[b]	
	Mean	3.4	85	2.8	87	1.9	86
γ-Tocopherol	1y	0.6[a]		0.3[a]		0.3[a]	
	2y	0.7[a]		0.5[a]		0.4[a]	

	Mean	0.6	15	0.4	13	0.3	14
Total tocopherols	1y	4.3[a]		2.3 [a]		2.0[a]	
	2y	4.0[a]		4.0[b]		2.6[b]	
	Mean	4.1		3.2		2.3	
T.E. (mg/100 g WB)		3.6		2.9		2.1	

Values are expressed as mean (n = 3). WB−wet basis. T.E. Tocopherol Equivalent [23] 1y 2018, 2y 2019. Different letters within the same column indicate a significant difference ($p < 0.05$)

Table 19. Content of thiamine and riboflavin in WEPs (mg/100 g WB) in the two harvest years.

Species		Thiamine	% RDA	Riboflavin	% RDA
S. asper	1y	0.12[a]		0.01[a]	
	2y	0.07[b]		0.01[a]	
	Mean	0.09	8	0.01	0.7
S. oleraceus	1y	0.10[a]		0.02[a]	
	2y	0.09[a]		0.01[a]	
	Mean	0.10	9	0.01	1.2
C. vesicaria	1 y	0.15[a]		0.01[a]	
	2y	0.12[b]		0.03[a]	
	Mean	0.13	12	0.02	1.4

Values are expressed as mean (n = 3). WB– wet basis. RDA Recommended Daily Allowance [26] 1y–2018, 2y–2019.Different letters within the same column indicate a significant difference ($p < 0.05$)

References for tocols are not available for *Crepis vesicaria*, while, for *Sonchus* species, similar results are reported by Morales et al. [16], Petropoulos et al. [17] and Sánchez-Mata and Tardío [1], with amounts of α-T going from 0.29–1.75 mg/100g in *Sonchus oleraceus*. Similar tocol amounts and T.E. are found in other green vegetables [33]. For

WEPs and some Asteraceae species, tocol contents are of the same or lower order of magnitude [1, 16]. Conforti et al. [34], in *S. oleraceus* and *S. asper*, found no tocols; this finding could be probably due to the different extraction method used, which does not include a saponification procedure and therefore it is not able to hydrolyze esters and eventually present bound forms of tocols. The Recommended Daily Allowance (RDA) for vitamin E is 12 mg/day [26]; therefore, 100 g of *Crepis vesicaria*, *S. asper* and *S. oleraceus* contribute approximately to about 30, 24 and 18% of the RDA, respectively, to be declared as a “source of vitamin E”. **Table 19** reports the amounts (mg/100 g WB) of thiamine and riboflavin of analyzed plants in the two harvest years. Good amounts of thiamine and low contents of riboflavin were found in all species. Some slightly significant ($p < 0.05$) differences for thiamine were found between years for *Crepis vesicaria* and *S. asper*, but for the few found amounts, these data need further investigation. Since the RDA for thiamine is of 1.1 mg/day [26], 200 g of all species contribute approximately to 15% of the RDA, so that to be declared “as a source of thiamine”. Data about B-complex vitamins in WEPs is very scarce, but contents in literature are quite in accordance with those of some green leafy vegetables [1, 35]. Similar data on thiamine (4–32 μg/100 g), but higher amounts of riboflavin (71–101 μg/100 g) were reported by Sánchez-Mata and Tardío [1] for *S. asper*.

Conclusion

The analyzed WEPs resulted as rich sources of fiber, carotenoids and tocols, such as to encourage an in depth investigation on the health potentiality of these plants and to justify their future commercial production, given the consumer and industry increasing demands for healthy foods. First results demonstrated the differences between different harvest years. Future researches are needed in order to better investigate the different compositional variations due the stage of maturity, climate or season, eventually farming practices, as well as the effect of the commonly used food processing on the content of nutritional compounds.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

Human and Animal Participants This article does not contain any studies with human or animal subjects.

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7.4 Results and discussion of *Spinacea Oleracea, Cicoria intybus, Beta vulgaris*

7.4.1 Nutritional composition

In **Table 20** the chemical composition of spinach, chicory and chard, expressed on wet basis (W.B.), is reported. The fat content was very low (0.2–0.6 g/100 g W.B.). The protein content of chard was 3.6 g/100 g W.B., higher than that of spinach and chicory (2.4 g/100 g W.B. and 1.8 g/100 g W.B., respectively). Spinach and chard had a very similar total fiber content (3.3 - 3,2 g/100 g W.B., respectively), with the prevalence of the insoluble fraction (2.6 - 2.5 g/100 g W.B.) to the soluble fraction (0.7 - 0.8 g/100 g W.B.). Chicory showed slightly lower values of total fiber, 2.0 g/100 g B.W. According to the European law (EC 1924/2006), spinach and chicory can be declared on the label with the claim "source of fiber", since they contain at least 3 g of fiber per 100 g.

Values are expressed as mean ± standard deviation (n = 3). W.B. –wet basis. Total fiber–sum of soluble and insoluble fiber.

Table 20. Proximate composition of green leafy vegetables (g/100 g W.B.).

Species	Moisture	Protein	Fat	Ash	Fiber		
					Soluble	*Insoluble*	*Total*
Sp. oleracea	92.2 ± 0.7	2.4 ± 0.0	0.2 ± 0.0	1.9 ± 0.0	0.7 ± 0.0	2.6 ± 0.0	3.3 ± 0.1
C. intybus	94.1 ± 0.8	1.8 ± 0.0	0.1 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	1.3 ± 0.0	2.0 ± 0.1
B. vulgaris	87.6 ± 0.4	3.6 ± 0.1	0.6 ± 0.1	1.8 ± 0.0	0.8 ± 0.1	2.5 ± 0.2	3.3 ± 0.2

The content of carotenoids (mg/100 g W.B.) in the investigated plants, is reported in **Table 21**. The mean of total carotenoid content did not show significant differences beetween spinach and chard (17.0 - 16.9 mg/100 g W.B.), while in chicory it was about 14.0 mg/100 g W.B. Lutein was the main carotenoid in spinach and chard (range about 8–9 mg/100 g W.B.), while β-carotene accounted for about 3–4 mg/100 g W.B. and was the main carotenoid in chicory (4 mg/100 g W.B.). Taking into account the Recommended Daily Allowance (RDA) for vitamin A, which is 800 µg/day (EC 1924/2006), 100 g of leaves contribute from 71% of the RDA in *B. vulgaris* to 99 % in C. intybus, so that all plants can be declared as a "source of vitamin A".

Table 21. Content of carotenoids in the analyzed green leafy vegetables (mg/100 g W.B.).

Compound	*Sp. oleracea*	*C. intybus*	*B.vulgaris*
Violaxanthin	1.5 ± 0.1	0.8 ± 0.0	1.6 ± 0.1
Neoxanthin	1.6 ± 0.1	1.1 ± 0.1	1.1± 0.1
Lutein	8.5 ± 0.4	6.1 ± 0.2	8.8 ± 0.4
Zeaxanthin	0.7 ± 0.0	0.5 ± 0.0	1.1 ± 0.1
α-Carotene	0.6 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
13-Cis-β-carotene	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.0
β-Carotene	3.1 ± 0..2	4.0 ± 0 .5	2.9 ± 0.1
9-Cis-β-Carotene	0.9 ± 0.0	0.8 ± 0.0	0.5 ± 0.0
Total carotenoids	17.0 ± 0.9	14.0 ± 0.7	16.9 ± 1.1
R.E. (μg/100 g WB)	600	742	567
% RDA (800 μg/100g W.B.)	75	99	71

Values are expressed as mean (n = 3). W.B.–wet basis. R.E. – Retinol Equivalent (EC 1924/2006).

Table 22 reports values of vitamin E activity provided by 100 g of product, expressed as Tocopherol Equivalent (T.E.) (mg/100 g W.B.). The main detected tocopherol was α-tocopherol, with amounts going from 2.1 - 2.5 mg/100 g W.B.; γ-tocopherol was detected in less amounts, 0.2 – 0.3 mg/100 g W.B. No tocotrienols were detected.

The Recommended Daily Allowance (RDA) for vitamin E is 12 mg/day (EC 1924/2006); 100 g of all leafy vegetable contribute approximately to more than 15% of the RDA, so to be declared as a “source of vitamin E”.

Table 23 reports the amounts (mg/100 g W.B.) of thiamine and riboflavin of analyzed plants. Good amounts of thiamine and low contents of riboflavin, total absent in chicory, were found in all species. Chard had the higher amounts of thiamine (15.9 mg/100 g W.B.). Since the RDA for thiamine is of 1.1 mg/day (EC 1924/2006), 200 g of all species contribute approximately to 15% of the RDA, while for chard 10 g contribute to 100%.

Table 22. Content of main tocols in green leafy vegetables (mg/100 g W.B.).

Compound	*Sp. oleracea*	*C. intybus*	*B.vulgaris*
α-Tocopherol	2.5 ± 0.24	2.3 ± 0.10	2.1 ± 0.09
γ-Tocopherol	0,3 ± 0.01	0,2 ± 0.01	0.2 ± 0.01
Total tocopherols	2.8 ± 0.02	2.5 ± 0.05	2.3 ± 0.02
T.E. (mg/100 g W.B.)	2.5	2.3	2.1
% RDA (12 mg/100 g W.B.)	21	19	17

Values are expressed as mean (n = 3). W.B.– wet basis. RDA Recommended Daily Allowance.

Table 23. Content of thiamine and riboflavin in WEPs (mg/100 g W.B.)

Species	**Thiamine**	**% RDA** (1.1 mg/100 g W.B.)	**Riboflavin**	**% RDA** (1.4 mg/100 g W.B.)
Sp. oleracea	0.16 ± 0.02	2	0.12 ± 0.01	1
C. intybus	0.01 ± 0.00	–	0.16 ± 0.02	2
B.vulgaris	15.86 ± 0.89	145	0.38 ± 0.04	3

Values are expressed as mean (n = 3). W.B.– wet basis. RDA Recommended Daily Allowance.

Loss or Gain of Lipophilic Bioactive Compounds in Vegetables after Domestic Cooking? Effect of Steaming and Boiling

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Abstract

Lipophilic antioxidants are essential components, which have been pointed as bioactive beneficial for human health. This study aimed at evaluating the effect of domestic cooking (boiling, steaming) on the main carotenoids (lutein and β-carotene) and tocols in four different green leafy vegetables: *Sonchus asper* L. Hill, Sonchus oleraceus L., *Spinacia oleracea* L. and *Cichorium intybus* L. The total content of the analyzed compounds was determined following the method of alkaline hydrolysis of the matrix and solvent extraction. The leaching of soluble solids after domestic cooking was found to determine a gain in the investigated bioactive compounds in the cooked vegetables, so to cause an apparent content increase in all leafy vegetables, when expressed as mg/100 g dry matter. Considering solid losses, all lipophilic compounds were not affected by boiling; on the contrary, steaming slightly significantly decreased the contents of lutein and β-carotene (on average 20 and 15%, respectively).

Keywords: domestic cooking; green leafy vegetables; solid loss; carotenoids; tocols.

Introduction

The consumption of vegetables and fruit is associated with different health effects against chronic disorders, due also to their amount of bioactive phytochemicals and micronutrients, such as flavonoids, vitamin C, folates (vitamin B9), tocols, carotenoids and xanthophylls. Carotenoids and tocols are an important group of bioactive compounds with an antioxidant activity and health-promoting properties [1,2]. Different carotenoids are precursors of vitamin A and are among the compounds having a great influence on the color of different foods [3,4]. Carotenoids can be present in flowers, fruit and vegetables in their free or ester forms. Lutein, β-cryptoxanthin, zeaxanthin and violaxanthin are the most frequent xanthophylls found as esterified forms in fruits and vegetables [4–6]. Different carotenoids, such as α, β-carotene and β-cryptoxanthin, lutein and epoxycarotenoids are provided in the diet by yellow/orange fruits and vegetables, but also by green leafy vegetables [4–8]. Tocols, also known as vitamin E, comprise two groups of vitamers, tocopherols and tocotrienols, occurring in eight forms: α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), δ-tocopherol (δ-T) and α-tocotrienol (α-T3), β-tocotrienol (β-T3), γ-tocotrienol (γ-T3) and δ-tocotrienol (δ-T3). Tocols have been demonstrated to prevent certain types of cancer, heart and other chronic diseases [2]. Their main sources are vegetable oils, but they are also found in a large amount in different vegetable products with significant nutritional contents [2,8,9]. Most of the green leafy vegetables are consumed in-house after removing no edible parts, washing, cutting and different domestic processes involving boiling, microwave cooking, steaming, stewing and frying. Heat treatments can cause, at different degrees, softening of the tissue, color change, aroma formation and inactivation of compounds considered as anti-nutritional, but also a damage in color, taste and nutritional value. In particular, during boiling, they can produce modifications in cellular structure and composition, the breakdown of food matrix (mainly formed of dietary fiber) that may cause the release in water of compounds with low molecular weight and solid losses [10]. Depending on the processing conditions, the cooking of vegetables can affect their bioactive compound contents, with a consequently significant decrease in nutrients and, therefore, of the nutritional quality [11]. Moreover, the changes in the natural barriers in which some nutrients can be involved may result in the release from the matrix of bioactive components or, for those more polar, their loss in cooking water [11]. Different papers have studied the effect of different domestic cooking procedures on several

phytochemicals (polyphenols, carotenoids, tocopherols, glucosinolates) and micronutrients (vitamins and minerals) on vegetables, with somehow contradictory results [11–16]. Being lipid soluble, carotenoids and tocols are not significantly lost into water-soluble mediums during processing. However, their content in vegetables can be significantly affected by domestic cooking processes in different ways [11–15]. The interactions between the two factors, vegetables and cooking procedures, were significant [12]. In some cases, a reduction was observed for their thermal lability and their sensitivity to oxidation. The extent of degradation is dependent on temperature, light, oxygen occurrence, pH, water activity and the interactions with other antioxidants [17–21]. Furthermore, the severity and length of heat treatment can induce different carotenoid losses/isomerization [22,23]. These phenomena could also depend on the structure and cellular organization of compounds in the food matrix. Moreover, published papers on fruits or vegetables show an increased stability of carotenoid esters compared to the corresponding not-esterified forms [4–6]. In other cases, an increase in compounds was found, and it was attributed to their improved extractability and bioavailability [11–15]. In the light of the importance of the in-house cooking treatments in our daily life, the aim of this paper was to a have deeper insight into the effect on the amounts of tocols and carotenoids of two domestic cooking processes, such as conventional boiling and steaming, in four different leafy vegetables. Since solid loss could affect the weight of the resulting cooked vegetables, the influence of the solid loss on the amounts of the analyzed compounds was also investigated.

Materials and Methods

Plant Material

Four different leafy vegetables, chosen on the basis of their wide diffusion in several traditional recipes of the Mediterranean diet [24], were investigated. They were two wild edible plants, belonging to *Sonchus* species, *Sonchus asper* L. Hill and *Sonchus oleraceus* L. (Asteraceae), and two more commercial ones, spinach (*Spinacia oleracea* L.) and chicory (*Cichorium intybus* L.). Plants of *Sonchus* species were collected during the years 2019–2020. The conditions of collection and handling are reported in [8]. *Spinacia oleracea* and *Cichorium intybus* were purchased from local markets. The non-

edible portion was discarded; from each sample, a minimum of 500 g of edible portion was gathered and cleaned by removing damaged parts and soil particles.

Cooking Conditions

Two different cooking methods were tested: conventional boiling and steaming. Cooking conditions were performed by preliminary experiments carried out for each vegetable, considering the minimum cooking time to achieve softness, palatability and taste, according to the consumption habits or to the recipe. Each plant batch was divided into three parts to have at least three repetitions in the experiments. A total of 100 g of leaves was chopped and boiled in a beaker in 1 L of water (1:10 food: water). For conventional boiling, a fresh portion was added to 1 L of boiling water and cooked for 10 min. For steaming, the portion of the vegetable was placed on a steaming rack over boiling water in a closed water bath for 10 min. The boiling water was drained off for 5 min. After cooking and draining, the cooked portions, the water samples and the fresh controls were freeze-dried (Genesis 25SES freeze dryer, VirTis Co., Gardiner, NY, USA), grounded with a refrigerated IKA A10 laboratory mill (IKA®-Werke GmbH & Co. KG, Staufen, Germany), carefully mixed and stored at −20 ◦C until analysis. The dried water residue was weighed in order to determine the soluble loss after cooking.

Chemicals and Reagents

Solvents were commercially obtained (Sigma Aldrich, St. Luis, MO, USA), at the highest quality, and used without further purification. All other used reagents were of analytical grade. Lutein was purchased from CaroteNature (Lupsingen, Switzerland); all-trans-β-carotene was from Sigma Chemicals (St. Luis, MO, USA). α-, β-, γ- and δ-Tocopherol standards were from Merck (Darmstadt, Germany); α-, β-, γ- and δ-Tocotrienol standards were purified, as reported in Panfili et al. [25]. Purity for all standards was above 95% (as certified by the suppliers).

Tocols and Carotenoids Extraction and Quantification

Fresh, freeze-dried plants and water residues were analyzed for moisture, according to the AOAC methods [26]. The procedure for tocols and carotenoids extraction was the saponification method reported in [25,27]. About 0.3 g of milled freeze-dried sample and residue of boiling water was weighed and placed in a screw-capped tube. Then, 5 mL of ethanolic pyrogallol (60 g/L), 3 mL of absolute ethanol, 1 mL of sodium chloride (10 g/L) and 2 mL of potassium hydroxide (600 g/L) for alkaline digestion were added. After nitrogen flushing for 1 min, the tubes were kept for 45 min in a 70 ◦C water bath and stirred every 5–10 min. After cooling, 15 mL of sodium chloride (10 g/L) were added. Compounds were extracted with 15 mL of n-hexane/ethyl acetate (9:1, v/v), until the organic layer was colorless (about three times). Organic layers were collected and evaporated to dryness. Carotenoids were analyzed through the combination of a normal (for xanthophylls) and a reverse phase (for carotenoids) HPLC method. An HPLC Dionex (Dionex, Sunnyvale, CA, USA) analytical system, consisting of a 50 µL injector loop (Rheodyne, Idex Health & Science, Northbrook, IL, USA) and a U6000 pump system was used. For normal phase (NP), samples were suspended in 2 mL of isopropyl alcohol (10%) in n-hexane. The mobile phase was 10% n-hexane: isopropyl alcohol (A) and 20% n-hexane: isopropyl alcohol (B), with the following gradient: 0–6 min (100:0), 16–25 min (50:50), 28–32 min (100:0), respectively, with a flow rate of 1.5 mL/min. The chromatographic separation of the compounds was achieved by means of a 250 × 4.6 mm i.d., 5 µm particle size, 100A Luna Phenomenex Si column (Phenomenex, Torrance, CA, USA) [27]. For the reverse phase (RP), the organic dry residue was dissolved in methanol: MTBE, 50:50 (v/v). Separation was performed, at a flow rate of 1 mL/min, by using a 5 µm, C30 YMC (Hampsted, NC, USA) stainless steel column (250 × 4.6 mm i.d.). The mobile phase was methanol: MTBE: water. The gradient profile is given in [28,29]. For tocol analysis, the dry residues were suspended in 2 mL of isopropyl alcohol (1%) in n-hexane, and the analysis was performed through a normal phase HPLC, as already reported for carotenoids and as in [25]. The fluorimetric detection of all tocols was performed by means of a Dionex RF 2000 spectrofluorimeter, at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Carotenoids were spectrophotometrically detected at 450 nm. Standards were spectrophotometrically quantified and identified through their spectral characteristic. Compounds were identified by comparison of their retention times and Uv/Vis spectra with known commercially available standard solutions. 2.5. Statistical Analysis The

samples were analyzed in triplicate. The results were reported as the average of three determinations. The ANOVA test was applied to data, by using a Statistical Software Package for Windows (SPSS Inc., Chicago, IL, USA). Significance of difference was defined at $p < 0.05$.

Results and Discussion

The amounts of the single carotenoids, expressed as mg/100 g dry matter (d.m.), in fresh and cooked samples, at the end of each domestic boiling and steaming treatment, for every investigated vegetable, are reported in **Table 24**. Results are expressed on a dry matter basis (d.m.), to allow a good comparison, taking into account possible moisture change.

Table 24. Contents of the main carotenoids and tocols in the investigated leafy vegetables before and after cooking (mg/100 g d.m.).

Carotenoids	Treatment	*S. oleraceus*	*S. asper*	*Sp. oleracea*	*C. intybus*
Lutein	Fresh	57.3 [a]	60.5 [a]	109.1 [a]	87.1 [a]
	Boiling	86.7 [b]	83.7 [b]	122.8 [b]	115.2 [b]
	Steaming	51.4 [a]	47.9 [c]	91.8 [c]	84.9 [a]
β–Carotene	Fresh	17.7 [a]	22.2 [a]	39.6 [a]	56.6 [a]
	Boiling	25.7 [b]	36.9 [b]	44.9 [b]	82.8 [b]
	Steaming	12.4 [a]	18.2 [c]	40.9 [b]	55.4 [a]
Tocols					
α–T	Fresh	19.7 [a]	20.5 [a]	32.3 [a]	33.1 [a]
	Boiling	31.7 [b]	27.4 [b]	41.0 [b]	43.3 [b]
	Steaming	22.6 [a]	18.1 [a]	31.3 [a]	39.6 [a]
γ–T	Fresh	3.3 [a]	2.7 [a]	4.7 [a]	14.1 [a]
	Boiling	5.3 [b]	4.7 [b]	6.4 [b]	15.8 [b]
	Steaming	4.1 [a]	3.9 [c]	4.7 [a]	10.9 [c]

For each compound different letters in the same column indicate a statistically significant difference at $p < 0.05$.

In all analyzed leafy vegetables, lutein and β-carotene were the main detected carotenoids, and they are the only ones discussed in this paper. Data on the other carotenoids are, therefore, not reported. In fresh samples, lutein ranged from about 60 mg/100 g d.m. in *Sonchus* species to about 100 mg/100 g d.m. in *Sp. oleracea*. Beta-carotene went from about 20 mg/100 g d.m. in Sonchus species to about 60 mg/100 g

d.m. in *C. intybus*. These data were confirmed by literature studies on *Sonchus* species [8,30] and other different green leafy vegetables [4,31]. Among tocols, only α-tocopherol (α-T), from about 20 mg/100 g d.m. in *Sonchus* species to about 33 mg/100 g d.m. in the others, and γ-tocopherol (γ-T), from about 3 to 14 mg/100 g d.m. in *C. intybus*, were detected. No tocotrienols were found. References for tocols in the investigated species are in accordance with those reported by different authors, for *Sonchus* [8,30] and for other green vegetables [32–34]. A general significant increase (mg/100 g d.m.) in all compounds as to fresh vegetables was found after the boiling treatment (Table 1). In particular, the increase in carotenoids ranged up to 50% for lutein (*S. oleraceus*), to about 65% for β-carotene (*S. asper*). A similar trend was observed for tocols, with increments of α-T going from 25% (*Sp. oleracea*) to 60% (*S. oleraceus*) and of γ-T ranging from about 12% (*C. intybus*) to about 75% (*S. asper*). Results on the effects of domestic cooking on the investigated liposoluble compounds are controversial [11–15,33,34]. Some authors reported losses of carotenoids and tocols after cooking, some others did not observe significant changes, some concluded that thermal processing increases compound concentrations. Regarding carotenoids, when lower temperatures were applied, they seemed more stable during water cooking. Boiling was reported by some authors to be the most destructive water cooking process, while steaming the least [13]. In different cases, particularly after water cooking, increased concentrations in comparison with the fresh uncooked samples were reported. This phenomenon was explained to be due to the enhanced carotenoid chemical extractability from the plant tissue after heating, following disintegration of the plant matrix, cellular breakage and dissociation of molecular linkages between food components, such as carotenoid–protein complexes of the chloroplasts [11–15]. The overall results are variable, since the compounds behavior during cooking could depend on the part of the cooked vegetable, the particle size of the vegetable, its shape and tissue structure and, as a consequence, on the plant under investigation [12–14]. There are relatively few studies on the processing, storage and cooking effects on vitamin E in fruits and vegetables, with controversial results, depending on the type of food and cooking time [11,32–34]. As already observed for carotenoids, some of these studies found, on average, higher levels of α-tocopherol than in the fresh products, which was suggested to be due to the increment of the chemical extractability of lipidic molecules through heat treatment [11,33,34]. Moreover, greater extractability has been usually associated to a greater bioavailability [35,36], even though, in most cases, this hypothesis was not

assessed through proper methods of investigation [36]. It is worth noticing that, in almost all papers, the observed greater extractability from the food matrix refers to free compounds determined after a solvent extraction method. In several cases, the extraction phase is followed by a saponification of the extract, in order to hydrolyze esters or to remove compounds that could interfere with the chromatographic analysis. The statement "greater extractability" after the cooking treatments implies that the extraction method used by the authors was not able to correctly quantify the contents of the investigated compounds, in an untreated matrix. Few papers use a method involving an alkaline hydrolysis (saponification) of the food matrix followed by solvent extraction, which, allowing the opening up of the cell wall matrix and releasing compounds that might be strongly linked to cellular components, can cause a more effective and complete extraction of compounds, thus giving more reliable and comparable results. By applying the cited method, not only free compounds or those de-esterified by saponification, but also compounds difficult to access to the solvent or linked to the food matrix are extracted. We have already proven this procedure for its reliability, and it has been successfully used for cereals and other vegetable samples [4,5,8,17,19,25,27]. The same method was applied in this paper for the extraction of lipophilic compounds from fresh and processed vegetables. For this reason, the observed increases reported in Table 1 after cooking could not be ascribed to a greater extractability from the plant tissue caused by thermal treatments. As already pointed out, the breakdown of the food matrix and the release of compounds, due to thermal treatments, can cause weight changes due to solid loss. Therefore, apart from moisture loss or gain, solid loss might also be taken into consideration [37]. Regarding the influence of solid losses, in an old paper by Baloch et al. [38], the incomplete extraction of pigments from raw vegetables and/or leaching of soluble solids during processing of the vegetables were considered as the possible explanations for the apparent increase in carotenoid content during processing. In fact, values were found to increment only if they were expressed on dry weight basis. The latest evidence was also discussed in a recent paper by Diamante et al., in colored cauliflowers [33]. The soluble solid losses in water from 100 g lyophilized samples after cooking are reported in **Table 25**.

Table 25. Soluble solid losses (g) in cooking water from 100 g of dry matter of the cooked samples

Vegetables	Fresh	Cooking water	
		Boiling	**Steaming**
S. oleraceus	100	36	5
S. asper	100	35	5
Sp. oleracea	100	21	4
C. intybus	100	23	10

Solid losses went from 21% in *Sp. oleracea* to 36% in *S. oleraceus*. As expected, for steamed vegetables, they were to a less extent (about 5% and 10% for *C. intybus*). The content of carotenoids and tocols of Table 1 was therefore corrected, either in processed foods, or in the cooking water, considering the solid loss, as reported in **Table 26** (*S. oleraceus*), **Table 27** (*S. asper*), **Table 28** (*Sp. oleracea*) and **Table 29** (*C. intybus*).

Table 26. Contents of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *S. oleraceus.*

Compounds	Fresh	Boiling			Steaming		
		Cooked (A)	Water (B)	A+B	Cooked (C)	Water (D)	C+D
	100 g	(64 g)*	(36 g)*	(100 g)§	(95 g)*	(5 g)*	(100 g)§
Carotenoids							
Lutein	57.3 [a]	55.5	0.5	56.0 [a]	48.8	n.d.	48.8 [b]
β–Carotene	17.7 [a]	16.5	0.1	16.6 [a]	11.9	n.d.	11.9 [b]
Tocols							
α–T	19.7 [a]	20.3	0.1	20.4 [a]	21.4	n.d.	21.4 [a]
γ–T	3.3 [a]	3.4	n.d.	3.4 [a]	3.9	n.d.	3.9 [a]

* Partition of 100 g of the dry matter of raw samples in cooked samples and in cooking water. § Sum of the contents in cooked samples and in cooking water (mg/100 g d.m.) Different letters in the same row indicate a statistically significant difference at $p < 0.05$. n.d.: not detectable.

Table 27. Contents of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *S. asper*.

Compounds	Fresh	Boiling			Steaming		
		Cooked (A)	Water (B)	A+B	Cooked (C)	Water (D)	C+D
	100 g	(65 g)*	(35 g)*	(100 g)§	(95 g)*	(5 g)*	(100 g)§
Carotenoids							
Lutein	60.5 [a]	54.4	0.5	54.9 [a]	45.5	n.d.	45.5 [b]
β–Carotene	22.2 [a]	24.0	1.7	25.7 [a]	17.3	n.d.	17.3 [b]
Tocols							
α–T	20.5 [a]	17.8	0.2	18.0 [a]	17.2	n.d.	17.2 [a]
γ–T	2.6 [a]	3.1	n.d.	3.1 [a]	3.7	n.d.	3.7 [a]

* Partition of 100 g of the dry matter of raw samples in cooked samples and in cooking water. § Sum of the contents in cooked samples and in cooking water (mg/100 g d.m.). Different letters in the same row indicate a statistically significant difference at $p < 0.05$. n.d.: not detectable.

Table 28. Content of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *Sp. oleracea.*

Compounds	**Fresh**	**Boiling**			**Steaming**		
		Cooked (A)	Water (B)	A+B	Cooked (C)	Water (D)	C+D
	100 g	(79 g)*	(21 g)*	(100 g)§	(96 g)*	(4 g)*	(100 g)§
				Carotenoids			
Lutein	109.1 [a]	97.0	2.1	99.1 [a]	88.2	n.d.	88.2 [b]
β–Carotene	39.6 [a]	35.5	1.7	37.2 [a]	39.3	n.d.	39.3 [a]
				Tocols			
α–T	32.3 [a]	32.4	0.2	32.6 [a]	30.0	n.d.	30.0 [a]
γ–T	4.7 [a]	5.0	n.d.	5.0 [a]	4.5	n.d.	4.5 [a]

* Partition of 100 g of the dry matter of raw samples in cooked samples and in cooking water. § Sum of the contents in cooked samples and in cooking water (mg/100 g d.m.). Different letters in the same row indicate a statistically significant difference at $p < 0.05$. n.d.: not detectable.

Table 29. Content of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *C. intybus.*

Compounds	**Fresh**	**Boiling**			**Steaming**		
		Cooked (A)	Water (B)	A+B	Cooked (C)	Water (D)	C+D
	100 g	(77 g)*	(23 g)*	(100 g) §	(90 g)	(10 g)	(100 g)
				Carotenoids			
Lutein	87.1 [a]	88.7	0.7	89.4 [a]	76.4	n.d.	76.4 [b]
β–Carotene	56.6 [a]	63.8	0.6	64.4 [a]	49.9	n.d.	49.9 [b]
				Tocols			
α–T	33.1 [a]	33.3	0.2	33.5 [a]	35.5	n.d.	35.5 [a]
γ–T	14.1 [a]	12.2	n.d.	12.2 [a]	9.9	n.d.	9.9 [b]

* Partition of 100 g of the dry matter of raw samples in cooked samples and in cooking water. § Sum of the contents in cooked samples and in cooking water (mg/100 g d.m.). Different letters in the same row indicate a statistically significant difference at $p < 0.05$. n.d.: not detectable.

A rather small, but significant, decrease due to steaming was observed for lutein (on average 20%) and β-carotene (on average 15%) in all vegetables, with the exception of β-carotene of Sp. oleracea. A more marked stability was found for tocols. The extent of the degradation of the investigated compounds could depend on temperature, available oxygen and oxidative enzymes. Since no effect was observed due to the boiling temperature, the same could be stated for steaming, where the temperature range is in the same order of magnitude (97–100 ◦C) [37]. During the steaming treatment, the adopted process conditions could not have been effective in having an enzymatic inactivation; therefore, the activities of different oxidative enzymes, such as peroxidase and lipoxygenase, may have influenced the levels of the analyzed carotenoids in the early stages of the process [39,40]. As already stated, it is difficult to assess from the literature a general effect of food processing. Different domestic processes have proven to have different impacts on carotenoids and tocols, due to their stability upon heating time and temperature, exposure to oxygen and light and the matrix in which they are involved. Data emerging from this work confirm the hypothesis that leaching of soluble solids could be the cause of the apparent increases in boiled samples. Apart from the paper by Baloch et al., 1977 [38], to our knowledge, up to now, there are no experimental papers demonstrating soluble leaching as one of the causes of the observed apparent increase in liposoluble compounds after the boiling treatment.

Conclusions

Data emerging from this research demonstrate that, in order to have reliable data of the effect on in-house processing on liposoluble pigments, a complete extraction procedure, together with the evaluation of the solid loss ought to be taken into consideration. In fact, under our experimental conditions, the use of the saponification procedure of the matrix followed by solvent extraction allowed a complete extraction of total tocols and carotenoids from the investigated vegetable samples. Therefore, the initially observed increments of bioactives after the applied in-house treatments could not be related to their higher extractability, due to the breakdown of the food matrix by the high temperatures. Leaching of soluble solids during processing could therefore be the cause of the observed increased amounts in boiled vegetables, when data are expressed as mg/100 g d.m. By considering solid losses, boiling did not significantly affect the main carotenoids and tocols in the investigated vegetables, while steaming had a small effect on their amounts. Further experiments are needed in order to investigate the nature of the solids solubilized in the cooking water, also in relation to different vegetables, with a different tissue structure. Finally, since the thermal lability of carotenoids could also be influenced by their chemical structure, the behavior of the single found compound, as a result of thermal treatments, should be evaluated.

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7.5 Effect of domestic cooking on *Beta vulgaris*

Table 30 shows the contents of the main carotenoids and tocols in the fresh and cooked samples, at the end of each domestic boiling and steaming treatment. Results are expressed as mg/100 g of dry matter (d.m.), to allow a good comparison, taking into account the possible moisture change. Data obtained were confirmed by literature studies (Fratianni et al., 2021; Sánchez-Mata et al., 2012). Like the other species investicated, also chard showed a general significant increase (mg/100 g d.m.) in all compounds as to fresh vegetables after the boiling treatment.

Table 30. *Content of the main carotenoids and tocols in fresh and in cooked Beta vulgaris* (d.m.).

Carotenoids	Treatment	*B. vulgaris*
Lutein	Fresh	71.5 [a]
	Boiling	98.2[b]
	Steaming	68.3[a]
β-carotene	Fresh	23.7[a]
	Boiling	29.0[b]
	Steaming	20.2[c]
Tocols		
α–T	Fresh	15.1 [a]
	Boiling	21.7 [b]
	Steaming	16.3 [a]
γ–T	Fresh	1.7[a]
	Boiling	3.0[b]
	Steaming	1.8[a]

For each compound different letters in the same column indicate a statistically significant difference at $p < 0.05$.

The soluble solid losses in the cooking water from 100 g lyophilized sample after cooking are reported in **Table 31**. Solid losses were 10% in boiled and to a less extent for steamed chard. The content of carotenoids and tocols of **Table 30** were therefore corrected, either in processed foods, or in the cooking water, considering the solid

losses, as reported in **Table 32**. By considering solid losses, boiling did not significantly affect the main carotenoids and tocols in the investigated chard, while steaming had a small effect on its amounts (decrease of lutein and β-carotene contents of 10% and 20%, respectively).

Table 31. Soluble solid losses (g) in cooking water from 100 g of dry matter of the cooked samples.

Vegetables	Fresh	Cooking water	
		Boiling	Steaming
S. oleraceus	100	30	10

Table 32. Contents of the main carotenoids and tocols in fresh vegetables (mg/100 g d.m.), in cooked samples (mg/g d.m.) and in cooking water (mg/g d.m.) of *B. vulgaris.*

Compounds	Fresh	Boiling			Steaming		
		Cooked (A)	Water (B)	A+B	Cooked (C)	Water (D)	C+D
	100 g	(70 g)*	(30 g)*	(100 g)§	(90 g)*	(10 g)*	(100 g)§
				Carotenoids			
Lutein	71.5 [a]	68.7	4.9	73.6 [a]	61.5	3.3	64.8 [b]
β−Carotene	23.7 [a]	20.3	1.4	21.7 [a]	18.2	1.0	19.2 [b]
				Tocols			
α−T	15.1 [a]	15.2	1.0	16.2 [a]	14.6	0.1	14.7 [a]
γ−T	1.7 [a]	2,1	0,1	2.2 [a]	1.6	0.1	1.7 [a]

* Partition of 100 g of the dry matter of raw samples in cooked samples and in cooking water. § Sum of the contents in cooked samples and in cooking water (mg/100 g d.m.) Different letters in the same row indicate a statistically significant difference at $p < 0.05$. n.d.: not detectable.

7.6 Hydrosoluble compounds

In all investigated green leafy vegetables, only boiling was responsible for a signicant loss of Total Polyphenol Content (TPC) and Total Flavonoid Content (TFC). In particular, the polyphenol content of the fresh plants ranged from 1.5 nmoles catechin/g f.w. in *Sp. oleracea* to 7.6 nmoles catechin/g f.w in *S. asper*. The boiling treatment caused a decrease in the TPC, with losses from 40% in *Sp. oleracea* to 80% in *S. asper* **(Figure 20)**. The total flavonoid content of the fresh plants varied from 2.6 nmoles catechin/g f.w. in *Sp. oleracea* to 5.0 nmoles catechin/g f.w. in *B. vulgaris*. The same behavior of TPC after boiling was observed for flavonoids **(Figure 21).** This was due to a release of phenolic/flavonoid compounds in the cooking water, as also confirmed by their presence in the boiling water.

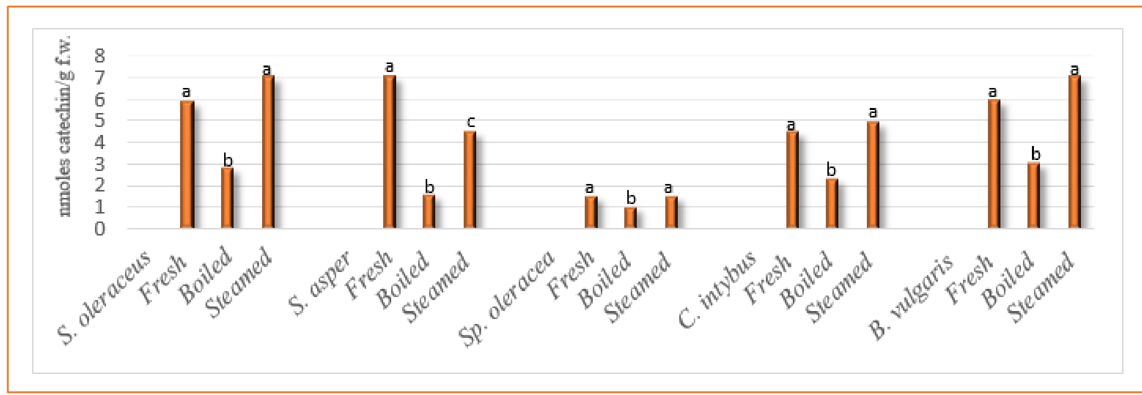

Figure 20. TPC contents (nmoles of catechin/g f.w.) in fresh and cooked plants. Different letters indicate a significant difference.

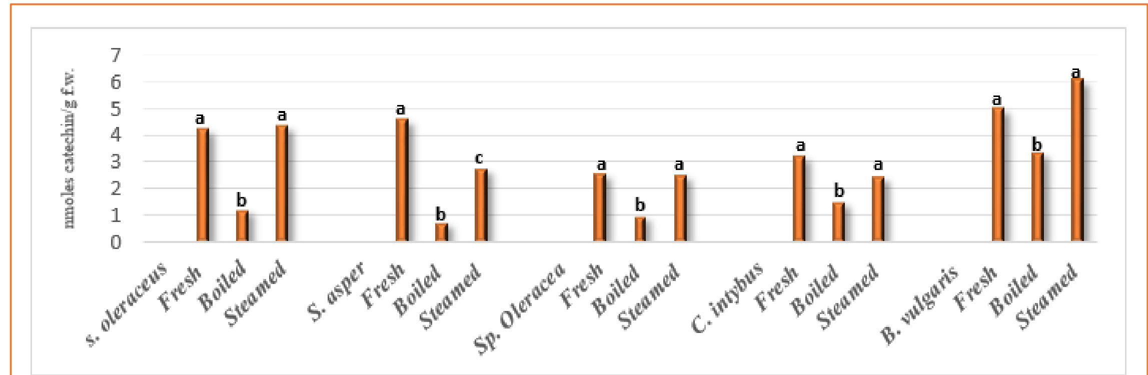

Figure 21. TFC contents (nmoles of catechin/g f.w.) in fresh and cooked plants. Different letters indicate a significant difference.

7.7 Antioxidant activity

The antioxidant activity in fresh samples is shown in **Figure 20**. *S. asper* had the highest values of antioxidant capacity (178899 nmoles trolox/g f.w.), while lower values were found in *C. intybus* (6952 nmoles trolox/g f.w.). In general, from the obtained data, it appeared that boiling caused the most important losses in antioxidant capacity, with a decrease profile similar to those of TPC and TFC.

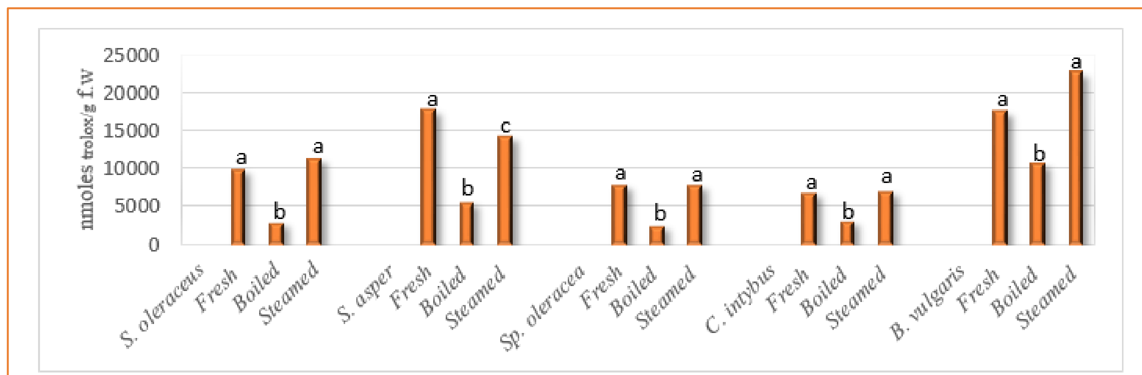

Figure 22. Antioxidant activity (nmoles of trolox/g f.w.) in fresh and cooked plants. Different letters indicate a significant difference.

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CHAPTER 8

Pasta enriched with green leafy vegetables

Introduction

Food and its manufactures are currently attracting significant and public interest due to extensive media coverage of diet-related diseases and their influence on health and wellbeing of communities. This has led to an increased consumer demand for nutritious foods with not only a balanced caloric content, but also with additional health-promoting functions, i.e. functional foods. This trend is promoted and endorsed by governments and consumers worldwide and it is generally accepted that healthy products of high quality and convenience need to be developed, through innovative multidisciplinary research programs. To date, the primary concern of the food industry has been to produce and provide the consumer with a safe food. However, while safety is still of paramount importance, the nutritional and caloric composition of foods is becoming equally important. In addition, if the food is to be considered a ‘‘functional food’’, it should also provide health benefits beyond basic nutrition (American Dietetic Association, 2004; Health Canada, 2004; International Life Sciences Institute, 1999; Thomas & Earl, 1994; Guaadaoui A. et al., 2014; Sangha A.K. et al., 2015).
In this context, the research activities of this Doctoral thesis ended with the development of a protocol for the production of pasta, in the optics of an innovative healthy food enriched with the investigated leafy vegetables, considering the found significant quantities of bioactive compounds. Pasta was enriched with freeze-dried leaves of chicory, chard and spinach and the nutritional and sensorial characteristics of the made final products were evaluated.
This was achieved through the following three steps:

1. study of optimization of product formulations;
2. study of the effects of green leafy vegetables inclusion on sensory and nutritional pasta quality;
3. quantitative analysis of bioactive compounds, in particular tocols and carotenoids, in final products.

8.1 Pasta in the world

Traditionally being an Italian product, pasta has become a worldwide consumed product due to its convenience, cost, palatability, nutritional value and long shelf life (Petitot at al., 2010). The most common method for the production of pasta is through extrusion. In this process, the flour is mixed with water (usually about 30 – 35% moisture) (Gianibelli al al., 2005; Torres et al., 2007), resulting in the formation of a dough that is forced through a die and then dried (Sozer at al., 2007). Due to its unique proteins that will form a very strong and visco–elastic network, wheat is the preferred cereal for the production of flour for pastamaking. Among wheat, durum wheat (*Triticum turgidum* L. var. *durum*) semolina is regarded as the best raw-material for the production of high quality pasta, due to its unique colour, flavour, and cooking quality (Abecassis at al., 1989; Lamacchia at al., 2010). The composition of durum wheat semolina can be divided into 3 main constituents (Petitot at al., 2010):

- starch, represents the main fraction being, varying between 70 and 80% of the total weight;
- proteins, reaching up to 15% of the total weight;
- small amounts of fiber, lipids, vitamins and minerals represent the remaining part.

The proteins in durum wheat semolina are a mixture of albumins, globulins, glutenins and gliadins. The last two are capable of interacting with each other and with other components, forming intra and intermolecular disulphide bonds that will result in the development of a three dimensional visco-elastic gluten network (Weiser, 2007; Petitot at al., 2010). Besides controlling the visco–elastic properties, protein content and composition also determine the quality of the flour and consequently the quality of pasta (such as firmness and extensibility) (Gianibelli at al, 2005; Majzoobi at al., 2012). Durum wheat semolina is regarded to be the best raw-material for the production of pasta because of its high protein content, resulting in the formation of a dense gluten network (Howard at al., 2011). Gluten is

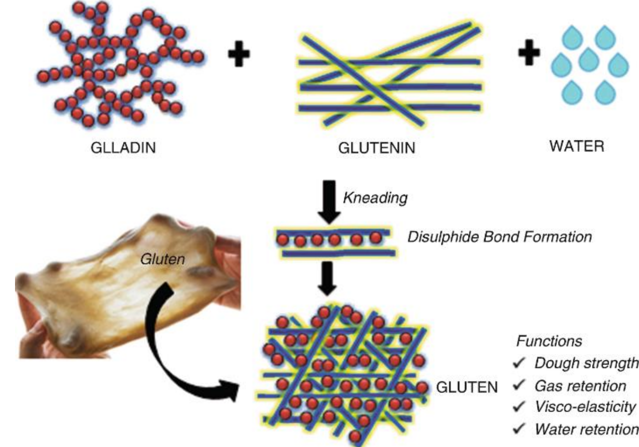


Figure 23. Formation of gluten in the dough.

responsible for the development of dough during mixing and extrusion, entrapping the starch granules in its network. This visco–elastic network restricts starch swelling, maintaining the structure of pasta during cooking, and thus preventing cooking losses (Abecassis at al., 1989). Besides this, gluten is also responsible for the elasticity and the "al dente" bite of pasta (Sozer at al., 2007). This stresses the importance of gluten since the quality of pasta is mostly determined by its textural properties and cooking quality (Edwars at al., 1993; Tudoricà at al., 2002).

8.2 Enriched pasta-like products

The enrichment of pasta products already began more than five decades ago with the addition of soy protein (Pulsen at al., 1961). Since then, enrichment, mostly with vegetable and legume flours, is common only in pasta made from durum wheat semolina. Several reasons for the enrichment of pasta have been pointed out in literature, such as nutritional improvement, use of local raw materials, use of cereal by-products, production of gluten-free pasta or development of products with additional health benefits (Marconi at al., 2001). The enrichment can have a negative effect, if the ingredients are not well balanced, on the structural properties of this type of products, as the replacement of gluten proteins will dilute the network and thereby weaken it (Gallegos-Infante at al., 2010). The quality of pasta is of a great importance to producers and consumers and includes cooking properties, i.e., firmness, stickiness, and overcooking tolerance, as well as water absorption, degree of swelling, and gelatinization rate (Manser, 1981). Cooking loss is the amount of material that detaches from the product; high quality products should have low cooking losses. With regard to processability and textural and sensorial properties, the pasta should have a low degree of stickiness and highrmeness. The enrichment of pasta above a certain concentration often results in a negative effect on the product properties, decreasing its quality (Brennan at al., 2007). In literature, the most reported problems of the enrichment of pasta-like products include a detriment in cooking quality, textural and sensorial properties (Zhao at al., 2005; Madhumitha at al., 2011). More specifically, the addition of flours different than wheat increases cooking losses, decreases firmness and increases stickiness of pasta. Moreover, usually, enriched products have a lower acceptability than pasta that has not been enriched (Edwards at al, 1995). These negative effects have been linked to the dilution of the network, since wheat flour or starch are replaced by

vegetable/legume flours that do not contain proteins capable of forming a network (Petitot at al., 2010).

8.3 Materials and methods

8.3.1 Ingredients

Freeze-dried leaves of chicoria, chard, and spinach, together durum wheat semolina (Divella, Rutigliano, Bari) bought at a local supermarket, were used as ingredients to develop enriched pastas. Moreover, commercial "paglia e fieno" pasta (Conad, Italia) was purchased in a local supermarket, to use it as a control. The control samples, semolina (paglia) and spinach semolina pasta (fieno), were called CM1 and CM2 respectively. The ingredients listed on their label are shown in **Table 33.**

Table 33. Control purchased samples.

Sample	Ingredients
CM1	Durum wheat semolina, fresh pasteurized eggs 28%.
CM2	Durum wheat semolina, fresh pasteurized eggs 28%, dehydraded spinach 0,9%.

8.3.2 Proximate Composition, Tocols and Carotenoids analysis

Methods of analysis have been reported in chapter 4.

8.3.3 Pasta making

Pasta samples were produced at “Parco Scientifico e Tecnologico” of the Molise Region (Campobasso, Italy). Four doughs were produced, enriched pastas were formulated in order to achieve almost 15% of Tocopherol Equivalents in the final products, considering tocol amounts in the raw ingredients:

- control sample (CTRL), made of 100% semolina flour;
- spinach samples (S1), where the semolina flour has been added with 15% of freeze-dried spinach;
- chicory samples (S2) where the semolina flour has been added with 15% of freeze-dried chicory;

- chard samples (S3) where the semolina flour has been added with 15% of freeze-dried spinach.

Dried ingredients (flour and freeze-dried vegetables) were first weighed and combined into a homogenous mixture. Subsequently, they were put in a professional bench mixer, "La prestigiosa s.r.l.", followed by addition of cold water that was slowly poured as the mixer kneaded. The crumbly dough mass was then kneaded for approximately 15 minutes and extruded through a bronze die. Finally, pasta was dried in a static dryer (Namad, Rome, Italy), at 30 °C for 48 hours. The pasta formulations are shown in **Table 34** and the process in **Figure 24**.

Table 34. Pasta formulations.

Samples	Semolina flour	freeze-dried vegetable	Water %
CTRL	100%	-	30%
S1	85%	15%	38%
S2	85%	15%	52%
S3	85%	15%	38%

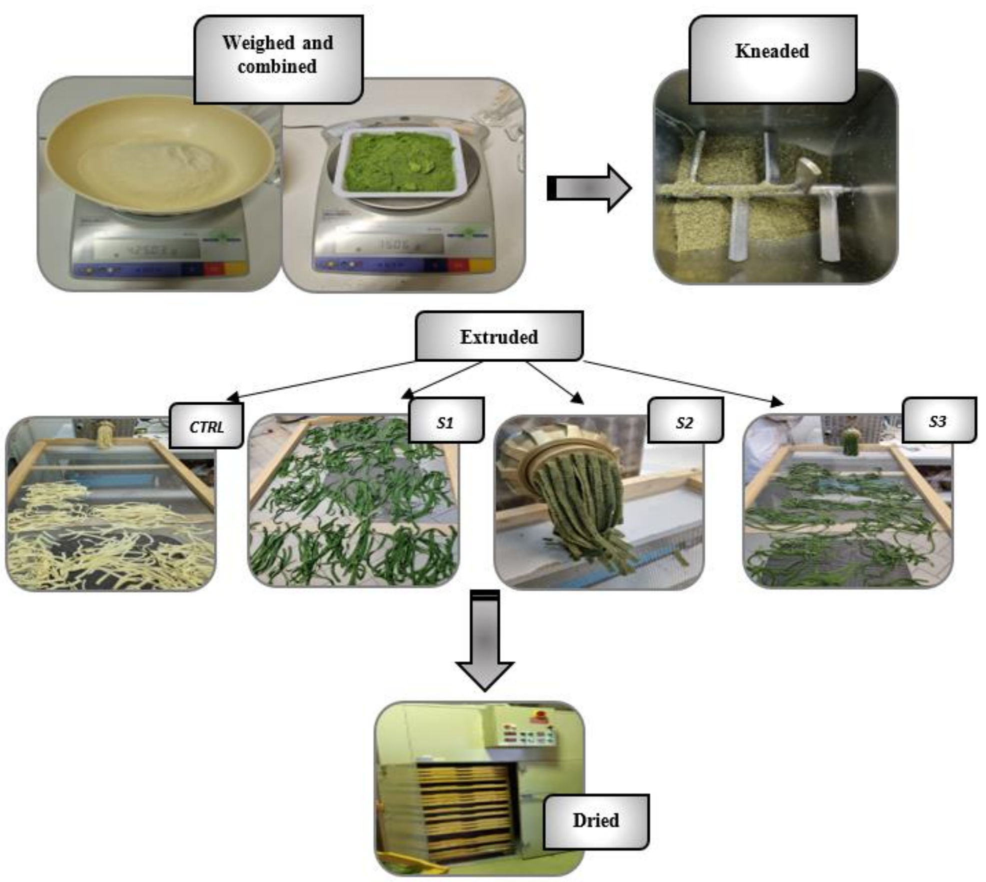


Figure 24. Preparation of pasta samples

8.4 Results and discussion

Pasta samples made with green leafy vegetables and semolina flour were investigated for composition, bioactives (carotenoid and tocols) and sensorial properties. The obtained results will be described separately for each step of the experimental design.

8.4.1 Chemycal composition

The proximate composition of commercial and developed pastas is shown in **Table 35**. Vegetable samples reported the higher ash content (in a range within 2.7-3.0 g/100 f.w.) as to 0.8 g/100 g f.w. of CTRL; while, CM1 and CM2 showed amounts of 1.0 – 1.3 g/100 g f.w., rispectively. Protein and fat content showed no significant changes as CTRL, CM1 and CM2. Regarding total dietary fiber it showed a higher content in experimental pasta S1, S2 and S3, ranging from 4.5 to 6.0 m/100 g f.w., so to be declared with "high o very high fiber content", respectively, according to the European law (EC 1924/2006).

Table 35. Proximate composition of dried pastas (g/100 g f.w.).

	Moisture	**Fat**	**Protein**	**Ash**	**Carbohydrate***	**Dietary Fiber**
S1	7.3 ± 0.37	0.3 ± 0.00	16.9 ± 0.01	3.0 ± 0.07	66.5 ± 0.00	6.0 ± 0.00
S2	9.0 ± 0.20	0.5 ± 0.78	14.5 ± 0.13	2.7 ± 0.42	68.8 ± 0.00	4.5 ± 0.00
S3	8.5 ± 0.76	0.3 ± 0.45	16.6 ± 0.03	2.8 ± 0.02	66.2 ± 0.00	5.8 ± 0.00
CTRL	8.9 ± 0.37	0.5 ± 0.01	14.6 ± 0.04	0.8 ± 0.01	71.9 ± 0.00	3.3 ± 0.00
CM1	9.8 ± 0.22	4.3 ± 0.01	14.3 ± 0.10	1.0 ± 0.03	68.1 ± 0.00	2.5 ± 0.00
CM2	10.4 ± 0.31	4.0 ± 0.06	14.7 ± 0.01	1.3 ± 0.03	66.9 ± 0.00	2.7 ± 0.00

S1: spinach pasta samples**; S2:** chicory pasta samples**; S3:** chard pasta samples**; CTRL:** control semolina pasta**; CM1:** commercial semolina pasta**; CM2:** commercial spinach and semolina pasta

8.4.2 Nutritional properties

Freeze-dried vegetabes were analyzed for their carotenoids and tocols content and afterwards their content was determined in the realized pasta samples.

Tocol composition in the realized pasta samples

Table 36 shows the contents of tocols of the produced pasta and the commercial pasta expressed in mg/100 g f.w. The total tocol content was significantly higher in pasta samples enriched with green leafy vegetables, in fact, while the CTRL had values of 1.5 mg/100 g f.w., S1, S2 and S3 pasta samples contained 6.1 - 2.0 - 3-6 mg/100 g f.w. In particular, sample S1 had a high content in α-T (6.1 mg/100 g), followed by sample S3 (3.6 mg/100 g). Samples CM1 and CM2 have similar amounts (respectively 3.3 - 3.8 mg/100 g f.w.). **Table 36** also reports values of vitamin E activity provided by 100 g of product, expressed as Tocopherol Equivalent (T.E.) (mg/100 g f.w.) (Sheppard, 1993). In particular, T.E. is 4.2 in S1 and 2.0 in S3. Taking into account the Recommended Daily Allowance (RDA) for vitamin E that is 12 mg/day (EU 1169/2011), 100 g of S1 and S3 contribute approximately to about 35% and 17% of the RDA, respectively, so to be declared as a “source of vitamin E”. Considering a portion of 80 g of pasta, the percentage of the RDA is 28% in S1 and 14% in S3.

Table 36. Tocol composition in the investigated pasta samples (mg/100 g f.w.).

Compounds	S1	S2	S3	CTRL	CM1	CM2
α–T	3.9 ± 0.15	0.9 ± 0.01	2.0 ± 0.05	0.1 ± 0.00	1.4 ± 0.01	1.9 ± 0.11
α–T3	0.1 ± 0.12	0.1 ± 0.00	0.2 ± 0.00	0.1 ± 0.00	0.2 ± 0.00	0.2 ± 0.02
β–T	0.2 ± 0.00	0.1 ± 0.00	0.1 ± 00.0	0.1 ± 0.00	0.1 ± 0.00	0.1 ± 0.00
γ–T	0.7 ± 0.05	0.6 ± 0.02	0.4 ± 0.04	0.1 ± 0.00	0.3 ± 0.00	0.4 ± 0.04
γ–T3	-	-	-	-	-	-
δ–T	-	-	-	-	-	-
β–T3	1.2 ± 0.15	0.5 ± 0.08	1.1 ± 0.21	1.1 ± 0.22	1.2 ± 0.21	1.2 ± 0.04
δ–T3	-	-	-	-	-	-
Total tocol	**6.1 ± 0.07**	**2.0 ± 0.11**	**3.6 ± 0.32**	**1.5 ± 0.23**	**3.3 ± 0.22**	**3.8 ± 0.13**
T.E. TOT	**4.2**	**1.1**	**2.0**	**0.3**	**1.7**	**2.1**
% RDA%	**35%**	**9%**	**17%**	**2%**	**14%**	**18%**
% RDA 80 g	**28%**	**7%**	**14%**	**2%**	**11%**	**14%**

S1: spinach pasta samples**; S2:** chicory pasta samples**; S3:** chard pasta samples**; CTRL:** control semolina pasta**; CM1:** commercial semolina pasta**; CM2:** commercial spinach and semolina pasta

Carotenoid composition in the realized pasta samples

Table 37 shows the carotenoid contents in the produced and commercial pasta samples, expressed in mg/100 g f.w. Regarding the total content of carotenoids, it was considerably higher in samples enriched with green leafy vegetables. In fact, S1, S2 and S3 showed a total content of carotenoids of 17.81 - 12.73 - 15.16 mg/100 g f.w, respectively; while, CTRL, CM1 and CM2 showed very low values. In particular, the most found compound was lutein, with values ranging from 8.6 to 10.5 mg/100 g f.w.; followed by β-carotene which was found in a high quantity in S3 (5.7 mg / 100 g f.w.). **Table 37** also reports values of vitamin A, expressed as Retinol Equivalent (R.E.) (mg/100 g f.w.) (EFSA 2015). Values of lutein ranged from traces to about 11 mg/100 g. Values similar to those of enriched pastas (S1, S2 and S3), could be associated with more than 50% reduction in risk for age-related macular degeneration (AMD) and cataract (Alves-Rodrigues and Shao). Taking into account the Recommended Daily Allowance (RDA) for vitamin A, which is 800 μg/day (EU 1169/2011), 100 g of pasta

samples contribute to from 40% of the RDA in S2 to about 151% in S3, so that 100 g of these pasta samples can be declared as a " source of vitamin A".

Table 37. Carotenoid composition in the investigated pasta samples (mg/100 g f.w.).

Compounds	S1	S2	S3	CTRL	CM1	CM2
Lutein	10.5 ± 0.29	8.6 ± 0.38	9.5 ± 0.52	0.01 ± 0.00	0.07 ± 0.02	1.0 ± 0.01
Zeaxanthin	0.3 ± 0.04	0.3 ± 0.00	0.2 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.1 ± 0.03
Violaxanthin	1.4 ± 0.29	0.6 ± 0.02	0.6 ± 0.44	-	-	-
Neoxanthin	1.7 ± 0.27	0.9 ± 0.04	1.1 ± 0.00	-	-	-
α-carotene	0.3 ± 0.02	0.2 ± 0.01	0.5 ± 0.03	-	-	0.03 ± 0.03
13cis-β-carotene	0.6 ± 0.01	0.3 ± 0.00	1.1 ± 0.05	-	-	0.1 ± 0.00
β-carotene	2.8 ± 0.14	1.7 ± 0.13	5.7 ± 0.65	-	0.01 ± 0.00	0.2 ± 0.02
9cis-β-carotene	0.2 ± 0.00	0.1 ± 0.00	0.3 ± 0.03	0.01 ± 0.01	-	0.02 ± 0.00
Total Carotenoid	**17.81**	**12.73**	**15.16**	**0.03**	**0.11**	**1.36**
R.E. (mg/100 g WB)	533	317	1050	0.8	1.7	43
% RDA (800 μg/100g)	67	40	151	0.1	0.2	54

S1: spinach pasta samples**; S2:** chicory pasta samples**; S3:** chard pasta samples**; CTRL:** control semolina pasta**; CM1:** commercial semolina pasta**; CM2:** commercial spinach and semolina pasta

8.4.3 Cooking quality

A sensory evaluation of the different produced pastas was carried out in order to evaluate the acceptability of the enriched products **(Table 38.)**. Parameters of stickiness, firmness, and bulkiness, which are generally used as qualitative parameters of semolina pasta, were considered. The sensory evaluation was performed in one day and the pasta products were cooked before being served. The optimal cooking time varied between 5:00 – 6:25 minutes. The results showed that it is possible to produce nutritionally valid pasta with excellent cooking quality, by only partially replacing semolina with the investigated vegetables. In fact, total score veried between 79 and 89.

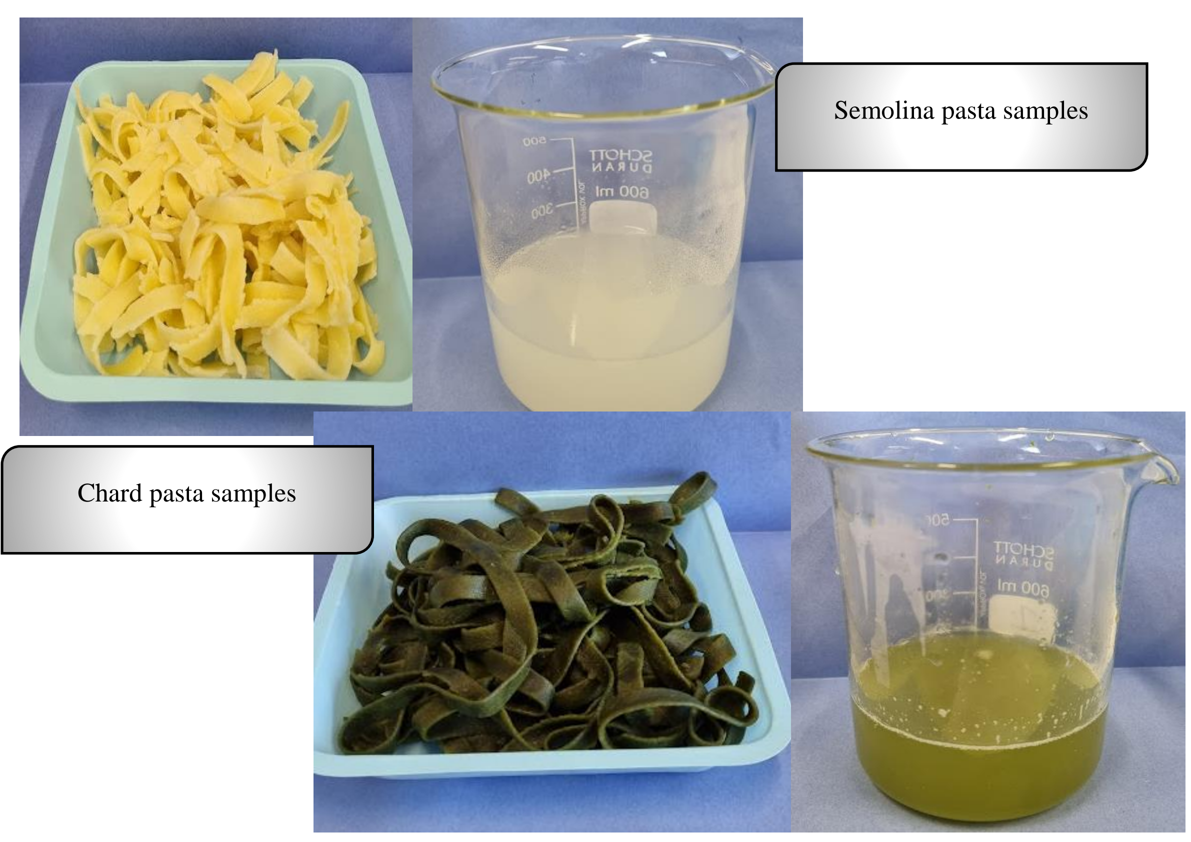
The addition of 15% freeze-dried vegetables in the formulations, in general, led to satisfactory values. In particular, S1 and S3 were found to have an excelent cooking quality, with a score > 80. Only that of sample S2 was rated slightly lower than the other samples, but resulted in a good cooking quality (total score >70 to 80).

Because of their high nutritional value, the investigated green leafy vegetables are an appropriate ingredient for the enrichment of pasta-like products; results obtained suggest that the incorporation of vegetables do not lead to undesirable taste and resulted in a good firmness and stickiness. A very large number of published studies on the enrichment of pasta products refer to nutritional improvement as their goal (Manthey at al., 2004). The fact that pasta is largely consumed worldwide is a good justification for the nutritional improvement of this type of product.

Table 38. Cooking quality of experimental pastas.

Sample	Cooking quality				Panel comments	Cooking time
	Stickiness	*Firmness*	*Bulkiness*	*Total score*		*(min)*
S1	87	90	90	89	Bright green color, pleasant and taste smell, optimal consistency	05:00
S2	80	78	80	79	Dark green color, herbaceous flavor, bitter taste and aftertaste, optimal consistency	05:15
S3	85	90	90	88	Intense green color, pleasant smell, sweet taste, optimal consistency	05:47
CTRL	80	85	80	82	Light yellow color, optimale consistency	06:25

S1: spinach pasta samples**; S2:** chicory pasta samples**; S3:** chard pasta samples**; CTRL:** control semolina pasta**.**


Semolina pasta samples
Chard pasta samples

Chicory pasta samples

Spinach pasta samples

8.5 Conclusions

Green leafy vegetables are a rich source of bioactive compounds and pasta is an ideal matrix to incorporate unconventional ingredients or raw materials and bioactive compounds. The objective of this activity was to incorporate freeze-dried vegetables powder as an ingredient to make dried pastas of high nutritional quality. Overall, using balanced formulations and appropriate technologies, leafy vegetables pastas are a good alternative food, with high nutritional quality and a good healthy properties. Since pasta is very appreciated by children, unlike vegetables in general, the incorporation of vegetables in pasta coul be also a healthy alternative for children who normally do not eat vegetables, and could be used as part of a strategy to fight obesity amongst children.

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CHAPTER 9

General conclusion

9.1 General conclusion and future perspectives

The topic of protective components in dietary vegetable-based foods is an immense issue to address. Indeed, there are numerous plant-based foods with several protective components within them and highly diversified technological processes applied to them. Thus, given the importance of vegetables and vegetable-based products in the human diet, the aim of the research was not only the qualitative and quantitative evaluation of the presence of bioactive compounds of different vegetable products, but also the evaluation of the influence of technological treatments on their content, the identification of product and process markers and, finally, the development of healthy foods enriched with the identified bioactive compounds. Interesting topics for discussion have emerged from the overall results. Globally, the results obtained in the first section can provide more information on the nutritional value of gluten-free minor cereals and pseudocereals, with a particular focus on some bioactive compounds, such as carotenoids, tocols, thiamine and riboflavin. All analyzed samples showed a high content of bioactive compounds. In the light of these results, this research is of a great importance in order to formulate new gluten free cereal-based products with a high nutritional value. Moreover, data coming from this study may be included in food nutrient databases on these products, to date limited, if not lacking.

Results emerging from the second section showed significant differences in the tocol and fatty acids profiles in laboratory made bakery products, which reflected the tocol and fatty acids composition of the specific fat/oil used as ingredient, providing the possibility to use these compounds as markers to identify the origin of the oils/fats used. Moreover, the obtained data can provide more information on the effects of technological treatments on tocols in biscuits.

Data coming from the third section on wild vegetables demonstrated that they are a high source of bioactive compounds, such as carotenes xanthophylls, tocols, fiber and thiamine, such as to encourage an in depth investigation on their health potentiality and to justify their future commercial production, given the consumer and industry increasing demands for healthy foods. The effect of the domestic boiling and steaming treatments on bioactive compounds (tocols, carotenoids, phenols) and or the antioxidant activity of the studied vegetables, were compared. For hydrosoluble compounds, the obtained results confirmed their loss in the cooking water. For liposoluble ones,

obtained data demonstrate that, in order to have reliable data of the effect on in-house processing on their content, a complete extraction procedure, together with the evaluation of the solid loss ought to be taken into consideration. Further experiments are needed in order to investigate the nature of the solids solubilized in the cooking water, also in relation to different vegetables, with a different tissue structure.

Given the positive obtained feedback, functional products were produced. In particular, a cereal product, such as pasta, was used as a vehicle of the beneficial substance coming from the studied vegetables. Each case study addressed proved that the investigated vegetables could be used as a high value food ingredient, allowing to better satisfying consumer demand for healthy food products, in a more sustainable perspective. Wild edible plants can represent, for our country, a strategic resource, a point of strength to which a number of positive values can be associated: ecological, nutritional, socio-cultural and agro-food. This could be true mainly during this delicate historical period that we are going through, due to the Covid-19 pandemic, which is leading us towards a new paradigm that supports local self-reliance and domestic farming production. Domestic and community vegetable patches, the traditional agro-ecosystems and farmer markets are shaping up as increasingly essential services. Within this socio-economic framework, the wild edible plants could constitute a precious extra food source for the present and future generations, so studies about these vegetables is highly worthwhile.

One of the major issues related to the research about WEPs is that most of the available studies have been carried on with a sector-specific approach. Therefore, a project involving the contribution of different research activities is strictly needed. Ethnobotanical studies could allow a collection of dispersed information about an "intangible" heritage, of inestimable value for Italy. Agronomical studies will be addressed to assess the suitability of cultivation of the selected species having promising nutritional values. The cultivation of WEP will promote sustainable agriculture by decreasing the pressure on the natural biodiversity of the territory, reducing the harvest of plants in the wild.

A nutritional evaluation, together with bioaccessibility/bioavailability studies, will enhance the knowledge of nutritional and health qualities of the selected WEPs, improving databases on the nutritional composition, content and biological accessibility of the bioactive compounds of these species. Finally, the study of the technological processes will acquire information on the feasibility of traditional and mild technologies to obtain processed products with high nutritional and sensorial properties and ready for

market placement, fostering the development of new agriculture and food productive sectors.