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Traditional crops and foods: documentation, analytical characterization, retention factors of bioactive compounds

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To my parents,

Patrizia and Bruno…

and to my grandmother,

Quinta … my favourite

“local informant”!!!

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Chapter 1

Introduction

1.1. Preface

This work was carried out within the research and documentation on traditional foods of plant origin, that is going on as a mainstream research line at the Food Plant Production and Quality Group of my department, since several years.

This work was started by me as participant in BaSeFood project (www.basefood-fp7.eu) and went on independently after the end of the project itself.

The experimental part of this thesis mainly deals with two topics, both carried out on different crops: **a)** the characterization of raw materials in term of the content of phytochemical compounds with potential health-promoting properties; **b)** the experimental determination of yield factors during traditional processing and phytochemical compounds retention factors during cooking.

The documentation part was carried out, following the BaSeFood scheme of qualitative surveys, in a specific area of north Tuscany. Following this part, a still on-going research line on wild leafy food plants of the *Asteraceae* family was set up.

The motivation of this organization and the milestones of its background are shortly explained later, in the introductory part.

Basically, the following points were considered:

- Traditional foods are since thirty years attracting the interest of industry and regulatory bodies as potential opportunities to manufacture value added foods, at different levels. For this, conspicuous efforts were devoted to formally define these foods.
- Evidence suggests however that traditional foods have some characteristics making such a formal definition rather far from representing their intrinsic nature.
- Traditional foods have been often associated with health promoting foods, without however any real evidence in support of this generalization.
- BaSeFood investigated traditional foods in their local context, giving further contribution to their allocation in term of local resources, variability, evolution and perceptions. The project also pointed out how the “traditional” and “health promoting” claims may belong to different spheres of perception.
- At the same time, the project allowed to allocate local raw materials, that were selected from in-depth investigation, of their analytical properties and the evolution of some of their phytochemical compounds following traditional processing schemes and cooking.

1.2. Different approaches to define traditional foods

1.2.1. *European legislation*

The previous European Community foodstuff legislation was characterized by common market objectives and harmonization approaches, with the main issue to overcome national "technical barriers" and promote trades. At the end of '80 years a general dissatisfaction of European State Member conveyed in a stronger demand of legal protection for their own typical products. So foodstuff legislation objectives were reformulated and mainly addressed to highlight topics such as food quality, consumer protection and support to small agricultural producers (Tosato, 2013).
In particular, as a result, the European Union (EU) issued two regulations with respect to traditional foods:

1) *Council Regulation No 510/06 of 20 March 2006 "On the protection of geographical indications and designations of origin for agricultural products and foodstuffs" (Commission of the European Communities, 2006a) (replacing the previous Council Regulation No 2081/92 of 14 July 1992 "On the protection of geographical indications and designations of origin for agricultural products and foodstuffs" (Commission of the European Communities, 1992a));*

2) *Council Regulation No 509/06 of 20 March 2006 "On agricultural products and foodstuffs as traditional specialities guaranteed" (Commission of the European Communities, 2006b) (replacing the previous Council Regulation No 2082/92 of 14 July 1992 "On Certificates of Specific Character for agricultural products and foodstuffs" (Commission of the European Communities, 1992b)).*

The introduction of the two regulations determined the fact that many European foods have been registered as "Protected Designation of Origin (PDO)" or "Protected Geographical Indication (PGI)". Whereas very few foods have been certified as "Traditional Speciality Guaranteed (TSG)", probably due to the lack of a definition of the term "traditional" in the previous regulation 2082/92, and a consequent inadequacy in the protection of producer and food, as also discussed by Trichopoulou *et al*. (2007).
Only recently the EU introduced a definition for the term "traditional", with special regard to foods, as reported following:

"*Traditional means proven usage in the Community market for a time period showing transmission between generations; this time period should be the one generally ascribed as one human generation, at least 25 years.*" (European Commission, 2006)

1.2.2. *Italian legislation*

Considering national legislation, a definition for traditional food products in Europe is given by the Italian Ministry of Agriculture, as following:

"*Agrifood products whose methods of processing, storage and ripening are consolidated with time (at least 25 years) according to uniform and constant local use.*" (Ministero Agricoltura, 1999)

Recently the 15th revision of the national list of Traditional Agrifood products (Gazzetta Ufficiale n. 168, 22 July 2015) has been published. The list, not including products registered as PDO or GPI, consists of 4881 traditional products, of which 68 are newly added. Tuscany shows the highest number, with 461 Traditional Agrifood products, followed by Campania (457), Lazio (393), Emilia-Romagna (378), and Veneto (370).
Based on the data reported on the ISTAT website (www.noi-italia.istat.it), at 31 December 2013, 261 Italian agro-food products (excluding the wine sector) obtained recognition PDO, PGI or TSG. So Italy shows the highest number of certifications at the EU level followed by France (208), Spain (173) and Portugal (123). In particular 26.9% of the total of Italian agro-food certified products are PDO, 17.1% are PGI, and the remaining 4.7% are TSG. The most widely represented products in Italy in 2013 included fruit, vegetables and cereals (101 products, with a majority of PGI), followed by cheeses, extra-virgin olive oil and meat preparations.

1.2.3. *The scientific approach*

The definition of traditional food has been scientifically analyzed from different approaches. Following are reported elaborated specific definitions of traditional foods from two former projects funded by the European Union, EuroFIR and TRUEFOOD.
A more academic, substantially static, and elaborative definition has been elaborated by the EuroFIR Network of Excellence (EuroFIR, 2007; Trichopoulou *et al.* 2006). EuroFIR aimed to provide data of traditional foods for inclusion in national food composition tables along with their

raw ingredients and recipes. The inclusion of traditional foods in national databases was necessary to accurately estimate population dietary intakes. The determination of macro and micronutrient composition of traditional foods could be important to elucidate their role in the traditional dietary pattern of a population. In fact many traditional foods are generally linked to health promoting effects, and this association is still poorly explored.

The EuroFIR approach has been based on a critical re-elaboration of previous definitions and regulations, focusing mainly on 2 needs: 1) to elaborate a definition useful in the research activities, and 2) to standardize the concept of traditional food, in view of their registration, within EU regulatory system, above discussed. In particular EuroFIR Network of Excellence (EuroFIR, 2007; Trichopoulou *et al.* 2006) defined "traditional" as:

"conforming to established practice or specifications prior to Second World War, transmitted from generation to generation."

And "traditional food" as:

"a food for a specific feature or features, which distinguish it clearly from other similar products of the same category in terms of the use of "traditional ingredients" (raw materials or primary products) or "traditional composition" or "traditional type of production and/or processing method."

Which definition includes also statements about traditional ingredients, traditional composition and traditional type of production and/or processing. In particular:

- "*traditional ingredients*" are represented by raw material or primary product that has been used in selected areas, still in use nowadays (even if their use was abandoned for a certain time and then reinstated); their characteristics are in accordance with both, national and European legislation;
- "*traditional composition*" is the list of ingredients that was first established prior to Second World War and passed down through generations (even if this composition was abandoned and then reinstated); when necessary is differentiated from the composition generally described for the wider group of foods to which the product belongs;
- "*traditional type of production and/or processing method*" is transmitted from generation to generation, has been applied prior to Second World War and remain in use (even if abandoned for a time); it may have undergone adjustments to comply with national or European legislation, or to incorporate updated technologies.

From what reported by Trichopoulou *et al*. (2007) the selected limit "prior to the Second World War" is intended as a reference time prior to the era of mass food production.

On the other hand TRUEFOOD aimed to improve quality and safety into traditional European food production by introducing innovation along the food chain, using a "fork to farm" approach. One of the main objectives of the project was the evaluation of consumer perceptions, expectations and attitudes with respect to 1) safety and quality characteristics of traditional foods, and b) innovations that could be introduced into the traditional food industry. So TRUEFOOD traditional food definition was generated by a quantitative survey mainly aimed at defining consumers' perception. The concepts of traditional food products and innovations in traditional food products were stressed, and their comparisons were carried out in six European countries (Belgium, France, Italy, Norway, Poland and Spain) throughout planning focus groups. A textual and semantic statistical analysis was used for a more objective approach in the elaboration of obtained data. Based on consumers' and manufacturers' opinions, for Guerrero *et al.* (2009) the most representative definition of traditional food seemed to be:

"a product frequently consumed or associated with specific celebrations and/or seasons, normally transmitted from one generation to another, made accurately in a specific way according to the gastronomic heritage, with little or no processing/manipulation, distinguished and known because of its sensory properties and associated with a certain local area, region or country."

In this survey, conduct within TRUEFOOD project, four main different dimensions were identified to express traditional food: habit and natural, origin and locality, processing and elaboration, and sensory properties.

By means of cross-sectional data collected through TRUEFOOD surveys, another study (Pieniak *et al*. 2009) investigated the association between traditional food consumption and motive for food choice. Structural equation modeling were used in the elaboration of data. In this study the natural content, related with emotional terms such as traditional and homemade, was positively associated with traditional food consumption, suggesting the current importance of the concept of food naturalness for consumers. Similar results were obtained for all the countries taken into account, even if traditional foods appeared closely connected to the culture and the population of the place where they are produced.

1.2.4. *Alternative concepts of "traditional foods"*

Recent efforts have been made to describe traditional foods, trying to find a single definition. Static and centralized approaches to define traditional foods, through standard concepts are reported in the previous paragraph. The logic of introducing standards, with a top-down communication approach guided by credence characters (which definition is reported below at paragraph 1.5), resulted a weak model. This has been also demonstrated by the fact that registration of real local scale food products was seldom undertaken by local growers or processors, being instead mostly pushed by public administrations and organizations with purposes of image promotion, rather than protection. Often, certification is likely perceived by local actors of the food chain as a way to protect larger-scale productions from foreign imitation.

Moreover, definitions provided by different actors along the food chain offer different perspectives, as also partially discussed above.

Alternative strategies to analyze the concept of traditional foods, somewhat deviating from the mainstream of formal definitions, moved from a top-down communication to a bottom-up approach, aiming to meet local consumers and producers' perceptions (Amilien and Hegnes, 2013; D'Antuono, 2013). The concept of traditional food, in this alternative approach, was brought out as being a modern construct: in fact, in the past, tradition was practically coincident with the term customs (Amilien and Hegnes, 2013). Moreover traditional is not seen as a fixed and static concept, but is intended as a dynamic notion composed of various factors, strictly correlated to place, time and culture. Amilien and Hegnes, (2013) describe the dynamic concept as a "sensitizing" one, using the description adopted by Blumer (1954), and hereafter reported, to describe this term:

"A definitive concept refers precisely to what is common to a class of objects, by the aid of a clear definition in terms of attributes or fixed bench marks. ... A sensitizing concept lacks such ... and gives the user a general sense of reference and guidance in approaching empirical instances. Whereas definitive concepts provide prescriptions of what to see, sensitizing concepts merely suggest directions along which to look. The hundreds of our concepts – like culture, institutions, social structure, mores, and personality – are not definitive concepts but are sensitizing in nature. ... They ... rest on a general sense of what is relevant. There can scarcely be any dispute over this characterization."

1.2.5. *The case of BaSeFood project: traditional foods and food systems*

1.2.5.1. *BaSeFood's synthesis and scopes*

BaSeFood – Sustainable exploitation of bioactive components from the Black Sea Area traditional foods – is a 3 year collaborative research programme (2009-2012), funded by 7th Framework Programme, aimed at local investigations of traditional foods (D'Antuono *et al.*, 2010).

The main goals of BaSeFood were to contribute and to investigate for a better understanding and integration of the concepts of health promoting foods and traditional foods, in order to create the knowledge base for a sustainable economic development in the area of production and processing of tradition healthy foods (www.basefood-fp7.eu).

During the European project investigations were carried out following a scheme of qualitative, cross-cultural, on-site surveys of traditional foods and plant raw material, including uses, cultural, diversity and conservation issues. The on-site investigations were conducted according to general qualitative survey methodologies, by face to face, in-depth interviews. Documentation sheets were obtained as result of this activity.

In particular, this kind of surveys were addressed to:

1) document on place all aspects of knowledge related to local foods;

2) investigate facts in natural, rather than in experimental settings;

3) retrieve evidence-based information, latent in diffuse knowledge, but often absent in official, formal scientific knowledge;

4) help generating, besides testing hypotheses;

5) allow cross-cultural comparison of the present status and perspectives of traditional food systems;

6) help programming and tuning possible future experimental research.

As a result 839 foods, 456 of which were reported as "main" foods and 383 as variants, were documented and described (D'Antuono, 2013). Variants represented different situations, in some case they were very similar to the basic food, in other cases they showed substantial variation. This fact, intimately connected to local variability and diversity of traditional foods, highlighted the difficulty of their classification with formal schemes.

Besides recipes also traditionally adopted processing were documented and described by means of flow charts.

Afterwards selected traditional crops and foods were nutritionally and microbiologically characterized. In particular bioactive compounds were investigated in raw materials and along

documented traditional processing and cooking ways, in order to determine technological-chain effects on the bioactive retentions during traditional food preparations.
Plant bioactives resulted a specific target of BaSeFood investigation. The characterization of plant raw materials was a pre-requisite for both the analysis of plant diversity and the determination of the evolution of these substances during food preparation and processing.

1.2.5.2. *BaSeFood's final remarks*

The main issues arising from BaSeFood activities were:

- local food systems are mainly referred to available local resources;
- the intrinsic variability of traditional foods in local contexts is directly linked to crops' availability and seasonality;
- sometimes traditional foods are characterized by the presence of a specific raw vegetable representing the main ingredient;
- some landraces have been or are actually in the process of recovering;
- traditional foods are still poorly analytically characterized;
- at local scale production modern technologies are employed in a different level of application;
- the transition from familiar production to the market requires a higher uniformity of raw materials and procedures, moving towards a standardization of end products.

If referring to a local context, generally comparable to a closed system and with short distance exchanges, the perception of traditional foods' value occurs spontaneously. In fact, local people perception is formed by a complex of perceived immaterial traits. On the other hand, a contemporary and urban context is associated to an open system. In this case consumers have almost completely lost direct contact with food production and the term "traditional" is a credence quality trait, generally communicated by other parties.
So, in a local traditional food context great importance assume local skills and knowledge, and one of the main challenges could be represented by the transmission of this capability to modern urban consumers.
This approach adopted in BaSeFood, defined as a bottom-up approach, was focused at the increasing awareness and re-evaluation of experience qualities; this could be an interesting option for future studies.

Moreover BaSeFood investigations highlighted the fact that the perceptions of specific health promoting effects do not represents a prominent aspect of traditional food values in local communities.
These main concepts have been described by D'Antuono (2013), in particular two main sentences are reported to conclude the study:

1) *"The intrinsic variability of traditional foods in local contexts, making them difficult to define in a formal way"*

2) *"The peculiar nature of traditional food systems, made of a mix of close and open systems traits"*.

1.3. Traditional crops and agricultural biodiversity

The term agricultural biodiversity has been defined in a FAO conference (1999) as the variety and variability of animals, plants and micro-organisms that are used directly or indirectly for food and agriculture, including crops, livestock, forestry and fisheries. Agricultural biodiversity is composed by the genetic diversity at three levels:
a) diversity of genes within species (intraspecific diversity),
b) among species (interspecific diversity),
c) by the diversity of agro-ecosystems (agro-biodiversity).
Traditional foods are generally produced by using traditional crops and products, so they are a good opportunity to maintain in the local communities the use of the available landraces, representing a very important contribute to preserve the agricultural biodiversity of that geographical area. However the characterization of biodiversity is critical to explore the variability of different plant foods, with respect to their nutrient and bioactive component content.
Johns *et al*. (2013) described agricultural biodiversity as a link between traditional food systems and contemporary development, social integrity and ecological health. In particular after the unhealthful consequences of modern diets, the authors proposed agro-biodiversity as a possible solution to contrast malnutrition, by means of a diet richer in micronutrients. Maintaining traditional food systems is associated by Johns *et al*. (2013) with a corresponding diversity of crops varieties, helpful against diet-related chronic disease. A clear example of this fact is represented by the Mediterranean diet (Bach-Faig *et al*., 2011).

Moreover agrobiodiversity could represent a good opportunity for smallholder farmers' participation as contributors and beneficiaries.

The role of biodiversity has been described by Wahlquist and Specht (1998) in one review, taking into account several aspects, as the role of diversity in human nutrition, and also the role of food in the evolution of the human species. The ways by which biodiversity confers health are described by McMichael (1993), underlying again the importance of biodiversity in contrasting malnutrition. Moreover a variety of food source is recognized as an important resource against climatic and pestilent disasters, invasive plants and animals, pathogens, and toxins, and for the extraction of therapeutic compounds. Exploring the diversity of plants, animals and ecosystems in an area appear to have beneficial effect on mental health as well, with a higher feeling of "belonging to the landscape".

As a consequence of this conceptual movement, several review works have been developed about the topic of biodiversity, food security and diets (Frison *et al.*, 2006; Toledo and Burlingame, 2006; Burlingame *et al.*, 2009; Frison *et al.*, 2011).

A high variability of raw traditional crops could be acceptable for home production in which most of the operations are manual. However commercial scale production generally calls for a high degree of standardization of raw materials (i.e. shape, maturity and harvest time). So, in many cases, the recovery of traditional crops is accompanied by a selection of types that better meet these standards.

1.4. Traditional foods: local crops, processing/cooking and recipe information

Traditional foods are sometimes perceived as healthy; however there is a lack of information regarding their composition to confirm this hypothesis (Trichopoulou *et al.*, 2007). The term "traditional food" include a myriad of different food species, and varieties within species; in the past no sufficient attention has been addressed to their characterization, being considered underutilized, therefore less important. However the information regarding the nutrient content of these foods, and the characterization of the local species used in their preparation, is very important in order to complete food composition databases, that can be used by different actors along the food chain, and for the valorization of minor crops (Burlingame *et al.*, 2009). These activities result very useful in particular for consumers that could discover or re-discover traditional foods, and for producers that could use the data in breeding programmes and also for the optimization of processing.

So the characterization of raw materials, being part of traditional foods, as local crops, with special regard to their content in bioactive compounds and their evolution during processing and cooking, is still necessary.

The valorization of local crops, taking into account the diversity and specific characteristics, such as the psychological push to consume, sensorial aspects, and health promotion, needs different approaches. First of all it requires the characterization of the within species variability, with particular attention to the content of bioactive compounds.

Also the analysis of the processing used to obtain traditional foods from raw materials is important to evaluate the technological chain effects on bioactives and to compare the different local-scale production flow charts. In this way it is possible to define the impact of the process' critical phases on the retention of phytochemical compounds with potential health promoting effects or impacts on the organoleptic characteristics.

In the same way the informations regarding recipes, such as name, preparation method, scale and other documentation, are important. A series of guidelines, useful for the harmonization of procedures to adopt for recipe information management, have been established during a workshop on "Recipe calculation and information" organized by the COST Action 99-EUROFOODS project. These guidelines are reported in a paper by Unwin (2000), where detailed definitions of the information required to identify, describe and document a recipe are provided. Moreover the author included some calculation, to take into account preparation losses of material and changes in weight during cooking, in order to obtain yield and nutrient retention factors.

Recipe information and calculation is becoming increasingly important to compile and to complete food databases and to assess food component intakes, a key element for food consumption and epidemiological studies.

1.5. Food quality

The concept of food quality is based on "objective" and "subjective" characters. In particular three types of food quality are based on objective characters:

1) product-oriented quality (physical and descriptive aspects of a food product),

2) process-oriented quality (the way the food product has been produced),

3) quality control (required standards of the food product to be approved and to be part of a quality category).

Finally user-oriented quality is based on subjective characters (Brunsø *et al*., 2002).

The four types of quality are interrelated, however in the specific user-oriented quality is affected by all other three types.

Analyzing subjective quality, a further distinction can be made (Grunert, 2002):

- Search quality cues: characters directly perceived, that can be ascertained before the purchase;
- Experience quality traits: characters perceived after experiencing the product;
- Credence quality traits: characters that cannot be perceived, but they need the communication from third parties.

An example of this distinction could be represented by the "traditional" character of food that is perceived as an experience quality trait in local context, in which people have a direct contact with food production. On the contrary in an urban context the "traditional" character is considered as a credence quality trait that need to be communicated by third parties, due to the loss of direct knowledge about food production.

The distinction between subjective quality characteristics has been handled by means of a multi-attribute models (due to its multidimensional nature), with the resulting distinction between intrinsic and extrinsic product attributes (Olson and Jacoby, 1972). Intrinsic factors are related to the product itself (i.e. colour, shape, sensorial properties such as texture and flavour, and presence of bioactive compounds with beneficial effect); extrinsic attributes refer to everything else (i.e. religious and cultural aspects, use of pesticides).

During the last decades health attributes, such as nutritional and health-promoting value, of food have become very important characters to many consumers, especially in developed countries.

One of the main factor determining this trend is the increased concern, especially in these countries, regarding the link between chronic-degenerative diseases and bad food habits (Rock *et al*., 2012). So health attributes are mainly linked to higher quality expectations and a growing concern about safety and risk-related issues.

1.5.1. *Changes in food habits and food processing*

Previous policies, spread worldwide, aimed to reduce hunger in developing countries, introduced high-energy cereal staples with a bigger yield of productivity, leading to a simplified diet and a lack of dietary diversity, in particular if considering micronutrients (Frison *et al*., 2006). The results of these choices, reported in a paper from the Food and Agriculture Organization (FAO, 2003), have been a relative decline of hungry people from developing countries and, at the same time, an

increase of health diseases, as obesity and hypertension, with the following coexistence, especially in developing countries, of hunger and malnutrition.
On the other side, in developed countries, changes in the society due to various phenomena, such as globalization, industrial development, urbanization, high-input industrial agriculture and long-distance between production and consumption sites, have determined changes in the structure of food production leading to an increasing share of processed and commercial foods. For example the absence of employed women from their homes for a large part of the day has resulted in the replacement of "domestic" food with "convenience" foods, such as easy-to-prepare or ready-to-eat. Again these changes led to a simplification of diets, with a negative impact on quality.
Moreover various food scares and crises, that occurred in the last decades, resulted in a general consumers' dissatisfaction and lack of trust in the food chain.
Following these facts, the necessity to recover and reintroduce traditional crops and traditional food systems into dietary habits has increased. This request has been emphasized also by the World Declaration and Plan of Action for Nutrition, during the International Conference on Nutrition (1992). Although this necessity was mainly due to the fact that in developed countries traditional food are perceived as an element of healthy lifestyle (as discussed also above, in paragraph 1.2.5.2.).

1.5.2. *Health effects of crops: bioactive compounds*

The protective role of vegetables and fruit consumption could be explained by the occurrence of bioactive compounds (phytochemicals) (Liu, 2003). Bioactive compounds, occurring naturally in plants, can have or not nutritional value and are defined in a previous report by Gry *et al*. (2007) as:

"inherent non-nutrient constituents of food plants and edible mushrooms with anticipated health promoting/beneficial effects and/or toxic effects when ingested".

Bioactive compounds are secondary metabolites of plants, covering many different compound classes and including large numbers of closely related compounds. These metabolites have important functions in plants, such as the interaction of the plants with the environment and the mechanisms of defense against others harmful and invasive organisms.
Bioactive compounds have been largely investigated because of their beneficial effects. Epidemiological studies have demonstrated that they can help in slowing the aging process and

reducing the risk of many chronic diseases, such as cardiovascular diseases and cancer (Tokuşoğlu and Hall, 2011).

1.5.3. *The fate of bioactive compounds during processing/cooking*

Recently an internet-deployed database, EUROFIR-Basis, has been developed combining food composition and biological effects data for plant-based bioactive compounds, as well as an up-to-date list of the major food plants consumed in Europe (Gry *et al*., 2007). The database includes data on approximately 200 bioactive compounds (carotenoids, phenolic compounds, flavonoids, lignans, stilbenes, capsacinoids, cysteine sulphoxides, glucosinolates, and phytosterols).
Moreover EUROFIR-Basis contains data on approximately 550 plants and some processed plant based foods are included as well (Kiely *et al*., 2010). In fact most dietary vegetables are eaten after cooking and the thermal treatment can produce a change in the bioactive content of cooked product. Many studies have evaluated the effect of different ways of cooking on the bioactive content, such as boiling, steaming, frying, baking and roasting. Palermo *et al*. (2013), in a review, tried to describe, from a general point of view, the effect of cooking on the phytochemical content of foods: the changes of bioactive compounds upon cooking were mainly due to two opposite phenomena, thermal degradation (reducing the concentration) and matrix effect (increasing the extractability). So the changes in bioactive concentration after cooking are strictly related to the initial concentration in raw material, the processing parameters (e.g. time and temperature), the food matrix, and the chemical nature of the examined compound.
Mathematical kinetic modelling represents a useful tool to simplify and predict complex reactions that can occurs during food processing or cooking (Van Boekel, 2009). Moreover, modelling the fate of bioactive compounds during processing or cooking could be useful in the comparison of different local populations and processing or cooking ways. Moreover the evaluation of kinetic model could contribute to better elucidate and highlight critical points, for the bioactive retention, along the flow chart of food production.

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Chapter 2

Scopes and Outline of the Thesis

The aim of the PhD research has been the characterization of plant raw materials used in the preparation of traditional foods, with special respect for the determination of their content of bioactive compounds and their retention factors during cooking and processing. These experimental activities were based on materials previously located and selected on the basis of on-place documentation, with questions related to traditional crops and foods. The activities have been developed using the following scheme:

Chapter 3. DOCUMENTATION AND SELECTION

A preliminary activity of documentation has been carried out in selected areas. The interviews conduct during the documentation have focused on: **1)** species for which characterization opportunity could emerge, by means of on-site investigations and with local stakeholders; **2)** local-scale processing for these species and their variability; **3)** critical points in the flow charts.
This activity was carried out as part of research activities. Moreover, based on documentation results, plant raw materials, and cooking or processing, were selected and taken into account for the following experimental part.

EXPERIMENTAL PART

Chapter 4. PRIMITIVE WHEATS

A previous documentation on primitive hulled wheats was carried out within the BaSeFood project in the Black Sea Area countries (Bulgaria, Georgia, Turkey), in Armenia and in Italy.
Local populations of *Triticum monococcum* and *T. dicoccum* (hulled wheats) from Bulgaria, Georgia, Turkey, Armenia and Italy were characterized for their content in bioactive compounds: tocopherols and tocotrienols, carotenoids, phytosterols, free and bound phenolic compounds (**Chapter 4.1**).

Glume removal and kernel crushing were considered as a characterizing and critical point of hulled wheat processing. Traditional processes, still used in Turkey and in Armenia, were compared with an updated plant found in Italy. Hulled wheat fractions were directly on-plant sampled and used to: **1)** calculate process yield factors (**Chapter 4.2**), and **2)** evaluate retention of bioactive compounds (**Chapter 4.3**) as affected by traditional hulled wheat processing.

Chapter 5. BRASSICACEAE

A previous documentation on kale (*Brassica oleracea* ssp. *acephala*) was carried out within the BaSeFood project in the Black Sea Area countries, Portugal and Italy. Kales were selected since they are at the same time a typical example of vegetable mostly used at local scale, and rich of putative health-promoting phytochemical compounds.
Local kale landraces from Turkey, Portugal and Italy, locally sampled or grown in a common environment, were characterized for their content of bioactive compounds: glucosinolates, phenolic compounds, carotenoids and chlorophylls (**Chapter 5.1**).

Part of my period abroad was carried out at the Food Quality and Design Group of Wageningen University. During the stay experimental works were carried out to: **1)** study the fate of kale bioactive compounds (glucosinolates and indole-derived products, ascorbic acid, carotenoids, total phenolic content and antioxidant activity) and visual quality traits (hue and saturation) following traditional cooking methods (boiling, steaming and stir frying) and time (**Chapter 5.2**); **2)** assess the effect of added ingredients (potato or corn starch, lentils proteins and onion) on glucosinolate thermal degradation in broccoli (*Brassica oleracea* L. var. *italica*) (**Chapter 5.3**).

Chapter 6. ASTERACEAE

The use of edible wild leafy vegetables of the *Asteraceae* family, and *Knautia integrifolia* of the *Dipsacaceae* family, were documented during my PhD activities in the area of the Tosco-Romagnolo Apennine. Subsequently, samples of several species were collected in the wild. During a part of the period abroad, carried out in the Research Centre for Agricultural and Food Biotechnology (University of Almería), the collected samples were characterized for their content

in phenolic compounds by means of UHPLC coupled to high resolution mass spectrometry (Orbitrap-MS) (**Chapter 6.1**).

Even if not reported as part of this thesis, edible wild leafy vegetables are also being characterized for their content of phytochemicals (sesquiterpene lactones, ascorbic acids, carotenoids and chlorophylls). Moreover, following the same scheme adopted for kale, retention studies during boiling and steaming have been carried out to investigate fate of phenolics, sesquiterpene lactones, carotenoids and chlorophylls following cooking.

Chapter 7. CONCLUSIONS

In the final chapter the main findings and their implications are summarized and discussed.

Chapter 3

Documentation

The specific documentation activities were rather limited during this PhD work, that was mainly focused on the experimental and analytical part. However, documentation of traditional foods and crops was carried out throughout on-site investigation in a selected area of north Tuscany. This allowed to locate wild greens as interesting research targets to be included in the experimental part. General qualitative surveys were carried out according methodologies previously adopted in BaSeFood (D'Antuono, 2013). The main layout and results of documentation are hereafter reported.

3.1. Materials and methods

Defining the subjects. A preliminary decision of food groups was done; the activity was mainly addressed to document leafy vegetables, in particular kale and turnip, and wild leafy vegetables of the *Asteraceae* family. However other crops were included, such as corn, pulses, onion, tomatoes and chestnuts. Among wild vegetables particularly interesting resulted the case of *Knautia integrifolia*, of the *Dipsacaceae* family, and wild fennel (*Foeniculum vulgare* subsp. *piperitum*).

Defining the boundaries The target geographic area for the documentation was the Italian north Tuscany, with special regard for the province of Arezzo (Valtiberina area), and the nearby part of the province of Perugia (Alta Valle del Tevere area), in Umbria (**Figure 3.1**). In some cases specific investigation areas were located through personal knowledge and available literature. For example, when considered kale and turnip, the documentation was extended to Casentino and Valdarno areas (province of Arezzo, Tuscany) and to Val di Vara (province of La Spezia, Liguria). In case of edible wild leafy vegetables the documentation was carried out in the Tosco-Romagnolo Apennine area (part of province of Arezzo, Tuscany, and part of province of Forlì-Cesena, Emilia-Romagna).

Defining a checklist of concepts. Checklist of concepts were the same as reported in D'Antuono (2013). Questions to be asked belonged to the following themes: 1) raw materials characteristics and growing; 2) exploitation; 3) local knowledge about uses; 4) food uses and recipes, including variants.

Figure 3.1. Selected area of north Tuscany for the documentation.

Finding key respondent. Key respondents were identified through personal and previous knowledge, or with the help of local organizations and associations. Chosen informants had a direct knowledge of local resources. The investigations were carried out by face to face interviews.

Data interpretation and synthesis. Collected data were organized by means of documentation sheets. In **Figures 3.2** and **3.3** are reported, respectively, an example of food documentation and source documentation sheet, obtained during BaSeFood activities.

HF10. Minestra di pane (informants: KIT16, KIT17, KIT18)
Italy, Toscana region, Arezzo province
Local language: as in Italian
English: literally: bread soup

A variant of minestra di pane (ribollita), Montevarchi, Italy

Ingredients
Palm tree kale or local Black kale leaves
Dry beans
Onion
Carrot
Potatoes
Leaf celery
Olive oil
Garlic
Rosemary
Sage
(Curly head cabbage, leaf beets)
Left-over bread

Amounts
The proportion of the two main ingredients, beans and kale leaves, varied a lot, depending on likes and availability. Roughly, it can be considered 1 kg clean kale leaves for 400 g dried beans. Potatoes as well are an important ingredient, often used in the same amounts as beans.

Procedure

Preparation of the beans
Pre-soak and boil the beans, as in HF8-HF9. In different ways of preparation, the beans can then be completely mashed or, a part of them, is left whole.

Preparation of the "soffritto"
As for HF8, but no animal fats are generally used, only olive oil and typically using finely cut onion, celery, sometimes carrots.

Preparation of the dish
Cut the kale leaves in pieces. Add the cut kale to the soffritto, together with potatoes. Add the mashed beans and their cooking water, then boil until the kale and potatoes are cooked. Adjust with water if necessary. At the end, olive oil flavoured with garlic, rosemary, sage and hot pepper can be added. Put the slices of left over bread in the oven or on an earth or metal pan, to make it a little crumbly, with a bit of olive oil. Soak the bread with some of the soup; then add new layer of bread and again soup. The soup should contain enough liquid to soak well the bread.

Notes to the recipe. The "zuppa di pane" is one of the staple dishes consumed in rural areas of Tuscany. It represented a way to use the bread left over and already a bit dry, since bread was prepared once a week or once each ten days. In some rural areas it was a real staple of the diet: it is reported (KIT17) that it represented a daily meal, alternated with "Pappa al pomodoro", another dense soup with bread, and the "farinata", a gruel made of wheat flour or, in mountain areas, the "polenta" of chestnut flour. The left over "zuppa di pane" was used again the following days, by putting it again on fire, in a pan or a pot, adding sometimes some oil, and boiled again: from here comes the name "Ribollita" with which this soup is nowadays mainly known.

Variants. Being as staple widespread in almost the whole Tuscany region, this soup has an almost endless number of variants, that are not easily defined. Many of them are simply due to the availability of one or more ingredients: the main group of variants are the following

HF10.1. Variant 1. The use of different leafy vegetables, besides or in substitution of black kale. Although black kale is the characterising ingredient, rather frequent is the use of curly head cabbage, frequently used traditionally together with kale, which tends to substitute kale in the

Figure 3.2. An example of a food documentation sheet (www.basefood-fp7.eu).

Acronym: KIT16
Name: Viviano Venturi
Function / profession: technician, co-maneger
Company / employer: Unione dei Comuni del Pratomagno, Radici (firm)
Type of organisation: public / private
City: Loro Ciuffenna (AR)
Address: Azienda Agricola Radici, srl, Via 7 Ponti Ponente, 18 - 52024 Loro Ciuffenna (AR)
Information: Mr. Venturi is an employee of the Union of municipalities of Pratomagno, but also manages, together with his wife, the firm Radici, a small scale firm manufacturing food products from local or anyway low input raw materials, often reproducing traditional recipes. He is also a farmer, growing a substantial part of products, that are also sold fresh at local zero miles markets. He is also the president of the local "Associazione agricoltori custodi (Association of custodian farmers), and the responsible of the Valdarno Slowfood section.

Acronym: KIT17
Name: Carolina Galli
Function / profession: house holder
Company / employer: independent
Type of organisation:
City: Loro Ciuffenna (AR)
Address: Loc. Poggio di Loro, Loro Ciuffenna (AR)
Information: Aged 78, Mrs. Galli spent all her life on place, in a family of farmers. She learn fro grandmother and mother local traditional recipes

Acronym: KIT18
Name: Quintilia Baldi
Function / profession: house holder, owner of a local restaurant
Company / employer: independent
Type of organisation:
City: Loro Ciuffenna (AR)
Address: Loc. Gorgiti, Loro Ciuffenna (AR)
Information: Aged 85, mrs Baldi has been working as a waiter and cook in a restaurant i n Vallombrosa. Then, together with husband, they set up a small food store in Rocca Ricciarda, the highest fraction of the Pratomagno mountains, evolving then in a local restaurant, where specialities of local food tradition are served.

Figure 3.3. Documentation of sources (respondents) (www.basefood-fp7.eu).

3.2. Results

The Italian area subject to documentation in this thesis, mainly located in the north-central Apennine, was interested by a relevant change in land uses during the last 50 years. Mountainous areas, such as the province of Arezzo in Tuscany and the province of Perugia in Umbria, assisted to a drastic migration of people to the city, with the consequent abandonment of agricultural lands, animal pastures and woods for the production of chestnuts and timber. The increase of forest area has been the main result of this trend; this fact, extended to the rest of Europe, has been also analyzed in a Scientific Symposium held in Freiburg, Germany (Piussi and Pettenella, 2000).

A clear example has been reported during our on-site documentation and is represented by the village of Marzana (municipality of Monte Santa Maria Tiberina), located in the province of Perugia at 828 m above sea level (**Figure 3.4**). The village, highly populated till the second World War, was subject to the abandonment since 1960s and 1970s, mainly due to the changes related to the industrial development and the necessity of the people to reach easily urban centers.

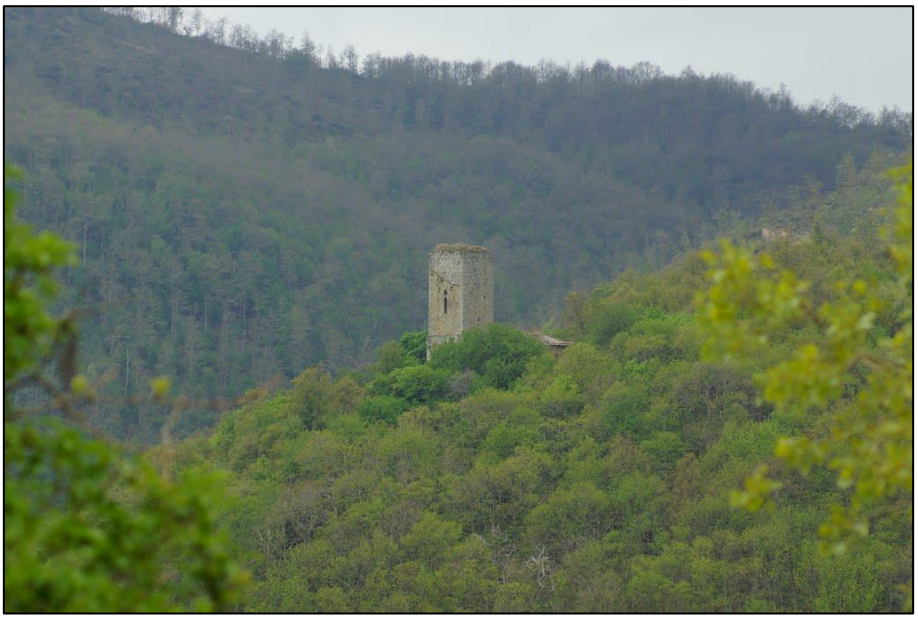


Figure 3.4. Ruins of Marzana's Tower (Perugia province).

At the same time we assisted to a sharp change in the agricultural practices, with a trend forward higher productivity crops. In particular the provinces of Arezzo and Perugia were interested by the replacement of traditional crops with cereals to destine to livestock, tobacco and, lately, medicinal plants.

Spontaneous afforestation of abandoned lands together with the changes of agricultural practices produced a decrease of biodiversity of this territory. However recently a bigger sensibility forward such issues, as traditional crops and foods and biodiversity, have led to a change of course with the intent of recovering ancient traditions linked to the territory.

Various organizations and associations were formed with the intention of recovering the genetic heritage of traditional endangered crops. A successful example is the case of "Nero di Toscana" kale, included in the list of endangered crop plants, by the Regional program of genetic resource conservation of the Tuscany region (as reported below, paragraph 3.2.1.1).

Knowledge in the past was transmitted from generation to generation, so in this study we decided to conduct on-site interviews to have a witness from local people or stakeholders, directly involved in these issues.

3.2.1. *Brassicaceae family*

Within the *Brassicaceae* family *Brassica oleracea* L. is a highly polymorph species. Among this species, the leafy forms resulted particularly interesting, also for a following experimental activity. Hereafter are reported the results obtained about two leafy *Brassica* forms: kale and turnip.

3.2.1.1. *Kale (Brassica oleracea L. var. acephala)*

Brassica oleracea L. includes different populations of kale (*Brassica oleracea* L. var. *acephala*), leafy forms. This *Brassica* is largely spread in the target areas and in other European countries (Netherlands, Portugal, Turkey), and mostly used for the preparation of traditional recipes. Kale retain the vegetative and growth pattern of wild types, and have been only little subject to breeding, with modern techniques.
In Italy a main kale growing area is located in the northern Apennine (Liguria and Tuscany regions), including inland valleys, and coastal and mountain sites.

Figure 3.5. Local palm tree kale in the Pratomagno area, province of Arezzo (Tuscany).

The so-called Italian Palm tree kale (**Figure 3.5**) is a type well established in traditional cropping systems in the Tuscany region, characterized for bio-agronomic traits (D'Antuono and Neri, 1998), and for the possibility of being grown as an industrial crop (D'Antuono *et al*., 2003). All the kale

types of Tuscany belong to kale population known as "Nero di Toscana" (Tuscany Black Kale), and this region is believed to be its centre of origin. "Nero di Toscana" is characterized by a very dark green colour (this is the reason of the name black kale), and by a marked curly leaf lamina.

Recently "Nero di Toscana" has been included in the list of endangered crop plants, by the Regional program of genetic resource conservation of the Tuscany region, according to the regional law on endangered crop genetic resources (Regione Toscana, 2004): after the evaluation of the central regional gene bank, acting as coordinating structure, local farmers may be admitted to financial support to become "Coltivatori custodi" (Custodian farmers) of local kale types. By entering the system, the Custodian farmers are committed to reproduce the seed of their accessions, according to defined protocols, and send a part of them to the regional gene bank, to be available for other farmers who may demand it. Up to now 15 Custodian farmers have been recognized for kale, and the list is available on the Regione Toscana website (www.germoplasma.arsia.toscana.it).

Traditionally local farmers produced their own seed, that was exchanged with neighbours. Following the re-discovery of this *Brassica* type, local seed companies started to produce seeds of black kale, and recently they committed seed multiplication to more specialized seed firms.

In Liguria the situation is quite different; local kale and leafy cabbages, with similar uses, can be divided in three main types:

- "Black kale", a kale widespread from the lowlands of La Spezia province to upper Val di Vara and to the neighbouring province of Genova; substantial morphological variation is observed for this type, leaf curliness varies, some populations show smoother leaves, whereas others have more markedly curly leaves;
- "Broccolo Lavagnino" (**Figure 3.6**), a type forming a small, loose head, with elongated pyramidal shape and smooth leaves; it is reported as being a very old crop in the coastal area of the province of Genova;
- "Cavolo Gaggetta", a sort of cabbage producing a small, loose head, of round shape; local crops of the coastal area, nowadays it becomes pretty rare to find.

In Liguria there is not any organized seed production of the local populations, but individual growers produce their own seeds, sometimes making selection of the better plants as well.

In small scale commercial growing systems, especially when referred to Tuscany, individual leaves are generally hand-picked from the base upwards, meanwhile the plant is still growing. For medium-large scale commercialization, and in general in Liguria, the whole top of the plant, including younger leaves, top part of the stalk and the apical bud, are collected and used together or separated.

Figure 3.6. Small scale plots of Broccolo Lavagnino in Val di Vara, province of La Spezia (Liguria).

Generally kale are harvested after a period of cold, because it is noted that they assume a better taste and are more easily cooked. So kale are one of the few fresh vegetables available during winter time. In the past, kales were used as an ingredient of main courses, combined with energetic ingredients, such as potatoes, bread crumbs, or corn meal.
Some examples of traditional recipes are:

- "*Minestra di pane*" or "*Ribollita Toscana*" (Tuscany region):

is a soup in which the proportion of the two main ingredients, kale leaves and beans, varies depending on likes and availability. The first step of this recipe consist in the preparation of the so called "soffritto", that is the base of many traditional Tuscan dishes: generally soffritto is made by finely chopping onion, celery, carrot and parsley (these ingredients are defined "odori" that means smells), frying them in a pan until golden, successively adding fresh tomatoes, if available, or preserved tomato sauce, and finally cooking for some minutes. Once the soffritto is ready kale leaves are added, together with potatoes, and then mashed beans and their cooking water are poured and cooked together, adjusting with water when necessary. At the end the soup is seasoned and left over bread can be added and soaked.

- "*Polenta e cavolo nero*" (**Figure 3.7**) (Liguria region):

kale leaves are boiled in water until they are softened; then corn flour is added and the mix is slowly cooked, adding water when necessary and stirring to avoid sticking at the pot bottom; at the end, when applicable, olive oil or lard and cheese were added.

Figure 3.7. "*Polenta e cavolo nero*"

- "*Crostoni con cavolo nero*" or "*Brustichino con cavolo nero*" (**Figure 3.8**) (Tuscany region):

bread slices are roasted and flavoured with garlic and olive oil; then boiled kale is put on the bread; sometimes the bread slices are soaked in the kale cooking water.

Figure 3.8. "*Crostoni con cavolo nero*"

3.2.1.2. *Turnip (Brassica rapa L.)*

Another plant of the *Brassicaceae* family, classified as *Brassica rapa* L., resulted very frequently cultivated in the central area of Italy. One important production area is located among Valtiberina, Casentino and Valdarno (Tuscany region), where the vegetable is commonly known as "Rapo del Valdarno" (Turnip tops of the Valdarno) or "Pulezza". It has a big round taproot, white or purple, that is not used for human nutrition but to feed animals; the edible parts are represented by leaves, buds, and stems (**Figure 3.9**).

It is sown in summer and harvested during winter, when the yellow florets start to develop. It is easily grown, not requiring irrigation or special treatments, so it is largely used as winter leafy vegetable. As discussed above for kale, it is generally grown at local scale, mainly for home consumption, and nowadays its commercialization is very limited. Considering Valdarno, Casentino and Valtiberina we can find around 100 farmers producing "Rapo del Valdarno", of which 10 are organic farmers.

Figure 3.9. Rapo del Valdarno (www.terraditoscana.com)

The leaves and the florets are mainly used to prepare stewed or side dishes. For these preparations turnip tops are firstly boiled in salted water and drained (in some cases after draining turnip tops are rolled with hands to form balls that can be kept for some days (**Figure 3.10**)).

Figure 3.10. Drained and rolled turnip tops, after boiling.

After boiling they are added in a pan, where garlic has been previously fried with lard (or olive oil), and stir-fried for some minutes. This recipe is considered a traditional and poor dish of the winter time; a tastier and "richer" variant is represented by "*Pulezze con salsicce*" (Turnip tops with sausages), when available, the leafy vegetable can be used to accompany pork meat.
Other ways to prepare turnip tops are their use in soups or filled pasta. In the case of soups they are generally added to the "*Minestra di pane*", as leafy vegetable when available, for example instead of kale, and boiled with other ingredients.
After boiling and draining, turnip tops can be mixed with ricotta cheese, pecorino or goat cheese, and nutmeg, and used for the filling of "*Ravioli*" pasta. This is a variant of the most common filling containing spinach as vegetable; in the past turnip tops were normally employed in case of not availability of spinach.

3.2.2. *Wild vegetables*

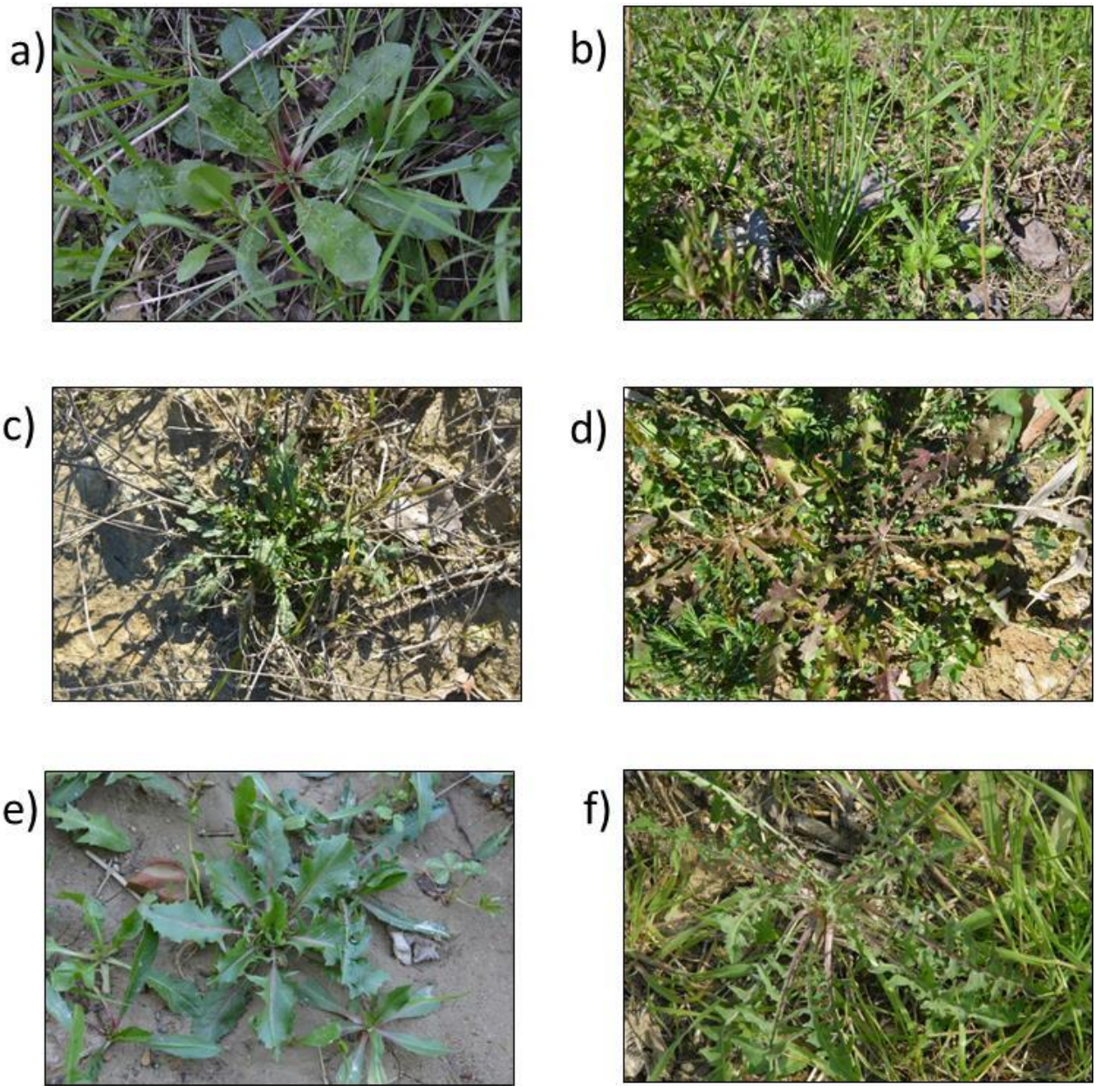
Collecting edible wild herbs was a very common practice in the past, especially among women and children of rural families.
Nowadays only few people are able to recognize different wild vegetables species before, and also after, flowering. In particular between younger generation this knowledge has been lost. However the interest of consumers towards this kind of vegetables is growing and several events in the last years are being organized, with the main target to spread the knowledge of these plants and their use in the kitchen.

3.2.2.1. *Wild leafy vegetables of the Asteraceae family*

Traditionally, various wild species belonging to the *Asteraceae* family were generally harvested and used as food. The use and preference of some species were closely connected to the geographic area. The recognition of the species was a common knowledge that was passed from generation to generation. Basal rosettes were the parts mainly consumed and they were gathered before flowering in uncultivated fields or along rivers or ditches.
Wild leafy vegetables collected at the beginning of spring time were mostly used to prepare fresh salads, after removing damaged and withered parts. From a survey carried out in the Tosco-Romagnolo Apennine area (provinces of Arezzo, Tuscany, and Forlì-Cesena, Emilia-Romagna) resulted that young leaves of different species of the *Asteraceae* family were preferred to prepare

mix of salads; between these: *Helminthia echioides* (L.), *Reichardia picroides* (L.), *Chondrilla juncea* L., *Cichorium intybus* L., *Crepis vesicaria* L., *Crepis leontodontoides* All., *Crepis sancta* (L.) Babc, *Hypochaeris radicata* L., *Sonchus asper* (L.) Hill, *Sonchus oleraceus* L., *Tragopogon pratensis* L. and *Taraxacum officinale* (**Figure 3.11**).

If gathered later, during summer, leaves started to become of unpleasant texture so they were consumed after cooking. The most typical way of cooking was boiling leaves in salted water; once ready, cooked leaves were drained and stir-fried in a pan with olive oil and garlic. Cooked leafy vegetables could be employed as side dishes or as a part of more elaborated foods: for examples filling for pasta, as in the case of ravioli, or pies.



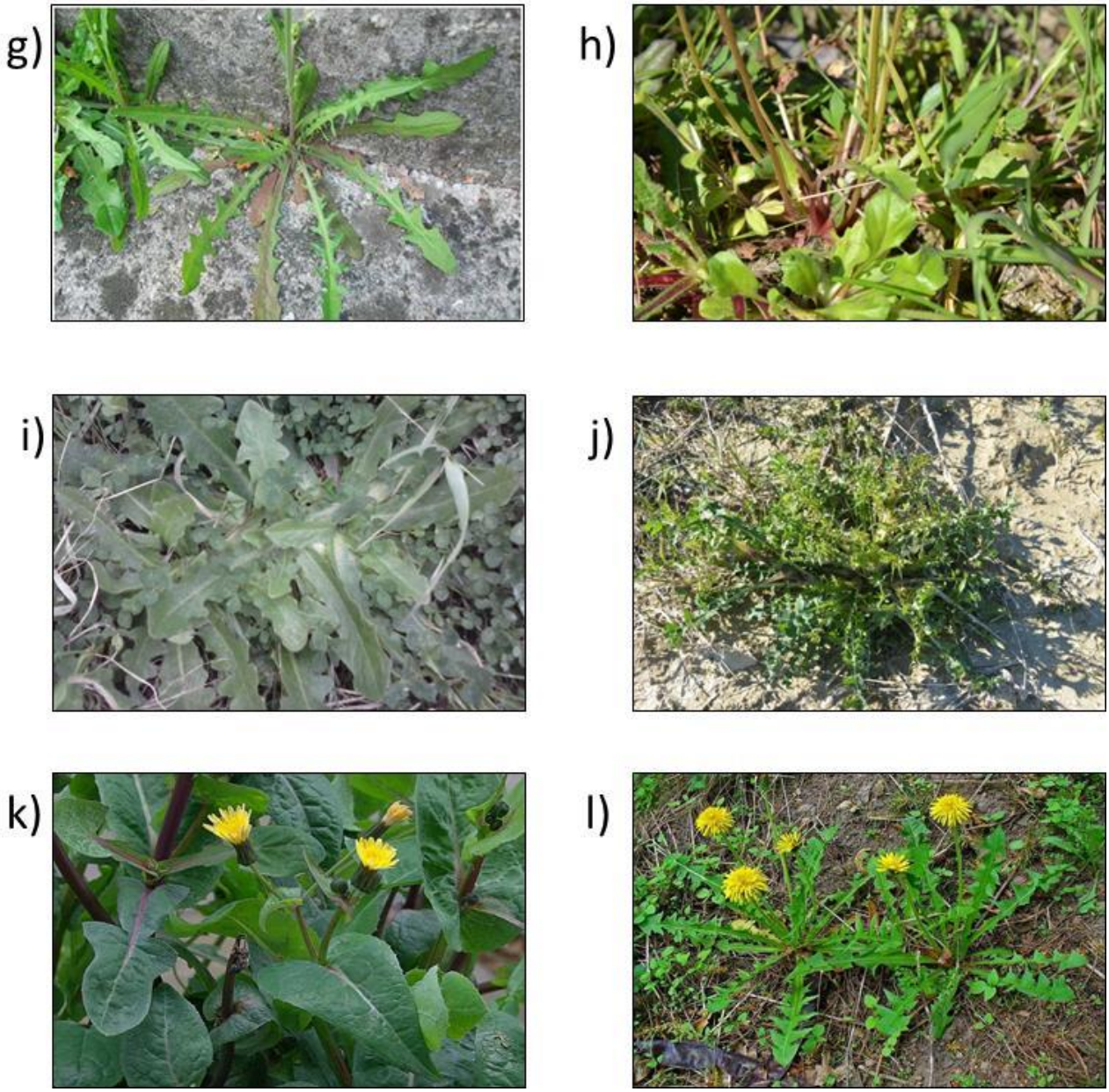


Figure 3.11. a) *Helminthia echioides* (L.); b) *Tragopogon pratensis* L.; c) *Reichardia picroides* (L.); d) *Chondrilla juncea* L.; e) *Cichorium intybus* L.; f) *Crepis vesicaria* L.; g) *Crepis leontodontoides* All.; h) *Crepis sancta* (L.) Babc; i) *Hypochaeris radicata* L.; j) *Sonchus asper* (L.) Hill; k) *Sonchus oleraceus* L.; l) *Taraxacum officinale* Weber.

3.2.2.2. *Other wild leafy vegetables*

Also other wild leafy vegetables, belonging to other families than *Asteraceae*, were collected in uncultivated lands and used as food after cooking. For example leaves of nettle and borage were widely gathered to prepare soups or filling of pastries.

In Valtiberina very interesting resulted the collection of young leaves of a species of *Dipsacaceae* family, *Knautia integrifolia* (L.) Bertol. (**Figure 3.12**). The plant, locally called "*gallinaccio*", grows typically along rivers and ditches, for the presence of water.

Figure 3.12. *Knautia integrifolia* (L.) Bertol.

It was generally harvested between the end of March and the beginning of April, anyway before flowering. It was preferably consumed after cooking, as side dish by repassing boiled leaves in a pan with olive oil and garlic; moreover it was added to soups or mixed with eggs to prepare frittata.

3.2.2.3. *Wild fennel*

Fennel (*Foeniculum vulgare* Mill.), of the *Apiaceae* family, is a typical aromatic plant of the Mediterranean area. The subspecies *Foeniculum vulgare* subsp. *piperitum*, the wild form of fennel, is a perennial plant with rigid and rather fleshy leaf lobes, characterized by the presence of terminal umbels often overtopped by lateral ones (usually with 4-10 rays and sharp-tasting), that generally grows in dry, rocky places (Conforti *et al*., 2006).

In most of cases *Foeniculum vulgare* subsp. *piperitum* is collected from the wild; cultivated forms are used as annual or biennal plants (**Figure 3.13**). The fruits are gathered at the end of summer, when their colour turns to light yellow. Fruits ripening is scalar, so several manual collection are made in the same season.

The use of wild fennel is common in traditional food recipes of the central and southern Italy; the plant is mostly used in meat or fish dishes (especially in case of fatty meats or fishes), cakes, canned fruits and spirits. Different parts of the plant are used: vegetative parts, fruits, and flowers.

Figure 3.13. Wild plant of *Foeniculum vulgare* subsp. *piperitum* (left), and cultivated forms (right).

Vegetative parts are largely used in traditional preparations. In particular they are used to season pork meat, for example in the case of "*Porchetta*", where boneless pork is filled with vegetative parts of fennel (also flowers can be used) and garlic, as flavoring, salt and pepper, together with lard fat. These parts are also used to fill other types of roasted meat (especially goose), in the same way as reported for "*Porchetta*".

Vegetative parts can be added to prepare vegetables, as zucchini, or mushrooms, during their cooking in a pan; moreover in the tradition they can be added to chestnuts during boiling in water.

Fennel fruits, less used in the central Italy, are generally added as flavouring agents to cakes and sweets. An example are "*Zuccherini*", typical dry cakes, with the shape of a ring (traditional prepared for weddings). In these recipes fruits of wild fennel represent a cheaper alternative to anise seeds.

With regard to central Italy, dried flowers are the most employed parts for traditional preparations, and are nowadays considered as a niche product.

In the town of Città di Castello, in Alta Valle del Tevere Umbra (province of Perugia, Umbria), two producers of dried flowers were found, both registered at the local organization Coldiretti. Cultivated plants for the production of dried flowers originate from wild plant of the area. Young plants are produced in local nursery and then transplanted during spring season. Selection of fruit-bearing plants for propagation is generally neglected, so the obtained crops are characterized by great plant variability within the same field. Harvest is mainly addressed to florets, anyway part of the fruits are harvested as food, or for propagation. The production can go on for 3-4 years. Manual harvesting starts in mid-June, from primary umbels, proceeding in the secondary and tertiary umbels till autumn. Umbels are collected when the flowers are open and appear yellow, but before fruit set; subsequently they are sun-dried, in greenhouses or covered by plastic sheets to avoid that

environmental moisture could damage the product. Once dried, umbels are manually threshed and sieved (**Figure 3.14**).

Figure 3.14. Sieving.

Locally flowers are used as flavoring for roasted meat, as pork, goose, duck and rabbit; generally a mixture of fennel flowers, garlic, salt and pepper is chopped and spread on the external part of the meat. Flowers are traditionally added to chicken and rabbit's entrails, but also in mushroom preparation.

Another typical example is the "*Finocchiona*", a cured pork salami from Valdarno (Tuscany). In the past "*Finocchiona*" was produced by using less valuable cut of meat, so the flavor was correct with the aroma of wild fennel flowers. However among interviewed producers of "*Finocchiona*" there is heterogeneity: someone uses flowers, others fruits, in some cases also a mixture of them.

Another example similar to that discussed above is represented by a kind of sausage called "*Sambudello*" in Tuscany and "*Mazzafegato*" in Umbria. Also in this case less valuable cut of pork meat are used (as tongue, cut of cheek and head, heart, ears) and flavored with garlic and fennel flowers.

3.2.3. *Other crops*

In the last years the Technical institute (Istituto Tecnico Agraria, Agroalimentare, Agroindustria "Alberto M. Camaiti"), located in Pieve Santo Stefano (Valtiberina), has been involved in a collaboration to recover the germplasm of ancient cultivars of fruit trees and plant species. The species analyzed within this project are reported in the "Banca Regionale del Germoplasma" (Germplasm Regional Bank), with a brief description of the morphological traits and the list of the

Custodian farmers of such species for the considered area. These data are available in Tuscany region website (www.germoplasma.arsia.toscana.it).

A survey conduct with the responsible of the project, prof. Gonnelli from the Technical institute, highlighted that, in the past, the Valtiberina area was characterized by the cultivation of different traditional crops.

Following are reported documented vegetables during the survey, with a special focus on those resulting interesting for the purpose of the present research study.

3.2.3.1. *Pulses*

Different types of pulses were present, strictly related to the production area (the name of the crop is often associated with the name of the area where it is typically grown). The investigation pointed out the presence of various traditional landraces; in Valtiberina area we can find between beans: "fagiolo dall'occhino", "fagiolo piattello della Valtiberina", and "fagiolo cappone della Valsovara"; and between chickpeas: "cece cappuccio", and "cece nano".

These pulses are generally sown in spring and harvested during summer; the pods are shelled once they are dry, and for this reason they are maintained in the open air after harvest. They are rustic crops and resistant to diseases, however they need a lot of manual work so nowadays their production is limited to family garden for home consumption, being replaced by more productive cultivars that require less manual labor (for example "fagiolo cannellino").

Once dried, pulses are traditionally stored in glass bottles, to preserve them during winter time.

A typical soup is locally called "*Patate e fagioli*" (Potatoes and beans), from the name of the two main ingredients (**Figure 3.15**).

Figure 3.15. "*Minestra patate e fagioli*"

For this preparation beans are firstly soaked overnight, in order to make easier their cooking. Once drained they are added to a soffritto, and boiled for long times together with potatoes. In some cases this dish is consumed with left over bread, or if available with small cut of pasta.

After boiling pulses can be used also to prepare stewed dishes. A very famous example is "*Fagioli all'uccelletto*" (Beans in the manner of birds or Tuscan style beans) in **Figure 3.16**: garlic is previously fried in olive oil and seasoned with sage, then boiled beans are added, covered with tomato sauce, and cooked for 20-30 minutes to mix flavours. Traditionally an earthenware pan is used to prepare the dish, in a way to cook it slowly.

Figure 3.16. "*Fagioli all'uccelletto*"

The use of chickpeas is very similar to that already reported above for beans. They can be used in soups or stewed dishes, after water soaking of chickpeas, generally for a night.

In the tradition a very popular and poor dish consumed during Christmas' Eve was "*Baccalà con i ceci*" (dried salted cod and chickpeas) in **Figure 3.17**.

Figure 3.17. "*Baccalà con i ceci*"

Boiled chickpeas are poured in a pan, together with dried salted cod (that is previously left in cold water for at least one day and then stir-fried); chickpeas and dried salted cod are subsequently covered with tomato sauce, cooked slowly, and seasoned with rosemary and garlic.

3.2.3.2. *Corn*

At the beginning of XX century most of the corn grown in the northern and central Italy was used for human consumption, as staple cereal, to prepare daily simple dishes. Locally adapted population were grown, with a wide range of variation, besides a very relevant internal heterogeneity. Different landraces were traditionally grown in Valtiberina, between these: "mais quarantino di Monteviale", "mais quarantino di Sansepolcro", "mais quarantino di Frassineto", "mais Villa di Petranera", "mais Villa di Poti", and "mais di Caprese Michelangelo". These local varieties are still cultivated in small scale farms and the seeds are selected individually by farmers, in a way to assure a sufficient stability of the main productive and quality characters. Sowing is generally done in spring and harvesting is still mostly done by hand in September. Whole spikes are manually picked from the plant, allowing a better selection from both the sanitary and the conformity points of view; in this phase the spikes that better reproduce the standard are preserved for the next year crop. After harvest the spikes are air-dried hung in bunches or put in open containers ("granaio"), and subsequently threshed. In the past, and today for some more traditional production areas, grains were milled according to needs by means of stone mills. Wholemeal flour obtained by corn milling was traditionally sieved, to separate a consistent part of the bran and of the embryo, which presence was not very appreciated to prepare foods. Nowadays more evolved local producers make use of cylinder mills and subsequent separation by sieving, allowing a better separation of the kernel parts. This scheme allows to obtain various outputs from processing: "bramata", coming from the vitreous part of the endosperm, the typical fraction used for polenta, "fioretto", coming from the inner floury part, and bran, that can be partially added again to obtain a partial whole corn meal.

Polenta is the most popular dish from corn flour; its preparation is easy and can be the basis of several composite dishes. Polenta is basically a mix of corn flour and water, slowly cooked traditionally in a copper pot in the fireplace (**Figure 3.18**). The proportion of flour and water and the time of cooking resulted variable, depending on local traditions. Part of polenta was consumed the same day of its preparation, whereas left over pieces were eaten the following days after being roasted.

Figure 3.18. Polenta traditionally cooked in a copper pot in the fireplace.

Depending on their availability, other ingredients could be added to polenta, alone or in combination; typical ingredients can be herbs and greens (see above "*Polenta e cavolo nero*"), legumes, cheese, fats, meat.

A very known variant in Valtiberina is represented by "*Farinata*" in which soaked beans are added to polenta and cooked together (**Figure 3.19**). The dish is seasoned with some garlic cloves and, if there is the possibility, with lard, that are added during cooking.

Figure 3.19. "*Farinata*", polenta with beans.

In Valtiberina corn flour was used also mixed with wheat flour and water to prepare a kind of pasta locally called "*Bringoli*" (**Figure 3.20**). The ingredients were manually kneaded and the dough was manually hand-rolled until thickness of big spaghetti. They were typically used to prepare soups, as in the case of "*Patate e fagioli*"; the recipe could be enriched by adding chopped lard. However the term "*Bringoli*" can create a bit of confusion. Today this term is mostly associated to a kind of pasta, prepared only with wheat flour, and served with "sugo finto", a sort of tomato sauce added to

a soffritto, without meat (due to poor availability of meat in the past). In the last years this recipe has been re-evaluated and several festivals are dedicated to "Bringoli", the most famous are located in the town of Anghiari (Tuscany) and Lisciano Niccone (Umbria).

Figure 3.20. "*Bringoli*"

3.2.3.3. *Onions*

In the flat area of Valtiberina onion represented an important species for the peasants' economy. The local landrace "Cipolla rossa della Valtiberina" was spread in all the valley for home consumption or for the market. Onions were sold in bunches in the local markets and also in the nearby regions of Marche and Emilia Romagna. Nowadays people from Sansepolcro, in Valtiberina area, are still called "cipollari", from "cipolla" that means onion, to witness the popularity of this crop.

Sowing in a sort of nursery in the open air, called "semensaio", begins in January, in a soil properly manured, generally with organic manure of the previous year. Transplanting can be made between the end of April and the end of May. Harvesting generally start after mid-August, when the leaves are withered. Once collected bulbs are stored in dark place to complete ripening, subsequently tied in bunches, with the shape of braids, and hung in ventilated, but protected from the frost, rooms.

The crop is at the base of most of the typical dishes of this area, and used as "odore" in the soffritto preparation.

Onions are consumed fresh in salads; one of the most typical examples is represented by "*Panzanella*" (**Figure 3.21**), a traditional summer dish in which soaked and drained left over bread was mixed with tomatoes, cucumber, onion and basil.

A simple way to consume onions during winter consisted in cooking them in the oven or under the embers in the fireplace. Roasted onions were then seasoned with olive oil, salt and pepper.

Figure 3.21. "*Panzanella*"

3.2.3.4. *Tomatoes*

Between tomatoes a very important role played the "Pomodoro perina a punta" or "Pomodoro da serbo", a small yellow tomato, that could be kept hanging and preserved during all the winter.

3.2.4. *Forest fruits*

The highest zones around the town of Caprese Michelangelo, Monterchi and Anghiari, in the province of Arezzo, are characterized by the presence of extensive and secular chestnut woods. Chestnut played a basic role for the rural economy in the past; the activities of traditional peasant families were split between the work in the fields, during summer, and in the woods during winter. The woods represented a resource not only for chestnut harvest, but they were used also as pasture for animals, for firewood and for gathering other forest fruits (mushrooms).

3.2.4.1. *Chestnuts*

Wild chestnut plants do not produce tasty fruits, locally called "selvarine", so chestnuts are harvested from grafted trees, called "marroni". In 2009 "Marrone di Caprese Michelangelo", a local chestnut harvested in the town of Caprese Michelangelo and part of the town of Anghiari, has been recognized as PDO (Protected Designation of Origin).
In the tradition, chestnut fruits were harvested in October and November, and sold in local markets. A large part of the fruits was dried in special stone buildings (seccatoi), that can still be found in some groves. Drying was carried out for about one month by a fire with a lot of smoke but no

flames. Once dried, chestnuts were moved to the watermills and milled for the production of flour used for the preparation of food.

Chestnut flour was used to prepare polenta, in the same way reported above in the case of corn flour polenta. After boiling, polenta was drained from part of the water and poured on the wooden work surface, subsequently worked with "rasagnolo" and cut in slices with a piece of string.

Chestnut flour is also used to produce sweets or cakes. An example is "*Baldino di castagne*" (mostly called "*Castagnaccio*" in other parts of the Tuscany), where chestnut flour is mixed with water, and then rosemary, olive oil, raisin and walnuts or pine nuts are added to the liquid dough. After mixing all the ingredients the cake is cooked on a pan (**Figure 3.22**).

Figure 3.22. "*Baldino di castagne*"

Fresh chestnuts are consumed as "*Caldarroste*" after their roasting on a specific pan, with holes on the basis to allow a better cooking; in the past it was very typical to roast chestnuts under the embers in the fireplace. A normal practice after peeling roasted chestnuts is their soaking in a glass of red wine. With the time fresh chestnuts loss their texture so they are preferably consumed after their boiling in water with peel ("*Balocie*"), or without ("*Monde*").

3.3. Discussion

As discussed also by D'Antuono (2013) traditional crops often represented the main ingredient in the preparation of traditional foods. Recipes could variate also in relation to the availability of ingredients, so in case of vegetables mainly based on their seasonality. Traditional foods prepared during particular celebrations, religious or not, were strictly connected to the season and availability of ingredients. Some examples:

- pastries, such as "Castagnole", were typical of Carnival times; they were baked and fried in pork fat, based on its availability after killing of the pig, usually practiced in January;
- traditionally during New Year's Eve was consumed boiled and stir-fried kale, a typical winter crop;
- during Easter times housewives prepared cakes containing eggs and dried grapes; in fact in spring time normally there was an abundance of eggs and grapes reached the perfect grade of dryness (however grapes were mostly dried to produce "*vin santo*", a sort of dessert wine that can vary in sweetness level).

Traditional crops are mainly cultivated by small local producers. In most of cases they use their own seeds or seeds exchanged with the neighboring and they sell the crops in local markets. This results in the short availability of these crops, strictly related to the seasonality, and in the great variation of landraces. The last point could be seen as:
- negative, the largest food production requires a minor variation of raw materials, so it could represent an obstacle to extend the production of these crops;
- positive, this variability could represent an opportunity to increase the availability of biodiversity; recently has been discussed the importance of maintaining the biodiversity, both to safeguard agricultural production and to guarantee the intake of minor compounds with the diet (Frison *et al*. 2006).

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EXPERIMENTAL PART

Chapter 4

Primitive wheats

Chapter 4.1

A comparative study of bioactive compounds in primitive wheat populations from Italy, Turkey, Georgia, Bulgaria and Armenia

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ABSTRACT

BACKGROUND: In recent years there has been a considerable interest in the consumption of ancient wheats, often referred to as having superior health promoting properties than modern cultivars. The BaSeFood project allowed to explore the use of primitive wheats in the Black Sea area region and in Italy, with special respect to emmer (*T. dicoccum*) and einkorn (*T. monococcum*), and to collect seed samples to be grown and compared for their bioactive content, together with some other primitive wheat genotypes (*T. timopheevi*, *T. palaeo-colchicum*, *T. macha*).

RESULTS: The data show that genotype was an important factor controlling phytochemical content. Variability range were as follows: lipids (18.0-28.5 g kg^{-1}), tocols (26.6-72.8 mg kg^{-1}), carotenoids (1.6-8.4 mg kg^{-1}), sterols (441-929 mg kg^{-1}), and phenolic compounds (819-1465 mg kg^{-1}) content (dry matter basis). The fraction of individual components, within each class, was also variable; however the species were well discriminated by their overall composition.

CONCLUSIONS: The present research represents a further contribution to the available literature about the analytical composition of primitive wheats, including the complete range of relevant bioactives and lesser investigated species. The data do not support an overall superiority of primitive forms, but evidenced interesting, potentially exploitable, between- and within-species variability.

Keywords: primitive wheats; bioactives; carotenoids; tocols; phytosterols; phenolic compounds.

INTRODUCTION

The term "primitive wheats" refers to ancient forms of different cultivated *Triticum* species. Within the broad meaning of this definition, all these forms are represented by populations not subject to any modern breeding or selection, and sometimes retaining characters of wild ancestors, such as individual variability, height, brittle rachis, low harvest index and, in some taxa, hulled kernels. They are sometimes reported as being adaptable to moderately adverse environmental conditions, like poor soils and water deficiency (Stagnari *et al.*, 2008). In modern agriculture, they have been replaced by varieties obtained by breeding, whereas primitive forms generally were kept in culture in marginal areas, where the productive advantage of new varieties was not so evident.

Wheat has been indicated as a potential source of several health-promoting components, namely tocopherols, tocotrienols, carotenoids, phenolic acids, flavonoids and phytosterols (Abdel-Aal and Rabalski, 2008; Ward *et al.*, 2008; Liu, 2007). Primitive forms are being often cited as richer sources of phytochemicals than modern varieties. Although there is no evidence for this statement (Shewry *et al.*, 2011), variability for several components has been detected, opening opportunities for its potential exploitation in a health-promoting perspective.

Previous studies have shown that wheat varieties vary for their carotenoid (Abdel-Aal *et al.*, 2007; 2002), tocopherol (Zhou *et al.*, 2004) and phenolic acids (Abdel-Aal *et al.*, 2001) composition.

Carotenoids have powerful activities against singlet oxygen generated from lipid peroxidation or radiation. β-carotene, α-carotene, and β-cryptoxanthin have provitamin A activity, while zeaxanthin and lutein are the major carotenoids in the macular region (yellow spot) of the retina in humans (Namitha and Negi, 2010). Generally, einkorn wheat has been recognized as a richer source of carotenoids, namely lutein, than other wheat species (Serpen *et al.*, 2008; Abdel-Aal *et al.*, 2007; 2002; Hidalgo *et al.*, 2006).

Phenolic compounds have antioxidant properties and may protect against degenerative disease in which reactive oxygen species are involved (Rhodes and Price, 1997). In some cases, emmer wheat has been indicated as richer in phenolics than einkorn wheat, also in connection with a high antioxidant activity, but often not in comparison with bread or durum wheat (Abdel-Aal and Rabalski, 2008; Serpen *et al.*, 2008; Carcea *et al.*, 2006).

Tocopherols and tocotrienols result unevenly distributed within the grain (tocotrienols primarily in the endosperm, whereas tocopherols in the embryo) (Hidalgo and Brandolini, 2008). They are known as vitamin E and their biological activity as antioxidants is due to the ability to donate phenolic hydrogen atoms to free radicals, thus breaking destructive chain reactions. Other beneficial

therapeutic properties of tocols include the ability to reduce serum cholesterol concentration and to inhibit the growth of certain cancer cells (Tiwari and Cummins, 2009).

Phytosterol, consumed with the diet, can influence cholesterol metabolism and the main health-promoting effect is lowering of serum cholesterol (Carr *et al.*, 2010). It has been demonstrated that although tetraploid and hexaploid wheat sterol profiles were qualitatively the same, they differed significantly on the basis of the relative amounts of specific compounds (Iafelice *et al.*, 2009; Pelillo *et al.*, 2003).

In several countries primitive wheats also represent the basis for the manufacture of local foods that are now being revalued after a long period of neglect. Their production survived and was recently resumed in some areas of Western Europe, where there is considerable interest in the consumption of ancient wheats, especially in organic, specialty, and health food markets (Jaradat *et al.*, 1996). In particular, the growing systems and uses of the hulled species emmer (*T. turgidum* L. *ssp. dicoccum* (Schrank ex Schübler) Thell.) and einkorn wheat (*T. monococcum* L.) have been the subject of detailed surveys, with special respect for Italy (D'Antuono, 1998; 1995; D'Antuono and Bravi, 1996; D'Antuono *et al.*, 1996; Jaradat *et al.*, 1996) and Turkey (Giuliani *et al.*, 2009), highlighting the present interest of consumers and stakeholders, making these species putative effective carriers for health-promoting components.

BaSeFood, a 3-year collaborative research project, funded by the 7th Framework Programme (www.basefood-fp7.eu), investigated several traditional foods of the Black Sea region, starting from specific plant raw materials. On-site investigation confirmed and updated the knowledge on emmer and einkorn wheat growing in Italy and Turkey. Also, still extensive use of emmer was documented in Armenia, and residual emmer wheat populations and attempts at einkorn wheat recovery were discovered in the Bulgarian Rodopi mountains. A detailed review of Georgian primitive wheat species was also done (Maisaia, 2009).

Within the scopes of the project, aimed at connecting the traditional and potential health-promoting characters of local foods, and in the perspective of the use and valorization of plant diversity, primitive wheat seed samples from the above-mentioned countries, with a prevalence of emmer and einkorn wheat, have been collected and grown in Italy, in order to evaluate the nature and content of selected phytochemicals (phenolic and lipid-associated compounds: tocopherols and tocotrienols, carotenoids, and phytosterols) in comparison with control modern bread, durum, einkorn and emmer wheat varieties.

MATERIALS AND METHODS

Seed samples

27 *Triticum* accessions, belonging to different species and including both local landraces and improved modern varieties, as controls, were considered in this study (**Table 4.1.1**).

Seeds of the local landraces from Armenia, Turkey, Bulgaria and the local populations from Italy were sampled in-place from farmers' current year harvest stocks. The samples from Georgia came from the ELKANA (BaSeFood partner) working collection. The Italian emmer and einkorn wheat commercial varieties Zefiro, Rosso rubino and Monlis were supplied by Prometeo s.r.l. (Urbino, Italy), whereas the commercial durum (Yelodur and Miradoux) and bread wheat (Kalango and Nogal) varieties were purchased from dealers.

A field experiment was set up during 2010-2011 at the research farm of the University of Bologna, located in Cadriano (latitude 44° 33' N, longitude 11° 21' E, 32 m above sea level), Italy. The soil of the site is classified as a fine silty, mixed, mesic. Udic Ustochrepts, and has silty loam texture, with 380, 375, and 245 g kg^{-1} of sand, silt, and clay, respectively. The pH (1:2.5 soil to water) is 7.9 and organic carbon is 8.5 g kg^{-1}.

The trials were sown on two dates - late fall (15 December 2010), and spring (18 March 2011) - in order to discriminate between winter and spring habit populations, in a 3-replication split-plot experimental scheme, with sowing dates in the main plots and genotypes in the sub-plots; sub-plot size was 5 m × 1.5 m. Harvest took place in July 2011 at grain full ripening stage, by means of a Wintersteiger experimental plot combine. The product was cleaned and preserved in adequate conditions. Glumes removal of hulled types was carried out by means of a testing husker from Otake Agricultural Co. (model FC-2K, Japan). Dehulled and free-threshed seed samples were packed in plastic bags and stored at 4°C until further analyses.

Chemicals

All chemicals and solvents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was obtained by an Elix 10 water purification system from Millipore (Bedford, MA, USA). Solvents employed in high-performance liquid chromatography (HPLC) were of chromatographic grade, from Sigma-Aldrich. Before use in HPLC, solvents were filtered through Nylon membrane filters (diameter 47 mm; pore dimension 0.45 μm) from GVS Filter Technology (Indianapolis, IN, USA) and sonicated at room temperature for 30 min.

Table 4.1.1. List of the accessions included in the field experiment.

Species, Tag [1]	**Botanical variety or cultivar**	**Type**[2]	**origin (location)**
Einkorn wheat *(Triticum monococcum* L., subsp. m*onococcum*)			
mG1		s, p	Georgia (Elkana collection)
mT2		s, p	Turkey (Isanghazı)
mT3		s, p	Turkey (Katalyazi Koyu)
mI4	cv. Monlis	s, v	Italy
Emmer wheat (*Triticum turgidum* L., subsp. *dicoccum* (Schrank ex Schübler) Thell.)			
eG1		s,p	Georgia (Elkana collection)
eT2		s,p	Turkey (Isanghazı)
eT3		s,p	Turkey (Katalyazi Koyu)
eI4		w, p	Italy (Leonessa)
eI5		s, p	Italy (Leonessa)
eI6		s, p	Italy (Monteleone di Spoleto)
eI7	cv. Zefiro	w, v	Italy
eI8	cv. Rosso rubino	s, v	Italy
eI9		w, p	Italy (Garfagnana)
eA10		s, p	Armenia (Fantan)
eA11		s, p	Armenia (Fantan)
eA12		s, p	Armenia (Vayotz Dzor)
eA13		s, p	Armenia (Rubik)
eB14		s, p	Bulgaria (Beden)
Zanduri wheat (*Triticum timopheevii* (Zhuk.) Zhuk. subsp. *timopheevii*)			
tG	var. *rubiginosum* Eritz	s, p	Georgia (Elkana collection)
Georgian emmer (*Triticum turgidum* L., subsp. *paleocolchicum* (Menabde) Á.Löve & D.Löve)			
pG		w, p	Georgia (Elkana collection)
Durum wheat (*Triticum turgidum* L., subsp. *durum* (Desf.) Husnot)			
dG1	var. *leucurum* Al. Desf.	w, p	Georgia (Elkana collection)
dG2	var. *leucurum* Al. Desf.	w, p	Georgia (Elkana collection)
dG3	var. *apulicum* Koern	s, p	Georgia (Elkana collection)
dC4	cv. Yelodur	s, v	-
dC5	cv. Miradoux	s, v	-
Macha wheat (*Triticum aestivum* L., subsp. *macha* (Dekapr. & Menabde) MacKey)			
hG		s, p	Georgia (Elkana collection)
Bread wheat (*Triticum aestivum* L., subsp. *aestivum)*			
aC1	cv. Kalango	w, v	-
aC2	cv. Nogal	w, v	-

[1] m: *T. monococcum*; e: *T. dicoccum*; t: *T. timopheevi*; p: *T. palaeo-colchicum*; d: *T. durum*; h: *T. macha*; a: *T. aestivum*.
G: Georgia; T: Turkey; I: Italy; A: Armenia; B: Bulgaria; C: Commercial.
[2] s: spring variety; w: winter variety; v: commercial selected variety; p: local landrace.

Standards of α-tocopherol, α-tocotrienol, lutein, β-carotene, ferulic acid, rutin, and 5α-cholestan-3β-ol, and sylilation reagents (pyridine, hexamethyldisilazane, trimethylchlorosylane) were from

Sigma-Aldrich; β-tocopherol was purchased from Supelco (Bellefonte, PA, USA), and zeaxanthine from AppliChem (Darmstadt, Germany).

Lipid extraction

Lipids were extracted from 50 g of milled grain according to Folch's method (Folch *et al.*, 1957), with some modifications as described by Boselli *et al.* (2001). After extraction with a chloroform-methanol mixture and removal of the organic solvent by a vacuum evaporator (bath temperature: ~40°C), the fat was stored in *n*-hexane-*iso*-propanol (4:1, *v/v*) at -20°C until further analyses.

Tocopherols and tocotrienols

About 25 mg of fat was dissolved in 2 mL *n*-hexane-*iso*-propanol (99:1, *v/v*). Tocopherols and tocotrienols quantification was performed by normal-phase HPLC, following Panfili *et al.* (2003). HPLC analyses were carried out on a HPLC apparatus from Jasco (Tokyo, Japan), equipped with two binary pumps (model PU-1580), an autosampler (model AS-2055 Plus), and an intelligent fluorescence detector (model FP-1520). Separation was achieved by a Luna Hilic 200A (150 mm × 3.0 mm i.d., 3 µm particle size) column from Phenomenex (Torrance, CA, USA). Mobile phase was *n*-hexane-ethyl acetate-acetic acid (97.3:1.8:0.9, *v/v/v*). Flow rate was 0.4 mL min^{-1}, and the injection volume was 10 µL. Fluorimetric detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. The analyses were carried out at 35°C. The data were processed by the software ChromNAV (version 1.16.02) from Jasco.

Peak identification was achieved by comparing retention times and spectra with standards and literature. Quantification was performed by external standard mode. From the stock solutions, prepared using the standards α-tocopherol, α-tocotrienol, and β-tocopherol dissolved in *n*-hexane-*iso*-propanol (99:1, *v/v*), 8 diluted solutions (0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 ppm) were obtained; each of them was injected three times.

Carotenoids

About 150 mg of fat was dissolved in 2 mL *n*-hexane-*iso*-propanol (90:10, *v/v*). Carotenoid quantification was performed by normal-phase HPLC, following Panfili *et al.* (2004). The analyses

were carried out by means of the same HPLC apparatus described for tocols, equipped with a diode array UV-visible detector (model MD-1510, quartz flow cell, 10 mm optical path). Chromatographic separation was performed by a Kromasil Si (250 mm × 4.6 mm i.d., 5 µm particle size) column. Mobile phase was *n*-hexane-*iso*-propanol (95:5, *v/v*). Flow rate was 1.5 mL min^{-1} and injection volume was 10 µL. Detection was achieved by means of a diode array detector set in the range of 350-500 nm. Chromatograms were recorded at 450 nm. The carotenoids were identified by their spectra and the comparison of peak retention times with standard compounds. The analyses were carried out at 35°C. The data were processed by the software ChromNAV (ver. 1.16.02) from Jasco.

The carotenoids were quantified by external standard mode, constructing calibration curves of β-carotene, lutein, and zeaxanthin dissolved in *n*-hexane-*iso*-propanol (90:10, *v/v*). From the stock solutions six diluted solutions (0.25, 0.5, 1, 5, 10 and 20 ppm) were prepared; each of them was injected three times.

Phytosterols

Phytosterol determination was carried out according to Iafelice *et al.* (2009). 0.5 mg of 5α-cholestan-3β-ol was added as internal standard to about 200 mg of extracted fat. The fat was subject to saponification at room temperature and the unsaponifiable fraction was extracted with diethyl ether. The organic extracts were washed once with 5 mL of 0.5 mol L^{-1} KOH and twice with 5 mL deionized water. After drying over anhydrous sodium sulfate, the extracts were filtered, concentrated under reduced pressure and dissolved in 2 mL *n*-hexane-*iso*-propanol (4:1, *v/v*). Sterol fractions were analyzed by GC after sylilation of sterols to the corresponding trimethylsilyl (TMS) derivatives, according to Sweeley *et al.* (1963). A Clarus 500 gas chromatograph from PerkinElmer (Norwalk, CT, USA) equipped with a flame ionization detector (FID) and a Zebron ZB-5 column (30 m length, 0.25 mm i.d., 0.25 µm film thickness) coated with diphenyl dimethyl-polysiloxane from Phenomenex were used. Helium was used as carrier gas. Other instrumental conditions were as follows: carrier flow 1.0 mL min^{-1}; split ratio 1:30; injector temperature 330°C; oven temperature 270 to 330°C at 3°C min^{-1}.

Identification of sterol compounds was achieved by mass spectral data obtained by GC-MS. GC was performed using a 6890N GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a MS detector, model 5973. Chromatographic conditions were the same as for GC-FID. The mass axis of the spectrometer was calibrated with perfluorotributylamine (PFTBA), the peak width

was 1 Th, and the instrumental precision was 0.1 Th over 8 h. The mass detector was used in the electron impact mode (EI), with an electron energy of 70 eV; the emission current was 10 mA and the scan rate 1 scan s^{-1}; the ion source temperature was 230°C, and the quadrupole temperature 150°C (Pelillo *et al.*, 2003).

Free and bound phenolic compounds

Free and bound phenolic compounds were extracted according to Van Hung and Morita (2008), Adom and Liu (2002) and Sosulski *et al.* (1982), with some modifications. Briefly, 0.5 g ground grain sample was extracted by sonication with 5 mL 80% (*v/v*) absolute ethanol for 10 min. After centrifugation, the supernatant was collected and the extraction repeated. The supernatant fractions were pooled, evaporated and reconstituted with 2 mL 0.3% (*v/v*) formic acid in methanol-water (1:1, *v/v*). Residual matter from free phenolic extraction was hydrolyzed by 2 mol L^{-1} NaOH for 90 min with continuous shaking at 60°C. The hydrolyzate was acidified to pH 2 with HCl and centrifuged to separate cloudy precipitate. The supernatant was extracted two times with *n*-hexane to remove free fatty acids; phenolic acids were then extracted five times with ethyl acetate. The ethyl acetate extracts were evaporated and redissolved in 2 mL 0.3% (*v/v*) formic acid in methanol-water (1:1, *v/v*). HPLC analysis were carried out using the same apparatus described for carotenoid determination. A Gemini-NX (150 × 3.0 mm i.d., 3 µm particle size) column from Phenomenex was employed. The analysis were carried out at 35°C. The flow rate was 0.5 mL min^{-1}, and the injection volume 10 µL. A gradient elution program was set using the following solvent system: mobile phase A: 1% acetic acid in water; mobile phase B: mobile phase A-acetonitrile (60:40, *v/v*). The gradient program was: from 0 to 8.4 min, 98% A; from 8.4 to 9.6 min, 98 to 94% A; from 9.6 to 12 min, 94 to 90% A; from 12 to 14.4 min, 90 to 83% A; from 14.4 to 22.8 min, 83 to 64% A; from 22.8 to 24 min, 64 to 61.5% A; from 24 to 31.8 min, 61.5 to 40% A; from 31.8 to 34.8 min, 40 to 0% A; from 34.8 to 46.8 min, 0 to 95% A; from 46.8 to 51.0 min, 95 to 98% A; from 51 to 58 min, 98% A as post-run. Each chromatogram was recorded at 280 nm whereas absorption spectra were recorded between 200 and 600 nm. The data were processed by the software Jasco-Borwin (version 1.50) from Jasco.

Phenolics were quantified by external standard mode, constructing calibration curves of two representative compounds of relevant phenolic classes: ferulic acid and rutin. Stock solutions were prepared in methanol-water (1:1, *v/v*) with 0.3% formic acid, and six diluted solutions (1, 5, 10, 50, 250 and 500 ppm) were obtained; each one was injected three times.

Phenolic identification was carried out on a LC system HP 1100 Series coupled with a MS detector (model G1946A) both from Agilent Technologies (Palo Alto, CA, USA). The mass spectrometer operated in both positive and negative atmospheric pressure ionization-electrospray source (API-ES) mode under the following operating conditions: drying gas temperature: 350°C; drying gas flow rate: 10.0 L min^{-1}; nebulizer pressure: 35 psig; capillary voltage: 3000 V; fragmentor voltage: 80 V; mass range: *m/z* 100-1400; scan mode: negative and positive. Data processing was performed by the software LC/MSD ChemStation from Agilent Technologies.

Statistic analyses

The data were primarily processed by means of analysis of variance. Since the winter type accessions gave no seed yield on spring sowing, the whole set of data was processed, in a first step, according to a nested design, considering the effects of sowing dates and genotypes, within sowing dates. The processing according to a complete factorial design, considering the effect of sowing date, genotype and their interaction was carried out, in a second step, only on spring types. Binary comparisons were carried out by means of the protected Fisher's LSD test.

Stepwise forward discriminant analysis was applied at the classification of the samples, using the species as categorical variable and the analytical data (amounts of the classes of components and relative amounts of individual components) as classifying variables.

All the analyses were carried out by means of the SYSTAT 10.0 package.

RESULTS AND DISCUSSION

Lipid content

Lipid content (**Table 4.1.2**) was significantly and consistently higher for spring (19.2-28.5 g kg^{-1}) than fall (18.0-26.5 g kg^{-1}) sowing. Significant variability was detected among accessions, however without any well-defined pattern due to species, except for a tendency of higher lipid content in einkorn wheat, likely connected to smaller seed size. Significant accession × sowing date interaction was also detected, with some accessions having rather stable high lipid content (eI6, eA10) and a few (dG3, mI4, and eA12) showing higher lipid content in fall sowing.

Tocopherols and tocotrienols

Also the average content of lipid-related tocols was significantly higher in spring than in fall sowing, with a mean value of 55.02 mg kg^{-1} dry matter (dm). However, no significant relation between lipid and tocol content was detected. Also in this case, no specific pattern was detected, because of high within-species variability and significant accession × sowing date interaction. Einkorn mT3 had the highest average tocol amounts, with higher values in fall sowing, whereas macha wheat hG and emmer eG1 had higher tocol content in spring sowing.

Four different tocols positional isomers were identified (**Table 4.1.2**): α-tocopherol (α-T) and β-tocopherol (β-T), and the two corresponding unsaturated tocotrienols, α-tocotrienol (α-T3) and β-tocotrienol (β-T3), in agreement with previous reports (Hidalgo *et al.*, 2009; 2006; Panfili *et al.*, 2003). Genotype affected the relative content of all the tocol components, whereas sowing date had slight effect.

Table 4.1.2. Lipid and tocol content of the investigated accessions and sowing dates. The acronyms used for each sample are the same as reported in **Table 4.1.1**.

Effects [1]			**Tocol components** [2]				
	Lipid	**Tocols**	**α-T**	**α-T3**	**β-T**	**β-T3**	**T3/T**
	(g kg^{-1} dm)	(mg kg^{-1} dm)	(mg mg^{-1} total tocols)				
Sowing date							
Fall	22.5	49.05	0.198	0.108	0.108	0.585	2.41
Spring	25.0	55.02	0.198	0.113	0.121	0.569	2.39
Significance (LSD)	** (1.0)	* (4.57)	ns	** (0.003)	** (0.006)	** (0.002)	ns
Accessions							
a. fall sowing							
mG1	24.6	46.64	0.198	0.167	0.075	0.560	2.67
mT2	23.2	46.62	0.156	0.189	0.053	0.601	3.77
mT3	21.7	73.90	0.154	0.174	0.057	0.615	3.74
mI4	26.5	47.42	0.192	0.185	0.069	0.554	2.83
eG1	21.7	48.66	0.184	0.095	0.151	0.570	1.99
eT2	18.0	53.91	0.213	0.129	0.131	0.527	1.90
eT3	23.6	43.86	0.205	0.160	0.093	0.542	2.38
eI4	22.0	46.32	0.199	0.103	0.104	0.594	2.30
eI5	21.2	40.68	0.238	0.096	0.139	0.528	1.66
eI6	26.5	49.36	0.230	0.106	0.143	0.520	1.69
eI7	20.0	58.58	0.177	0.075	0.082	0.666	2.87
eI8	22.7	44.32	0.239	0.100	0.153	0.508	1.56

eI9	23.9	59.38	0.177	0.074	0.089	0.660	2.76
eA10	25.2	60.81	0.234	0.082	0.154	0.531	1.58
eA11	23.7	45.57	0.244	0.087	0.155	0.514	1.51
eA12	23.0	39.14	0.234	0.107	0.151	0.508	1.60
eA13	24.3	44.96	0.236	0.091	0.150	0.523	1.59
eB14	20.0	39.06	0.229	0.105	0.139	0.527	1.73
tG	23.5	56.22	0.156	0.115	0.070	0.659	3.44
pG	22.7	34.34	0.178	0.082	0.095	0.646	2.68
dG1	21.6	38.27	0.214	0.093	0.104	0.589	2.17
dG2	22.0	66.33	0.139	0.072	0.086	0.703	3.45
dG3	22.8	39.41	0.254	0.100	0.097	0.549	1.85
dC4	22.6	51.28	0.164	0.108	0.077	0.652	3.16
dC5	23.0	47.51	0.192	0.077	0.105	0.626	2.37
hG	21.0	48.65	0.145	0.080	0.084	0.691	3.38
aC1	19.3	52.93	0.194	0.091	0.125	0.591	2.14
aC2	18.8	45.41	0.194	0.081	0.106	0.619	2.34
b. spring sowing							
mG1	28.5	47.16	0.193	0.163	0.064	0.580	2.89
mT2	27.0	58.25	0.138	0.190	0.050	0.622	4.33
mT3	27.7	63.37	0.152	0.190	0.058	0.600	3.77
mI4	20.3	38.70	0.166	0.172	0.073	0.589	3.18
eG1	25.5	65.01	0.185	0.091	0.167	0.558	1.85
eT2	24.6	55.94	0.235	0.120	0.124	0.522	1.79
eT3	25.3	55.24	0.222	0.131	0.115	0.532	1.97
eI5	24.3	44.01	0.226	0.096	0.160	0.517	1.59
eI6	27.5	57.70	0.219	0.098	0.152	0.531	1.70
eI8	28.5	63.31	0.229	0.097	0.160	0.515	1.57
eA10	25.5	58.41	0.240	0.077	0.168	0.515	1.45
eA11	23.6	45.42	0.241	0.076	0.183	0.501	1.36
eA12	19.2	41.39	0.231	0.098	0.146	0.525	1.65
eA13	27.0	60.27	0.231	0.082	0.174	0.514	1.48
eB14	24.1	53.45	0.229	0.091	0.156	0.524	1.61
tG	25.9	66.56	0.125	0.118	0.060	0.697	4.42
dG3	19.6	32.60	0.263	0.108	0.115	0.513	1.65
dC4	24.2	74.27	0.145	0.099	0.087	0.669	3.30
dC5	28.2	52.57	0.171	0.077	0.104	0.648	2.64
hG	24.2	66.73	0.125	0.076	0.096	0.703	3.52
Significance (LSD)	** (5.0)	* (22.09)	** (0.019)	** (0.015)	** (0.010)	** (0.029)	** (0.23)
Accession × sowing (spring types)	ns	Ns	** (0.018)	ns	** (0.011)	* (0.029)	** (0.23)

[1] Significance: *: $p \leq 0.05$; **: $p \leq 0.01$; ns: non significant; values in parenthesis: LSD, $p = 0.05$.
[2] α-T: α-tocopherol; α-T3: α-tocotrienol; β-T: β-tocopherol; β-T: β-tocotrienol.

The predominant tocol resulted β-T3, showing a higher variability during the spring sowing and ranging between 0.501 and 0.703 mg mg^{-1} total tocols. The highest amounts were reached by the Italian eI7 and eI9, and the Georgian tG and hG; also the modern *T. durum* varieties showed high relative β-T3 contents.

The second structure, in order of abundance, was α-T, whose relative content was, on average, higher in emmer wheat and in some durum wheat accessions than in diploid einkorn wheat and in the hexaploid bread and macha wheats.

T. monococcum accessions resulted the richest in α-T3 (0.163-0.190 mg mg^{-1} total tocols). The relative β-T content was higher in all the *T. dicoccum* samples, regardless of origin; lower amounts were detected in *T. durum* and *T. aestivum*, while the lowest values were observed in *T. monococcum*.

The lowest tocotrienol/tocopherol ratio (T3/T) was found in *T. dicoccum*; for all the other samples the ratio was greater than 2, reaching values higher than 3.5 for the Turkish *T. monococcum* samples, and the tetraploid tG (*T. timopheevi*, var. *rubiginosum*) and the hexaploid hG (*T. macha*), in agreement also with the data previously reported by Hidalgo *et al.* (2006).

Carotenoids

Carotenoid content (**Table 4.1.3**) was highly affected by the species.

T. monococcum grains had the highest concentrations, among hulled species (6.6-8.2 mg kg^{-1} dm, fall sowing; and 4.7-7.9 mg kg^{-1} dm, spring sowing), but equivalent contents were detected in the *T. durum* samples (5.8-6.8 mg kg^{-1} dm, fall sowing; and 5.4-8.4 mg kg^{-1} dm, spring sowing), with special regard for the commercial varieties, which are nowadays specifically selected for intense yellow colour. Significant within species variability and accession × sowing date interactions were also detected.

The identified pigments were lutein (LUT), zeaxanthin (ZEA), β-cryptoxanthin (β-CRY), and the sum of α- and β-carotene (α+β-CA), in agreement with previous studies (Abdel-Aal *et al.*, 2007; Panfili *et al.*, 2004). An unidentified minor peak represented ~5-10% of all pigments; a similar peak was observed by Panfili *et al.* (2004), and Hidalgo *et al.* (2010), and tentatively identified as the *cis* isomer of lutein (LUT iso), described by Humphries and Khachik (2003).

Lutein was the most abundant compound in all the samples, showing the highest relative contents in *T. monococcum*, *T. timopheevi* and *T. durum* (0.567-0.785 mg mg^{-1} total carotenoids). *T. dicoccum* showed the lowest relative lutein content, but the highest of zeaxanthin (0.103-0.272 mg mg^{-1} total

carotenoids). The highest amounts of β-cryptoxanthin were found in the hexaploid *T. aestivum* and *T. macha* (0.085-0.132 mg mg^{-1} total carotenoids). Sowing date little affected carotenoid profile.

Table 4.1.3. Carotenoid content of the investigated accessions and sowing dates. The acronyms used for each sample are the same as reported in **Table 4.1.1**.

Effects [1]	**Total**	**Carotenoid components** [2]				
	carotenoids	**(α+β)-CA**	**β-CRY**	**LUT**	**ZEA**	**LUT iso**
	(mg kg^{-1} dm)	(mg mg^{-1} total carotenoids)				
Sowing date						
fall	3.54	0.096	0.050	0.628	0.150	0.072
spring	3.80	0.097	0.052	0.593	0.181	0.074
Significance (LSD)	* (0.28)	ns	ns	** (0.005)	** (0.004)	** (0.001)
Accessions						
a) *fall sowing*						
mG1	7.39	0.097	0.039	0.676	0.121	0.067
mT2	8.22	0.055	0.034	0.776	0.083	0.051
mT3	6.65	0.053	0.031	0.785	0.078	0.053
mI4	6.92	0.088	0.047	0.714	0.090	0.060
eG1	2.15	0.111	0.050	0.547	0.231	0.078
eT2	2.04	0.099	0.063	0.590	0.171	0.076
eT3	4.90	0.067	0.034	0.738	0.103	0.058
eI4	2.14	0.110	0.052	0.616	0.146	0.076
eI5	1.79	0.121	0.054	0.531	0.212	0.082
eI6	1.97	0.123	0.068	0.500	0.221	0.088
eI7	2.08	0.098	0.043	0.640	0.144	0.076
eI8	2.31	0.111	0.048	0.544	0.224	0.074
eI9	2.00	0.098	0.051	0.617	0.153	0.082
eA10	2.56	0.115	0.043	0.535	0.236	0.071
eA11	2.61	0.112	0.051	0.545	0.216	0.076
eA12	2.19	0.109	0.045	0.594	0.179	0.073
eA13	2.35	0.113	0.047	0.561	0.205	0.074
eB14	1.63	0.109	0.055	0.562	0.192	0.082
tG	4.90	0.067	0.034	0.738	0.103	0.058
pG	2.17	0.080	0.088	0.537	0.159	0.081
dG1	3.99	0.089	0.021	0.718	0.100	0.073
dG2	2.69	0.096	0.042	0.625	0.159	0.078
dG3	4.54	0.079	0.029	0.647	0.183	0.062
dC4	5.75	0.090	0.023	0.759	0.068	0.060
dC5	6.78	0.107	0.028	0.729	0.078	0.059
hG	2.14	0.087	0.100	0.535	0.153	0.065
aC1	2.17	0.080	0.088	0.537	0.159	0.081
aC2	2.42	0.098	0.132	0.500	0.172	0.032

b) *spring sowing*						
mG1	7.19	0.088	0.041	0.673	0.143	0.054
mT2	7.86	0.053	0.042	0.755	0.090	0.060
mT3	7.24	0.055	0.045	0.754	0.086	0.060
mI4	4.73	0.075	0.037	0.689	0.120	0.079
eG1	2.48	0.109	0.059	0.483	0.272	0.077
eT2	2.73	0.106	0.059	0.554	0.207	0.074
eT3	2.96	0.092	0.049	0.617	0.168	0.073
eI5	1.74	0.121	0.066	0.451	0.270	0.091
eI6	1.95	0.129	0.070	0.476	0.231	0.094
eI8	2.02	0.117	0.067	0.499	0.224	0.094
eA10	2.60	0.111	0.057	0.497	0.259	0.075
eA11	2.29	0.116	0.053	0.495	0.259	0.077
eA12	1.95	0.108	0.047	0.555	0.216	0.074
eA13	2.64	0.113	0.055	0.497	0.255	0.080
eB14	1.88	0.119	0.066	0.495	0.230	0.090
tG	4.43	0.075	0.039	0.780	0.038	0.068
dG3	3.01	0.086	0.039	0.567	0.242	0.066
dC4	5.34	0.091	0.029	0.741	0.071	0.068
dC5	8.38	0.092	0.030	0.751	0.061	0.065
hG	2.59	0.080	0.085	0.532	0.175	0.069
Significance (LSD)	** (1.38)	** (0.008)	** (0.009)	** (0.025)	** (0.020)	** (0.008)
Accession × sowing (spring types)	ns	** (0.008)	** (0.009)	** (0.024)	** (0.019)	** (0.008)

[1] Significance: *: $p \leq 0.05$; **: $p \leq 0.01$; ns: non significant; values in parenthesis: LSD, $p = 0.05$.
[2] (α+β)-CA: (α+β)-carotene; β-CRY: β-cryptoxanthin; LUT: lutein; ZEA: zeaxanthin; LUT iso: lutein isomer.

Phytosterols

Total sterol content (**Table 4.1.4**) was higher than 500 mg kg^{-1} dm, except for one commercial *T. aestivum* (aC2, 441 mg kg^{-1} dm). Samples from the spring sowing were, on average, richer in these compounds, with special regard to the Turkish emmer and einkorn wheat (755-828 mg kg^{-1} dm) and the modern *T. durum* varieties (703-929 mg kg^{-1} dm).

Several compounds were identified: campestanol (ST2), sitostanol (ST6) and avenastanol (ST8), saturated sterols typically found in wheats; campesterol (ST1), β-sitosterol (ST5) and avenasterol (ST7), the correspondent unsaturated forms, which together with stigmasterol (ST3) are included in the group of Δ^5-sterols; Δ^7-campesterol (ST4), Δ^7-avenasterol (ST10) and Δ^7-sitosterol (ST9), in the group of Δ^7-sterols; finally citrostadienol (ST11), a 4-methyl-Δ^7-sterol.

Table 4.1.4. Sterol content of the investigated accessions and sowing dates. The acronyms used for each sample are the same as reported in Table **4.1.1.**

Effects [1]	**Total sterols** (mg kg^{-1} dm)	**Sterol components** [2] (mg mg^{-1} total sterols)									
		ST1	**ST2**	**ST3**	**ST4**	**ST5**	**ST6**	**ST7**	**ST8**	**ST9**	**ST11**
Sowing date											
fall	638.9	0.226	0.110	0.021	0.013	0.387	0.125	0.035	0.006	0.016	0.019
spring	726.4	0.214	0.104	0.021	0.015	0.395	0.127	0.038	0.006	0.019	0.020
Significance (LSD)	** (34.7)	** (0.006)	** (0.002)	** (0.001)	ns	** (0.001)	** (0.003)	ns	* (0.002)	ns	ns
Accessions fall sowing											
mG1	623.2	0.220	0.108	0.013	0.023	0.362	0.110	0.053	0.007	0.018	0.028
mT2	658.5	0.198	0.111	0.011	0.027	0.382	0.136	0.038	0.005	0.020	0.027
mT3	629.1	0.208	0.108	0.011	0.028	0.383	0.121	0.044	0.004	0.019	0.027
mI4	710.8	0.228	0.112	0.011	0.027	0.355	0.126	0.042	0.007	0.019	0.024
eG1	604.8	0.261	0.068	0.017	0.009	0.449	0.085	0.033	0.005	0.016	0.015
eT2	500.8	0.212	0.104	0.024	0.012	0.402	0.140	0.029	0.005	0.016	0.018
eT3	690.5	0.208	0.107	0.021	0.019	0.392	0.133	0.034	0.005	0.018	0.022
eI4	642.9	0.211	0.127	0.025	0.011	0.367	0.117	0.065	0.008	0.023	0.014
eI5	586.8	0.227	0.098	0.025	0.011	0.400	0.116	0.037	0.005	0.021	0.019
eI6	746.7	0.230	0.084	0.027	0.013	0.427	0.107	0.030	0.005	0.018	0.019
eI7	639.8	0.227	0.115	0.025	0.008	0.339	0.129	0.073	0.010	0.017	0.018
eI8	652.0	0.229	0.093	0.027	0.014	0.410	0.118	0.029	0.004	0.018	0.017
eI9	708.2	0.221	0.141	0.024	0.011	0.358	0.114	0.049	0.005	0.017	0.021
eA10	695.7	0.227	0.093	0.016	0.008	0.420	0.120	0.039	0.005	0.014	0.017
eA11	682.4	0.225	0.097	0.016	0.008	0.422	0.128	0.032	0.006	0.013	0.016

eA12	684.2	0.203	0.116	0.021	0.010	0.434	0.132	0.023	0.006	0.011	0.014
eA13	672.5	0.224	0.101	0.018	0.008	0.419	0.125	0.033	0.005	0.012	0.016
eB14	543.9	0.204	0.095	0.027	0.021	0.411	0.135	0.025	0.005	0.017	0.019
tG	591.2	0.215	0.094	0.015	0.011	0.392	0.134	0.029	0.007	0.019	0.028
pG	660.0	0.177	0.080	0.024	0.008	0.374	0.217	0.044	0.006	0.017	0.015
dG1	664.6	0.242	0.131	0.028	0.015	0.350	0.117	0.028	0.006	0.020	0.018
dG2	635.1	0.246	0.169	0.027	0.010	0.360	0.117	0.021	0.004	0.010	0.009
dG3	675.4	0.234	0.151	0.025	0.010	0.359	0.117	0.025	0.006	0.013	0.017
dC4	683.2	0.239	0.133	0.027	0.007	0.357	0.136	0.029	0.004	0.012	0.014
dC5	614.8	0.227	0.154	0.021	0.010	0.340	0.134	0.034	0.005	0.014	0.018
hG	571.6	0.246	0.087	0.027	0.012	0.414	0.114	0.031	0.006	0.015	0.017
aC1	661.8	0.268	0.097	0.018	0.013	0.402	0.117	0.014	0.007	0.010	0.016
aC2	440.8	0.280	0.113	0.021	0.007	0.362	0.112	0.024	0.010	0.008	0.021
spring sowing											
mG1	800.1	0.213	0.107	0.014	0.022	0.357	0.124	0.051	0.007	0.018	0.029
mT2	828.5	0.193	0.103	0.012	0.027	0.379	0.137	0.045	0.005	0.020	0.030
mT3	808.7	0.198	0.103	0.013	0.029	0.398	0.129	0.032	0.006	0.020	0.027
mI4	554.3	0.214	0.104	0.010	0.028	0.357	0.139	0.046	0.006	0.020	0.026
eG1	769.2	0.254	0.060	0.015	0.010	0.464	0.077	0.039	0.005	0.019	0.015
eT2	755.4	0.207	0.105	0.027	0.014	0.392	0.140	0.036	0.005	0.022	0.015
eT3	816.4	0.203	0.104	0.028	0.027	0.388	0.131	0.041	0.007	0.022	0.016
eI5	672.5	0.207	0.091	0.027	0.013	0.422	0.123	0.035	0.006	0.022	0.018
eI6	741.7	0.217	0.088	0.026	0.014	0.418	0.118	0.030	0.005	0.021	0.021
eI8	755.7	0.215	0.098	0.027	0.014	0.404	0.131	0.027	0.005	0.021	0.019
eA10	656.0	0.221	0.090	0.018	0.008	0.422	0.120	0.041	0.005	0.018	0.017
eA11	620.8	0.215	0.093	0.017	0.009	0.424	0.117	0.050	0.005	0.018	0.016
eA12	537.6	0.212	0.115	0.024	0.010	0.416	0.130	0.028	0.005	0.013	0.015
eA13	753.1	0.227	0.095	0.019	0.009	0.411	0.117	0.042	0.006	0.016	0.018
eB14	805.7	0.187	0.100	0.028	0.012	0.417	0.139	0.038	0.006	0.022	0.017
tG	754.9	0.206	0.096	0.014	0.013	0.380	0.142	0.030	0.007	0.021	0.031

dG3	626.3	0.218	0.149	0.028	0.012	0.350	0.125	0.031	0.006	0.017	0.018
dC4	702.6	0.221	0.133	0.030	0.008	0.358	0.135	0.040	0.006	0.015	0.014
dC5	929.0	0.215	0.153	0.025	0.013	0.336	0.135	0.038	0.006	0.017	0.020
hG	640.3	0.234	0.085	0.024	0.014	0.407	0.127	0.031	0.006	0.018	0.021
Significance (LSD)	** (167.5)	** (0.029)	** (0.012)	** (0.006)	** (0.002)	** (0.007)	** (0.016)	** (0.013)	** (0.011)	** (0.001)	** (0.007)
Accession × sowing (spring types)	** (163.1)	* (0.029)	* (0.010)	** (0.006)	** (0.002)	ns	ns	* (0.010)	* (0.009)	* (0.001)	ns

[1] Significance: *: $p \leq 0.05$; **: $p \leq 0.01$; ns: non significant; values in parenthesis: LSD, $p = 0.05$.
[2] ST1: campesterol; ST2: campestanol; ST3: stigmasterol; ST4: Δ7-campesterol; ST5: β-sitosterol; ST6: sitostanol; ST7: avenasterol; ST8: avenastanol; ST9: Δ7-sitosterol; ST11: citrostadienol.

β-sitosterol was the predominant compound in all samples, followed by campesterol. The *T. dicoccum* accessions from Armenia, Bulgaria and Georgia were characterized by the highest β-sitosterol relative content (0.411-0.464 mg mg^{-1} total sterols), while *T. durum* showed the lowest content (0.336-0.360 mg mg^{-1}). High percentages of campesterol were detected in the *T. aestivum* and *T. durum* samples, and in Georgian eG1 (*T. dicoccum*) and hG (*T. macha*).

Among saturated sterols, campestanol ranged around 0.090-0.120 mg mg^{-1}, reaching values of 0.130-0.150 mg mg^{-1} for *T. durum*. Sitostanol content was around 0.120-0.130 mg mg^{-1}, except for pG (*T. palaeo-colchicum*), a winter variety, where it exceeded that of campesterol, with a relative content of 0.217 mg mg^{-1}. *T. monoccum* was richer than the other species in the minor classes of Δ^7-sterols and 4-methyl-Δ^7-sterols.

Phenolic compounds

Total (TPC), free (FPC) and bound (BPC) phenolic content (**Table 4.1.5**), on average, were unaffected by sowing date. In both sowings the lowest phenolic amount was found in Georgian tG (*T. timopheevi*). Among the other species no defined clean pattern was detected, because of within-species variability and accession × sowing date interaction. However, hexaploid types showed a tendency to high phenolic content.

Bound phenolics, associated with cell wall components (BPC), contributed to over 0.670 mg mg^{-1} to total phenolic content, confirming previous findings (Okarter *et al.*, 2010; Adom *et al.*, 2003). BPC reached values around 0.850-0.890 mg mg^{-1} TPC in *T. monococcum*, and 0.780-0.830 mg mg^{-1} TPC in the commercial types. Similar relative contents, around 0.740-0.800 mg mg^{-1} TPC, were found in *T. durum* and *T. macha*, while the lowest amounts were detected in *T. dicoccum* especially considering the Georgian, Armenian and Bulgarian samples (0.679-0.726 mg mg^{-1} TPC). The BPC/FPC ratio confirmed what was illustrated above, with values higher than 6 in *T. monococcum*, where BPC reached 0.850-0.890 mg mg^{-1} TPC, around 2 in *T. dicoccum* samples, and intermediate values in the other species.

BPC were mainly composed of phenolic acids. A total of 10 phenolic acids (including isomeric structures) were identified, among the following: vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, sinapic acid, two isomeric forms of ferulic acid and three isomers of dihydroferulic acid. Moreover we could identify 2 phenolic aldehydes: *p*-hydroxybenzaldehyde and vanillin.

Table 4.1.5. Phenolic content of the investigated accessions and sowing dates. The acronyms used for each sample are the same as reported in **Table 4.1.1.**

						Phenolic acids [2]				
Effects [1]	**TPC**	**BPC**	**BPC/ FPC**	**FPC4**	**FPC5**	**p-COU**	**FER**	**DHF1**	**DHF2**	**DHF3**
	(mg kg^{-1} dm)	(mg mg^{-1} TPC)		(mg mg^{-1} FPC)		(mg mg^{-1} BPC)				
Sowing date										
fall	1046.3	0.767	3.75	0.093	0.298	0.098	0.758	0.015	0.014	0.030
spring	1021.6	0.758	3.52	0.103	0.277	0.101	0.745	0.014	0.013	0.031
Significance (LSD)	ns	ns	ns	** (0.0032)	** (0.0020)	** (0.0007)	ns	** (0.0015)	** (0.0005)	** (0.0007)
Accessions										
fall sowing										
mG1	1080.4	0.886	7.81	0.130	0.240	0.104	0.758	0.009	0.010	0.019
mT2	1058.8	0.875	6.98	0.124	0.216	0.231	0.655	0.006	0.005	0.015
mT3	1105.2	0.883	7.63	0.124	0.197	0.282	0.598	0.006	0.007	0.016
mI4	1058.2	0.898	8.80	0.117	0.178	0.165	0.708	0.007	0.006	0.016
eG1	1015.1	0.712	2.52	0.106	0.268	0.113	0.756	0.013	0.013	0.031
eT2	1145.2	0.761	3.31	0.094	0.318	0.163	0.697	0.011	0.012	0.026
eT3	1248.4	0.846	5.53	0.103	0.285	0.294	0.585	0.008	0.008	0.019
eI4	937.6	0.750	3.03	0.089	0.377	0.088	0.786	0.015	0.013	0.032
eI5	994.1	0.682	2.21	0.081	0.319	0.078	0.795	0.010	0.009	0.023
eI6	1052.0	0.684	2.17	0.079	0.322	0.100	0.773	0.011	0.009	0.024
eI7	881.7	0.740	2.88	0.091	0.400	0.082	0.774	0.014	0.010	0.029
eI8	1069.6	0.691	2.25	0.081	0.338	0.095	0.763	0.011	0.010	0.029
eI9	911.1	0.749	3.13	0.086	0.354	0.065	0.786	0.014	0.010	0.029
eA10	944.7	0.679	2.17	0.099	0.274	0.084	0.778	0.012	0.010	0.027
eA11	981.3	0.711	2.49	0.089	0.274	0.068	0.802	0.013	0.010	0.028

eA12	962.4	0.711	2.48	0.088	0.312	0.087	0.779	0.012	0.013	0.030
eA13	937.7	0.708	2.43	0.091	0.279	0.071	0.810	0.012	0.011	0.028
eB14	1061.2	0.679	2.13	0.073	0.341	0.085	0.778	0.010	0.009	0.025
tG	831.6	0.756	3.12	0.099	0.286	0.089	0.763	0.015	0.015	0.034
pG	957.9	0.680	2.13	0.096	0.327	0.083	0.788	0.013	0.012	0.028
dG1	1090.4	0.740	2.85	0.078	0.344	0.046	0.787	0.027	0.027	0.051
dG2	971.8	0.781	3.60	0.073	0.269	0.025	0.797	0.035	0.031	0.053
dG3	1007.3	0.776	3.46	0.077	0.307	0.027	0.792	0.025	0.023	0.042
dC4	1165.0	0.788	3.72	0.079	0.273	0.026	0.782	0.025	0.032	0.051
dC5	1045.1	0.795	3.94	0.087	0.355	0.018	0.791	0.028	0.032	0.053
hG	1136.8	0.735	2.86	0.117	0.298	0.077	0.787	0.016	0.012	0.027
aC1	1466.0	0.833	5.04	0.071	0.323	0.052	0.800	0.014	0.019	0.035
aC2	1178.2	0.806	4.18	0.096	0.271	0.043	0.769	0.018	0.020	0.036
spring sowing										
mG1	1062.3	0.872	6.81	0.140	0.196	0.117	0.720	0.009	0.008	0.020
mT2	1075.9	0.864	6.40	0.139	0.196	0.252	0.626	0.007	0.007	0.017
mT3	907.0	0.848	5.23	0.126	0.185	0.145	0.714	0.006	0.006	0.020
mI4	1015.3	0.878	7.16	0.127	0.148	0.155	0.692	0.008	0.007	0.018
eG1	890.6	0.718	2.55	0.112	0.257	0.107	0.768	0.013	0.012	0.033
eT2	1011.1	0.718	2.72	0.091	0.323	0.115	0.747	0.011	0.011	0.032
eT3	1161.1	0.735	2.78	0.091	0.324	0.198	0.652	0.009	0.010	0.025
eI5	1004.1	0.686	2.23	0.091	0.324	0.095	0.769	0.011	0.010	0.028
eI6	1038.2	0.683	2.18	0.083	0.311	0.088	0.765	0.012	0.010	0.026
eI8	978.6	0.697	2.32	0.086	0.328	0.093	0.762	0.010	0.009	0.025
eA10	884.3	0.719	2.60	0.106	0.290	0.105	0.756	0.011	0.009	0.028
eA11	884.6	0.716	2.54	0.100	0.281	0.093	0.745	0.009	0.008	0.034
eA12	960.1	0.726	2.66	0.098	0.309	0.107	0.760	0.010	0.010	0.028
eA13	973.9	0.681	2.16	0.096	0.283	0.088	0.782	0.011	0.010	0.027
eB14	975.4	0.702	2.37	0.087	0.312	0.063	0.816	0.011	0.008	0.026
tG	819.8	0.802	4.07	0.124	0.219	0.091	0.747	0.014	0.013	0.034

dG3	1064.2	0.792	3.82	0.088	0.285	0.027	0.765	0.027	0.024	0.044
dC4	1301.0	0.800	4.09	0.078	0.276	0.020	0.743	0.032	0.038	0.057
dC5	1181.1	0.776	3.47	0.088	0.389	0.023	0.759	0.036	0.037	0.068
hG	1206.5	0.738	2.83	0.121	0.268	0.044	0.804	0.019	0.018	0.038
Significance (LSD)	** (159.6)	** (0.049)	** (1.13)	** (0.0156)	** (0.0098)	** (0.0036)	** (0.0549)	** (0.0072)	** (0.0024)	** (0.0034)
Accession × sowing (spring types)	ns	* (0.049)	** (1.17)	** (0.0149)	** (0.0097)	* (0.0034)	* (0.0584)	ns	** (0.0025)	** (0.0034)

[1] Significance: *: $p \leq 0.05$; **: $p \leq 0.01$; ns: non significant; values in parenthesis: LSD, $p = 0.05$.

[2] TPC: total phenolic compounds; FPC: free phenolic compounds; BPC: bound phenolic compounds; BPC/FPC: bound phenolic compounds/ free phenolic compounds; FPC4 and FPC5: isomeric forms of apigenin-6-C-arabinoside-8-C-hexoside; p-COU: *p*-coumaric acid; FER: ferulic acid; DHF1, DHF2, DHF3: dihydroferulic acid isomers.

Ferulic acid was the predominant phenolic acid in all the samples, in accordance also with other studies (Dinelli *et al.*, 2011; Serpen *et al.*, 2008; Adom *et al.*, 2003). In most cases ferulic acid accounted for 0.750-0.800 mg mg^{-1} BPC. Sowing date did not affect its relative content. Among genotypes, a tendency towards lower percentages (0.585-0.747 mg mg^{-1} BPC), was observed for all the Turkish samples and for the Italian *T. monococcum* mI4. The lower ferulic acid percentages were in all cases counterbalanced by higher *p*-coumaric acid relative amounts, the second more abundant bound phenolic compound.

The sum of the three isomers of dihydroferulic acid resulted more abundant in the *T. durum* samples, reaching the highest percentages in the modern varieties (0.130-0.140 mg mg^{-1} BPC).

Flavones resulted the most representative free phenolics (FPC); this class was represented by glycosidic forms of apigenin, in agreement with Dinelli *et al.* (2011).

The effect of variety was highly significant, with *T. dicoccum* showing the highest relative FPC content (0.150-0.320 mg mg^{-1} TPC), and *T. monococcum* (0.110-0.160 mg mg^{-1} TPC) the lowest. On average, free phenolics were about 60-65% lower in *T. monococcum* than in *T. dicoccum*.

Accession profiling on the basis of discriminant analysis

Discriminant analysis made a further contribution to profiling the accessions considered, at species level. **Table 4.1.6** reports the correlation coefficients between the five extracted discriminant factors (DF), and the retained analytical variables by the stepwise discriminant procedure.

DF1 explained 42.4% among species variance, clearly discriminating *T. durum* and *T. aestivum* and, partially, the hexaploid *T. macha* from all the other primitive types (**Figure 4.1.1**). DF1 was negatively related to α-T3 relative content, together with that of some phenolics, like *p*-coumaric acid and FPC5, and sterols, like avenastanol and citrostadienol. It was positively related to other sterols and phenolic compounds, specifically, Δ7-campesterol and campestanol, and two isomeric forms of dihydroferulic acid and FPC4.

DF2 explained a further 22.0% variance, efficiently separating the hexaploid (*T. aestivum* and *T. macha*) from the tetraploid and the diploid species (**Figure 4.1.1**). DF2 was positively related to the relative amounts of β-cryptoxanthin, and campestanol, and to the sterol:stanol ratio, whereas it was negatively related to stigmasterol relative content.

The other discriminant functions (DF3-DF5) contributed to a better specific separation within the diploid and tetraploid primitive genotypes.

Table 4.1.6. Correlations between the discriminant factors for the different species and the independent variables used in the analysis.

	Discriminant factors [1]				
Compounds [2]	**DF1**	**DF2**	**DF3**	**DF4**	**DF5**
BPC	0.068	0.115	**-0.641 ****	0.096	-0.057
FPC	0.519 **	0.255 **	**0.606 ****	0.002	-0.132
(α+β)-CA	0.302 **	0.058	**0.678 ****	0.249 **	0.059
B-CRY	0.157	**0.871 ****	0.100	-0.016	0.032
LUT iso	0.045	0.041	**0.643 ****	-0.074	0.124
α-T3	**-0.727 ****	-0.196	-0.433 **	0.157	-0.035
ST2	**0.490 ****	0.368 **	-0.352 **	0.156	0.157
ST3	0.438 **	**-0.555 ****	-0.403 **	0.085	-0.044
ST4	**0.706 ****	-0.012	0.369 **	0.000	-0.217 **
ST7	-0.116	-0.164	0.226 **	**-0.575 ****	-0.054
ST8	**-0.395 ****	-0.145	0.116	0.123	-0.224 **
ST9	-0.028	0.217 **	-0.169	-0.160	**0.330 ****
ST11	**-0.433 ****	-0.285 **	-0.409 **	-0.219	0.319 **
p-COU	**-0.674 ****	-0.052	-0.467 **	-0.163	0.175
FER	**0.454 ****	0.118	0.198	-0.167	0.018
DHF1	-0.135	-0.222	**0.532 ****	0.012	0.309 **
DHF2	**0.789 ****	-0.365 **	-0.279 **	-0.278 **	-0.091
DHF3	**0.767 ****	-0.358 **	-0.346 **	-0.243 **	-0.006
FPC4	**0.579 ****	0.223 **	0.179	-0.430 **	0.042
FPC5	**-0.740 ****	0.111	-0.364 **	-0.141	-0.247 **
BPC/FPC	-0.515 **	-0.125	**-0.697 ****	0.088	-0.028
T3/T	-0.361 **	-0.068	**-0.601 ****	-0.501 **	-0.097
STE/STA	-0.157	**0.395 ****	0.181	0.246 **	0.020
Explained variance	0.424	0.220	0.185	0.094	0.050

[1] ****** significant correlation for $p \leq 0.05$; bold characters: highest correlation in each row.

[2] BPC: bound phenolic compounds; FPC: free phenolic compounds; (α+β)-CA: (α+β)-carotene; β-CRY: β-cryptoxanthin; LUT iso: isomer of lutein; α-T3: α-tocotrienol; ST2: campestanol; ST3: stigmasterol; ST4: Δ7-campesterol; ST7: avenasterol; ST8: avenastanol; ST9: Δ7-sitosterol; ST11: citrostadienol p-COU: *p*-coumaric acid; FER: ferulic acid; DHF1: dihydroferulic acid, isomer 1; DHF2: dihydroferulic acid, isomer 2; DHF3: dihydroferulic acid, isomer 3; FPC4 and FPC5: isomeric forms of apigenin-6-C-arabinoside-8-C-hexoside; BPC/FPC: bound phenolic compounds/ free phenolic compounds; T3/T: sum of tocotrienols/ sum of tocopherols; STE/STA: sum of sterols/ sum of stanols.

DF3 explained 18.5% variance, discriminating *T. palaeo-colchicum* and, to a lesser extent, the *T. dicoccum* accessions, from the other species. DF3 was positively related to the relative content of some pigments, like (α+β)-carotene and the lutein isomer, and to the total FPC and DHF1 amounts, and negatively correlated with total BPC, and the BPC/FPC and T3/T ratios.

DF4, explaining 9.4% variance, separated the two Georgian primitive species *T. palaeo-colchicum* and *T. timopheevi*; *T. macha*, another Georgian primitive wheat, was collocated in an intermediate position. DF4 was negatively correlated primarily to avenasterol (ST7), FPC4 and T3/T ratio.

The last discriminant factor, DF5, explained 4.9% variance. It discriminated the two hexaploid grains *T. aestivum* and *T. macha* and, again, *T. timopheevi* from the other diploid genotypes. DF5 had its main relation with the two sterols Δ7-sitosterols and citrostadienols, besides DHF1.

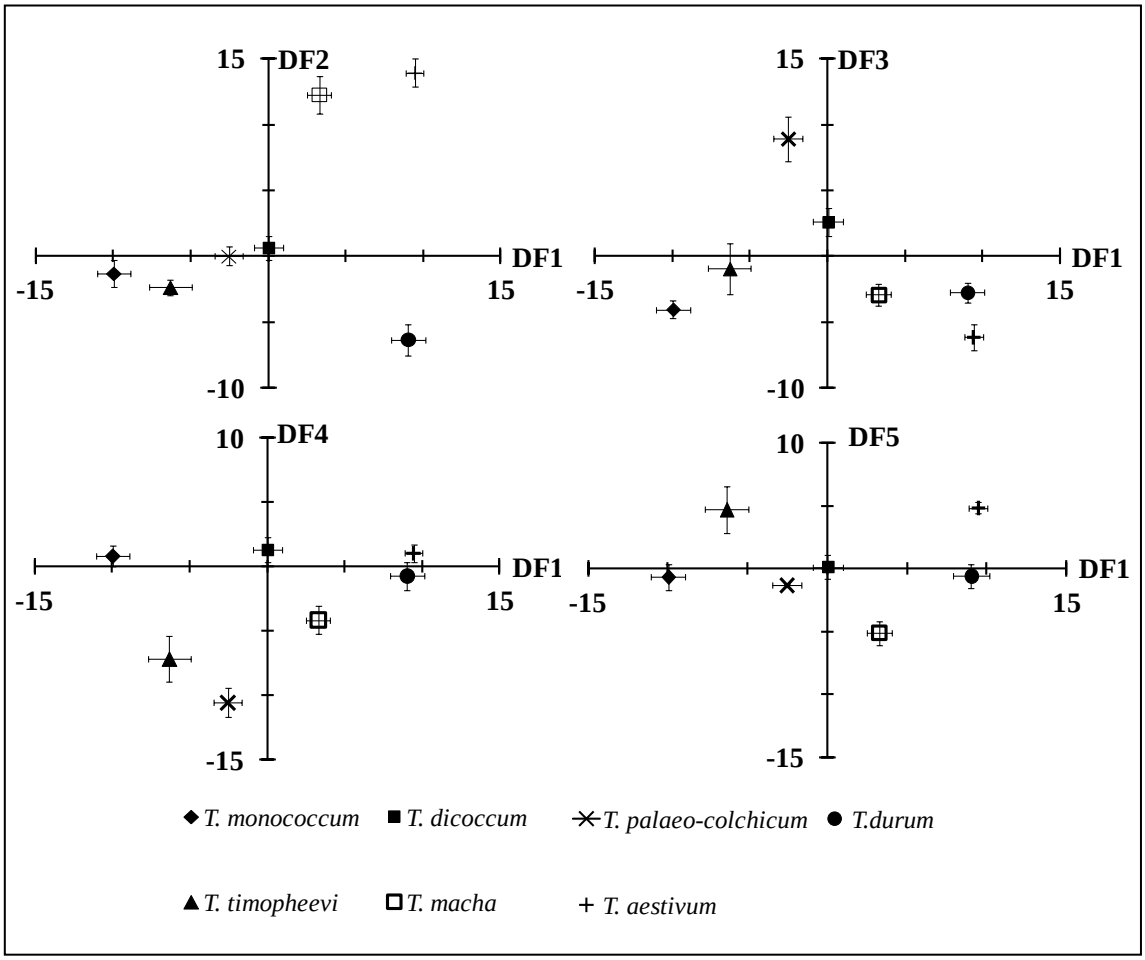

Figure 4.1.1. Score plot of discriminant factors (DF) analysis.

CONCLUSIONS

The present research contributed to further knowledge of wheat grain bioactive composition, with main focus on hulled einkorn and emmer wheats, and including for the first time some species such as macha, Georgian emmer and zanduri wheats.

The data do not support the often reported assumption of higher bioactive compound content in primitive forms, with respect to modern wheat cultivars, which were, however, present with a low

number of control samples. Indeed, einkorn wheat was confirmed to be particularly rich in carotenoids, but equivalent amounts were detected in specifically selected durum wheat cultivars.
However, quite ample within-species variability was detected, indicating that individual populations may be of some interest for specific compounds.
As a whole, the data suggest that the present interest in primitive wheats, based on immaterial perceptual and sometimes sensory traits, can be partially supported by a more in-depth knowledge of their chemical composition.
The data also indicate that, besides major components, the discrimination between taxonomical groups is determined by a complex of compounds present in minor amounts, especially within the phenolic and sterol families.

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Chapter 4.2

An on-site comparative study of yield factors during glume removal, a primary step in the traditional processing of hulled wheats

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ABSTRACT

BACKGROUND: Glume removal represents the preliminary step of hulled wheat grain processing for human consumption. Einkorn and emmer wheat are two hulled species, cultivated in marginal areas, from the Mediterranean to the Caucasian region, and the subject of investigation in this paper. An experiment has been carried out to compare the yield of the traditional process, used in Turkey and Armenia, and an updated Italian procedure, by means of on-plant samplings.

RESULTS: Crushed grains represent a product from all the plants examined. However, the relative amounts of fractions of different size and use, according to each country, varied consistently. The main Italian product is whole pearled grain. The calculated yield factors varied in the following ranges: de-hulling: 0.73-0.81 kg kg^{-1}; food product: 0.82-0.96 kg kg^{-1}; overall yield: 0.56-0.95 kg kg^{-1}, excluding unwanted losses, and 0.42-0.74 kg kg^{-1}, including unwanted losses.

CONCLUSIONS: This first comparative assay of hulled wheat processing showed that, not considering the kind of product obtained, glume removal can be carried out with good efficiency in continuously operating traditional plants. Modern schemes, however, allow yielding of intact kernels. Some yield factors were highly affected by the available technology, especially when the process is operating discontinuously.

Keywords: einkorn; emmer; glume removal; hulled wheats; processing; yield factors; bulgur.

INTRODUCTION

Hulled wheats include some *Triticum* taxa that retain the glumes during the threshing process, unlike the nowadays more common free threshing types; the product of hulled wheat threshing is therefore the whole spikelet, generally containing 1 or 2 kernels, according to the species.

Apart from some minor species, such as Georgian emmer, zanduri wheat and macha wheat, the three main cultivated hulled species are diploid einkorn wheat (*Triticum monococcum* L. subsp. *monococcum*), tetraploid emmer wheat (*T. turgidum* L. subsp. *dicoccum* (Schrank ex Shubler) Thell) and hexaploid spelt (*T. aestivum* L., subsp. *spelta* (L.) Thell). The latter is present with relict populations in NW Spain (Peña-Chocarro, 1996) and is being cultivated all over Europe with some recently selected varieties. Einkorn and emmer wheat are traditional crops in several southern European countries, and have been the subject of cross-country, comparative investigation within the BaSeFood programme (D'Antuono, 2009), especially with respect to Italy, Turkey and Armenia. BaSeFood (Sustainable exploitation of bioactive components from the Black Sea Area traditional foods) is a 3-year collaborative research project funded by the 7th Framework Programme, aimed at investigating plant origin traditional foods from the Black Sea Area, with special focus on the exploitation of their potential health-promoting properties, in relation to their bioactive component content.

In Italy the situation of traditional emmer wheat growing areas and uses has been the subject of detailed surveys (D'Antuono and Bravi, 1996). Briefly, emmer wheat was cultivated in relict areas along the whole Apennines. In all traditional areas emmer wheat progressively disappeared, except in Garfagnana (northern Tuscany) and in some areas of central Italy, centred in Monteleone di Spoleto. In these two areas the production of emmer wheat has been the subject of a recovery, in the frame of organic traditional products, resulting in the registration of PGI (Protected Geographical Indication), for the Garfagnana emmer, and PDO (Protected Denomination of Origin), for the Monteleone emmer. Meanwhile, emmer wheat production has also spread in several non-traditional areas, driven by market demand. At present, although emmer wheat is still consumed in traditional areas, its main importance is as a commercial niche market crop.

Einkorn and emmer wheat were reported as neglected and almost disappearing crops in some Turkish provinces in the Black Sea area (Karagöz, 1996). More detailed surveys indicated hulled wheats as still important crops in local economies (Giuliani *et al.*, 2009), however especially referring to emmer wheat, whereas BaSeFood surveys indicated that mainly einkorn wheat is used as human food in the same areas. In the Kastamonu and Sinop provinces einkorn (locally siyez) is still a relevant human food staple; it is mainly used to prepare bulgur, a typical cooked grain, which

has recently attracted relevant worldwide appreciation (Bayram and Öner, 2007). To obtain bulgur the cleaned spikelets are boiled and then sun dried; only when required for food preparation the dried spikelets are dehulled, ground, and finally sifted into distinct size particles for different culinary uses (Kadakal *et al.*, 2007). Bulgur production and consumption have increased because of its low cost, shelf-life, ease of preparation and good nutritional value (Ünal and Sacilik, 2009). Einkorn wheat bulgur processing and consumption in Turkey are still mainly local, with only initial attempts for a commercial production.

In Armenia, emmer wheat was largely cultivated from the end of the 3rd millennium (Dorofeev *et al.*, 1979), and nowadays it is still extensively grown (Zaharieva *et al.*, 2010). Surveys conducted within BaSeFood confirmed that emmer wheat, locally grown on a fairly large scale using traditional methods, is still a local staple food consumed at home or sold in local markets, without any apparent link to any concept of new or organic products (D'Antuono and Darbinyan, 2009).

For all hulled wheats, the preliminary, essential step for human consumption is glume removal. This traditional process is strikingly similar in the three countries, but the nature of the final product is affected by the available technology (D'Antuono *et al.*, 2011).

Traditionally, the first step of glume removal is grain crushing by means of a stone mill, which separates the glumes from the grain - however generally also crushing the grain into pieces. Glumes are then removed by ventilation and groats of different size are separated by sifting.

Local mills in Turkey are mostly small-size, electrically powered on-farm plants with stone diameter and weight varying from 70 to 100 cm and 70 to 150 kg, respectively (D'Antuono *et al.*, 2011; Giuliani *et al.*, 2009). This type of mill has a compression and rubbing action, the latter assuring glume removal (Ünal and Sacilik, 2009). The main differences between processing schemes occurs after crushing. In this phase, to separate the glumes and chaff from kernel groats, a flow of air can be used, sometimes accompanied by manual sweeping or sieving to increase efficiency. Moreover, the process can be continuous or discontinuous; groat sieving conforms to some common criteria, whether done mechanically or manually, or operated continuously or discontinuously.

In Armenia, the processing flow chart is basically similar, except for the fact that processing plants are generally of larger size and capacity, are collectively managed and normally operate continuously.

In Italy, two processing schemes were traditionally applied. In central Italy, grain crushing used to be carried out exactly in the way illustrated for the other two countries. In Garfagnana, on the contrary, where a floury winter emmer type is used, glume removal was accomplished by stone mills with stones that were rather far apart; these removed glumes with minimal grain breaking,

resulting in, on the contrary, a partial removal of the pericarp (pearling), by a process very similar to that used to process barley as human food (Jadhav *et al.*, 1998). Pearled grains gained popularity in recent years. Therefore, the process in Garfagnana has remained unchanged, except for the adoption of more modern and efficient machines. Conversely, emmer processing in central Italy was updated, with the adoption of dehulling machines capable of removing the glumes with very limited grain crushing. Whole kernels are then mostly pearled by means of a specific machine, whereas only a minor part is crushed, to obtain the traditional product: medium-coarse groats, called "farricello".

BaSeFood was aimed at characterizing local raw materials and traditional foods for their bioactive content and their role in local food systems, and also by comparing situations in different cultural and social contexts. From the above description, glume removal appeared to clearly affect the composition of the final product, compared to the whole kernel, with possible effects on content of nutrients and bioactive compounds. Moreover, the process efficiency could vary according to the flow chart scheme. Production yield analysis has been introduced as a useful tool to optimize raw material processing (Somsen and Capelle, 2002). It has been however also pointed out, however, that processors generally, at best, know their actual yields, but they are hardly aware of maximum potential yields, for which process optimization needs to be referred to (Somsen *et al.*, 2004). An experiment aimed at comparing the processing efficiency in local plants and final chemical composition of obtained fractions was therefore planned. Traditional schemes of Turkey and Armenia were compared with the updated processing scheme adopted in central Italy. The results of process efficiency and yield factors during glume removal are the subject of this research.

MATERIALS AND METHODS

Materials

Fractions from primary (dehulling) emmer and einkorn wheat processing were sampled from traditional plants in Italy, Turkey (two plants) and Armenia during summer and fall 2010. The complete sample and acronym list is reported in **Table 4.2.1**.

Table 4.2.1. List of the samples taken into account during on-plant sampling.

Tag	Description
Turkey (TR1)	
TR1BL	whole cooked spikelets, fed into the mill
TR1F4	keşkeklık, mostly not crushed kernels remaining above the 1st sieve
TR1F5	pilavlık, coarse groats remaining above the 2nd sieve
TR1F6	çorbalık, fine groats remaining above the 3rd sieve
TR1F8	kısırlık, semolina-like material recovered by the last manual sieving
TR1W	final wastes
Turkey (TR2)	
TR2BL	whole cooked spikelets, fed into the mill
TR2F4-F5	keşkeklık, mostly not crushed kernels above the 1st sieve + pilavlık, coarse groats remaining above the 2nd sieve
TR2F6	çorbalık, fine groats remaining above the 3rd sieve
TR2F8	kısırlık, semolina-like material recovered by the last manual sieving
TR2W	final wastes
Armenia (AR)	
ARF5	clean spikelets
ARF6	whole dehulled kernels
ARF7	coarse groats, for human consumption
ARF8	fine groats and semolina-like fraction, mainly for animal feed
Italy (IT)	
ITF1	clean spikelets
ITF2	small or broken grains, for crushing
ITF3	medium-size whole grains, for pearling
ITF4	large grains, to be used as such, or for crushing
ITF6	pearled output, for packaging
ITF7	medium-size groats (farricello), for packaging
ITIW	initial waste (from dehulling)
ITSP	waste, from crushing
ITPR	waste, from pearling

Traditional plants in Turkey

In Turkey, sampling was carried out in two traditional plants of the Kastamonu province. In both cases the materials sampled were einkorn spikelets that have been already subject to the pre-boiling procedure, locally adopted for the preparation of bulgur (see the flow chart represented in **Figure 4.2.1**).

At the end of this preparation the dried spikelets were put into sacks while awaiting stone mill crushing. The two fractions (TR1BL and TR2BL) are therefore cooked, and the dried spikelets represented the input for milling, in both Turkish plants (TR1 and TR2).

Figure 4.2.2 represents the flow chart of TR1. The dried spikelets (TR1BL) were crushed by the stone mill and the product was dropped to the floor, passing through an air flow, to remove part of the glumes. A part of the residual glumes from the output was swept away by a broom and the residual material was again passed through the air flow.

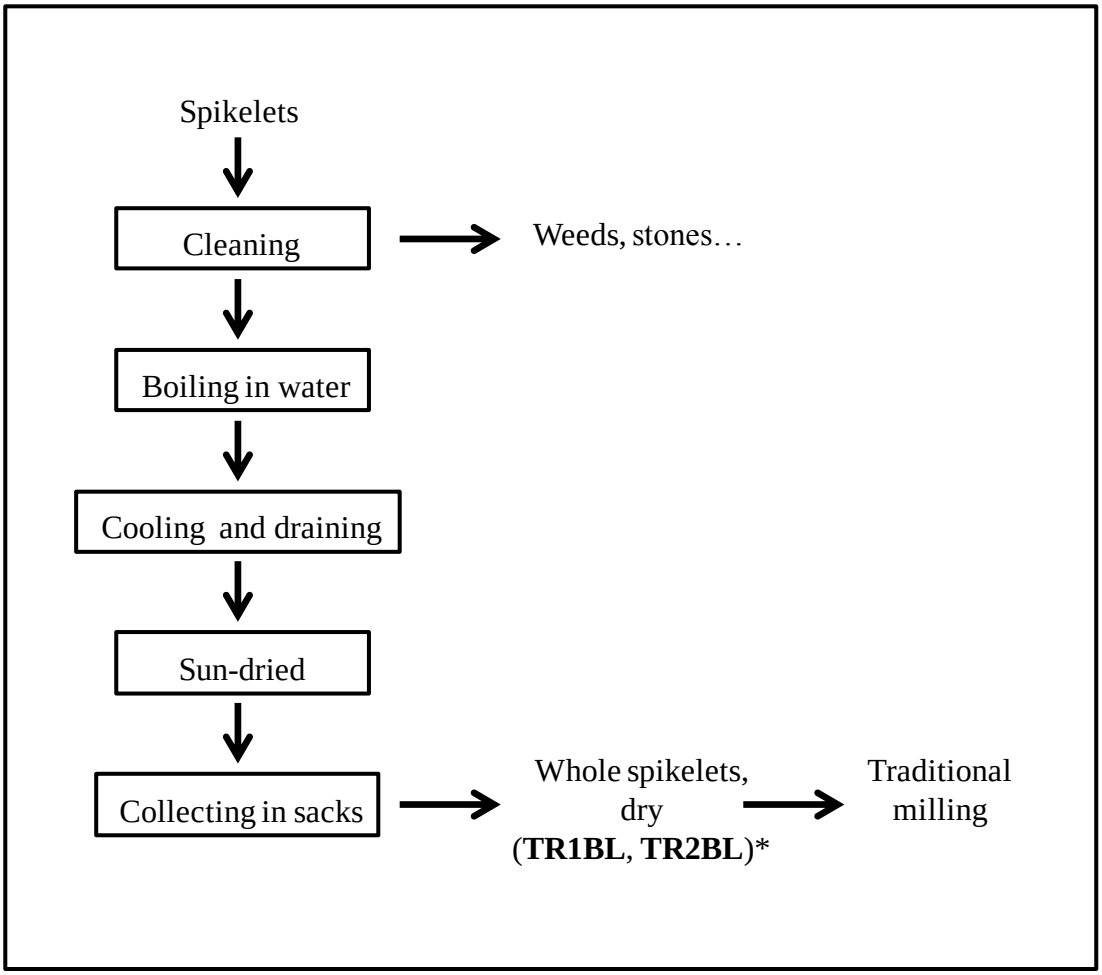

Figure 4.2.1. Flow chart of a typical procedure of einkorn wheat spikelet cooking and drying, for bulgur preparation in Turkey.

***TR1BL**: dried einkorn spikelets, Turkish plant 1; **TR2BL**: dried einkorn spikelets, Turkish plant 2.

The final material, represented by clean, whole dehulled and crushed grains and intact spikelets, was brought to the mechanical, electrically powered sieving system, consisting of three sieves in series. The material remaining above the first sieve, made mainly of unbroken grains, grains still in the glumes and glumes, was refilled, cleaned as reported above, and fed again into the sieves.

At the end of the whole process, the material remaining above the first sieve, mainly composed of unbroken dehulled grains, was kept separate, representing a fraction named keşkeklık (TR1F4).

The material passing through the first sieve and remaining above the second sieve, made of coarse groats, winnowed to remove the residual glumes, dust and other possible alien material, represented the bulgur used for a typical recipe, the pilavlık (TR1F5).

The material passing through the second sieve and remaining above the third (fine groats), also subject to further cleaning, represented the çorbalık fraction (TR1F6), traditionally used as an ingredient of meatballs ("köftelık" bulgur) or soups (çorbalık bulgur).

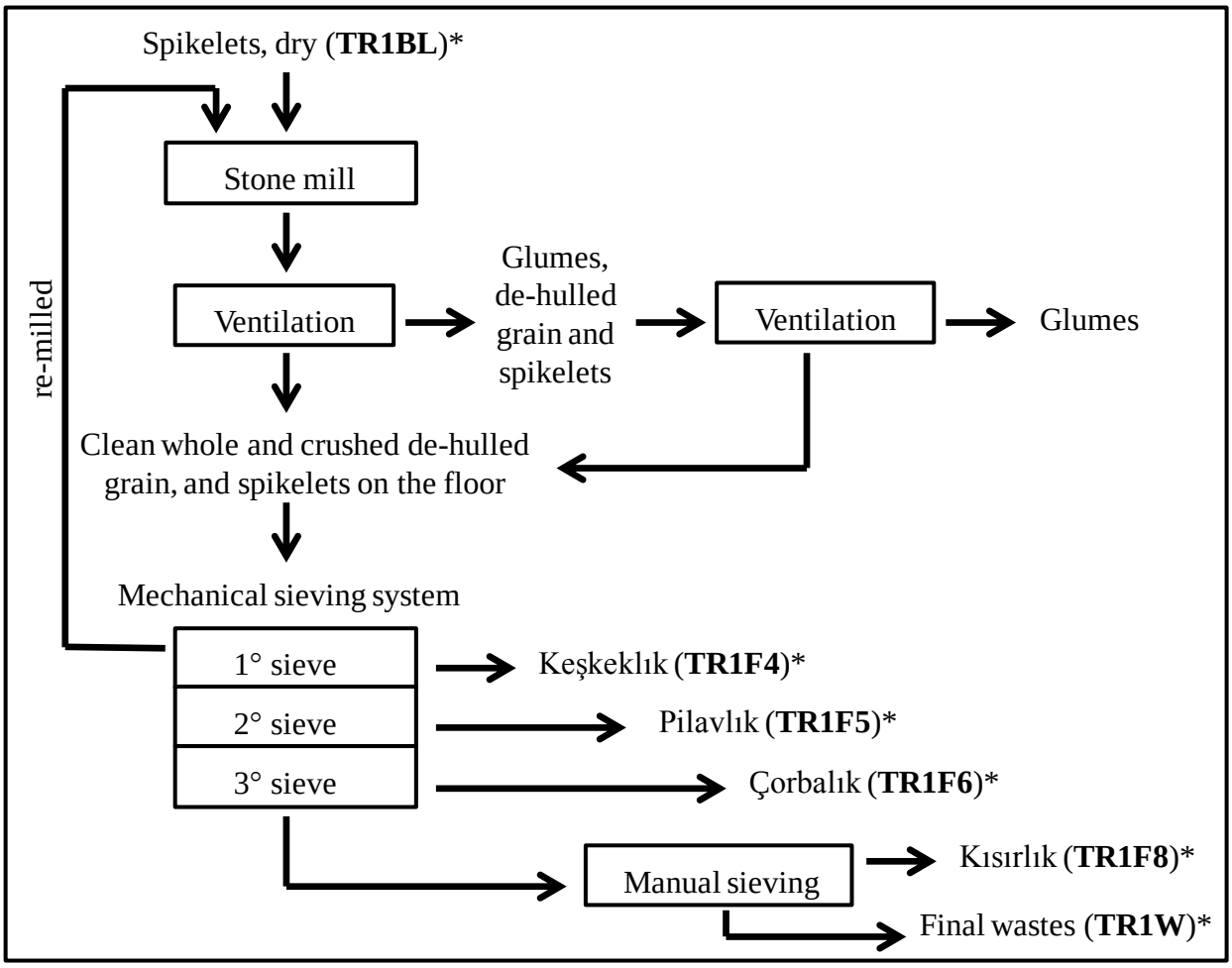

Figure 4.2.2. Schematic flow chart of the traditional processing Turkish plant one (TR1).

***TR1BL**: dried spikelets, fed into the mill; **TR1F4**: keşkeklık, mostly unbroken grain remaining above the 1° sieve; **TR1F5**: pilavlık, coarse groats remaining above the 2° sieve; **TR1F6**: çorbalık, fine groats remaining above the 3° sieve; **TR1F8**: kısırlık, fine semolina-like, recovered by manual sieving; **TR1W**: final wastes, material passing through the last sieve.

The material flowing to the floor from the third mechanical sieve still contained some fine grain that was recovered by manual sieving. This last phase yielded the "kısırlık" (TR1F8) - a further, very fine, semolina-like product for human consumption - and the final waste (TR1W), to feed animals.

Turkish plant two (TR2, **Figure 4.2.3**) was also discontinuous.

Milling was carried out as in TR1, but the output product, represented by crushed and whole kernels, intact spikelets and kernels and glumes, was collected in sacks, without any sorting or ventilation. This material was then further processed by manual sieving, carried out by women, using a series of four sieves, used in sequence; the net of these was made of natural rope, with almost square holes of variable diameter, but the process substantially repeated what was observed for the metallic sieves employed in TR1.

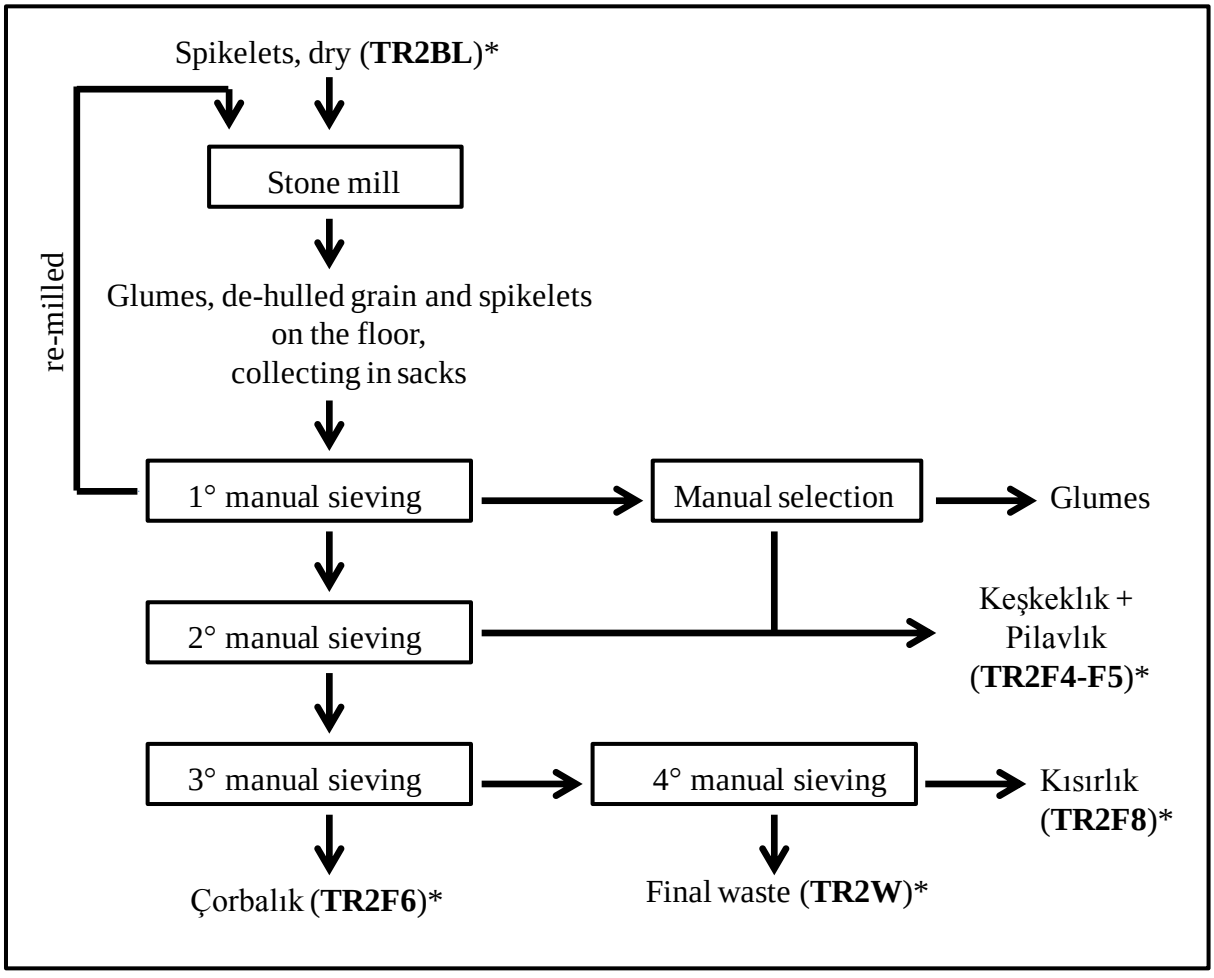

Figure 4.2.3. Schematic flow chart of the traditional processing Turkish plant 2 (TR2).

***TR2BL**: dried spikelets, fed into the mill of the plant 2 (TR2); **TR2F4-F5**: keşkeklık, mostly not crushed grain remaining above the 1° sieve, and pilavlık, coarse groats remaining above the 2° sieve; **TR2F6**: çorbalık, fine groats remaining above the 3° sieve; **TR2F8**: kısırlık, fine semolina-like, recovered by the last manual sieving; **TR2W**: final wastes, material passing through the last sieve.

During the first sieving the glumes floated to the top and were manually removed. The material remaining above the first sieve, was remilled. After the second milling, a very small amount of whole, dehulled grain remained above the first sieve and it was therefore added to the material remaining above the second sieve; thus from TR2 we had a single product (TR2F4-F5), represented by a mixture of prevailing pilavlık (F5) and limited keşkeklık (F4) fractions. The third manual sieve separated the çorbalık fraction (TR2F6), whereas the material passing through was subjected to a fourth sieving, from which the "kısırlık" fraction (TR2F8) and the finale waste (TR2W) were obtained.

Traditional plant in Armenia

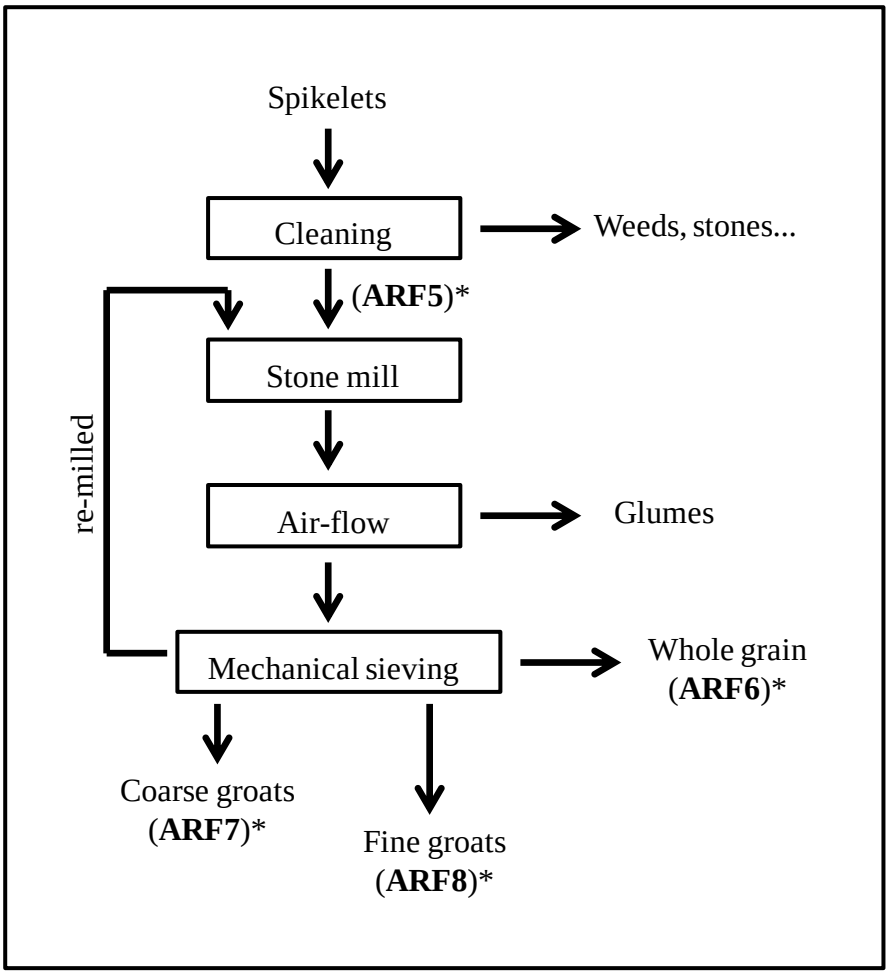

Figure 4.2.4. Schematic flow chart of the traditional processing Armenian plant.

***ARF5**: raw emmer wheat spikelets, fed into the mill; **ARF6**: whole, dehulled grain; **ARF7**: coarse groats for human consumption; **ARF8**: fine groats, to feed animals.

In Armenia the sampling was carried out in a local community processing plant in Kaputan, Kotayk region (central Armenia); the process flow chart is represented in **Figure 4.2.4**.
Input raw material was in this case represented by uncooked emmer wheat spikelets. After the preliminary operations of removing weeds, straw and other material, the cleaned spikelets (ARF5) were fed into the stone mill. At the output an air flow blew the glumes away, out of the processing plant, and the broken kernels were mechanically conveyed to the sieving phase, also working mechanically and continuously. From the sieves the following fractions were obtained: spikelets still in glumes, that were remilled, whole dehulled kernels (ARF6), coarse groats (ARF7), and fine groats (ARF8). The whole grains and coarse groats grains are used for human consumption, while the fine groats and semolina-like parts are mostly employed as animal feed.

Processing plant in Italy

In Italy the sampling was carried out in a modern plant in Monteleone di Spoleto (Umbria region), carrying out glume removal in a first phase, and kernel pearling and crushing in two subsequent, independent steps (**Figure 4.2.5**). The input material was raw emmer wheat spikelets.
The cleaned input material (ITF1) was fed into a dehulling equipment in which glumes were removed by a rotating Teflon device. The separated glumes were blown away by an air flow, and the other material was sorted by sieving into the following fractions:

- broken or very small seeds, destined for crushing (ITF2);
- medium-size seeds, destined for pearling (ITF3);
- large seeds for crushing, in this specific case (ITF4);
- an initial waste consisting of very little broken seeds, to produce flour to feed animals (ITIW);
- still intact spikelets, that were remilled.

Dehulling was therefore followed by two distinct processes: the pearling and the crushing.
The pearling used abrasive discs to partially remove the pericarp, producing the pearled seeds (ITF6) and a waste composed exclusively of bran (ITPR).
Crushing was performed by means of hammer mills, producing rather fine groats (ITF7), representing the typical traditional product, called "farricello" and a waste, consisting of fine material (ITSP), for animal feed.

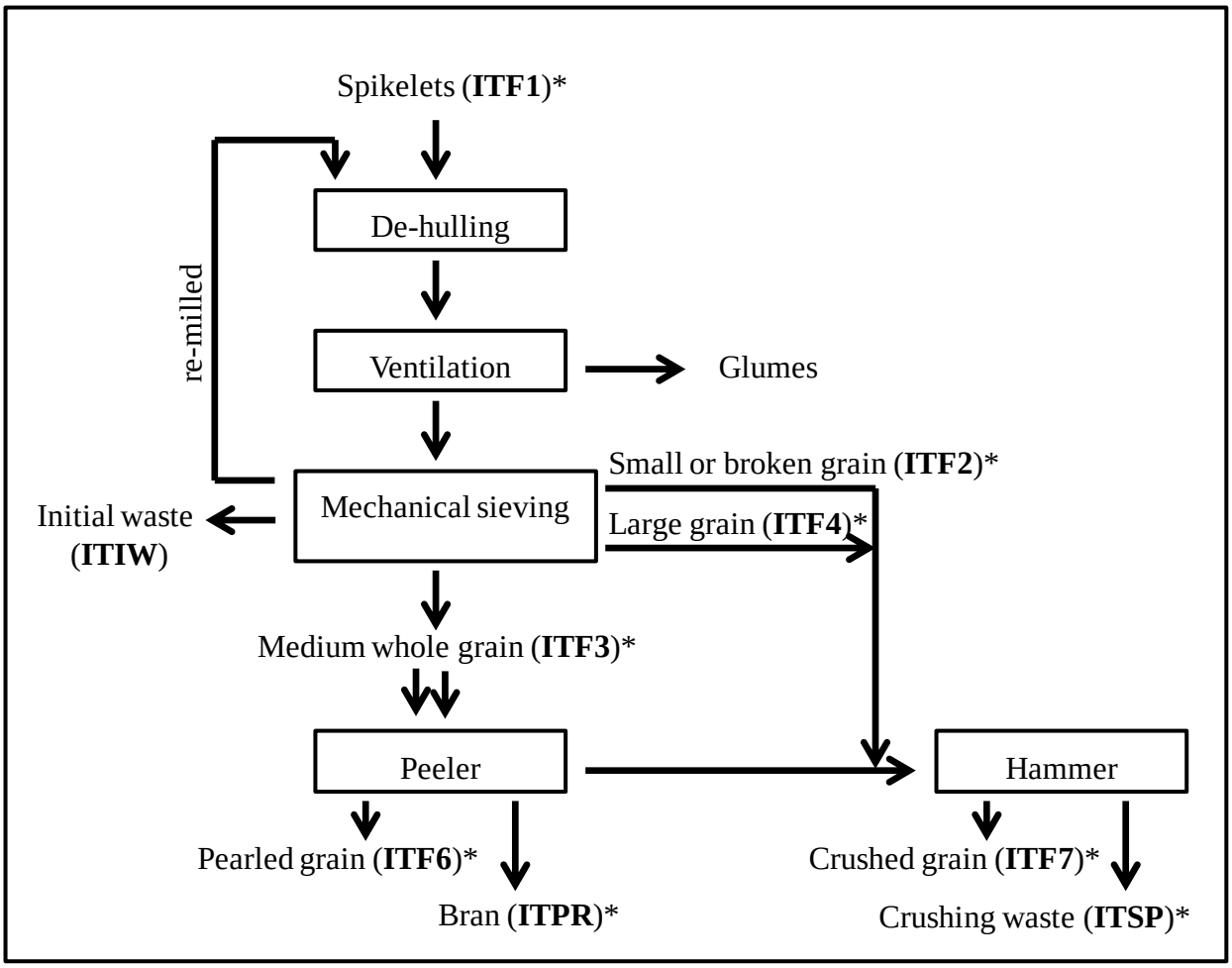

Figure 4.2.5. Schematic flow chart of the modern plant in Monteleone di Spoleto (Italy).

***ITF1**: raw emmer wheat spikelets, for dehulling; **ITF2**: small or broken grain, for crushing; **ITF3**: medium and whole grain, for pearling; **ITF4**: large grain for crushing; **ITIW**: initial waste, very small-size particles to feed animals; **ITF6**: pearly output, for packaging; **ITF7**: broken end, for packaging; **ITPR**: waste from pearling, bran; **ITSP**: waste from crushing.

On-plant sampling

The plants were preliminarily visited in order to organize the sampling, assure the consistency of the raw materials and procedures used, and determine the adequate amount of initial material to assure correct plant functioning; this was found to be about 100 kg in Italy and Armenia, 30 kg in Turkish TR1 and 15 kg in Turkish TR2. The initial input material and the whole output of each processing fraction were weighed on place. Subsamples of about 0.5 kg were randomly taken and brought to Cesena labs, Italy, where further measurements aimed at yield factor quantification were carried out. Three complete replications of the processing scheme and sampling were carried out, except in Turkish plant 2, where only two replications were possible due to the length of manual

operations. Yield factors of single processing steps were calculated as the ratio between the output and input of the specific step, whereas overall yield factors were calculated as the ratio between the specific step output and the initial input material.

Laboratory determinations

Samples of 10 g of spikelets grain from each plant were dehulled manually in 3 replicates, the grain being weighed after glume removal. This operation was aimed at determining the theoretical maximum dehulling yield and the number of grains per spikelet.
Thousand-kernel weight was determined from subsamples of 100 dehulled whole kernels, with 5 replications

Statistics

The yield factors of each processing step were calculated as the ratio between the output and the input products.
The data were processed by means of analysis of variance; multiple comparisons were carried out according to Tukey's HSD. All statistical processing were carried out by means of the SYSTAT 10.0 package.

RESULTS AND DISCUSSION

As previously illustrated, although this study considered the common process of glume removal from hulled wheat spikelets, the four plants considered differed in some respects. In particular, in the two Turkish plants pre-cooked einkorn wheat spikelets were processed, whereas raw emmer wheat spikelets were used in the Armenian and Italian plants. Moreover, the Italian plant did not use stone mills, being organized according to a two-step flow chart, with unbroken kernels as the primary product, to be further processed subsequently into groats or pearled grains. The Turkish and Armenian plants, although differing in size and automation level, adopted the traditional flow chart, in which glume removal and kernel crushing occurred simultaneously, as a consequence of the stone milling itself.

Figure 4.2.6 represents the comparative partitioning of the different sorts of product, with respect to dehulled kernel. In Turkish plant 1, the fraction represented by whole kernels (TR1F4) was kept separate and represented, as a mean, 0.09 kg kg^{-1} of all products. However, to make comparison possible with Turkish plant 2 (TR2), the sum of this fraction (keşkeklık) and coarse groats (pilavlık) are reported in **Figure 4.2.6** (TR1F4-F5 and TR2F4-F5). In both TR1 and TR2 the total amounts of whole kernels and coarse groats represented the more abundant processing fraction; however, in TR1 this fraction reached 0.69 kg kg^{-1}, a significantly higher amount than TR2 (0.43 kg kg^{-1}). Fine groat (çorbalık) percentage was significantly higher in TR2, whereas semolina-like fraction ("kısırlık") did not differ significantly. TR2 also had a significantly higher fraction of waste, not destined to human food.

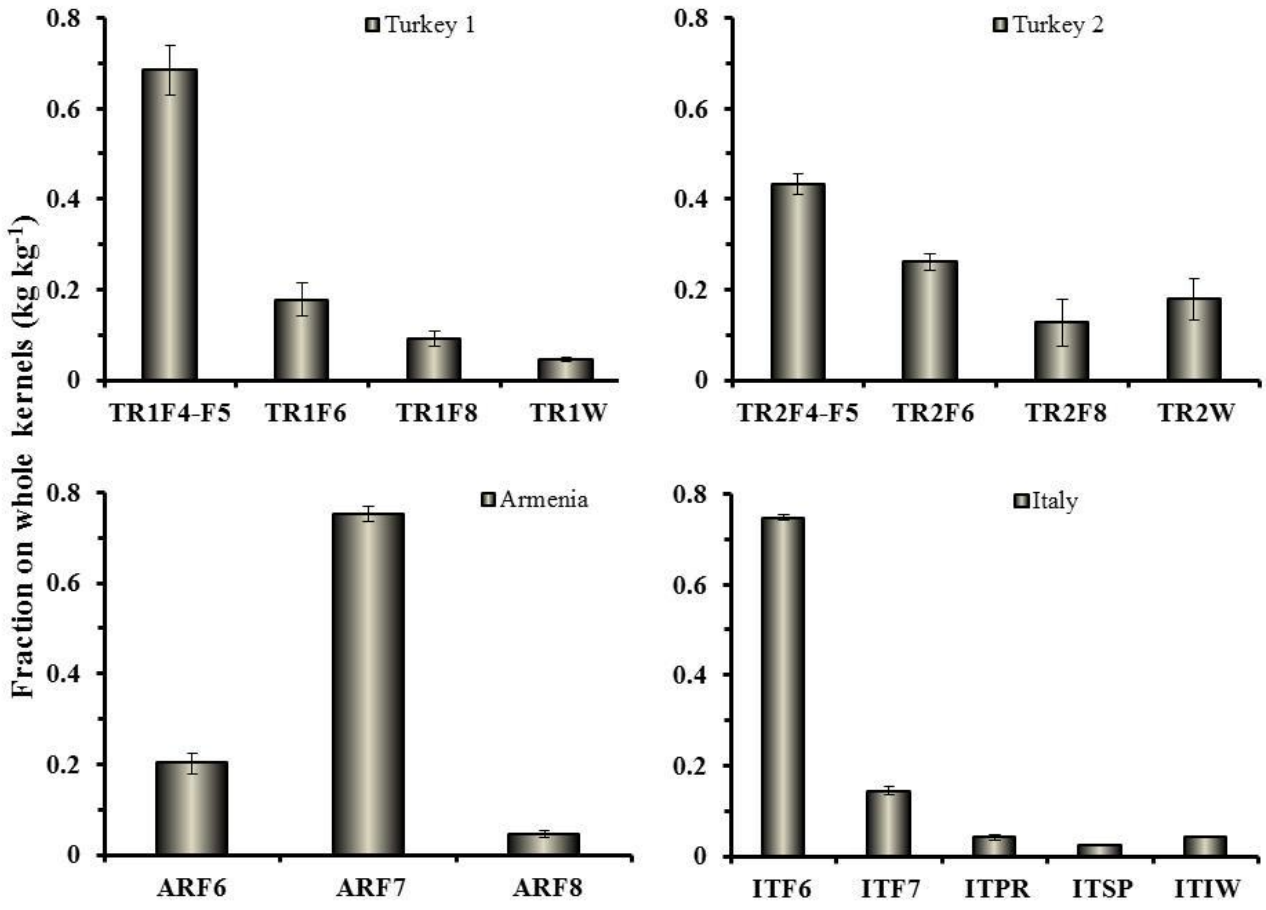

Figure 4.2.6. Partitioning of the different product fractions (acronyms as in flow charts, **Figures 4.2.1-4.2.5** and **Table 4.2.1**) with respect to whole-grain dehulled kernels.

In the Armenian plant, a total of 0.20 kg kg^{-1} was left unbroken after stone milling; this figure, although higher with respect to 9% of TR1, is, however, hardly comparable in relation to process efficiency, since the initial input material was different. The coarse groats represented, however, the predominant product (0.75 kg kg^{-1}). The very fine fraction, not considered as human food, amounted at only 0.05 kg kg^{-1}.

In the Italian case the situation was quite different. In fact, glume removal was aimed at yielding whole kernels as intermediate product; the global yield of whole kernels (ITF3 and ITF4) was indeed around 0.90 kg kg^{-1}, with only the remaining 10% represented by broken or very small kernels.

In the process examined, both the ITF2 fraction (small or broken kernels) and the large kernels (ITF4) went to crushing, yielding the medium-size groats called "farricello" (ITF7), representing only overall 0.14 kg kg^{-1}. On the contrary, pearled grains ITF6 (medium whole kernel (ITF3), after pearling) represented 0.75 kg kg^{-1}.

The sum of all wastes (sum of the initial waste of dehulling, and the wastes from crushing and pearling) accounted for 0.10 kg kg^{-1}.

Despite the differences in the product obtained, the four plants can be better compared at the level of process yield factors, representing process efficiencies, taking into account also unwanted wastes. In **Table 4.2.2** the processing yield factors are reported, for the two steps of glume removal and subsequent grain processing. This phase was represented by groat sieving and sorting in Turkey and Armenia, and by grain crushing, sieving and sorting, and pearling, in Italy.

The yield of glume removal was determined at plant level (YF2), partitioned in the two components of wanted (glumes: YF3) and unwanted (material lost: YF4) losses and also by manual dehulling (YF1). Manual dehulling yields were mainly dependent on the nature of raw materials: in fact, they were significantly higher in emmer wheat than in Turkish einkorn wheat, because of the higher kernel/glume ratio and, within emmer wheat, significantly higher in the Italian than in the Armenian plant, because of the higher average kernel weight and number of kernels per spikelet of the input product.

Wanted dehulling losses did not deviate significantly from manual data, except for Turkish plant TR1, whereas unwanted losses were highest in the Italian plant, intermediate in the two Turkish plants and lowest in the Armenian one. As a result, significant differences were detected between reference manual and global mechanical dehulling yields, however, with significant interaction with the processing plants; whereas, manual and mechanical dehulling yields were not different in the Armenian plant; in the other cases possibilities for improving process efficiency did exist, in particular with respect for the Italian and the Turkish TR1.

During the subsequent phases of grain processing, material losses (YF5) were high in the two Turkish plants, especially with respect to TR2, which were operated manually and in a typical discontinuous way. The losses due to the production of sortings not useful as human food (YF6) amounted at little less than 0.05 kg kg^{-1} except for the Turkish TR2, where they were significantly higher, at about 0.18 kg kg^{-1}. The global yield during grain processing (YF7) was therefore lower in the two Turkish plants. The yield of bran removal during pearling (YF8) is reported only for the Italian plant, with wanted losses amounting at about 0.05 kg kg^{-1}.

Table 4.2.2. Yield factors and initial grain product characters

	Processing plant *(wheat species)* [2]			
	Turkey - TR1	**Turkey - TR2**	**Armenia - AR**	**Italy - IT**
Grain characters	*(einkorn wheat)*	*(einkorn wheat)*	*(emmer wheat)*	*(emmer wheat)*
grains per spikelet (n)	1.00±0.01 b	1.00±0.01 b	1.88±0.07 a	1.95±0.01 a
mean kernel weight (mg)	27.4±0.69 b	25.6±0.60 b	33.9±1.17 a	35.2±0.40 a
Yield factors (kg kg^{-1}) [1]				
a. dehulling (glume removal)				
YF1 (manual)	0.759±0.003 b	0.732±0.004 c	0.768±0.010 b	0.815±0.004 a
YF2 (mechanical global)	0.674±0.013 c	0.713±0.001 c	0.780±0.016 a	0.729±0.004 b
YF3 (mechanical wanted)	0.710±0.005 c	0.747±0.001 b	0.784±0.017 a	0.806±0.002 a
YF4 (mechanical unwanted)	0.949±0.011 b	0.956±0.004 b	0.995±0.002 a	0.905±0.006 c
b. grain processing				
YF5 (unwanted: losses)	0.903±0.049 b	0.713±0.001 c	1 a	0.995±0.007 a
YF6 (unwanted: non food)	0.954±0.004 a	0.821±0.033 b	0.955±0.008 a	0.957±0.002 a
YF7 (unwanted: global)	0.861±0.047 b	0.586±0.025 c	0.955±0.008 a	0.952±0.004 a
YF8 pearling (wanted)	---	---	---	0.949±0.006
c. global (dehulling + grain processing)				
YF9 (all unwanted wastes)	0.817±0.005 b	0.560±0.003 c	0.949±0.001a	0.862±0.001 b
YF10 (YF9 + wanted dehulling)	0.580±0.040 b	0.419±0.018 c	0.744±0.014 a	0.695±0.007 b

[1] **YF1**: manual dehulling yield factor; **YF2**: global mechanical dehulling yield factor: wanted + unwanted losses; **YF3**: mechanical dehulling yield factor: wanted losses: glumes; **YF4**: mechanical dehulling yield factor: unwanted losses: material lost in process; **YF5**: grain processing, unwanted: loss of material; **YF6**: grain processing, unwanted: material not destined to human food; **YF7**: grain processing, unwanted: total; **YF8**: grain processing, wanted: bran from pearling (only in Italy); **YF9**: all unwanted losses; **YF10**: all unwanted losses + glumes (material not useful).

[2] different letters, within row, indicate significant between-plant differences according to analysis of variance and Tukey's HSD test

Finally, the sum of total unwanted losses (YF9) reflects the lower average efficiency of the two Turkish plants. When also wanted losses due to glume removal are considered in the final yield factor (YF10) Turkish plant TR1 lowers its efficiency due to poor efficiency in glume removal.

This research represented the first intercultural and technical comparison between different hulled wheat processing schemes. Therefore, the possibility of comparison with available literature is very small. Traditional stone crushing and selection, when carried out in apparently obsolete, but yet continuously operating plants, like the Armenian one, had an overall efficiency comparable to that of technologically advanced plants. The regulation of product assortment, however, represented by the fractions of different-sized groats and whole kernels, is less flexible in the traditional processing scheme, being heavily dependent on the less easily modifiable part, i.e. the stone mill, as reported also in other investigations carried out in Turkey (Bayram and Öner, 2005). On the contrary, obtaining unbroken whole kernels as a primary product, as in the Italian plant, leaves a considerable degree of flexibility in further processing, allowing choice of the optimal share between whole intact kernels, pearled grains and groats.

CONCLUSIONS

The comparative documentation of hulled wheat processing, carried out as a background of the experimental section, revealed that the apparently simple operation of glume removal has remained virtually unchanged for centuries, apart from the updating of accessory technology, being carried out by means of stone mills. The simultaneous glume removal and kernel crushing occurring during this process determined the fact that groats represented the ordinary traditional human food product from hulled wheats.

Only in Italy did market demand and the availability of new devices determine a clear shift towards the production of pearled whole kernels, making the traditional medium-texture groats (farricello) a small share of the products obtained.

The yield efficiency of the two traditional processing plants was substantially different, especially with respect to the phases following glume removal, in which the still relevant manual labour inputs required could not completely avoid unwanted losses, in the form of products less suitable for human consumption.

The two-stage processing flow chart of the Italian plant assured the greatest flexibility, best suited for a market-oriented, yet sustainable, traditional hulled wheat production scheme, which may also

promote the preservation of these crops to the benefit of local communities and the on-site preservation of local landraces.

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Chapter 4.3

Assessing the effect of traditional hulled wheat processing on bioactive compound retention

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To be submitted

ABSTRACT

Removing husks and grain crushing are the necessary preliminary operations to make hulled wheats, such as einkorn and emmer wheat, suitable to human consumption. Traditionally glume removal is carried out by means of stone mills, simultaneously causing the breaking of kernels. During this process parts of the kernel are selectively lost, determining the loss of associated phytochemicals. The aim of this study was to compare the effect of traditional processes still used in Turkey and Armenia, with a more modern plant located in Italy.

On-plant samplings were carried out in two plants from Turkey, on einkorn wheat bulgur, and in Armenia and in Italy, on emmer wheat. Whole and broken seeds from different sieving stages were sampled and analyzed for their content of phytochemical compounds; for the Italian plant also pearling process was evaluated.

Whole kernels showed higher lipid and phytochemical contents, with respect to the correspondent processed fractions, especially for tocopherols of which very low contents were detected in the Turkish plants, probably due to the previous preparation of bulgur. Carotenoid content was mainly affected by genotype.

Pearling resulted have a lower effect on the content of phytochemicals; however the correspondent waste fraction (bran) showed very high amounts of lipids and other compounds, especially phytosterols and phenolics.

All the plants showed similar retention of phytochemical upon processing; significant differences in bioactive composition, with higher losses occurring in smaller kernel fractions, mainly due to the loss of the germ, were, on the contrary, detected.

Keywords: hulled wheat; einkorn; emmer; glume removal; tocols, carotenoids; phytosterols; phenolic compounds.

INTRODUCTION

Einkorn and emmer wheat are hulled wheat species grown as traditional crops in several south European countries, which uses have been recently the subject of cross-country, comparative investigation within BaSeFood programme (D'Antuono *et al.*, 2009; Giambanelli *et al.*, 2013b).

The preliminary operation, necessary to make hulled wheat grain suitable to human consumption, is the removal of the glumes. The historical and traditional ways to carry out this operation have been described for various areas (D'Antuono and Bravi, 1996; Giuliani *et al.*, 2009; Peña-Chocarro, 1996). Basically the lack of technologies allowing an easy separation of the glumes from kernel, largely determined the kind of edible product obtained.

Traditionally spikelets are crushed by means of stone mills: this operation removes the glumes but also breaks the kernel in pieces of different size, originated from various kernel parts, that are subsequently sorted by sieving.

Teguments and embryo parts may represent an important source of minor compounds. The nature and content of specific cereals bioactive compounds, and their putative health benefits have been the subject of numerous periodic reviews (Liu, 2007; Ward *et al.*, 2008; Andersson *et al.*, 2014). However no specific experiments have been carried out about the effect this process, except for some contributions, with different purposes, mainly addressed to evaluate equipment and milling properties (Unal and Sacilik, 2009; Unal, 2009). The only references that could give an indication about the distribution of bioactives in different fractions are the ones dealing with the milling of other cereals, where it clearly appears how the distribution of kernel phytochemical is connected to the parts in which they are mainly contained: embryo residues are rich of sterol and tocols, and bran of phenolics and tocols (Gram *et al.*, 1972; Ko *et al.*, 2003; Lampi *et al.*, 2004; Tiwari *et al.*, 2009; Steadman *et al.*, 2001).

Process efficiency and yield factors of hulled wheats traditional processing, during glume removal and kernel breaking, has been described in a previous work (Giambanelli, 2013a). The present study was aimed at investigating the fate of phytochemical compounds associated to the kernel parts generated by the typical hulled wheat processing.

In the present study processed and unprocessed einkorn and emmer wheats, directly sampled on local plants, were analyzed for their content in minor compounds (tocols, carotenoids, phytosterols, and phenolic compounds). In particular, removing husks and grain crushing were selected as the first unit operation to be considered for the comparison of local processing from Turkey, Armenia and Italy. In case of the Italian plant also the pearling process was taken into account for sampling.

MATERIALS AND METHODS

Materials

The fractions resulting from glume removal and kernel breaking processing were directly sampled on-place from traditional plants in Italy, Turkey (two plants) and Armenia, during summer and fall 2010. In the Italian plant also the obtained fractions from pearling process were sampled and included in the experiment. The complete list of the samples is reported in **Table 4.3.1**; the processing diagrams and the resulting process fractions have been already described in a previous study (Giambanelli *et al.*, 2013a).

Table 4.3.1. List of the samples considered during on-plant sampling and analytically characterized. • Initial raw materials (whole kernels).

Tag	Fraction description
Turkey (TR1)	
•TR1BL	manually dehulled whole cooked spikelets, fed into the mill
TR1F4	keşkeklık, almost not broken kernels remaining above the 1st sieve
TR1F5	pilavlık, coarse groats remaining above the 2nd sieve
TR1F6	çorbalık, fine groats remaining above the 3rd sieve
TR1F8	kısırlık, semolina-like material recovered by the last manual sieving
Turkey (TR2)	
•TR2BL	manually dehulled whole cooked spikelets, fed into the mill
TR2F4-F5	keşkeklık, almost not broken kernels above the 1st sieve + pilavlık, coarse groats remaining above the 2nd sieve
TR2F6	çorbalık, fine groats remaining above the 3rd sieve
TR2F8	kısırlık, semolina-like recovered by the last manual sieving
Armenia (AR)	
•ARF5	manually dehulled whole kernels
•ARF6	whole dehulled kernels
ARF7	coarse groats, for human consumption
ARF8	fine groats and semolina-like fraction, mainly for animal feed
Italy (IT)	
•ITF1	manually dehulled whole kernels
•ITF2	small or broken grains, for crushing
•ITF3	medium-size whole grains, for pearling
•ITF4	large grains, to be used as such, or for crushing
ITF5	broken kernels from pearling, for crushing
ITF6	pearled output, for packaging
ITF7	medium-size groats (farricello), for packaging
ITSP	waste, from crushing
ITPR	waste, from pearling

Briefly TR1BL, TR2BL, ITF1, and ARF5 were samples of einkorn and emmer spikelets, representing the input of milling in each plant; for this experiment spikelets were manually dehulled. During process the following outputs were sampled:

- in plant 1 from Turkey einkorn spikelets (TR1BL) were crushed by means of stone mills and the following products were obtained: TR1F4, TR1F5 and TR1F6, larger or unbroken, medium and smaller broken kernels, respectively, remaining above the sieves, and TR1F8, semolina-like material passing through the last sieve;
- in plant 2 from Turkey einkorn spikelets (TR2BL) were crushed by means of stone mills and the following products were obtained: TR2F4-F5 and TR2F6, larger or unbroken + medium and smaller broken kernels, respectively, remaining above the sieves, and TR2F8, semolina-like material passing through the last sieve;
-in the Armenian plant emmer spikelets (ARF5) were crushed by means of stone mills and the following products were obtained: ARF6, whole kernels, dehulled by process, and ARF7 and ARF8, respectively, coarse and fine groats, obtained by crushing;
- in the Italian plant, emmer spikelets (ITF1) were firstly fed into a dehulling equipment to remove glumes, and the material was sorted by sieving in the following fractions: ITF2 and ITF4, respectively, smaller and larger whole kernels, destined to crushing, and ITF3, medium whole kernels, destined to pearling. In the Italian plant, crushing was carried out by means of hammer mills to produce: ITF7, rather fine groats, and ITSP, a waste consisting of fine material. The other step of pearling used abrasive discs to partially remove the pericarp, producing: ITF6, pearled seeds, ITF5, broken kernels by pearling and destined to the crushing, and ITPR, a waste, exclusively composed of bran.

Chemicals

All chemicals and solvents were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO). Deionized water was obtained by an Elix 10 water purification system from Millipore (Bedford, MA). Solvents employed in high performance liquid chromatography (HPLC) were of chromatographic grade, from Sigma-Aldrich. Before use in HPLC, solvents were filtered through Nylon membrane filters (diameter: 47 mm, pore dimension: 0.45 μm) from GVS Filter Technology (Indianapolis, IN) and sonicated at room temperature for 30 min. Standards of α-tocopherol, α-tocotrienol, lutein, β-carotene, ferulic acid, rutin, and 5α-cholestan-3β-ol, and sylilation reagents (pyridine, hexamethyldisilazane, trimethylchlorosylane) were from Sigma-Aldrich; β-tocopherol

was purchased from Supelco (Bellefonte, PA), and zeaxanthine from AppliChem (Darmstadt, Germany).

Lipid extraction

Lipids were extracted from 50 g of milled sample according to Folch method (1957), with some modifications as described by Boselli *et al.* (2001). Lipid extracts were stored in *n*-hexane/*iso*-propanol 4:1 (*v/v*) at -20°C until further analyses.

Tocopherols and tocotrienols

Tocopherols and tocotrienols quantification was performed by normal phase HPLC, following Panfili *et al.* (2003), with some modification as reported in Giambanelli *et al.* (2013b). Tocopherols and tocotrienols were measured by HPLC coupled to fluorescence detector with a Luna Hilic 200A (150 mm × 3.0 mm i.d., 3 µm particle size) column from Phenomenex (Torrance, CA). Mobile phase was *n*-hexane/ethyl acetate/acetic acid 97.3:1.8:0.9 (*v/v/v*). Flow rate was 0.4 mL min^{-1}, and the fluorimetric detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. The data were processed by the software ChromNAV (ver.1.16.02) from Jasco.

Identification was done by comparing retention times and spectra with standards and literature. Quantification was performed by external standard mode, preparing standard solutions of α-tocopherol, α-tocotrienol, and β-tocopherol; 8 diluted solutions (0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 ppm) were obtained; each of them was injected three times.

Carotenoids

Carotenoids quantification was performed by normal phase HPLC, following Panfili *et al.* (2004). The analyses were carried out by means of the same HPLC apparatus described for tocols, coupled to a diode array UV-Vis detector, with a Kromasil Si (250 mm × 4.6 mm i.d., 5 µm particle size) column. Mobile phase was *n*-hexane/*iso*-propanol 95:5 (*v/v*), and the flow rate was 1.5 mL min^{-1}. Detector was set at 450 nm, recording the spectrum in the range of 350-500 nm. The carotenoids

were identified by their spectra and the comparison of peak retention times with standard compounds.

Quantification was carried out by external standard mode, constructing calibration curves of β-carotene, lutein, and zeaxanthin. From the stock solutions six diluted solutions (0.25, 0.5, 1, 5, 10 and 20 ppm) were prepared; each of them was injected three times.

Phytosterols

Phytosterol determination was carried out according to Iafelice *et al.* (2009). Briefly, 5α-cholestan-3β-ol was added as internal standard to the extracted fat. After saponification at room temperature, the unsaponifiable fraction was extracted with diethyl ether. The organic extracts were washed once with 5 mL KOH 0.5 N, and twice with 5 mL deionized water. After drying over anhydrous sodium sulfate, the extracts were filtered, and dissolved in 2 mL *n*-hexane/*iso*-propanol 4:1 (*v/v*). Sterol fractions were analyzed by GC after sylilation of sterols to the corresponding trimethylsilyl (TMS) derivatives, according to Sweeley *et al.* (1963). A Clarus 500 gas chromatograph from Perkin-Elmer (Norwalk, CT) equipped with a flame ionization detector (FID) and a Zebron ZB-5 column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) coated with diphenyl-dimethyl-polysiloxane from Phenomenex were used. Helium was used as carrier gas. Other instrumental conditions were as follows: carrier flow: 1.0 mL min^{-1}; split ratio: 1:30; injector temperature: 330°C; oven temperature: 270 to 330°C at 3°C min^{-1}.

Identification of sterol compounds was achieved by mass spectral data obtained by GC-MS. Gas-chromatography was performed using a 6890N GC system (Agilent, Palo Alto, CA, USA) equipped with a MS detector, model 5973. Chromatographic conditions were the same for the GC-FID, whereas MS conditions were the same as reported by Pelillo *et al.* (2003).

Free and bound phenolic compounds

Free and bound phenolic compounds were extracted according to Van Hung and Morita (2008) and Adom and Liu (2002), with some modifications. Briefly, ground grain samples were extracted twice by sonication with an ethanol/water 4:1 (*v/v*) solution for 10 min. The supernatant fractions were pooled, evaporated and reconstituted with 2 mL 0.3% (*v/v*) formic acid in methanol/water 1:1 (*v/v*) and analyzed for the content in free phenolic compounds.

Residual matter from free phenolic extraction was hydrolyzed at 60°C, by NaOH 2N for 90 min, with continuous shaking. The hydrolyzate was acidified to pH 2 with HCl and centrifuged. The supernatant was extracted two times with *n*-hexane to remove free fatty acids; phenolic acids were then extracted five times with ethyl acetate. The ethyl acetate extracts were evaporated, re-dissolved in 2 mL 0.3% (*v/v*) formic acid in methanol/water 1:1 (*v/v*) and analyzed for the content of bound phenolic compounds.

HPLC analysis were carried out using the same apparatus described for carotenoid determination, with a Gemini NX (150 × 3.0 mm i.d., 3 µm particle size) column from Phenomenex. The flow rate was 0.5 mL min^{-1}, and the injection volume 10 µL. A gradient elution program was set using the following solvent system, as already reported by Verardo *et al.* (2008): mobile phase A: 1% acetic acid in water; mobile phase B: mobile phase A/acetonitrile 60:40 (*v/v*). The gradient program was: from 0 to 8.4 min, 98% A; from 8.4 to 9.6 min, 98 to 94% A; from 9.6 to 12 min, 94 to 90% A; from 12 to 14.4 min, 90 to 83% A; from 14.4 to 22.8 min, 83 to 64% A; from 22.8 to 24 min, 64 to 61.5% A; from 24 to 31.8 min, 61.5 to 40% A; from 31.8 to 34.8 min, 40 to 0% A; from 34.8 to 46.8 min, 0 to 95% A; from 46.8 to 51.0 min, 95 to 98% A; from 51 to 58 min, 98% A as post-run. Each chromatogram was recorded at 280 nm whereas absorption spectra were recorded between 200 and 600 nm.

Phenolics were quantified by external standard mode, constructing calibration curves, in methanol/water 1:1 (*v/v*) with 0.3% formic acid, of two representative compounds of relevant phenolic classes, ferulic acid and rutin. Six diluted solutions (1, 5, 10, 50, 250 and 500 ppm) were prepared; each one was injected three times.

Phenolic identification was carried out by means of a liquid chromatography system HP 1100 Series coupled with a MS detector (mod. G1946A) both from Agilent Technologies (Palo Alto, CA, USA). The mass spectrometer operated both in positive and negative atmospheric pressure ionization-electrospray source (API-ES) mode under the following operating conditions: drying gas temperature: 350°C; drying gas flow rate: 10.0 L min^{-1}; nebulizer pressure: 35 psig; capillary voltage: 3000 V; fragmentor voltage: 80 V; mass range: *m/z* 100-1400; scan mode: negative and positive. Data processing was performed by the software LC/MSD ChemStation from Agilent Technologies.

Statistical analyses

Bioactive content data were processed using analysis of variance (ANOVA), using processing fractions as experimental factors. Comparisons were carried out by means of the protected Fisher's LSD test. Statistical analyses were carried out by means of the SYSTAT® package (San Jose, CA, USA).

RESULTS AND DISCUSSION

Phytochemical compounds evolution and distribution following processing

Total lipids

Lipid content in starting raw materials, represented by whole kernel fractions, ranged between 23.7-31.5 g kg^{-1} d.m.

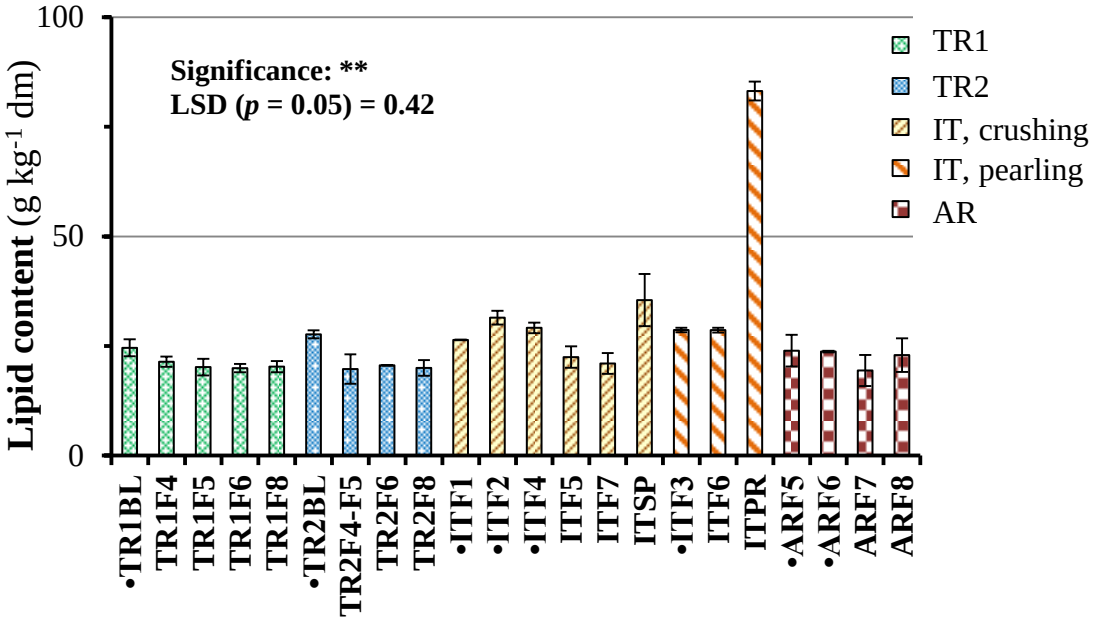

Figure 4.3.1. Lipid content (g kg^{-1} dm). • Initial raw material (whole kernels).

The differences between species (einkorn and emmer wheat) were not relevant, whereas the Italian samples showed significantly higher lipid content, especially if referring to smaller kernel fraction

(ITF2), because of their higher embryo to whole kernel ratio (**Figure 4.3.1**). In crushed fractions (**Figure 4.3.1**) lipid content generally decreased in all cases, except for the Armenian plant, since embryos are separated from the kernels and are generally lost with wastes. Lipid content of crushed fractions were quite similar among all crushed kernel fractions, ranging between 19.4-22.9 g kg^{-1} dm. The highest value was found for ARF8, fine groats and semolina-like fraction, with a content similar to the correspondent whole grain (ARF5).

Tocols

Emmer and einkorn wheat substantially differed for tocol content (**Figure 4.3.2**). Einkorn wheat bulgur samples from Turkey showed very low tocol contents, also in comparison with raw einkorn wheat data reported in literature (Hidalgo *et al.*, 2006; Hidalgo and Brandolini, 2008).

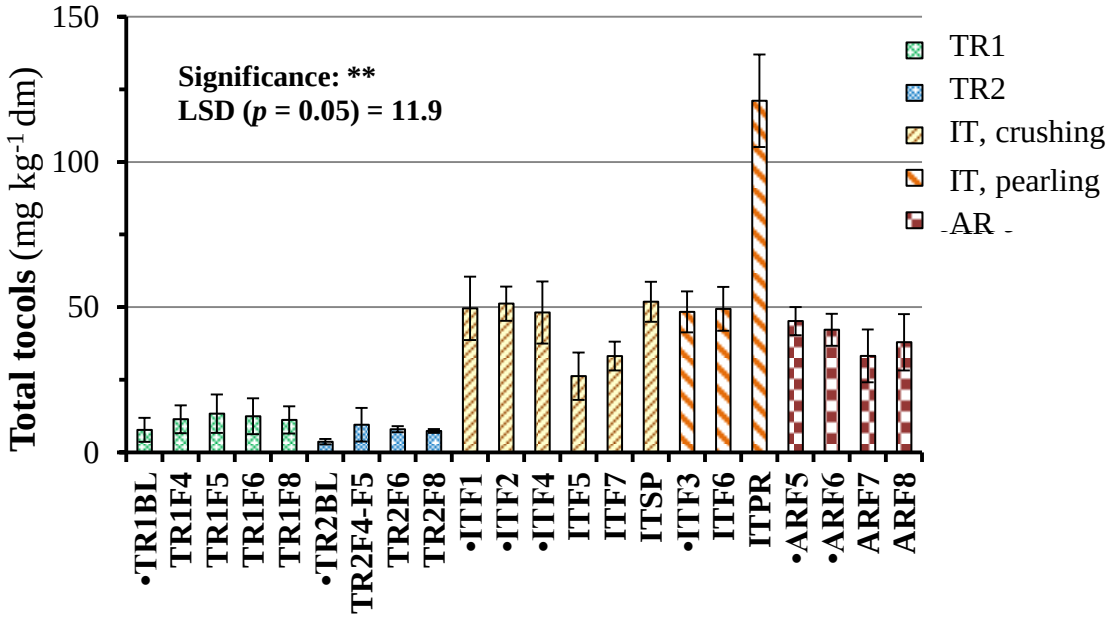

Figure 4.3.2. Tocol contents (mg kg^{-1} dm). • Initial raw materials (whole kernels).

This difference, in absence of specific data, could be attributed to the processes of cooking and sun-drying used to obtain bulgur; in particular thermal damage and the effect of light could have drastically decreased the content of this class of compounds. Tocol content of emmer wheat whole kernels ranged between 45.2-51.2 mg kg^{-1} dm, with slightly higher amounts in Italian whole kernels, especially if referring, also in this case, to ITF2, the fraction consisting of smaller kernels.

Four tocols were detected in emmer wheat whole kernels: α-tocopherol, β-tocopherol, α-tocotrienol and β-tocotrienol (**Table 4.3.2**). This latter resulted the most abundant compound, representing 52.0-58.4% of tocols. α-tocopherol represented 19-22% of tocols; minor amounts were detected for α-tocotrienol and β-tocopherol; moreover these compounds were not identified in einkorn wheat bulgur samples.

Table 4.3.2. Tocol relative composition (% on total tocols). • Initial raw materials (whole kernels).

Sample	α-tocopherol	α-tocotrienol	β-tocopherol	β-tocotrienol
	% on the total			
•TR1BL	3.4	-	-	35.3
TR1F4	2.0	-	-	51.8
TR1F5	1.5	-	-	42.1
TR1F6	1.6	-	-	41.5
TR1F8	1.9	-	-	54.5
•TR2BL	8.9	-	-	72.9
TR2F4-F5	2.2	-	-	55.1
TR2F6	3.0	-	-	97.0
TR2F8	3.4	-	-	96.6
•ITF1	20.8	9.5	16.5	53.2
•ITF2	20.0	10.7	13.3	56.0
•ITF4	21.4	9.5	12.5	56.6
ITF5	4.9	4.3	11.9	78.9
ITF7	7.8	11.9	5.3	75.0
ITSP	22.8	9.0	16.1	52.0
•ITF3	19.0	10.0	12.6	58.4
ITF6	18.4	9.9	12.6	59.1
ITPR	32.8	9.6	17.9	39.7
•ARF5	22.0	8.1	17.8	52.0
ARF6	14.6	9.0	10.5	66.0
ARF7	11.9	9.6	8.3	70.2
ARF8	19.2	8.5	13.5	58.7
Significance[1]	**	**	**	**
LSD	9.9	1.2	1.6	10.4

[1] Significance: **, $p \leq 0.01$; LSD, $p = 0.05$.

Tocol content of emmer wheat crushed kernel fractions was lower compared with the correspondent whole kernels (**Figure 4.3.2**), ranging between 33.2-37.9 mg kg^{-1} dm. In addition, also the composition of tocols resulted different from that of input material (**Table 4.3.2**). In particular, the relative α-tocopherol and β-tocopherol contents decreased in medium size crushed fractions (ITF7 and ARF7) to about half the amounts of whole kernels. Consequently β-tocotrienol relative amount

increased to 70.2-75% of tocols, whereas non-significant changes were observed for α-tocotrienol. Again, ARF8, fine groats and semolina-like fraction, showed total and relative tocol contents similar to the correspondent whole grain (ARF5).

The results confirm the fact that tocols are differently distributed in the kernel, as already reported by Hidalgo and Brandolini (2008).

It was not possible to detect a clear pattern of tocol content and profile of einkorn wheat bulgur following processing, due to the low detected tocol content.

Carotenoids

Carotenoid content (**Figure 4.3.3**) of manually dehulled whole kernels was higher in Turkish einkorn wheat bulgur samples (3.3 and 3.7 mg kg^{-1} dm respectively in TR1BL and TR2BL) than in emmer wheat from Armenia and Italy (1.4 and 1.6 mg kg^{-1} dm in ARF5 and ITF1, respectively).

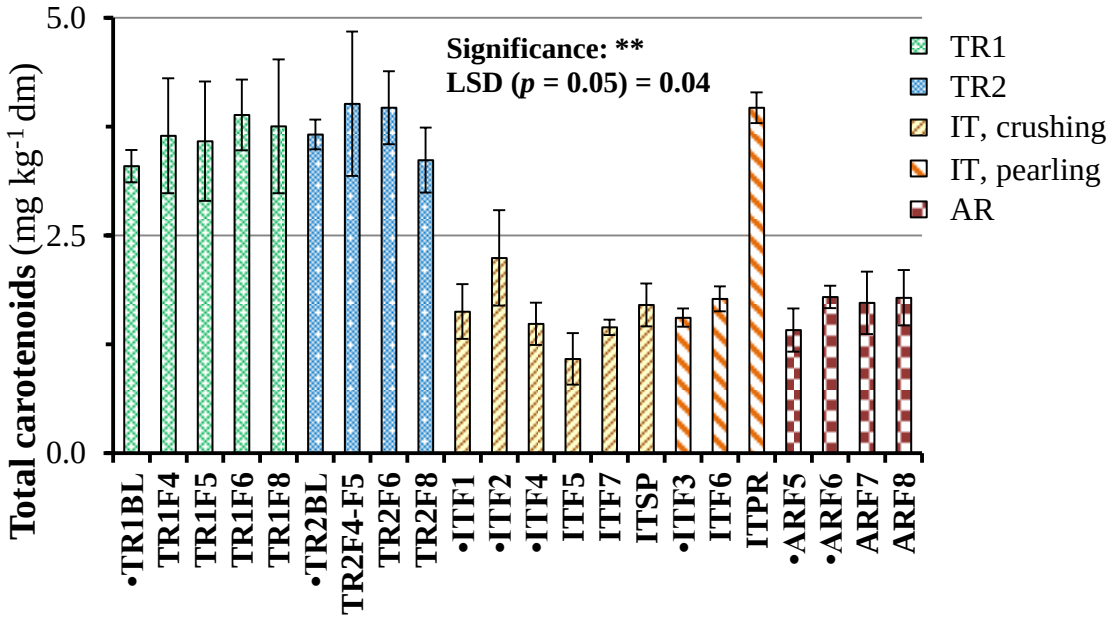

Figure 4.3.3. Carotenoid contents (mg kg^{-1} dm). • Initial raw materials (whole kernels).

These results were in agreement with previous works, reporting a higher carotenoid content for einkorn wheat (Abdel-Aal *et al.*, 2007; Giambanelli *et al.*, 2013b). Among emmer wheat whole materials from the Italian plant, the fraction with smaller kernels, ITF2, showed the highest

carotenoid content (2.2 mg kg^{-1} dm). In all whole kernel samples α and β-carotene, zeaxanthin, lutein and one isomeric form of lutein were identified (**Table 4.3.3**). β-cryptoxanthin was identified and quantified in einkorn wheat.

Table 4.3.3. Carotenoid relative content (% on total carotenoids). • Initial raw materials (whole kernels).

Sample	α+β-carotene	β-criptoxanthin	Lutein	Zeaxanthin	Lutein (isomer)
			% on the total		
•TR1BL	4.8	4.6	72.6	7.3	10.6
TR1F4	3.7	4.2	75.2	6.3	10.6
TR1F5	3.3	4.0	76.0	6.0	10.7
TR1F6	3.3	4.0	75.4	6.0	11.2
TR1F8	3.3	3.9	75.4	6.0	11.4
•TR2BL	4.7	4.4	73.7	7.4	9.8
TR2F4-F5	3.5	3.3	76.3	6.8	10.1
TR2F6	3.3	3.4	76.4	6.6	10.3
TR2F8	3.9	3.6	74.4	6.2	11.9
•ITF1	11.2	-	61.0	22.2	5.6
•ITF2	8.6	-	56.9	26.9	7.6
•ITF4	10.8	-	53.2	28.4	7.6
ITF5	13.8	-	61.6	15.5	9.2
ITF7	7.7	-	62.3	21.0	8.9
ITSP	11.7	-	52.2	27.6	8.5
•ITF3	9.4	-	55.4	27.7	7.4
ITF6	8.7	-	57.9	25.5	7.9
ITPR	20.9	-	37.6	35.0	6.5
•ARF5	13.6	-	56.5	22.6	7.3
ARF6	8.2	-	67.6	15.7	8.5
ARF7	7.5	-	68.5	15.1	8.9
ARF8	9.1	-	65.1	17.0	8.8
Significance[1]	**	ns	**	**	**
LSD	1.0		2.2	1.3	0.9

[1]Significance: **, $p \leq 0.01$; ns, non significant; LSD, $p = 0.05$.

Also the composition of pigments were mainly affected by genotype. Lutein resulted the most abundant carotenoid in all whole fractions, with the highest relative contents in einkorn (72.6-73.7% of carotenoids), in agreement with previous works (Abdel-Aal *et al.*, 2002). On the contrary emmer wheat whole kernels showed quite relatively higher zeaxanthin and α and β-carotene contents, ranging respectively between 22.2-28.4% and 8.6-13.6%.

Carotenoid content was subject to an apparent increase in einkorn wheat bulgur from Turkey, following crushing and sieving (**Figure 4.3.3**): in fact the highest carotenoid contents were obtained

for coarse and fine groats fraction remaining above the sieves, in particular TR1F4, TR1F5 and TR1F6 in plant 1, TR2F4-F5 and TR2F6 in plant 2. The finest semolina-like material (TR1F8 and TR2F8) had a lower carotenoid concentration, especially in plant 2, whereas in plant 1 the amount was still higher if compared with the correspondent whole fraction.

Einkorn crushed bulgur fractions showed a slight increase of lutein relative amount, and a decrease of α and β-carotene and zeaxanthin relative amounts (**Table 4.3.3**), whereas non-significant differences were observed for β-cryptoxanthin. Emmer crushed fractions showed a slight increase of carotenoid content in the case of the Armenian plant, whereas non-significant changes were observed for the Italian crushing process. Carotenoid relative composition was affected by crushing, with a decrease of zeaxanthin and an increase of lutein in crushed fractions, especially in the Armenian plant.

Phytosterols

High phytosterol amounts were detected in the Italian whole grain fractions (**Figure 4.3.4**), ranging between 815-967 mg kg^{-1}, with the highest value for the small seed raw material sample (ITF2). Armenian emmer whole grain and Turkish einkorn bulgur had significantly lower phytosterol contents (656-733 mg kg^{-1}).

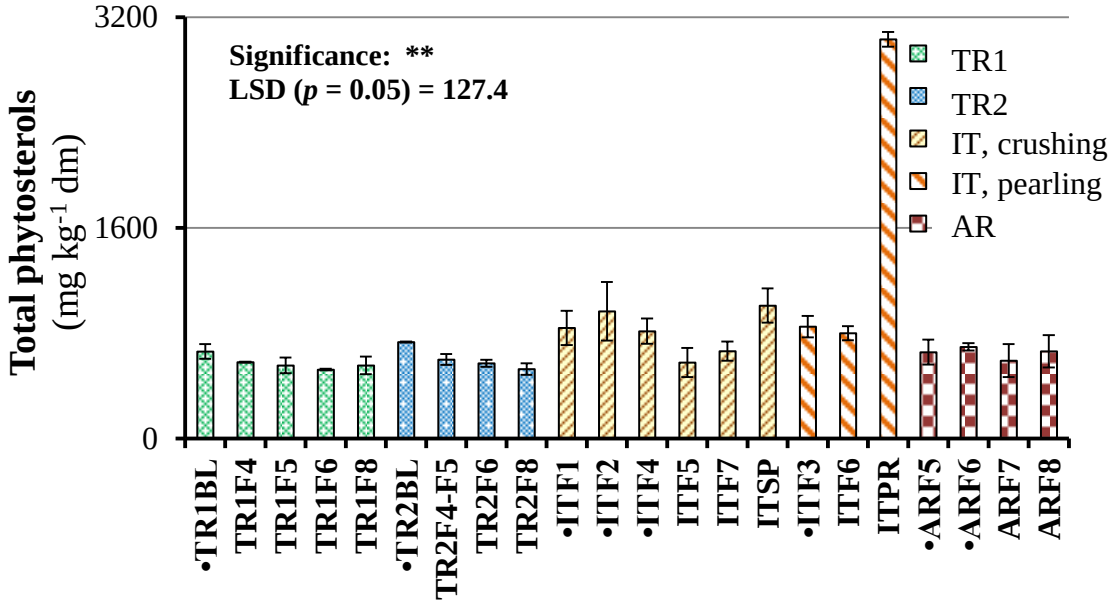

Figure 4.3.4. Phytosterol contents, expressed as mg kg^{-1} of dry matter. • Initial raw materials (whole kernels).

The following compounds were identified: campestanol, sitostanol and avenastanol, saturated sterols typically found in wheats; campesterol, β-sitosterol and avenasterol, the correspondent unsaturated forms that, together with stigmasterol, are included in the group of Δ^5-sterols; Δ^7-campesterol, Δ^7-avenasterol and Δ^7-sitosterol, in the group of Δ^7-sterols; finally citrostadienol, a 4-methyl-Δ^7-sterol. These compounds were already detected in a previous work on primitive wheat characterization (Giambanelli *et al.*, 2013b). β-sitosterol resulted the most abundant compound in all whole kernel fractions (34.2-42.8% of phytosterols), followed by campesterol (18.3-20.8%), sitostanol (11.3-13.0%), and campestanol (7.6-8.3%) (**Table 4.3.4**). In general emmer wheat whole fractions showed higher relative contents of β-sitosterol than einkorn wheat, that was characterized by higher sitostanol and other minor phytosterols contents.

Table 4.3.4. Phytosterol composition (expressed as percentage on total phytosterol content). • Initial raw materials (whole kernels).

Sample	ST1	ST2	ST3	ST4	Stanols	Δ5-sterols	Δ7-sterols	4-methyl-Δ7-sterols
				% on total phytosterols				
•TR1BL	18.3	8.3	35.7	13.0	23.0	58.5	10.4	8.1
TR1F4	17.9	9.5	33.5	14.0	24.9	56.1	10.9	8.1
TR1F5	18.0	9.3	32.6	14.9	25.7	54.3	11.4	8.6
TR1F6	17.5	9.5	32.5	14.9	26.1	54.0	11.3	8.6
TR1F8	17.7	9.6	32.4	14.3	25.5	54.7	11.2	8.5
•TR2BL	20.3	7.8	34.2	11.9	21.2	59.2	11.5	8.0
TR2F4-F5	20.3	8.7	31.5	13.4	23.5	55.6	12.1	8.8
TR2F6	20.2	8.8	31.1	12.6	22.8	56.2	12.2	8.8
TR2F8	19.5	8.4	32.7	12.8	23.0	56.6	11.6	8.9
•ITF1	20.9	7.8	39.2	11.4	20.3	65.8	8.1	5.2
•ITF2	20.8	7.6	40.5	11.3	20.1	66.2	8.5	5.2
•ITF4	20.3	7.6	41.8	11.3	20.2	67.7	7.5	4.6
ITF5	18.8	8.8	41.1	13.0	22.8	64.5	7.2	4.9
ITF7	19.9	9.1	38.6	13.1	23.4	63.0	8.5	5.1
ITSP	20.3	6.9	42.5	11.0	19.2	67.6	8.0	5.1
•ITF3	20.6	7.8	40.2	11.5	20.6	65.8	8.4	5.2
ITF6	20.5	7.7	41.1	11.6	20.4	66.3	8.2	5.1
ITPR	20.8	6.9	38.0	10.0	19.6	66.7	8.2	5.5
•ARF5	19.9	7.9	42.8	12.5	21.6	68.3	5.8	3.8
ARF6	18.7	8.6	43.1	13.4	23.2	67.2	6.1	3.4
ARF7	18.4	8.7	43.6	13.4	23.2	67.3	6.1	3.4
ARF8	18.6	7.7	45.4	12.8	21.7	69.1	5.9	3.3
Significance[1]	*	**	**	**	**	**	**	**
LSD	2.2	0.5	0.2	0.2	1.8	1.4	1.4	0.5

ST1: campesterol; ST2: campestanol; ST3: β-sitosterol; ST4: sitostanol.
[1] Significance: *, $p \leq 0.05$; **, $p \leq 0.01$; LSD, $p = 0.05$.

Crushing determined a generalized phytosterol content decrease (**Figure 4.3.4**). In particular the highest phytosterols variations were observed for the finest einkorn wheat bulgur material in plant 2, from Turkey (TR2F8), and for crushed emmer wheat kernels from the Italian plant (ITF7), with losses around 28 and 22% of the initial unprocessed whole kernel content, respectively. Phytosterol composition was mainly affected by genotype, as reported above; in fact, following crushing only a slight relative increment (around 2-3%) of compounds with a saturated structure was observed (**Table 4.3.4**).

Total phenolic compounds

Total phenolic compounds (TPC), ranged between 815-1107 mg kg^{-1} dm (**Figure 4.3.5A**). Manually dehulled emmer wheat whole grains (ITF1 and ARF5) had higher TPC content, than einkorn wheat bulgur (TR1BL and TR2BL). BPC represented the most abundant compounds in all whole kernel fractions (**Figure 4.3.5B**), especially in einkorn wheat bulgur with 83.2-83.9% of TPC, with respect to 61.0-70.6% in emmer wheat.

Several phenolic acids, belonging to BPC, were identified and quantified: *p*-coumaric and sinapic acid, two isomeric forms of ferulic acid and three isomers for dihydroferulic acid (**Table 4.3.5**).

Ferulic acid was the most abundant phenolic acid representing 78.8-84.1% of BPC. The highest relative contents were observed for the Italian emmer wheat samples, in particular ITF3 and ITF4, representing respectively, medium size and larger whole kernel fractions.

The highest TPC decrease following processing was observed for emmer wheat crushed fractions, with about 25% losses in thinner kernel fractions (**Figure 4.3.5A**). A lower decrease occurred in einkorn wheat crushed bulgur, also considering the thinner fractions. In particular for plant 2 a 3% TPC loss was calculated for the semolina-like material (TR2F8); however for this plant the lowest final TPC was observed for fraction TR2F6, consisting of fine groats remaining above the last sieve. Following crushing BPC contents evolved in the same way as discussed for TPC, with higher losses for emmer wheat processing.

No relative BPC changes were observed in processed einkorn wheat bulgur (**Figure 4.3.5B**). On the contrary, an increase of relative BPC amounts were found in emmer crushed fractions, in both the Italian and the Armenian plants.

Ferulic acid resulted the main compound in all crushed samples, representing 70-88% of BPC; the percentage of ferulic acid resulted higher for emmer wheat fractions, where it was subject to increase in the crushed fractions with respect to the correspondent whole seeds (**Table 4.3.5**). The

lowest relative ferulic acid contents was observed in plant 2 from Turkey, for semolina-like material (TR2F8), at the same time this fraction resulted the richest of *p*-coumaric acid, with a relative content of 20% BPC, compared to 3-9% in all the other samples, with a decreasing trend following process. The different composition in phenolic acids could be attributed to an unequal distribution of these compounds in the caryopses.

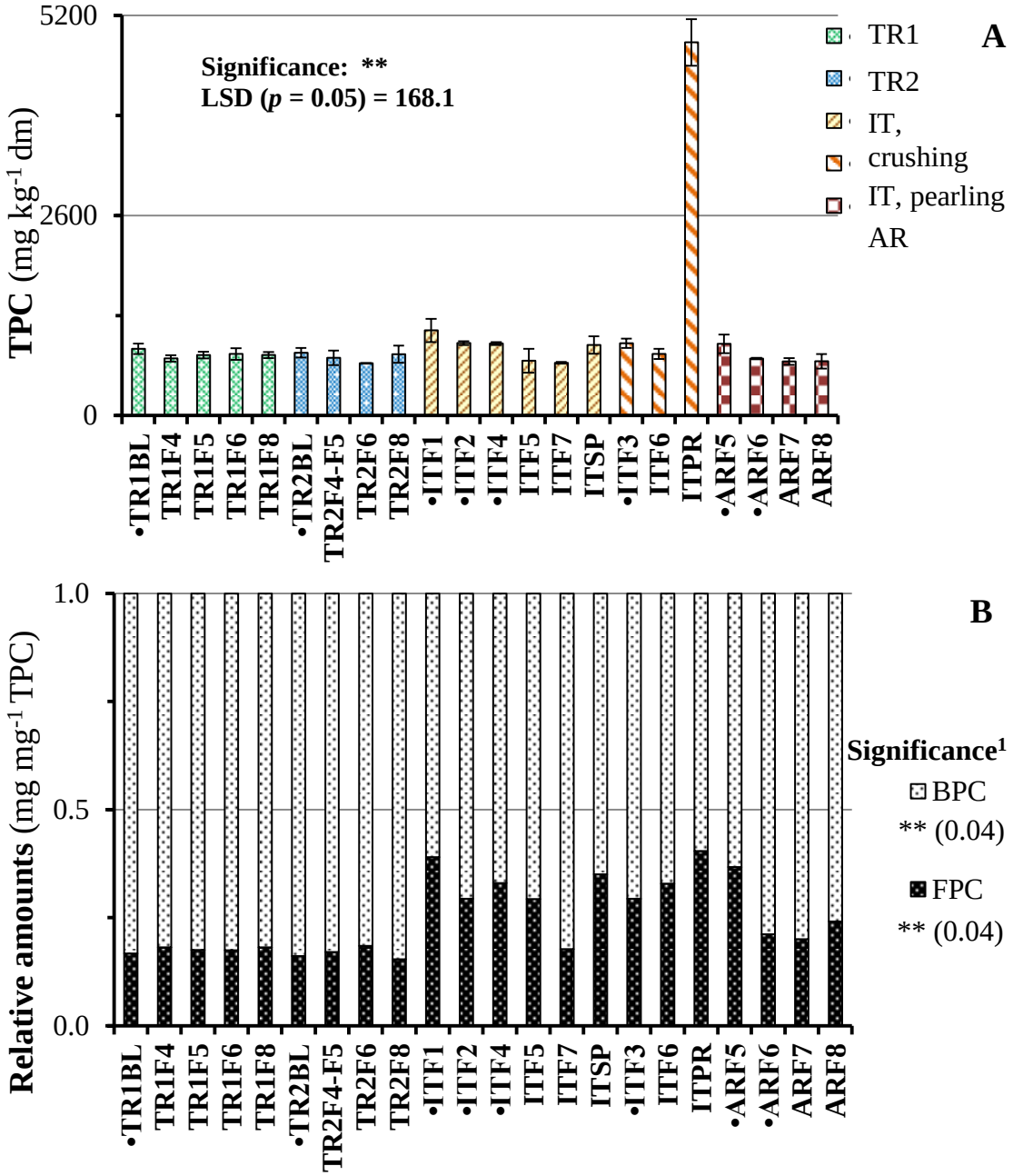

Figure 4.3.5. Total phenolic content (TPC, mg kg^{-1} dm) (A); Bound (BPC) and Free (FPC) phenolic relative contents (B). • Initial raw materials (whole kernels).

[1] value in parenthesi: LSD, p = 0.05.

Table 4.3.5. Composition in phenolic acids (expressed as percentage of BPC). • Initial raw materials (whole kernels).

Sample	PCA	FEA	FEA_i	SIA	DFA_{i1}	DFA_{i2}
			phenolic acids, % on BPC			
•TR1BL	8.9	79.0	6.5	2.3	0.6	2.0
TR1F4	6.6	81.5	7.5	2.3	0.3	1.7
TR1F5	6.5	82.2	6.8	2.3	0.4	1.6
TR1F6	6.4	81.8	7.2	2.1	0.4	1.7
TR1F8	6.4	81.1	8.2	2.1	0.4	1.5
•TR2BL	8.6	78.7	5.8	3.1	0.8	2.5
TR2F4-F5	6.7	78.8	9.0	2.7	0.6	1.9
TR2F6	8.4	75.6	11.6	2.4	0.3	1.6
TR2F8	20.3	69.6	5.8	2.2	0.4	1.5
•ITF1	9.2	81.9	2.4	2.9	0.8	2.6
•ITF2	6.3	83.4	3.7	1.9	1.2	2.9
•ITF4	5.3	84.1	4.8	1.7	1.1	2.6
ITF5	3.7	89.1	2.5	2.3	0.9	1.6
ITF7	3.5	86.5	5.2	1.6	0.8	2.1
ITSP	4.3	85.3	4.8	1.8	0.9	2.4
•ITF3	6.0	83.2	4.4	1.9	1.1	2.9
ITF6	2.9	86.8	4.7	1.4	0.8	3.2
ITPR	24.0	60.5	3.7	1.2	2.3	3.3
•ARF5	9.1	79.7	6.1	1.8	1.3	1.8
ARF6	3.4	86.9	4.5	0.7	1.0	2.9
ARF7	2.9	87.5	4.4	0.8	1.0	2.9
ARF8	2.8	88.1	4.3	0.8	0.9	2.7
Significance[1]	**	**	**	**	**	**
LSD	1.6	3.0	2.0	0.5	0.3	0.7

PCA: *p*-coumaric acid; FEA: ferulic acid; FEA_i: ferulic acid isomer; SIA: sinapic acid; DFA_{i1}: dehydroferulic acid isomer 1; DFA_{i2}: dehydroferulic acid isomer 2.

[1] Significance: **, $p \leq 0.01$; LSD, $p = 0.05$.

Following crushing, emmer wheat was subject to drastic FPC reduction, around 50% of the initial content for ARF8 and 72% for ITF7. The lowest FPC losses were observed in einkorn wheat bulgur from Turkey (**Figure 4.3.5B**), likely because of a migration and stabilization of these compounds following cooking for bulgur preparation.

Phytochemical compounds evolution following pearling

As explained in materials and methods, in the Italian plant also a process of pearling, consisting of the removal of the outer parts of the pericarp, was carried out, which materials were also considered in this work.

The pearled kernels (ITF6) from the Italian plant maintained a lipid content very similar to that of the input kernels (ITF3), composed of medium-size whole grains (**Figure 4.3.1**). On the contrary, the pearling by-product, represented by broken kernels destined to crushing (ITF5), showed a decreased lipid content, with values similar to those reported for the crushed seed fraction (ITF7).

A similar trend was observed for tocols (**Figure 4.3.2**). No changes of total tocol content were observed for ITF3, whereas a very high loss of these compounds was found for ITF5 (26.3 g kg^{-1} dm). Moreover ITF5 showed also a substantial different tocol composition (**Table 4.3.2**) with the highest β-tocotrienol relative amount (78.9%), and lowest saturated tocol relative amounts.

A slight increase of carotenoid content was observed for ITF6 (**Figure 4.3.3**), with respect to the correspondent input kernels, whereas a decrease was found for ITF5, showing the lowest value among all analyzed fractions; at the same time the highest α and β-carotene contents (13.8%) were detected in this sample (**Table 4.3.3**).

Following pearling a decrease of phytosterol content was observed for both fractions ITF5 and ITF6; however the loss was more evident in the first one (**Figure 4.3.4**). No significant changes of phytosterol composition were observed (**Table 4.3.4**).

Pearling led to a minor decrease of TPC (**Figure 4.3.5A**), with 15 and 25% losses, respectively for ITF6 and ITF5. In ITF6 the highest decrease was associated to BPC, with a loss around 3% the relative amount detected in the input kernels (**Figure 4.3.5B**). *p*-coumaric acid relative content decreased in both pearling fractions, with a consequent increase of ferulic acid relative amounts (**Table 4.3.5**).

Wastes from the two-step procedure adopted in the Italian plant

Two kinds of wastes, produced in the Italian plant, were taken into account during this study: ITSP, a waste consisting of the finest material produced by crushing, and ITPR, the waste from pearling, mainly composed of the bran part of the caryopses. The two samples resulted in all cases very rich sources of lipid and other phytochemical compounds, with contents in most cases higher than all other fractions. In particular the waste from pearling, ITPR, mainly composed of bran, showed the

highest lipid contents, 83.2 g kg^{-1} dm, more than 3 times higher than the amount observed in other fractions (**Figure 4.3.1**).

ITPR also had the highest tocols, reaching 121.0 mg kg^{-1} dm (**Figure 4.3.2**). A very different tocol composition was observed for this fraction (**Table 4.3.2**), in which the lowest β-tocotrienol (39.7%) and the highest α-tocopherol (32.8%) and β-tocopherol (17.9%) relative amounts were detected.

Carotenoid content for ITPR reached amounts around 4 mg kg^{-1} dm (**Figure 4.3.3**), similar to the values found in einkorn wheat. ITPR had similar amounts of both lutein and zeaxanthin, with 44 and 35% respectively, but showed the highest relative amount for the sum of α and β-carotene (21%) (**Table 4.3.3**), whereas in all the other samples this sum was lower than 14%. Moreover an additional peak was detected for ITPR, eluting near β-cryptoxanthin; in this part of the chromatogram some peaks were previously observed by Panfili *et al.* (2004) and are probably isomers of β-cryptoxanthin.

Wastes from the Italian emmer processing plant (ITPR and ITSP) resulted the richest phytosterol fractions (**Figure 4.3.4**), with 3032 and 1010 mg kg^{-1}, respectively, in agreement with data reported by Piironen *et al.* (2002), regarding sterol contents of different wheat milling products and bran. ITPR and ITSP, reached the highest percentages of unsaturated sterols (80-81%) (**Table 4.3.4**), mainly because of the presence of these fractions in the germ (Piironen *et al.*, 2002). Wheat germ is recognized as the richest part in unsaturated sterols, so it can adversely affect flour quality, and this is one of the main reasons for which it has to be efficiently separated during flour production (Brandolini and Hidalgo, 2012).

The waste from pearling resulted extremely rich of TPC (**Figure 4.3.5A**), with an increment around 5 times the content calculated in the correspondent whole fraction (ITF3). ITPR showed the lowest percentage of BPC (**Figure 4.3.5B**), although with the highest amount in absolute terms. Phenolic acid composition of ITPR resulted quite different from other Italian emmer wheat fractions and similar to what reported above for TR2F8 (**Table 4.3.5**), characterized, in particular, by the lowest ferulic acid (60%) and the highest *p*-coumaric acid (24%) relative amounts.

ITPR resulted the richest sample of FPC (**Figure 4.3.5B**), with 1960 mg kg^{-1}, 7 times the content observed in the input kernel fraction (ITF3).

DISCUSSION

Generally, following crushing, lipid, tocol, phytosterol and phenolic contents of einkorn and emmer fractions were subject to decrease. In all the plants lipid content and lipid associated bioactive

compounds resulted more affected by crushing, that led to the major losses in the embryo parts of the kernels. The highest losses of tocol and phytosterol contents, following crushing, were observed for emmer wheat from the Italian plant; in fact, this plant, even if fed with a whole kernel fraction (input of the process) richer in all these compounds, produced a final crushed output with similar or smaller amounts than other traditional processing plants. Carotenoids content was mainly affected by genotype.

The Armenian plant determined a lower decrease of bioactive compounds following crushing. Between the Turkish plants, the plant 2 caused higher losses, probably due to the relevant manual labour required following glume removal; also in a previous work (Giambanelli *et al.*, 2013a) the highest amount of unwanted losses, consisting of product less suitable for human consumption, was detected for this plant.

In emmer wheat bulgur from Turkey, the crushed fractions showed lower decreases of phenolic compounds, with respect to emmer crushed fractions from the other processing units. This fact could be a consequence of the cooking to obtain bulgur that could have determined the migration of phenolics inside the kernel and their conversion into more stable forms, as it occurs for parboiling process (Paiva *et al.*, 2014).

Semolina-like material, consisting of the finest fractions, mainly destined to feed animal, in many cases showed similar bioactive contents to correspondent whole kernel fractions, probably due to the fact that germ parts, separated during kernel breaking, were retained in these fractions.

Pearling did not produce significant decreases of bioactives in the pearled output. Again the fraction of broken kernels, generated by pearling and destined to crushing, showed the lowest lipid and bioactive contents, with similar amounts to those found for ITF7 (output from crushing); however the resulting waste (ITPR), containing mainly the bran part of the caryopses, showed the highest contents of all bioactive compounds, especially phytosterols and phenolic compounds.

CONCLUSIONS

This experiment represents a first approach to follow the evolution of specific potentially bioactive components (tocols, carotenoids, phytosterols and phenolic compounds) upon einkorn and emmer wheat processing. In particular removing husks and crushing were selected as unit operations, starting from a traditional food perspective. In case of the Italian plant also the presently more valued pearling operation was considered.

Following crushing, the updated process adopted in Italy, mostly readapted to meet market demand, showed lipid and bioactive compounds retention similar to the other traditional plants considered in the study. Minor variations were observed for the output obtained from pearling, and this process, at the same time, produced a waste very rich of bioactive compounds, that could be exploited for other uses.

Concluding all the plants showed similar trends following crushing, with the highest bioactive decreases in smaller kernel fractions, mostly due to the loss of the germ part.

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Chapter 5

Brassicaceae

Chapter 5.1

Comparison of leafy kale populations from Italy, Portugal, and Turkey for their bioactive compound content: phenolics, glucosinolates, carotenoids, and chlorophylls

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ABSTRACT

BACKGROUND: Kales are primitive leafy *Brassica oleracea* L. forms, widespread in local farming systems of several European countries and employed in the preparation of traditional recipes. Kales are also potential sources of healthy bioactive phytochemical components. The present study compared the bioactive compound content of kale populations from Italy, Portugal, and Turkey, either from local sources or grown in an experimental field.

RESULTS: Total phenolics, glucosinolates (GLS), carotenoids, and chlorophylls were in the range 8310-38110, 755-8580, 135-2354, 1740-16924 mg kg^{-1} dry matter, respectively. On average, locally harvested samples showed a total GLS content about twice as high as populations from the experiment. Conversely, pigments were significantly more abundant in experimental than in local kales, owing to the higher soil fertility. Portuguese samples showed higher phenolic and GLS amounts than Italian and Turkish kales, whereas some of the Italian samples were the richest in carotenoids.

CONCLUSION: This paper represented the first cross-country comparison of local kale accessions, with respect to bioactive compound amounts. Both geographic origin and growing environment appeared as remarkable and discriminating factors in determining bioactive levels in leafy kales, with possible effects on their health-promoting and sensorial attributes.

Keywords: kales; glucosinolates; phenolics; pigments; growing environment; geographic origin.

INTRODUCTION

Vegetables from the *Brassicaceae* family are among the most commonly grown and consumed worldwide. *Brassica oleracea* L. includes cabbage, broccoli, cauliflower, kales, collards, Brussel sprouts, kohlrabi, and Chinese broccoli (Lin and Harnly, 2009). Brassica vegetables are rich in several dietary bioactive compounds, including water-soluble phenolics, ascorbic acid, and glucosinolates (GLS), as well as lipid-soluble tocopherols and carotenoids, contributing to defense mechanisms against oxidative stress (Podsędek, 2007; Nilsson *et al.*, 2006; Kurilich *et al.*, 1999). GLS represent the most highly characterizing phytochemicals of *Brassica* vegetables, since their breakdown products isothiocyanates, thiocyanates, and nitriles have been suggested to be responsible for protective effects against carcinogenesis (Herr and Büchler, 2010; Chan *et al.*, 2009; Halkier and Gershenzon, 2006).

Kales are non-heading leafy primitive *Brassica oleracea* forms, the closest to wild ancestors (Hanelt, 1998). Different kale types are traditional crops of several European countries (Italy, Netherlands, Portugal, Scotland, Spain, Turkey). In Europe they are substantially neglected by breeding and no modern varieties exist. Kales are cool-season, frost-hardy vegetables, tolerating temperatures as low as -15°C (Olsen *et al.*, 2010). Exposure to frost determines a soluble sugar increase and flavour improvement (Hagen *et al.*, 2009).

Kales from different areas share similar aspects in their utilization, and are rooted in local farming and food systems, although generally lesser known outside them. They are ingredients of several traditional and artisanal foods, such as soups, fillings of pastries, and side dishes. They are mostly directly retailed by peasants in local groceries or street markets (D'Antuono *et al.*, 2010).

Kales showed the highest levels of chlorophyll-associated carotenoids, tocopherols, ascorbic acid, and total antioxidants among *Brassica* and other vegetables (Lefsrud *et al.*, 2007; Pfendt *et al.*, 2003; Halvorsen *et al.*, 2002; Kurilich *et al.*, 1999). Kales have also been reported to exhibit the highest antioxidant capacity in comparison to Brussels sprouts, broccoli flowers, cauliflower, and cabbage (Cao *et al.*, 1996). Furthermore, kales represent the most relevant potential dietary source of the glucobrassicin/indole-3-carbinol system, that is being investigated as a protective agent against cancer development and other immune and hormone-related diseases (Sarıkamış *et al.*, 2008; Higdon *et al.*, 2008; 2007; Velasco *et al.*, 2007). Because of these characteristics, kales are very interesting crops, from a health-protective and promoting perspective.

Few studies have been carried out for the characterization of local populations. Sarıkamış *et al.* (2008) investigated the GLS profile and content in Turkish kale genotypes from the Black sea region at two plant developmental stages. Ayaz *et al.* (2008) identified and quantified phenolic

acids in 10 seed lots of Turkish kales. Cartea *et al.* (2008) determined total GLS and GLS profile and studied the influence of growing seasons in 148 kale varieties from Galicia (northwestern Spain) and five commercial varieties. Italian kale populations were also preliminarily characterized (D'Antuono *et al.*, 2007; Romani *et al.*, 2003).

Other studies dealt with the investigation of the effect of environmental factors on GLS content of a local kale variety from northwestern Spain (Velasco *et al.*, 2007), the effect of developmental stage on pigment content of kale cv. 'Winterbor' (Lefsrud *et al.*, 2007), and the comparison of GLS, phenolic, and carotenoid content in *Brassica* genotypes, including kale (Nilsson *et al.*, 2006).

Finally, phenolics were characterized and indentified by high-performance liquid chromatogpraphy-mass spectrometry (HPLC-MS) in a few kale samples (Olsen *et al.*, 2010; 2009; Schmidt *et al.*, 2010; Lin and Harnly, 2009).

Up to now, no comprehensive comparative studies on the content of the main phytochemical classes (carotenoids, chlorophylls, GLS, and phenolics) in kale accessions from different geographic origin have been carried out. The present investigation was therefore aimed at comparing Italian, Portuguese, and Turkish kale populations either from local sources or cultivated in a common environment, assessing their phenolic, glucosinolate, carotenoid, and chlorophyll amounts, in order to evaluate the effect of both the growing environment and geographic origin on bioactive content. A total of 40 accessions, 25 of which were directly sampled from local sources, and 15 obtained from local seed samples and then grown in a common environment in Italy, were included in this study.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals and solvents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was obtained by an Elix 10 water purification system from Millipore (Bedford, MA, USA). Solvents employed in HPLC were of chromatographic grade and purchased from Sigma-Aldrich. Before use in HPLC, solvents were filtered through Nylon membrane filters (diameter 47 mm, pore dimension 0.45 μm) from GVS Filter Technology (Indianapolis, IN, USA) and sonicated at room temperature for 32 min. Standards of β-carotene, chlorophyll *a*, chlorophyll *b*, lutein, rutin, sinapic acid, and sinigrin, sulfatase enzyme and Sephadex® anion exchanger were from Sigma-Aldrich.

Samples

Local populations

Mature non-senescent leaves were randomly sampled in triplicate from local crops or markets during winter 2010/2011 in Italy (9), Portugal (9), and Turkey (7), for a total of 25 samples (hereafter referred to as "local"). Samples were refrigerated, after discarding damaged parts, and the edible aerial part was cut in small pieces (~1 cm), frozen at -18°C overnight, freeze-dried within one week, ground and stored at -18°C until bioactive extraction.

Experimental trial

Seeds of 15 kale landraces or local varieties were obtained from local sources (local seed companies, farmers) in Italy (7), Portugal (2), and Turkey (6). Seeds were planted in nursery in August 2010 in Cesena (Italy), and the seedlings were transplanted to a field located in Martorano (Cesena, Italy) in a three-replication randomized block design (September 2010). Mature leaves were harvested and processed in December 2010 (hereafter referred to as "experimental"), frozen at -18°C overnight, freeze-dried within one week, ground and kept at -18°C until bioactive extraction. The complete list of the accessions considered is reported in **Table 5.1.1**.

Glucosinolate (GLS) and phenolics extraction and determination

GLS and phenolics were simultaneously extracted with water-methanol mixtures according to D'Antuono *et al.* (2008). Extracts were recovered in plastic centrifuge tubes and stored at -18°C until further analyses.

Determination of GLS

Purification of enzyme sulfatase by solid phase extraction (SPE)

An appropriate amount of sulfatase from *Helix pomatia* (Type H-1, lyophilized powder, ≥10,000 units g^{-1} solid), to which was added 3 mL of deionized water and 3 mL of absolute ethanol, was weighed in a screw-cap plastic centrifuge tube in order to obtain a final concentration of 650 units mL^{-1}. After shaking for 1 min, the enzyme was centrifuged at 3373 × *g* (10 min, 5°C). The

supernatant fraction, to which was added 6 mL of absolute ethanol, was transferred to a second tube, and centrifuged at 3373 × *g* (10 min, 5°C).

Table 5.1.1. Name and geographic origin of local and experimental kale accessions.

Accession working tag [1]	Name	Country of origin	Collection site/Origin
ITA-L1	Nero di Toscana	Italy	San Giustino Valdarno, Arezzo
ITA-L2	Nero di Toscana	Italy	Faeto, Arezzo
ITA-L3	Nero di Toscana	Italy	Chiassaia, Arezzo
ITA-L4	Nero di Toscana	Italy	Pian di Loro, Arezzo
ITA-L5	Nero lavagnino	Italy	Varese Ligure, La Spezia
ITA-L6	Broccolo lavagnino	Italy	Lommeglia Toceto, La Spezia
ITA-L7	Nero lavagnino	Italy	Val di Vara, La Spezia
ITA-L8	Broccolo lavagnino	Italy	Chiavari, Genova
ITA-L9	Nero lavagnino	Italy	Chiavari, Genova
ITA-E1	Nero laciniato	Italy	Commercial (Cesena)
ITA-E2	Nero lavagnino	Italy	Chiavari, Genova
ITA-E3	Nero laciniato – Hortus	Italy	Commercial (Cesena)
ITA-E4	Gaggetta – Gargini sementi	Italy	Commercial (Lucca)
ITA-E5	Nero foglia larga	Italy	Lucca
ITA-E6	Nero lavagnino, Foglia liscia	Italy	Lavagna
ITA-E7	Nero lavagnino, Foglia bollosa	Italy	Lavagna
POR-L1	Couve galega	Portugal	Barqueiros
POR-L2	Couve galega	Portugal	Barqueiros
POR-L3	Couve galega	Portugal	Barqueiros
POR-L4	Couve galega	Portugal	Barqueiros
POR-L5	Couve galega	Portugal	Barqueiros
POR-L6	Couve galega	Portugal	Meixedo
POR-L7	Couve galega	Portugal	Montalegre
POR-L8	Couve galega	Portugal	Parafita
POR-L9	Couve galega	Portugal	Montalegre
POR-E1	Gama clasica; couve galega lisa (a sementeira)	Portugal	Commercial
POR-E2	Couve galega lisa (flora lusitana)	Portugal	commercial
TUR-L1	Kara lahana	Turkey	Giresun
TUR-L2	Kara lahana	Turkey	Giresun
TUR-L3	Kara lahana	Turkey	Trabzon
TUR-L4	Kara lahana	Turkey	Trabzon
TUR-L5	Kara lahana	Turkey	Dereli
TUR-L6	Kara lahana	Turkey	Giresun
TUR-L7	Kara lahana	Turkey	Giresun
TUR-E1	Kara lahana	Turkey	Giresun
TUR-E2	Kara lahana	Turkey	–
TUR-E3	Kara lahana	Turkey	Trabzon
TUR-E4	Yasar kan genis yaprahli Dargia	Turkey	–
TUR-E5	Kara lahana	Turkey	Yaprak
TUR-E6	Kara lahana	Turkey	Orhan

[1] ITA, POR, and TUR: Italian, Portuguese, and Turkish samples, respectively. L and E: local and experimental samples, respectively.

The supernatant was then discarded and the residual, to which was added 2 mL of deionized water, was shaken for 1 min and sonicated at room temperature for 2 min until complete dissolution. Enzyme solution was loaded onto a disposable Pasteur pipette previously filled with glass wool (~0.5 cm) and to which 330 µL of a water suspension of diethylaminoethyl (DEAE) A-25 Sephadex® anion exchanger (c = 80 mg mL^{-1}), preliminarily conditioned with 2 mL of Na-acetate buffer (pH 4.0), was added. Buffer was prepared from acetic acid (c = 0.02 mol L^{-1}) and sodium acetate trihydrate (c = 0.02 mol L^{-1}). Purified sulfatase was eluted in a plastic microtube and stored at -18°C until GLS desulphatation.

GLS purification and enzymatic desulphatation

GLS were purified from phenolics by solid phase extraction (SPE). Crude extract (750 µL) was transferred in a disposable Pasteur pipette previously filled with glass wool (~0.5 cm) and to which was added 330 µL of a water suspension of DEAE A-25 Sephadex® anion exchanger (c = 80 mg mL^{-1}), preliminarily conditioned with 2 mL Na-acetate buffer (pH 4.0). After loading onto SPE columns, samples were washed with 2 mL of Na-acetate buffer, added with 120 µL purified sulfatase and incubated at 39°C for 16 h to allow enzymatic desulphatation of GLS to the corresponding desulpho derivatives (DS-GLS). DS-GLS were eluted by 1.5 mL of deionized water and stored at -18°C until further analyses.

HPLC analyses

Before HPLC analyses, the samples were filtered in HPLC glass vials through Nylon syringe filters (diameter 13 mm; pore dimension 0.45 µm). HPLC analyses were carried out on an HPLC apparatus from Jasco (Tokyo, Japan), equipped with two binary pumps (model PU-1580), an autosampler (model AS-2055 Plus) and a diode array UV-visible detector (model MD-1510, quartz flow cell, 10 mm optical path). GLS elution was carried out in gradient mode employing the following solvent system: mobile phase A: water-acetonitrile 99:1 (v/v); mobile phase B: water-acetonitrile 20:80 (v/v). The gradient program was: from 0 to 22.3 min, 96.2 to 65.8% A; from 22.3 to 28.0 min, 65.8% A; from 28 to 29.8 min, 65.8 to 96.2% A; from 29.8 to 41 min, 96.2% A as post-run. The flow rate was 0.4 mL min^{-1} and the injection volume was 10 µL. Data were processed by the software ChromNAV (ver. 1.16.02) from Jasco. Each chromatogram was recorded at 229 nm whereas absorption spectra were recorded between 200 and 400 nm. A Gemini-NX 3 µm C18 (150 × 3.0 mm id, 3 µm particle size) column from Phenomenex (Torrance, CA, USA), equipped with an

HPLC guard cartridge system holding cartridges Gemini-NX C18 (4 × 3.0 mm) from Phenomenex was employed. The analyses were carried out at 35°C.

GLS quantification by external standard

GLS were quantified by external standard mode, constructing a calibration curve (r^2 = 0.999) of DS-sinigrin. A stock solution was prepared dissolving 20.3 mg sinigrin with ~5 mL of deionized water in a 10 mL volumetric flask, sonicating for two min and making the volume to the mark, for a final concentration of 2.0300 mg mL^{-1}. From the stock solution, four diluted solutions (0.0508, 0.2538, 0.5075, and 1.0150 mg mL^{-1}) were prepared. Each solution underwent in duplicate the same enzymatic desulphatation procedure previously described. Solutions containing DS-sinigrin were then analyzed by HPLC in two replications according to the formerly reported analytical conditions. The relative response factors of individual GLS were considered according to the *Official Journal of the European Communities* (Oil seed, 1990).

Quantification of phenolics by HPLC

Phenolic determination was performed on the crude extracts recovered from freeze-dried material by hydro-alcoholic mixtures. Phenolic elution was carried out by HPLC in gradient mode employing the following solvent system: mobile phase A: 0.5% (*v/v*) formic acid in methanol-water 5:95 (*v/v*); mobile phase B: 5% (*v/v*) formic acid in methanol-water 90:10 (*v/v*). The gradient program was: from 0 to 43 min, 95 to 55% A; from 43 to 44.2 min, 55 to 0% A; from 44.2 to 50.2, 0% A; from 50.2 to 51.4 min, 0 to 95% A, from 51.4 to 60.4 min, 95% A as post-run. The flow rate was 0.5 mL min^{-1}, and the injection volume was 12 µL. Data were processed by Jasco-Borwin software (ver. 1.50) from Jasco. Each chromatogram was recorded at 330 nm, whereas absorption spectra were recorded between 200 and 400 nm. A Gemini-NX 3 µm C18 (150 × 3.0 mm id, 3 µm particle size) column from Phenomenex, equipped with a HPLC guard cartridge system holding cartridges Gemini-NX C18 (4 × 3.0 mm) from Phenomenex, was employed. The analyses were carried out at 35°C. Phenolics were quantified by external standard mode, constructing calibration curves of two representative compounds of relevant phenolic classes. Hydroxycinnamic acid derivatives and flavonols were quantified at 330 nm using as reference compounds sinapic acid and rutin, respectively. Stock solutions were prepared in methanol. Diluted solutions containing both compounds were prepared in methanol-water 2:3 (*v/v*) and analyzed by HPLC in three replications.

Concentration ranges were: 0.0005-0.0505 mg mL^{-1} (seven calibration points, r^2 = 0.999), and 0.0025-0.2010 mg mL^{-1} (seven calibration points, r^2 = 0.999) for sinapic acid and rutin, respectively.

Identification of GLS and phenolics by HPLC-MS

GLS and phenolics identification was carried out on a HP 1100 Series LC system equipped with a diode array detector and coupled with a mass spectrometer detector (model 1100 MSD Series G1946A), both from Agilent Technologies (Palo Alto, CA, USA). Data processing was performed by the software LC/MSD ChemStation software (Rev. A.08.03) from Agilent Technologies. The mass spectrometer operated in atmospheric pressure ionization-electrospray source (API-ES) mode under the following operating conditions. *Glucosinolates*: drying gas (nitrogen) temperature, 350°C; drying gas flow rate, 10.0 L min^{-1}; nebulizer pressure, 50 psig; capillary voltage, 4000 V; fragmentor voltage, 100 V; mass range, *m/z* 50-800, scan mode: positive. *Phenolics*: drying gas temperature, 350°C; drying gas flow rate, 10.0 L/min; nebulizer pressure, 35 psig; capillary voltage, 3000 V; fragmentor voltage, 100 V; mass range, *m/z* 100-1400; scan mode negative. A total of 51 phenolic compounds were identified comparing peak mass spectra with those reported in former investigations (Olsen *et al.*, 2010; 2009; Schmidt *et al.*, 2010; Lin and Harnly, 2009). GLS were identified comparing mass spectra with those described by D'Antuono *et al.* (2008).

Quantification of carotenoids and chlorophylls

Carotenoids and chlorophylls were extracted from freeze-dried tissue according to Lefsrud *et al.* (2007), starting from 50 mg lyophilized material and employing tetrahydrofuran (THF) stabilized with butylated hydroxytoluene (BHT, c = 25 mg L^{-1}) as extracting solvent. Dried extracts were recovered in a plastic microtube with 2 mL of acetone and stored at -18°C. Before HPLC analyses, samples were centrifuged at 15000 × *g* (10 min, 10°C) and filtered in HPLC glass vials, equipped with 100 µL inserts, through regenerated cellulose (RC) syringe filters (diameter, 4 mm; pore dimension, 0.20 µm) from Phenomenex. HPLC analyses were carried out on the same HPLC apparatus employed for GLS and phenolics determination. Pigment elution was carried out in gradient mode employing the following solvent system: mobile phase A: water; mobile phase B: acetone. The gradient program was: from 0 to 5.1 min, 35% A; from 5.1 to 9.1 min, 35 to 10% A; from 9.1 to 11.9 min, 10% A; from 11.9 to 13.7 min, 10 to 0% A; from 13.7 to 16.8 min, 0% A;

from 16.8 to 17.7 min, 0 to 35% A; from 17.7 to 23 min, 35% A as post-run. The flow rate was 0.8 mL min^{-1} and the injection volume 2.5 µL. Data were processed by the software ChromNAV software (ver. 1.16.02) from Jasco. Each chromatogram was recorded at 431 and 450 nm, whereas absorption spectra were recorded between 400 and 650 nm. A Kinetex 2.6 µm C18 (75 × 4.6 mm id, 2.6 µm particle size) column from Phenomenex, equipped with an HPLC guard cartridge system holding cartridges Gemini C18 (4 × 3.0 mm) from Phenomenex, was employed. Analyses were carried out at 30°C. Pigments were quantified by external standard mode, constructing calibration curves of the most representative compounds. Lutein, β-carotene and chlorophyll *b* were quantified at 450 nm, whereas chlorophyll *a* was quantified at 431 nm. Standard solutions at different concentrations were prepared in acetone and analyzed by HPLC in three replications. Concentration ranges were: 0.0001-0.2000 (seven calibration points, r^2 = 0.987), 0.0005-0.2060 (six calibration points, r^2 = 0.999), 0.0005-0.5000 (six calibration points, r^2 = 0.997), and 0.0005-0.2000 (six calibration points, r^2 = 0.999) mg mL^{-1} for lutein, β -carotene, chlorophyll *a*, and chlorophyll *b*, respectively.

Recovery and sensitivity method evaluation

Recovery trials were carried out for bioactive classes spiking freeze-dried kale samples with three different amounts of standard compounds before extraction. Recovery evaluation was performed in triplicate for each added amount and calculated as a percentage, comparing the amount of each standard determined by HPLC and the known amount added before performing extraction. Sinapic acid, sinigrin, lutein, β-carotene, chlorophyll *a* and *b* were employed as reference compounds. Rutin was not used to evaluate phenolic recovery since, even though not detected in kale leaves, it co-eluted with other interfering phenolic compounds. The limit of detection (LOD) and the limit of quantification (LOQ) of different compounds were determined from calibration curves and corresponded to 3 × S/N and to 7 × S/N, respectively, where S/N is the signal-to-noise ratio.

Statistics

A nested factorial analysis of variance (ANOVA) scheme was used for data processing, including the effects: a) geographic origin (Italy, Portugal and Turkey), b) type of crop (locally harvested or from the experiment), c) their binary interaction, and d) accession within origin × type. The latter was included since the local and experimental accessions were not the same. The Tukey's honest

significant difference test was used for multiple comparisons. An overall synthesis of data pattern was obtained by means of a stepwise forward discriminant analysis. The six binary combinations of origin and growing environments were used as grouping variables and the total and relative amounts of the analyzed components as classification variables. All the analyses were carried out by means of SYSTAT 10.0 software (Systat Software, Chicago, IL, USA).

RESULTS AND DISCUSSION

Evaluation of analytical method performance: sensitivity, and recovery

LOD were at levels of 2.1, 41.6, 1.5, 2.7, 4.6, and 3.5 mg kg^{-1} dry matter (d.m.) for sinapic acid, sinigrin, lutein, β-carotene, chlorophyll *a*, and chlorophyll *b*, respectively, whereas for the same compounds LOQ were at levels of 5.0, 97.1, 3.5, 6.4, 10.8, and 8.2 mg kg^{-1} d.m., respectively. The lower sensitivity for sinigrin in comparison to other standard compounds was due to a peak-broadening effect that involved GLS peaks eluting first in HPLC traces. Recoveries were satisfactory for all compounds considered and were in the range 93.8-98.6, 79.1-94.1, 74.0-89.3, 90.0-106.1, 88.8-93.7, and 82.2-95.0 for sinapic acid, sinigrin, lutein, β-carotene, chlorophyll *a*, and chlorophyll *b*, respectively. On the whole, methods showed a good repeatability and precision, since the relative standard deviation associated to recovery values was lower than 5% for each compound at every trials.

Phenolic compounds

The extraction procedure, originally developed for glucosinolates (D'Antuono *et al.*, 2008), was suitable also for the simultaneous extraction of phenolics from kale leaves. The high temperature employed did not lead to significant phenolic losses.

A total of 51 phenolic compounds were identified (**Table 5.1.2**). 9 phenolics were non-acylated quercetin, kaempferol, and isorhamnetin glycosides, 33 were flavonol glycosides acylated with *p*-coumaric, caffeic, ferulic, hydroxyferulic, and sinapic acid, 4 were non-glycosylated hydroxycinnamic acids, and 5 were glycosylated phenolic acid derivatives.

Total phenolic content (**Table 5.1.3**) was affected by geographic origin, with Portuguese kales showing the highest amount, followed by Turkish and Italian samples. Phenolic content was significantly higher in experimental than in local samples only for Turkish accessions.

Table 5.1.2. Name and amount range of individual identified phenolics.

Tag	Phenolic class [1]	Compound name [2]	Amount range (mg kg^{-1} d.m.)
PH1	HA	CAFFEOYLQUINIC ACID	52-683
PH2	HA	*p*-COUMAROYLQUINIC ACID	0-188
PH3	HA	*p*-COUMAROYLQUINIC ACID	0-425
PH4	HA	FERULOYLQUINIC ACID	0-105
PH5	FLA	Q-3-DG-7-GL	46-2226
PH6	FLA	Q-3-CAF-DG-7-GL	233-1134
PH7	FLA	K-3-DG-7-GL	477-9780
PH8	HA	SIN-GL	48-620
PH9	FLA	K-3-DG-7-DG + K-3-HYDROFER-DG-7-DG	649-3424
PH10	FLA	Q-3-SIN-DG-7-GL + K-3-CAF-DG-7-DG	93-2112
PH11	FLA	Q-3-FER-DG-7-GL + Q-3-SIN-DG-7-DG	0-1283
PH12	FLA	K-3-CAF-DG-7-GL + Q-3-FER-DG-7-DG	0-485
PH13	FLA	K-3-SIN-DG-7-GL + K-3-SIN-DG-7-DG	1019-7760
PH14	FLA	K-3-FER-DG-7-GL	135-3139
PH15	FLA	K-3-FE-DG-7-DG + K-3-FE-TG-7-DG + K-3-COU-DG-7-GL	175-1997
PH16	FLA	K-3-COU-DG-7-DG	0-217
PH17	FLA	K-3-COU-TG-7-DG + K-3-GL-7-GL	0-215
PH18	FLA	K-3-CAF-DG-7-GL ISO	0-538
PH19	FLA	Q-3-COU-DG-7-GL + K-3-CAF-DG + K-3-TG	0-379
PH20	FLA	K-3-SIN-DG-7-GL ISO	0-471
PH21	FLA	K-3-COU-DG-7-GL ISO	0-708
PH22	FLA	K-3-COU-DG	0-1711
PH23	FLA	K-3-HYDROFER-DG-7-GL ISO	0-1047
PH24	FLA	I-GL	0-176
PH25	FLA	Q-3-DG	0-598
PH26	FLA	K-3-DG	0-142
PH27	FLA	K-3-SIN-DG	0-617
PH28	FLA	K-3-DG	0-754
PH29	FLA	K-3-COU-DG-7-GL ISO	0-736
PH30	FLA	Q-3-DISIN-TG-7-DG + Q-3-DIFER-TG-7-DG + K-3-COU-DG	80-1149
PH31	FLA	Q-3-DISIN-TG-7-GL + K-3-SIN-TG-7-DG	44-1689
PH32	FLA	K-3-DISIN-TG-7-DG	91-966
PH33	FLA	K-3-DISIN-TG-7-GL	176-4899
PH34	FLA	K-3-FER-SIN-TG-7-DG	0-1370
PH35	HA	DISIN-DG	162-655
PH36	HA	SIN-FER-DG	73-525
PH37	HA	TRISIN-DG	22-200
PH38	HA	DISIN-FER-DG	21-115

[1] FLA: flavonols; HA: hydroxycinnamic acids.

[2] Q: quercetin; K: kaempferol; I: isorhamnetin; GL: glucoside; DG: diglucoside; TG: triglucoside; CAF: caffeoyl; COU: *p*-coumaroyl; FER: feruloyl; DIFER: diferuloyl; HYDROFER: hydroferuloyl; SIN: sinapoyl; DISIN: disinapoyl; TRISIN: trisinapoyl; ISO: isomeric form.

Table 5.1.3. Effect of geographic origin and type of cultivation on the total amount and relative content of phenolics.

Source of variation [1]	**Total phenolics (mg kg^{-1} d.m.)** [2]	**Relative amounts (mg mg^{-1})**											
		FLA [3]	HA [3]	PH6	PH7	PH9	PH10	PH11	PH13	PH14	PH15	PH32	PH33
Geographic origin													
ITA	14089c	0.886c	0.114a	0.034a	0.085c	0.102°	0.053a	0.028a	0.236a	0.061c	0.071a	0.044a	0.041c
POR	28146a	0.945a	0.055c	0.028b	0.198a	0.079b	0.041b	0.026b	0.172c	0.081b	0.046c	0.008c	0.066a
TUR	18505b	0.927b	0.073b	0.028b	0.143b	0.089b	0.035c	0.027ab	0.231b	0.119a	0.062b	0.024b	0.052b
Significance [4]	**	**	**	**	**	**	**	**	**	**	**	**	**
Sample type													
LOCAL	20306a	0.928a	0.072b	0.029a	0.145a	0.081b	0.041b	0.034a	0.208b	0.070b	0.054b	0.031a	0.063a
EXPERIMENTAL	17796a	0.903b	0.097a	0.032a	0.121a	0.101°	0.047a	0.022b	0.228a	0.099a	0.069a	0.026b	0.040b
Significance	NS	**	**	NS	NS	**	**	**	*	**	**	**	**
Origin × Type [5]													
ITA-L	15016a	0.906a	0.094b	0.032a	0.093a	0.087b	0.054a	0.039a	0.241a	0.038b	0.057b	0.046a	0.052a
ITA-E	13493a	0.874b	0.126a	0.035a	0.079a	0.111°	0.053a	0.021b	0.232a	0.076a	0.079a	0.042a	0.033b
POR-L	28542a	0.949a	0.051a	0.029a	0.205a	0.071b	0.040a	0.032a	0.171a	0.072b	0.042b	0.008a	0.079a
POR-E	27257a	0.936a	0.064a	0.026a	0.183a	0.098°	0.043a	0.013b	0.175a	0.103a	0.055a	0.007a	0.036b
TUR-L	16520b	0.928a	0.072a	0.026a	0.133a	0.085°	0.026b	0.029a	0.214b	0.107b	0.064a	0.041a	0.056a
TUR-E	19663a	0.927a	0.073a	0.029a	0.149a	0.092°	0.041a	0.025a	0.242a	0.125a	0.062a	0.014b	0.050a
Significance	**	**	**	*	**	**	**	**	**	**	**	**	**

[1] ITA, POR, and TUR: Italian, Portuguese, and Turkish samples, respectively. L and E: local and experimental samples, respectively.
[2] Different letters within the same column and source of variation denote significant differences ($p \leq 0.05$).
[3] FLA: flavonols; HA: hydroxycinnamic acids; for individual phenolic names see **Table 5.1.2**.
[4] *: $p \leq 0.05$; **: $p \leq 0.01$; NS: not significant.
[5] Different letters indicate significant differences between samples (local or experimental), within geographic origin ($p \leq 0.05$).

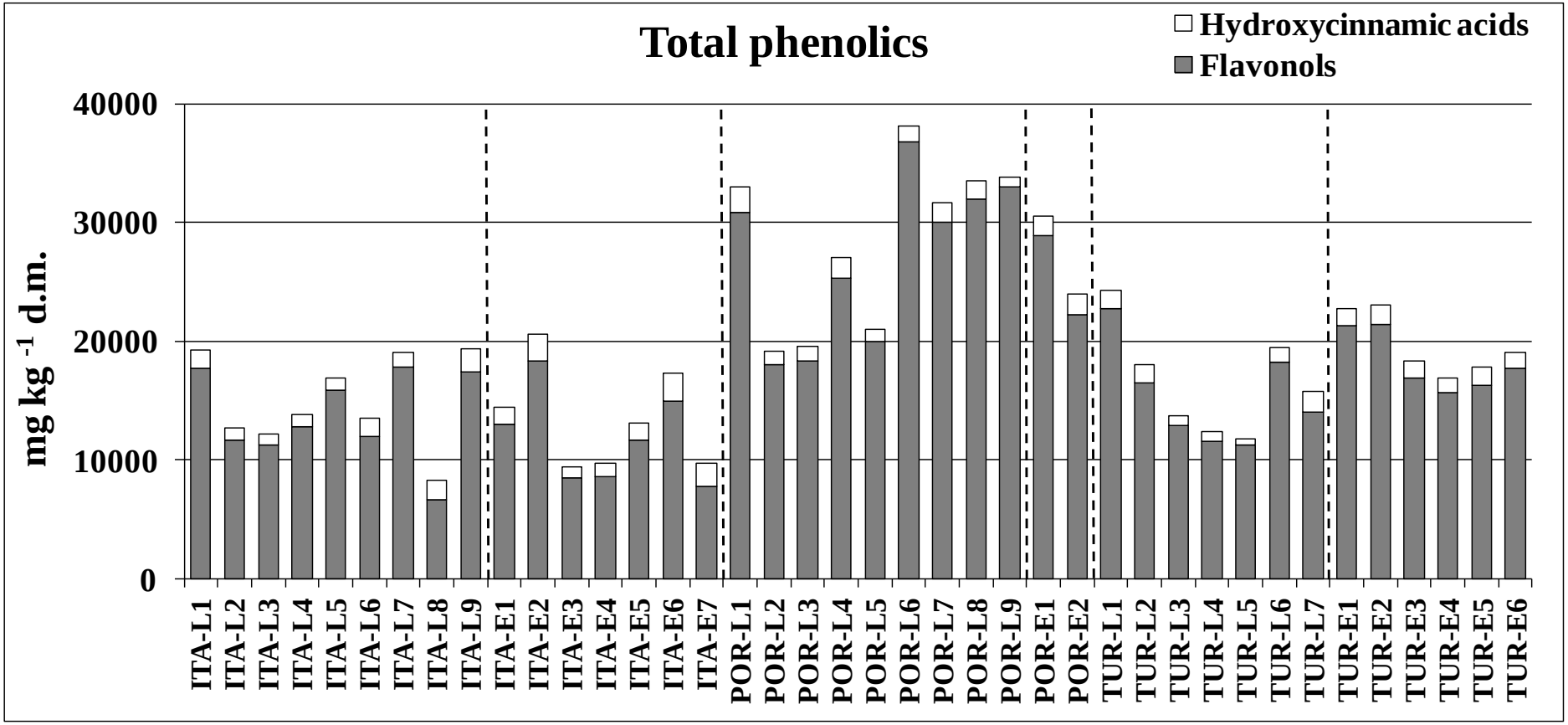

Figure 5.1.1. Effect of individual samples on total phenolic amount. Data are expressed as mg kg^{-1} d.m. ITA, POR, and TUR stand for Italian, Portuguese, and Turkish samples, respectively. L and E stand for local and experimental samples, respectively.

Significant differences among individual samples, (**Figure 5.1.1**) within the same origin and type were also detected. This variability, particularly remarkable within local kales, seemed to be sample specific. In fact, no apparent relation was determined between phenolic amount and other plant characteristics such as the dark-green colour of Italian samples from Tuscany. The high within-origin variability did not affect the differences previously illustrated. In fact, the variability range of Portuguese samples barely overlapped with that of Turkish and Italian accessions.

Flavonols were more abundant than hydroxycinnamic acids, accounting for over 80% phenolics in all samples. Geographic origin slightly affected the relative amount of the two phenolic classes, with Portuguese kales showing the highest flavonol fraction, amounting to about 95% total phenolics, followed by Turkish and Italian samples. A significant geographic origin × growing environment interaction was detected, due to a higher relative flavonol content in local than in experimental samples from Italy. The hydroxycinnamic acids pattern was complementary to that of flavonols. A significant variability was also detected in the relative hydroxycinnamic acid content within the Italian local kales, that ranged from 0.056 (ITA-L5) to 0.200 (ITA-L8) mg mg^{-1}.

Because of the high number of identified phenolics, only relative amounts of the more relevant ones are reported in **Table 5.1.3**. Some compounds coeluted in HPLC traces. Five phenolics represented on average about 50% of total identified compounds: PH7 (K-3-DG-7-GL), PH9 (K-3-DG-7-DG and coeluting K-3-HYDROFER-DG-7-DG), and PH13 (K-3-SIN-DG-7-GL and coeluting K-3-SIN-DG-7-DG).

PH13, including sinapic acid tri- and tetraglucosides, accounted on average 22% of total phenolics. Italian accessions had the highest PH13 fraction, followed by Turkish and Portuguese populations, with the latter showing less than 20% of these compounds. The significant difference between experimental and local samples was only due to an origin × types interaction, since in Turkish experimental samples PH13 relative content was 13% higher than in local samples.

PH7, a kaempferol triglucoside, was on average the second most abundant phenol. It was significantly more abundant in Portuguese than in Italian and Turkish samples. The growing conditions (local *vs.* experimental samples) did not affect PH7 fraction.

PH9 relative content was slightly, although significantly, higher in Italian than in Portuguese and Turkish accessions. Experimental samples showed a higher PH9 fraction than local accessions, with significant differences among samples from Italy and Portugal.

Analytical results on kale phenolics are quite scarce. The qualitative phenolic composition detected in our data is substantially in agreement with Olsen *et al.* (2009) who reported an average of 67% flavonols, with K-3-SIN-DG-7-DG as major component, accounting for 19% of total phenolics in curly kale. A less abundant hydroxycinnamic acid fraction was detected by Ayaz *et al.* (2008) in

leaves of Turkish kales where phenolic acids represented less than 1% of total phenolics determined by the Folin-Ciocalteau spectrophtometric method.

From a quantitative standpoint, data presented by Romani *et al.* (2003) agreed with the present study. Flavonol content was in the range 750-2000 and 500-1000 mg kg^{-1} fresh weight (f.w.) in Italian black kale from sandstone and calcareous marl, respectively. Total flavonols were determined at lower levels by Hagen *et al.* (2009). In curly kale grown in Norway, flavonols were in the range 6500-7000 mg kg^{-1} d.m. during 6 weeks of cold storage. Olsen *et al.* (2009) found higher phenolic amount in a Norwegian curly kale, where flavonols and hydroxycinnamic acids ranged from 4910 to 8310 mg kg^{-1} f.w. and from 1480 to 2500 mg kg^{-1} f.w., respectively. In the present investigation, total phenolic amount, expressed on fresh basis, was in the range 785-3430, 2313-5509, and 1386-3069 mg kg^{-1} f.w., for Italian, Portuguese, and Turkish samples, respectively.

Glucosinolates (GLS)

Quantified GLS were : glucoiberin (GI), sinigrin (SIN), and gluconapin (GN) among aliphatic GLS, glucobrassicin (GB), methoxyglucobrassicin (MGB), and neoglucobrassicin (NGB) among indolic GLS. Other GLS were not detectable, or determined in trace amounts.

The highest total GLS amount was determined in Portuguese kales (**Table 5.1.4**), followed by Turkish and Italian samples. Growing environment significantly and consistently affected total GLS content, that was in local samples at least double that in experimental kales, within the same origin.

A high variability was observed within local accessions (**Table 5.1.5**), in particular within Italian kales, whose total GLS content ranged from 1103 (ITA-L7) to 8580 (ITA-L8) mg kg^{-1} d.m., the latter being the highest amount among all accessions. Relevant variability was also found within Portuguese and Turkish locally sampled kales.

Origin significantly affected GLS profile. Aliphatic GLS fraction was consistently higher in Portuguese accessions, where it accounted on average for over 50% total GLS. Conversely, indolic GLS represented over 90% total GLS in the Italian and Turkish populations.

The significant origin × type interaction was due to a particularly higher relative indolic GLS content of Portuguese experimental samples, with respect to local ones. A complementary trend was assessed for aliphatic GLS.

Table 5.1.4. Effect of geographic origin and type of cultivation on the total amount and relative content of glucosinolates (GLS).

Source of variation [1]	**Total GLS (mg kg^{-1} d.m.)** [2]	**Relative amounts (mg mg^{-1})**							
		Aliphatic GLS	Indolic GLS	GI [3]	SIN [3]	GN [3]	GB [3]	MGB [3]	NGB [3]
Geographic origin									
ITA	1961b	0.074b	0.926b	0.053b	ND	0.021b	0.839a	0.061b	0.025b
POR	3760a	0.539a	0.461c	0.174a	0.365	ND	0.362b	0.056b	0.043a
TUR	1673c	0.040c	0.960a	0.013c	ND	0.027a	0.835a	0.115a	0.010c
Significance [4]	**	**	**	**	**	**	**	**	**
Sample type									
LOCAL	3344a	0.297a	0.703b	0.104a	0.153a	0.040	0.611b	0.059b	0.032a
EXPERIMENTAL	1406b	0.068b	0.932a	0.038b	0.030b	ND	0.820a	0.094a	0.018b
Significance	**	**	**	**	**	**	**	**	**
Origin × Type [5]									
ITA-L	2699a	0.153a	0.847b	0.099a	ND	0.054	0.790b	0.031b	0.026a
ITA-E	1487b	0.023b	0.977a	0.023b	ND	ND	0.871a	0.080a	0.025a
POR-L	4582a	0.609a	0.391b	0.183a	0.426a	ND	0.284b	0.051a	0.056a
POR-E	1910b	0.381b	0.619a	0.153a	0.228b	ND	0.538a	0.066a	0.015b
TUR-L	2582a	0.081a	0.919b	0.008a	ND	0.074	0.802b	0.105b	0.011a
TUR-E	1144b	0.016b	0.984a	0.016a	ND	ND	0.854a	0.120a	0.010a
Significance	**	**	**	**	**	**	**	**	**

[1] ITA, POR, and TUR: Italian, Portuguese, and Turkish samples, respectively. L and E: local and experimental samples, respectively.
[2] Different letters within the same column and source of variation denote significant differences ($p \leq 0.05$).
[3] GI: glucoiberin, SIN: sinigrin, GN: gluconapin, GB: glucobrassicin, MGB: 4-methoxyglucobrassicin, NGB: neoglucobrassicin.
[4] *: $p \leq 0.05$; **: $p \leq 0.01$; NS: not significant; ND: not detectable.
[5] Different letters indicate significant differences between samples (local or experimental), within geographic origin ($p \leq 0.05$).

Table 5.1.5. Effect of individual samples on the total amount and relative content of glucosinolates (GLS).

Accession working tag [1]	**Total GLS (mg kg^{-1} d.m.)** [2]	**Relative amounts (mg mg^{-1})**							
		Aliphatic GLS	Indolic GLS	GI [3]	SIN [3]	GN [3]	GB [3]	MGB [3]	NGB [3]
ITA-L1	1329c	ND	1.000a	ND	ND	ND	0.896ab	0.044a	0.060a
ITA-L2	1885c	0.223ab	0.777abc	0.223a	ND	ND	0.706bcd	0.042a	0.028bc
ITA-L3	1410c	ND	1.000a	ND	ND	ND	0.950a	0.029a	0.021bcd
ITA-L4	1317c	ND	1.000a	ND	ND	ND	0.935a	0.037a	0.028bc
ITA-L5	3568b	0.310ab	0.690bc	ND	ND	0.310a	0.650cd	0.025a	0.015cd
ITA-L6	3326b	0.408a	0.592c	0.408a	ND	ND	0.516d	0.040a	0.036b
ITA-L7	1103c	0.174b	0.826ab	ND	ND	0.174b	0.773abc	0.028a	0.025bcd
ITA-L8	8580a	0.262ab	0.738bc	0.262a	ND	ND	0.711bc	0.018a	0.009d
ITA-L9	1772c	ND	1.000a	ND	ND	ND	0.970a	0.018a	0.012cd
ITA-E1	1409abc	0.063a	0.937a	0.063a	ND	ND	0.838ab	0.081bc	0.018c
ITA-E2	1970a	ND	1.000a	ND	ND	ND	0.927a	0.065bc	0.008c
ITA-E3	1080bc	ND	1.000a	ND	ND	ND	0.905a	0.054c	0.041b
ITA-E4	1373abc	ND	1.000a	ND	ND	ND	0.915a	0.051c	0.035b
ITA-E5	780c	ND	1.000a	ND	ND	ND	0.906a	0.079bc	0.015c
ITA-E6	1697ab	ND	1.000a	ND	ND	ND	0.889a	0.103ab	0.008c
ITA-E7	2100a	0.100a	0.900a	0.100a	ND	ND	0.720b	0.127a	0.053a
POR-L1	5165ab	0.483b	0.517a	0.183ab	0.300c	ND	0.381ab	0.047ab	0.089b
POR-L2	4360bc	0.313b	0.687a	0.086b	0.227c	ND	0.490a	0.076a	0.120a
POR-L3	5238ab	0.514b	0.486a	0.171ab	0.343c	ND	0.348ab	0.065ab	0.073bc
POR-L4	4544b	0.480b	0.520a	0.137ab	0.342c	ND	0.401a	0.058ab	0.062c
POR-L5	4710b	0.396b	0.604a	0.140ab	0.256c	ND	0.459a	0.060ab	0.084b
POR-L6	3217cd	0.806a	0.194b	0.300a	0.507b	ND	0.110c	0.062ab	0.022de
POR-L7	4741b	0.760a	0.240b	0.222ab	0.539ab	ND	0.177bc	0.035ab	0.028d
POR-L8	6275a	0.868a	0.132b	0.215ab	0.653a	ND	0.107c	0.015b	0.010e
POR-L9	2987d	0.858a	0.142b	0.193ab	0.665a	ND	0.086c	0.044ab	0.012de
POR-E1	2510a	0.489a	0.511b	0.198a	0.291a	ND	0.460b	0.040b	0.011a
POR-E2	1311b	0.272b	0.728a	0.108a	0.164b	ND	0.616a	0.093a	0.018a

TUR-L1	1075c	ND	1.000a	ND	ND	ND	0.867ab	0.128b	0.006b
TUR-L2	2823b	ND	1.000a	ND	ND	ND	0.880ab	0.112b	0.008b
TUR-L3	1955bc	ND	1.000a	ND	ND	ND	0.940a	0.031cd	0.029a
TUR-L4	2156bc	ND	1.000a	ND	ND	ND	0.936a	0.047cd	0.017ab
TUR-L5	4520a	0.384a	0.616b	ND	ND	0.384a	0.593c	0.019d	0.004b
TUR-L6	4340a	0.185a	0.815ab	0.053	ND	0.132b	0.720bc	0.087bc	0.008b
TUR-L7	1203c	ND	1.000a	ND	ND	ND	0.682bc	0.314a	0.004b
TUR-E1	1219ab	ND	1.000a	ND	ND	ND	0.875a	0.109ab	0.016a
TUR-E2	1016ab	ND	1.000a	ND	ND	ND	0.856a	0.128ab	0.015a
TUR-E3	1585a	ND	1.000a	ND	ND	ND	0.905a	0.091b	0.004a
TUR-E4	903ab	ND	1.000a	ND	ND	ND	0.843a	0.142a	0.015a
TUR-E5	755b	ND	1.000a	ND	ND	ND	0.854a	0.141a	0.006a
TUR-E6	1383ab	0.094	0.906a	0.094	ND	ND	0.792a	0.109ab	0.006a

[1] ITA, POR, and TUR: Italian, Portuguese, and Turkish samples, respectively. L and E: local and experimental samples, respectively.
[2] Different letters along the same column denote significant differences with the same origin and type ($p \leq 0.05$). ND: not detectable.
[3] GI: glucoiberin, SIN: sinigrin, GN: gluconapin, GB: glucobrassicin, MGB: 4-methoxyglucobrassicin, NGB: neoglucobrassicin.

Significantly relevant GLS profile variability was determined, especially within local samples. In 4 out of 9 Italian local populations aliphatic GLS were not detected, but they accounted for over 15% total GLS in the remaining accessions. Similarly, aliphatic GLS were not detected in most Turkish local samples, representing however 19 and 38% total GLS in TUR-L5 and TUR-L6, respectively.

Within Portuguese kales, differences were assessed between local samples from the site of Barqueiros (from POR-L1 to POR-L5) and other local populations.

SIN and GB were the most abundant individual GLS and accounted on average for over 80% total GLS. SIN fraction was significantly affected by growing environment: in Portuguese kales its relative amount was almost double in local than in experimental populations.

GB was the main GLS in Italian and Turkish kales, which did not differ for their average relative GB amounts. GB relative content was higher in experimental than in local samples, in particular for Portuguese accessions, as indicated by the significant origin × type interaction.

MGB was the third most abundant GLS, with an average relative content of 8% total GLS. Its relative amount was significantly higher in Turkish than in Italian and Portuguese populations. A significant origin × type interaction was determined by the higher MGB relative content of the experimental Italian and Turkish samples with respect to local accessions.

Former studies agree with these findings, with respect to a high relative amount of aliphatic GLS in kales from Portugal and northwestern Spain. Aliphatic GLS accounted from 44 to 92% total GLS in a local kale population from northern Spain, as determined by Velasco *et al.* (2007) at different plant developmental stages. In the work of Cartea *et al.* (2008), aliphatic GLS amounted on average at 69% GLS in 153 kale varieties grown in northwestern Spain. Conversely, Sarıkamış *et al.* (2008) detected a prevalence of indolic GLS (93-97% total GLS) in 101 Turkish kale genotypes. Quantitative results herein determined agreed with findings of Nillson *et al.* (2006) who reported 3426-3661 mg sinigrin equivalent kg^{-1} d.m. total GLS content in one curly kale cultivar from Sweden. Higher amounts of total GLS were determined in formerly cited studies: 3359-17739 mg sinigrin equiv. kg^{-1} d.m. by Velasco *et al.* (2007), 4372-20982 mg of sinigrin equiv. kg^{-1} d.m. by Cartea *et al.* (2008), and 13720-24223 mg sinigrin equiv. kg^{-1} d.m. by Sarikamiş *et al.* (2008).

Pigments

Pigments identified were lutein, β-carotene, chlorophyll *a* and chlorophyll *b*. Minor carotenoids accounted for less than 5% total pigments.

The highest amount of carotenoids was determined in Italian kales, and a consistent difference was also assessed between Turkish and Portuguese accessions (**Table 5.1.6**). Growing conditions significantly affected the carotenoid amounts, which were, on average, five times higher in experimental than in local samples. This clear pattern was not affected by the origin × type interaction, although significant.

Total carotenoid content seemed to be also in relation to their profile. In fact, the highest lutein fraction was determined in Portuguese accessions, followed by Turkish and Italian samples. Lutein was also significantly more abundant in local (over 76% total carotenoids, on average) than in the experimental samples. This general pattern was not affected by the origin × type significant interaction, even if the relative dominance of lutein was particularly evident in Turkish local samples. A complementary pattern was verified for β-carotene.

A high variability was assessed among Italian kale samples (**Figure 5.1.2**). In particular, within experimental populations, significant differences were found between the dark-leaved populations from Tuscany, normally referred to as black kales or palm-tree kales (ITA-E1, ITA-E3, ITA-E4, and ITA-E5) and the other Italian samples. No significant variability of carotenoid content was detected, conversely, within Portuguese and Turkish accessions.

Total chlorophyll content was affected by sample origin and growing conditions, with a pattern similar to carotenoids. (**Table 5.1.6**).

In fact, Italian samples showed the highest chlorophyll amount, followed by Turkish and Portuguese accessions. Experimental samples also showed an almost three times higher chlorophyll level than local accessions. This pattern was common to all origins, despite a significant origin × type interaction.

Among individual accessions, the highest chlorophyll content was assessed in the dark-leaved Italian populations from Tuscany (ITA-E1, ITA-E3, ITA-E4, and ITA-E5) (**Figure 5.1.3**).

A high variability was also assessed within Italian local kales where total chlorophylls ranged from 1740 to 8342 mg kg^{-1} d.m. No differences were assessed among Portuguese and Turkish samples.

Chlorophyll *a* represented on average about 60% total chlorophylls. Slight, although sometimes significant, variability of chlorophyll composition was detected, with growing conditions as the main factor of variation. A higher relative chlorophyll *a* content in experimental than in local populations was verified.

Table 5.1.6. Effect of geographic origin and type of cultivation on the total amount of carotenoids and chlorophylls and the relative content of individual compounds.

Source of variation [1]	**Total carotenoids (mg kg^{-1} d.m.)** [2]	**Relative amount (mg mg^{-1})**		**Total chlorophylls (mg kg^{-1} d.m.)**	**Relative amount (mg mg^{-1})**	
		Lutein	Beta-carotene		Chlorophyll *a*	Chlorophyll *b*
Geographic origin						
ITA	1321a	0.536c	0.464a	10042a	0.673b	0.327a
POR	631b	0.615a	0.385c	5406c	0.680a	0.320b
TUR	959b	0.583b	0.417b	8130b	0.713a	0.287b
Significance [3]	**	**	**	**	**	**
Sample type						
LOCAL	325b	0.763a	0.237b	4243b	0.643b	0.357a
EXPERIMENTAL	1623a	0.410b	0.590a	11655a	0.727a	0.273b
Significance	**	**	**	**	**	**
Origin × Type [4]						
ITA-L	429b	0.711a	0.289b	4598b	0.602b	0.398a
ITA-E	1895a	0.424b	0.576a	13543a	0.718a	0.282b
POR-L	281b	0.705a	0.295b	3431b	0.654b	0.346a
POR-E	1419a	0.411b	0.589a	9849a	0.738a	0.262b
TUR-L	248b	0.906a	0.094b	4832b	0.680b	0.320a
TUR-E	1373a	0.394b	0.606a	10054a	0.733a	0.267b
Significance	**	**	**	**	**	**

[1] ITA, POR, and TUR: Italian, Portuguese, and Turkish samples, respectively. L and E: local and experimental samples, respectively.
[2] Different letters within the same column and source of variation denote significant differences ($p \leq 0.05$).
[3] *: $p \leq 0.05$; **: $p \leq 0.01$; NS: not significant.
[4] Different letters indicate significant differences between samples (local or experimental), within geographic origin ($p \leq 0.05$).

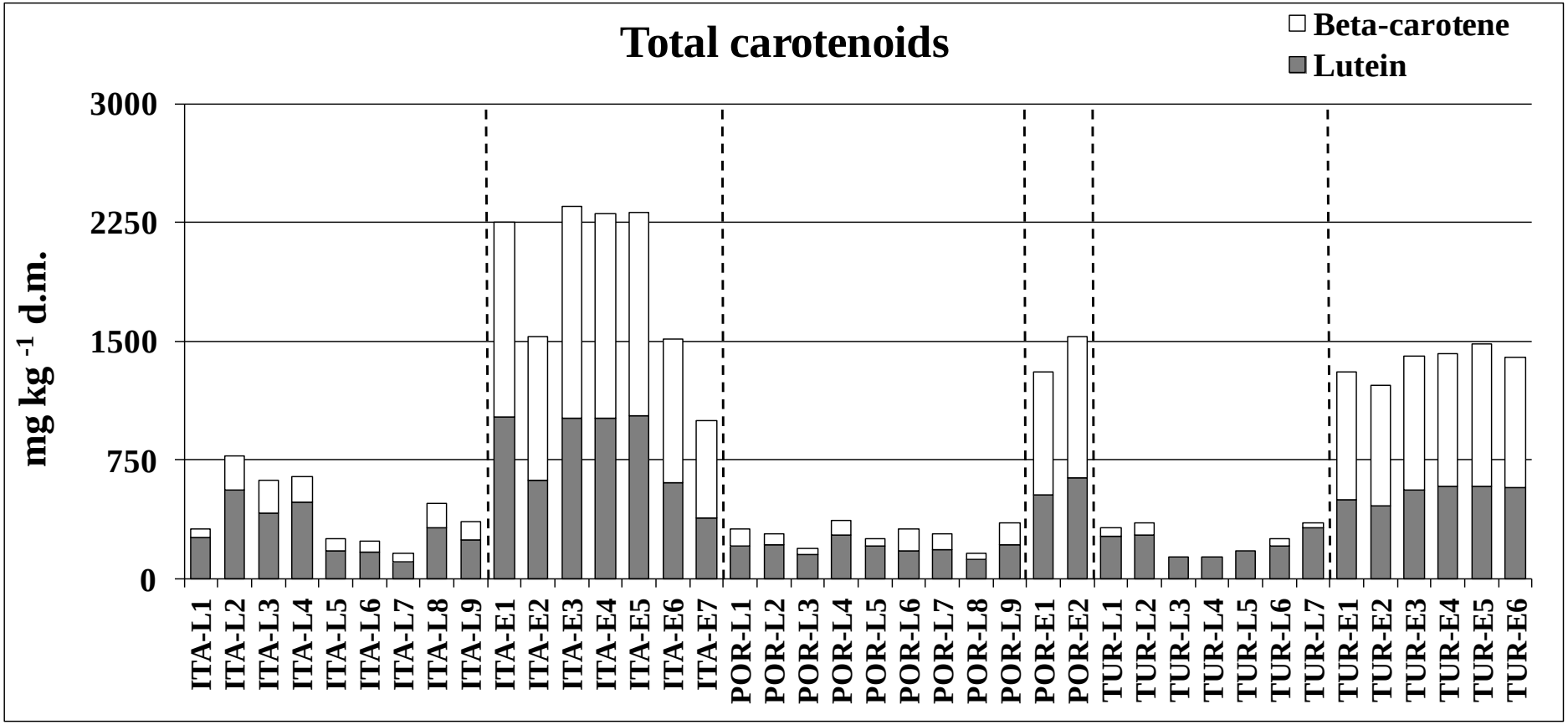

Figure 5.1.2. Effect of individual samples on total carotenoid amount. Data are expressed as mg kg^{-1} d.m. ITA, POR, and TUR stand for Italian, Portuguese, and Turkish samples, respectively. L and E stand for local and experimental samples, respectively.

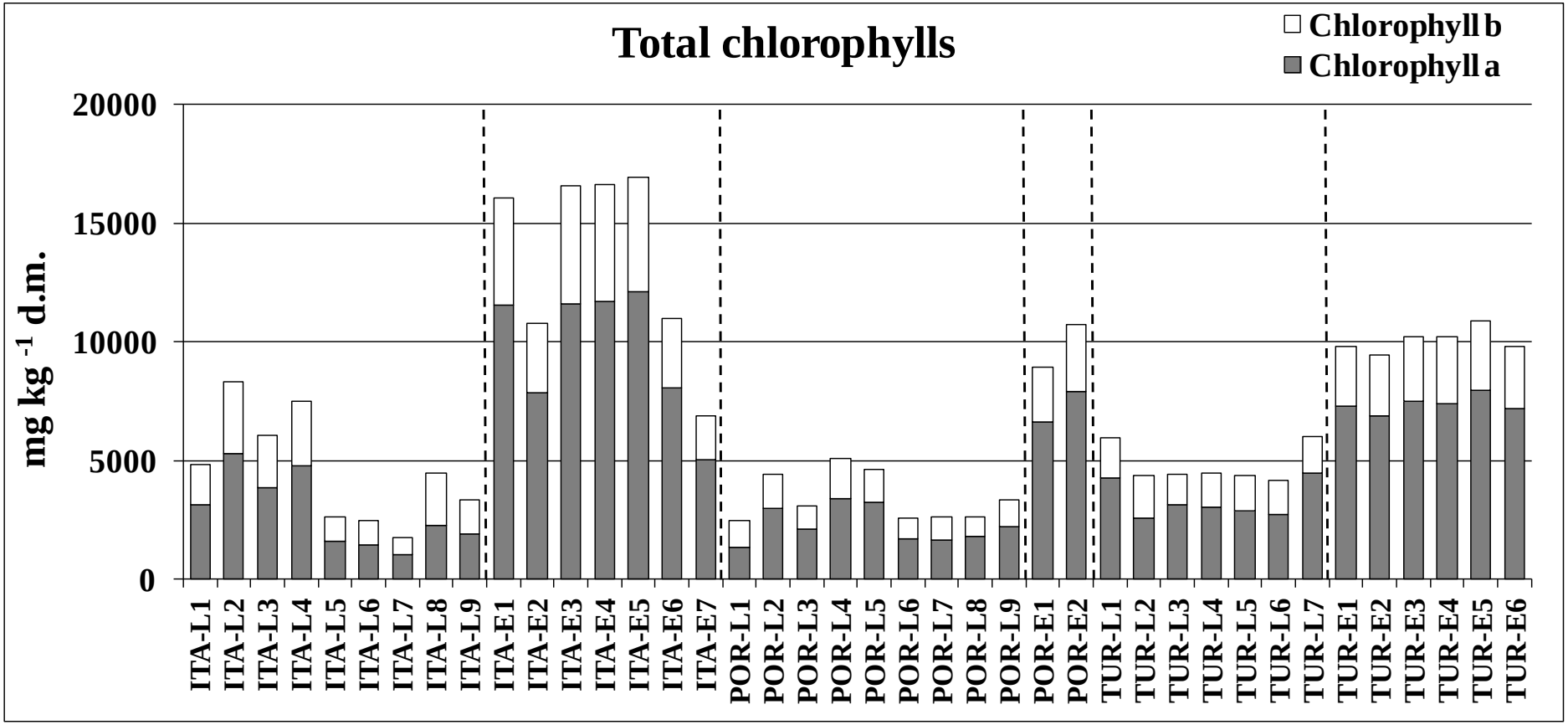

Figure 5.1.3. Effect of individual samples on total chlorophyll amount. Data are expressed as mg kg^{-1} d.m. ITA, POR, and TUR stand for Italian, Portuguese, and Turkish samples, respectively. L and E stand for local and experimental samples, respectively.

Data of Lefsrud *et al.* (2007) are in the range of our findings. Total carotenoids, total chlorophylls, and relative lutein content were in the range 1200-1870 mg kg^{-1} d.m., 13671-22398 mg kg^{-1} d.m., and 0.54 to 0.60 mg mg^{-1}, respectively, in a kale sample at different maturity stages. Slightly higher carotenoid content, also compared to the richest Italian accessions, was reported by Nilsson *et al.* (2006) in a curly kale sample grown in Sweden, with lutein and β-carotene at 2789 and 585 mg kg^{-1} d.m., respectively. Furthermore, in contrast to our results, other xanthophylls than lutein and β-carotene amounted to about 30% of total carotenoids. In two kale accessions, Kurilich *et al.* (1999) determined lower β-carotene amounts, ranging from 259 to 317 mg kg^{-1} d.m.

De Azevedo and Rodriguez-Amaya (2005) found total carotenoid amounts similar to our experimental kales in a cultivar ('Manteiga') grown in Brazilian conventional and organic farms. Carotenoids were expressed on a fresh basis and ranged from 127 to 152 mg kg^{-1} f.w., where in our experimental kales they were at levels of 116-374 mg kg^{-1} f.w. Violaxanthin and neoxanthin, not detected in the present research, amounted together from 32 to 41% of total carotenoids.

Accession profiling on the basis of discriminant analysis

Discriminant analysis allowed a further contribution to kale accession profiling on the basis of their geographic origin and growing conditions.

Figure 5.1.4 presents the score plots of the samples, whereas **Table 5.1.7** reports the correlations among total and relative phytochemical amounts and the five discriminant factors (DF).

DF1, explaining 49.3% among group variance, is clearly linked to pigment composition. In fact, it shows the highest correlations with total carotenoid content (negative), lutein (positive) and chlorophyll *a* (negative) relative amounts. Among GLS, SIN has the highest correlation (positive) with DF1. PH9 is the only major phenolic compound correlated with DF1, whereas some minor phenolics such as PH8, PH28 and PH36 (less than 3% of total phenolics each) show important connections with DF1. As illustrated in **Figure 5.1.4A**, DF1 discriminates experimental from local samples, respectively located on the negative and positive side of DF1 axis. In fact, experimental populations were characterized by a much higher carotenoid amount, lower lutein and a higher chlorophyll *a* relative contents, in comparison to locally cultivated accessions. Within type of sample, Italian kales are also discriminated from other populations along DF1, because of their higher total carotenoid content. A further (though minor) contribution to DF1 discrimination is also given by PH8 and PH9, whose relative fractions are higher in experimental than in local populations.

Table 5.1.7. Correlations among analytical variables and discriminant factors (DF).

Variables [1]	**Correlations to discriminant factors**				
	DF1	DF2	DF3	DF4	DF5
PH5	0.269	-0.054	-0.117	0.513	0.081
PH8	-0.622	0.218	-0.109	-0.090	0.014
PH9	-0.508	0.278	-0.192	-0.039	-0.087
PH14	-0.238	-0.304	0.104	-0.686	0.153
PH25	-0.005	-0.083	-0.093	-0.059	-0.559
PH26	0.215	-0.092	0.071	0.233	-0.186
PH28	0.364	-0.369	-0.143	-0.498	-0.128
PH30	0.169	0.070	-0.285	0.147	-0.155
PH36	-0.393	0.392	-0.496	0.059	0.103
PH37	-0.003	0.424	-0.076	0.364	0.040
PH38	-0.026	0.455	-0.266	0.023	0.260
SIN	0.369	-0.749	-0.188	0.334	-0.081
MGB	-0.248	-0.065	0.047	-0.498	0.251
NGB	0.225	-0.277	-0.172	0.449	0.148
Total carotenoids	-0.905	0.054	-0.160	-0.041	-0.027
Lutein	0.926	0.203	-0.154	-0.044	0.079
Chlorophyll *a*	-0.598	-0.252	-0.142	-0.442	0.042
% EV [2]	*49.3*	*34.8*	*10.9*	*4.3*	*0.7*

[1] PH: relative fraction of identified phenolic compounds (for individual names see **Table 5.1.2**); MGB: 4-methoxyglucobrassicin, NGB: neoglucobrassicin.
[2] *% EV*: percentage of variance among groups explained by each factor.

DF2, accounting for 34.8% variance, is mainly related to GLS and phenolic profile (**Figure 5.1.4A**). In fact, DF2 has the highest negative correlation to SIN fraction and shows important correlations with the relative amounts of PH14 and minor phenolics such as PH28, PH36, PH37, and PH38.
Sample location over DF2 reflects differences related to kale origin, accounting for the separation of Portuguese kales on the negative side of DF2 axis from the Italian and Turkish samples.
The discriminant factors DF3 to DF5 only add some information connected to the effects of origin × type of crop interaction, on group discrimination.
DF3 (10.9% variance explained) is correlated with minor phenolic PH36, and to a lesser extent with PH30 and PH38. DF3 did not add relevant contribution to further discrimination (**Figure 5.1.4B**). It separates the Turkish from the Italian and Portuguese experimental samples in a pattern differing from DF1 and DF2.
DF4 (4.3% variance explained) shows the highest correlations with some phenolics (PH5, PH14, PH28) and minor indolic GLS MGB and NGB. DF4 enables a clear distinction of Turkish populations within local samples. (**Figure 5.1.4C**).

DF5, explaining for less than 1% of variance, is highly related to PH25 fraction, determining a separation of Portuguese experimental samples (**Figure 5.1.4D**).

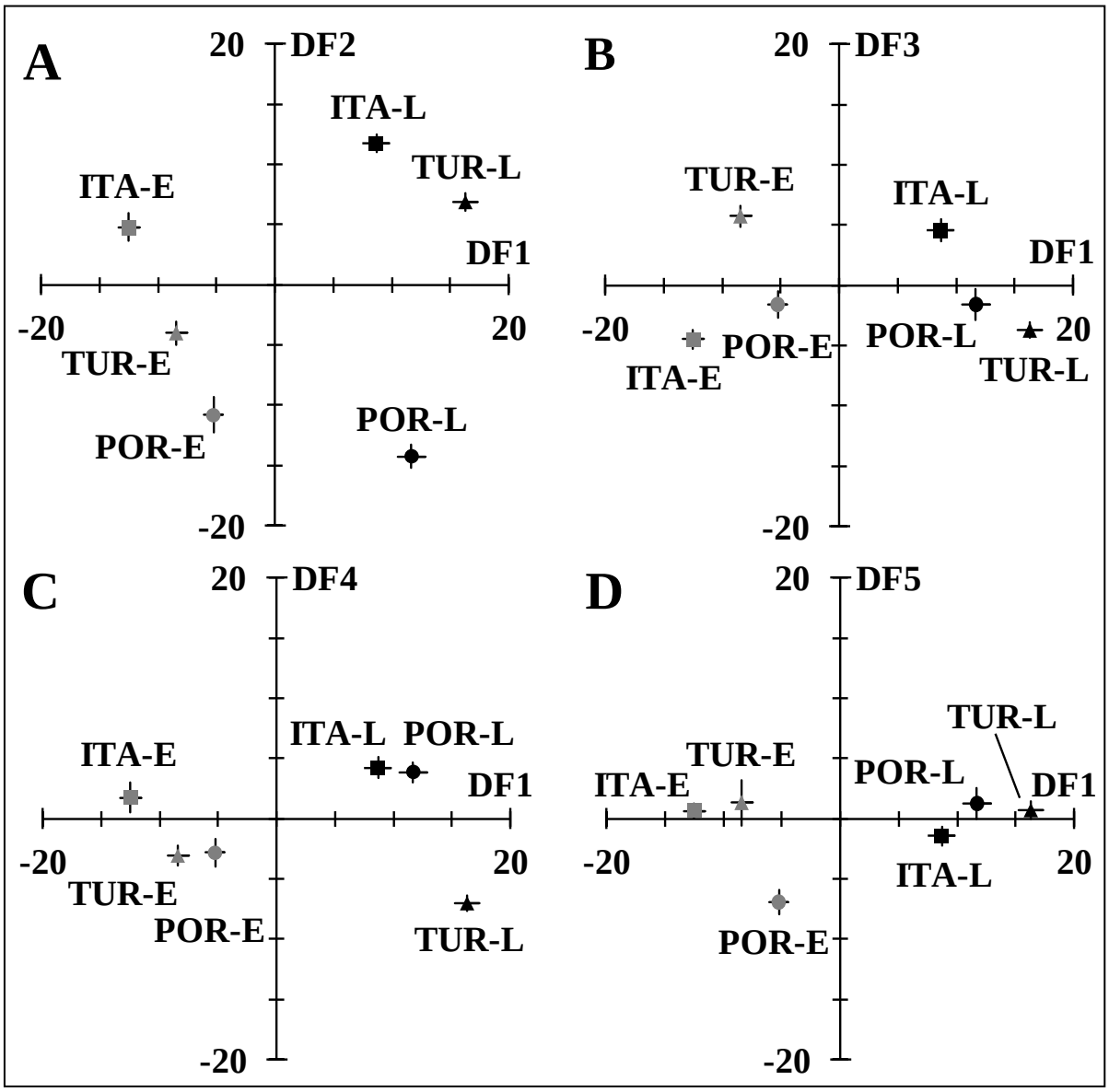

Figure 5.1.4. Loading of discriminant factors (DF) to analytical variables. ITA, POR and TUR stand for Italian, Portuguese, and Turkish samples, respectively. L and E stand for local and experimental samples, respectively. Horizontal and vertical bars represent DF standard deviations.

CONCLUSION

The first cross-country comparison of local kale types has been carried out with regard to their bioactive content: phenolics, GLS, carotenoids, and chlorophylls.

The results confirm that kales are the more readily available source of indolic GLS, especially with respect for Turkish and Italian accessions. The ample variability detected among populations, especially for phenolics and GLS, allows the possibility of selecting for specific components. Growing conditions of good soil fertility boosted pigment content but lowered total GLS amount. Stress conditions do not therefore affect the amounts of all potentially health-promoting bioactive compounds equally.

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Chapter 5.2

The kinetic of key phytochemical compounds of kale landraces (*Brassica oleracea* ssp. *acephala*) as affected by traditional cooking methods

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ABSTRACT

BACKGROUND: Kales are often a key ingredient of traditional foods, containing high amount of indolic glucosinolates (precursors of indole-3-carbinol and ascorbigen), carotenoids and phenolics. The present trend to associate traditional foods crops with health promoting properties, suggested to investigate the degradation kinetic of three kale landraces' phytochemicals, subjected to boiling, steaming and stir frying.

RESULTS: Boiling determined substantial losses, due to leaching of hydrophilic compounds. Glucosinolates decreased to represent 20% of their initial values after 10 minutes in Nero di Toscana; their decay followed a second order degradation kinetics. Phenolic content in leaves + cooking water remained unchanged, whereas however, their antioxidant capacity was reduced. Carotenoid content increased during the first minutes of boiling. Steaming showed the highest retention of phytochemicals, with often slight linear decreases, represented by a zero order degradation kinetic, having however a strong effect on colour. Stir frying produced high losses for all measured compounds; also β-carotene reduced its content to 10-23%, independently on variety. Conversion values for indole-derived compounds ranged from non-detectable to 23.5%.

CONCLUSION: Kale variety strongly affected observed degradation rates, because of a different glucosinolate composition and leaf structure. This research also allowed to add information to kinetic degradation of kale phytochemical compounds upon cooking.

Keywords: kale (*Brassica oleracea* ssp. *acephala*); glucosinolates; carotenoids; total phenolic compounds; cooking; retention.

INTRODUCTION

Brassica oleracea L. includes vegetables grown and consumed worldwide. Among this family kale (*Brassica oleracea* ssp. *acephala*) represent a leafy vegetable, generally grown in several European countries.

Brassica vegetables have been widely investigated in the last years for their richness in healthy phytochemicals. Kale, in particular, represent an important source of ascorbic acid, carotenoids, phenolics and glucosinolates (GLS) (Becerra-Moreno *et al.*, 2014; Aires *et al.*, 2012; Lefsrud *et al.*, 2007); moreover they show the highest antioxidant activity among other *Brassicas* (Podsędek, 2007).

GLS are particularly important because of the anticancer properties of their metabolic products (Verkerk *et al.*, 2009). Kale are the richest source of indolic GLS, and glucobrassicin (GB) represents one of the most abundant indolic GLS, although in different amounts (D'Antuono *et al.*, 2007; Velasco *et al.*, 2007). Indole-3-carbinol (I3C) has been identified as the major breakdown product from GB degradation, after the enzymatic action of myrosinase (Chevolleau *et al.*, 1997; Latxague *et al.*, 1991). Also ascorbigen (indol-3-ylmethyl-ascorbate, ABG), the result of the reaction of I3C with ascorbic acid (AA), (Aleksandrova *et al.*, 1992) possess cancer chemopreventive properties (Bonnesen *et al.*, 2001). In a previous work Hrnčiřík *et al.*(2001) calculated low conversion values of GB into ABG during homogenization of *Brassicas*, demonstrating that pH values of fresh *Brassica* vegetables differ from optimal pH for ABG formation. Kale is rich in ABG precursors so it could be interesting to evaluate their conversion in indole-derived compounds during processing.

Kale leaves represent a characteristic ingredient in the preparation of traditional dishes such as soups, gruels, side dishes and green filled pastries. The most common ways of kale preparation are water boiling, with or without a starchy matrix (e.g. potato, as it is for the Dutch dish "stamppot"), steaming and stewing in a pan with oil. In some cases, the preparation of traditional dishes involves cooking in water for very long times (e.g. the Italian "ribollita").

During heating of *Brassica* vegetables several mechanisms take place affecting the GLS content, such as thermal degradation, inactivation of enzyme myrosinase, and leaching of GLS and breakdown products (Nugrahedi *et al.*, 2015; Dekker *et al.*, 2000). Mathematical models could represent a useful tool to predict relevant quality aspects of food, such as nutrient content (Van Boekel, 2008). Several studies have investigated and modelled the trend of GLS during thermal treatment (Hennig *et al.*, 2012; Sarvan *et al.*, 2012).

Other works have evaluated the effect of thermal treatment or method of preservation on the content of bioactives in kale (Korus, 2013; Palermo *et al.*, 2013; Sikora and Bodziarczyk, 2012; Korus and Lisiewska, 2011).

In this study three varieties of kale, representative of Northern (the Dutch "Boerenkool") and Southern Europe (the Italian "Nero di Toscana" and "Broccolo Lavagnino") were studied. The main objective was to evaluate the fate and retention of various bioactive and antioxidant compounds (glucosinolates, I3C, ABG, carotenoids) and underlying mechanisms for three types of traditional prepared kale varieties. The applied modelling approach facilitates the comparison of the fate of these compounds between different cooking ways. Moreover, the effect of cooking on the formation of I3C, ABG and their precursors were investigated.

MATERIALS AND METHODS

Chemicals

Solvents used for chromatographic analyses were of high-performance liquid chromatography (HPLC) grade (Biosolve, Valkenswaard, The Netherlands). DEAE Sephadex A-25, indole-3-carbinol, lutein, β-carotene, gallic acid, ascorbic acid, tris-2-carboxyethyl phosphine, *meta*-phosphoric acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and trolox were bought from Sigma-Aldrich (Zwijndrecht, The Netherlands). The internal standard glucotropaeolin was purchased from the Laboratory of Biochemistry, Plant Breeding and Acclimatization Institute (Radzikow, Błonie, Poland). *Tert*-butylhydroquinone and orthophosphoric acid were supplied by Merck Millipore (Amsterdam, The Netherlands).

Samples and experimental plan

Three kale varieties were used for cooking studies: the Dutch "Boerenkool", and the Italian "Broccolo Lavagnino" and "Nero di Toscana". Boerenkool and Nero di Toscana seeds were purchased from seed firms, whereas Broccolo Lavagnino seeds were provided from a local Italian farmer. Sowing was done in cardboard alveolated trays on March 26, 2014; the seedlings were then transplanted in a field located in Wageningen (The Netherlands), on April 12. Harvest took place 7 weeks later; the leaves were selected, kept at 4°C and immediately processed.

The experimental treatments included three cooking systems: boiling, steaming and stir-frying, combined with four cooking times in the case of boiling and steaming (5, 10, 30 and 60 minutes), and three cooking times (5, 10 and 20 minutes) for stir-frying. All the cooking experiments were conducted in triplicate.

Sample processing

Kale leaves, at the same maturity grade and removed from damaged parts, were cut in stripes (2 cm width). A part of cut raw leaves was frozen overnight at -20°C, subsequently freeze-dried (Christ Alpha 1-4 LD Freeze Dryer, SciQuip Ltd, Newton, United Kingdom) and considered as control. 50 g of kale leaves were used for each cooking trial.

In the boiling experiment raw leaves were immersed in 1 L of boiling water and occasionally stirred with a ladle during cooking; kale leaves and water cooking were sampled after 5, 10, 30 and 60 minutes. Steaming process was performed using a steam oven by Miele (The Netherlands), and putting 50 g of raw leaves in the specific tray; again kale leaves were sampled after 5, 10, 30 and 60 minutes. For stir-frying 10 mL of sunflower oil were pre-warmed in a pan; raw leaves were added after 2 minutes, when the oil had reached the temperature of 140°C, spread in all the surface of the pan and stirred regularly; kale leaves were sampled after 5, 10 and 20 minutes. Cooked products, drained from the water in case of boiling, were cooled on ice for 2 minutes, and exactly weighed.

As already mentioned for the control ones, samples of kale were then stored overnight at -20°C, and freeze-dried till constant weight. Freeze-dried material was ground to a fine powder by using a Waring blender (model 34BL99, Dynamics Corp. of America, New Hartford, CT, USA), and stored at -20°C till further analyses.

The remained volume of water used for boiling was measured and a part was collected and stored at -20°C till further analyses.

Glucosinolate extraction and analyses

The method already described by Oliviero *et al.* (2012) was used with some modifications. 0.1 g of freeze-dried sample was extracted using methanol preheated at 75°C, in order to inactivate endogenous myrosinase. 200 μL of 3 mM glucotropaeolin solution were added as internal standard and samples were incubated in a water bath of 75°C for 20 min. Samples were subsequently centrifuged, and supernatants were collected and re-extracted twice with hot 70% methanol. The

extracted glucosinolates were desulphated and the separation was conducted using a LiChrospher® 100 RP-18 column 5μm (250 × 4.6 mm), furnished of a proper guard column. Elution from the HPLC column was performed by a gradient of water (A) and acetonitrile (B), as follows: from 0 to 2 min, 0% B; from 2 to 7,5 min, 0-8% B; from 7,5 to 14 min, 8-25% B; from 14 to 18 min, 25% B; from 18 to 20 min, 25-0% B; from 20 to 25 min, 0% B as post-run. The flow rate was 1 mL min^{-1} and the injection volume was 20 μL. Detection of the desulphoglucosinolates was performed at ʎ = 229 nm.

Determination of indole-GLS derived compounds: ascorbigen (ABG) and indole-3-carbinol (I3C)

ABG and I3C were simultaneously extracted as reported by Peñas *et al.* (2012). Freeze-dried powders were analyzed with slight modification of previously published methods (Matthäus and Fiebig, 1996). 1 g of sample was hydrolyzed in water (c = 50 mg mL^{-1}) for 4 hours in the dark and shaking periodically. Solutions were centrifuged at 3396 × *g* at 4°C for 15 minutes and supernatants were extracted with dichloromethane. Extracts were filtered through a paper filter filled with sodium sulphate, dried in rotavapor and recovered in 1 mL of acetonitrile. Before injection in HPLC samples were filtered through a 0.20 μm RC membrane filter (Phenomenex) into an HPLC amber vial. Samples of cooking water were treated in the same way as reported above, without the hydrolyzation step.

Separation was conducted using the same column and HPLC system already described for GLS extraction. Elution from the HPLC column was performed by a gradient of water (A) and acetonitrile (B), as follows: from 0 to 15 min, 20-100% B; from 15 to 20 min, 100% B; from 20 to 25 min, 20% B as post-run. The flow rate was 1 mL min^{-1} and the injection volume was 50 μL. Detection was performed at ʎ = 280 nm.

Carotenoids

Carotenoids were extracted from freeze-dried samples according to Lefsrud *et al.* (2007), and Ferioli *et al.* (2013). 50 mg of lyophilized material were extracted using 2.5 mL of tetrahydrofuran (THF) stabilized with butylated hydroxytoluene (BHT; c = 25 mg mL^{-1}). The extraction with stabilized THF was repeated three times more; the collected supernatants were dried with rotavapor and recovered in THF-methanol, 1:1 (*v/v*). Before HPLC analyses, samples were centrifuged at

15000 × *g* (10 minutes, 10°C). HPLC analyses were carried out on a UltiMate 3000 UHPLC system from Thermo Fisher Scientific equipped with a RD diode array detector. Separation was conducted using a Vydac RP C18 5.0 μm 250 × 4.6 mm column, maintained at 25°C using a thermostatic column compartment. Eluents were A: 88% methanol, 10% Milli-Q water, 2% THF, and 0.1% triethylamine (TEA), and B: 92.5% methanol, 7.5% THF, and 0.1% TEA. The flow rate was 0.7 mL min^{-1} and the gradient was 0% B for 12 minutes; from 12 to 15 minutes, 0-100% B; from 15 to 30 minutes, 100% B; from 30 to 40 minutes, 100-0% B; from 40 to 45 minutes, 0% B as post-run. Eluted carotenoids were determined at 452 nm. β-carotene and lutein stock solutions were prepared and test dilutions were prepared in the range 0.001-0.400 mg mL^{-1}.

Total ascorbic acid determination

Total ascorbic acid concentration was calculated as the sum of ascorbic acid and dehydroascorbic acid, according to Wechtersbach and Cigic (2007), with some modification as reported by Jin *et al*. (2014). 0.5 g of freeze-dried sample were extracted with 5 mL of *meta*-phosphoric acid (MPA) *tert*-butylhydroquinone (THBQ) solution (3 g 100 g^{-1} MPA, 1 mmol L^{-1} THBQ in Milli-Q water). The supernatant was collected in a new tube and the pellet was re-extracted with 5 mL of the MPA-THBQ solution, twice; the supernatants, collected in the same tube, was exactly weighed. To determine ascorbic acid, the extract was centrifuged at 12326 × *g* for 10 minutes at 4°C, filtered and injected in HPLC. To determine the total ascorbic acid, 15 μL of tris-2-carboxyethyl phosphine solution (1 mol L^{-1} in Milli-Q water) was added to 1.485 mL of the extract, incubated in the dark for 20 minutes and analyzed by HPLC. Separation was conducted using a Varian Polaris C18-A column 5μm (150 × 4.6 mm), with a Varian ChromSep SS 10 × 3.0 mm guard column. The flow rate was 1 mL min^{-1} and the injection volume was 20 μL. Elution was performed using 0.2% orthophosphoric acid in Milli-Q water, for a run time of 5.5 minutes.

A stock standard solution of ascorbic acid in MPA-THBQ solution was prepared; test solutions were diluted in the range 1.56-200 μg mL^{-1} in MPA-THBQ solution and used for the calibration curve.

Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu method, as described by Heimler *et al*. (2006). Phenolic compounds were extracted using the same hydro-alcoholic mixture

obtained from GLS extraction. To 125 µL of the extract, 0.5 mL of distilled water and 125 µL of the Folin-Ciocalteu reagent were added. After mixing it was kept in the dark for 6 minutes; after that 1.25 mL of a 7% Na_2CO_3 solution was added and the final volume was adjusted to 4 mL with distilled water. The mixture was kept in the dark for 90 minutes and then absorption at 760 nm was determined by UV-Vis spectrophotometer (Cary 50 BIO, Varian, Inc., Palo Alto, California, USA) against water as a blank.

A gallic acid stock solution was prepared in methanol; test solutions were prepared in the range 0.020-0.500 mg mL^{-1} in methanol and used for the calibration curve. Total phenolics were expressed as gallic acid equivalents (GAE, mg gallic acid kg^{-1} of kale, dry weight (dw)).

Antioxidant activity

Total antioxidant activity was determined in the same hydro-alcoholic extract obtained from GLS extraction, as described above. The activity of cooked samples was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, according to Brand-Williams *et al.* (1995). 0.1 mL of hydro-alcoholic extract was added to 3.9 mL of a 6×10^{-5} mol L^{-1} methanolic $DPPH^{\bullet}$ solution. Samples were incubated in a water bath at 25°C, during 30 minutes, then absorption at 515 nm was determined by UV-Vis spectrophotometer (Cary 50 BIO, Varian, Inc., Palo Alto, California, USA).

A trolox stock solution was prepared in methanol; test solutions were prepared in the range 0.020-0.200 mg mL^{-1} in methanol and used for the calibration curve.

Values obtained for antioxidant activity were expressed as mg of trolox equivalents kg^{-1} of kale (dw).

Colour determination

Colour of samples was determined as reported by Bongoni *et al*. (2014). Powder samples were placed into the cuvette of ColorFlex meter (Hunter Associates Laboratory, USA), with the lid closed and measured at two angles for the following parameters: L* (lightness), a* (redness) and b* (yellowness). The following parameters were calculated, according to Mc Guire (1992):

1) $C^* = \sqrt{a^{*2} + b^{*2}}$ (colour saturation, or chroma)
2) $H = \left| tan^{-1}\left(\frac{b^*}{a^*}\right)\right|$ (hue)

Data processing

The data of all traits were subject to three-way ANOVA, including variety, way of cooking and cooking time, and their interactions, as experimental factors.

The patterns of individual traits as a function of cooking time, within variety and cooking way, were fitted by means of degradation kinetic equations, of different order, according to the scheme already illustrated elsewhere (Giambanelli *et al.*, 2015), when applicable. Briefly models of order 0, 1, 2, and n, where order n was estimated as a parameter, were fitted to data and compared by means of the corrected Akaike information criterion (Burnham and Anderson, 2002).

For data fitting, the content of individual compound relative to time zero (raw material) were used, except for colour traits. In case in which no adequate fitting was obtained by kinetic models, the significance of differences between varieties and cooking ways, within cooking time, were assessed by means of the protected Fisher's LSD test.

All the statistical analyses were carried out by means of the SYSTAT® package.

RESULTS AND DISCUSSION

Raw material profile

The values of phytochemical compounds contents and colour traits of raw materials are reported in **Table 5.2.1**.

Total glucosinolate content ranged between 4.19-6.96 mmol kg^{-1} dw, with Nero di Toscana showing the highest value, and Boerenkool the lowest.

The glucosinolate profile included 7 compounds: the aliphatic glucosinolates glucoiberin (GIB), sinigrin (SIN) and glucoraphanin (GR), and the indolic glucosinolates glucobrassicin (GB), 4-hydroxy-glucobrassicin (H4GB), 4-methoxy-glucobrassicin (M4GB), and neo-glucobrassicin (NGB). Indolic GLS accounted for 62-80%, with the highest percentage detected in Boerenkool. GB represented the most abundant GLS (47-51% on the total amount of GLS), except for Nero di Toscana (30%) where neo-glucobrassicin was prevailing (32%). Aliphatic GLS were more abundant in Nero di Toscana (38%) that also showed the highest sinigrin percentage (18%), while Broccolo Lavagnino and Boerenkool showed a higher glucoraphanin and glucoiberin content (about 13%).

Table 5.2.1. Phytochemical compounds and colour traits in raw kale samples.

	Landraces			
	BL	**NT**	**BO**	**Significance[a] (LSD)[b]**
		mean ± SD		
Total Glucosinolates (mmol kg^{-1})	5.30 ± 0.36	6.96 ± 0.26	4.19 ± 0.15	** (0.81)
Relative contents (mmol mmol^{-1} total glucosinolates)				
Glucoiberin	0.067 ± 0.003	0.104 ± 0.009	0.129 ± 0.008	** (0.022)
Sinigrin	0.081 ± 0.007	0.176 ±0.009	0.059 ± 0.004	** (0.022)
Glucoraphanin	0.128 ± 0.007	0.100 ± 0.001	0.010 ± 0.002	** (0.014)
4-hydroxy-glucobrassicin	0.006 ± 0.001	0.007 ± 0.001	0.008 ± 0.001	** (0.002)
Glucobrassicin	0.510 ± 0.019	0.294 ± 0.035	0.469 ± 0.015	** (0.078)
4-methoxy-glucobrassicin	0.007 ± 0.001	0.002 ± 0.000	0.026 ± 0.001	** (0.004)
Neo-glucobrassicin	0.201 ± 0.003	0.315 ± 0.034	0.298 ± 0.012	** (0.066)
Aliphatic glucosinolates	0.276 ± 0.017	0.381 ± 0.001	0.199 ± 0.003	** (0.032)
Indolic glucosinolates	0.724 ± 0.017	0.619 ± 0.001	0.801 ± 0.003	** (0.032)
Indol-Glucosinolates derived compounds (mg kg^{-1})				
Ascorbigen	15.60 ± 0.41	6.18 ± 1.16	5.67 ± 1.25	** (2.38)
Indole-3-carbinol	21.53 ± 1.80	5.04 ± 1.07	16.63 ± 2.11	** (2.29)
Total Ascorbic acid (mg kg^{-1})	5629 ± 1547	8087 ± 595	5301 ± 65	** (935)
Relative contents (mg mg^{-1} total ascorbic acid)				
Ascorbic acid	0.544 ± 0.001	0.657 ± 0.029	0.237 ± 0.005	** (0.115)
Dehydro-ascorbic acid	0.456 ± 0.001	0.343 ± 0.029	0.763 ± 0.005	** (0.022)
Total Carotenoids (mg kg^{-1})	3330 ± 351	3836 ± 927	3124 ± 425	ns
Relative contents (mg mg^{-1} total carotenoids)				
Neoxanthin	0.153 ± 0.016	0.150 ± 0.008	0.132 ± 0.006	ns
Violaxanthin	0.186 ± 0.054	0.148 ± 0.011	0.179 ± 0.031	ns
Lutein	0.384 ± 0.003	0.386 ± 0.012	0.359 ± 0.021	ns
β-carotene	0.277 ± 0.073	0.316 ± 0.009	0.330 ± 0.004	ns
Total phenolic content (mg kg^{-1})	19543 ± 475	17501 ± 330	22325 ± 187	** (3762)
Trolox equivalent antioxidant capacity (mg kg^{-1})	12016 ± 145	9548 ± 23	13177 ± 283	** (3221)
Colour traits				
L*	46.04 ± 0.34	45.00 ± 0.18	43.73 ± 0.10	** (0.60)
a*	-9.09 ± 0.05	-8.52 ± 0.04	-8.27 ± 0.06	** (0.13)
b*	25.82 ± 0.28	22.88 ± 0.04	21.39 ± 0.12	** (0.46)
Hue	109.40 ± 0.09	110.43 ± 0.07	111.14 ± 0.04	** (1.69)
Saturation	27.38 ± 0.28	24.41 ± 0.05	22.93 ± 0.13	** (1.57)

Legend:
[a] Significance: **: $p \leq 0.01$; [b] LSD, $p = 0.05$.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Total ascorbic acid (TAA), calculated as the sum of ascorbic (AA) and dehydro-ascorbic acid (DAA), ranged between 5301-8087 mg kg^{-1} dw, in raw leaves, with Nero di Toscana showing the highest value. Similar figures are reported in Becerra-Moreno *et al.* (2014). The three landraces also differed for their AA and DAA relative contents: in Boerenkool DAA prevailed (76%), whereas AA represented 54 and 66% of TAA in Broccolo Lavagnino and Nero di Toscana, respectively.

Indole-3-carbinol (I3C) and ascorbigen (ABG) were identified and quantified as indole GLS breakdown products. I3C is formed upon hydrolysis of GB. ABG is formed by a condensation reaction of I3C with ascorbic acid (AA). Raw Broccolo Lavagnino had the highest initial contents for both compounds.

Total carotenoid content was in the range 3124-3836 mg kg^{-1} dw, without significant differences among landraces. β-carotene and lutein were identified as the most abundant compounds, violaxanthin and neoxanthin as minor compounds. The relative lutein and β-carotene contents were rather similar, without differences among landraces, as for minor compounds, that accounted for 13-18% of total carotenoids.

Total phenolic content (TPC) ranged between 17501-22325 mg gallic acid kg^{-1} dw, with the highest value for Boerenkool. These values are comparable with previous results (Fiol *et al.*, 2013), but lower compared with the amounts detected on kale by Ismail *et al.* (2004). Likewise the highest antioxidant activity was observed in raw Boerenkool leaves (13177 mg Trolox kg^{-1} dw).
Hue values indicate a greyish-green colour for all landraces, with lower yellow component and lightness in Nero di Toscana.

Evolution of analytical and colour traits during cooking

Glucosinolates. **Figures 5.2.1** and **5.2.2** report the patterns of total, aliphatic, indolic glucosinolates, and glucobrassicin, as a function of cooking time, together with the values of the best fit kinetic parameter (k), when applicable. In the case of boiling, two separate graphs report the patterns of compounds in the leaf matrix only and in leaf matrix+boiling water.
Data analysis indicated significant interactions between all the experimental factors, meaning that the evolution of compound content as a function of cooking time differed between both cooking systems and varieties.

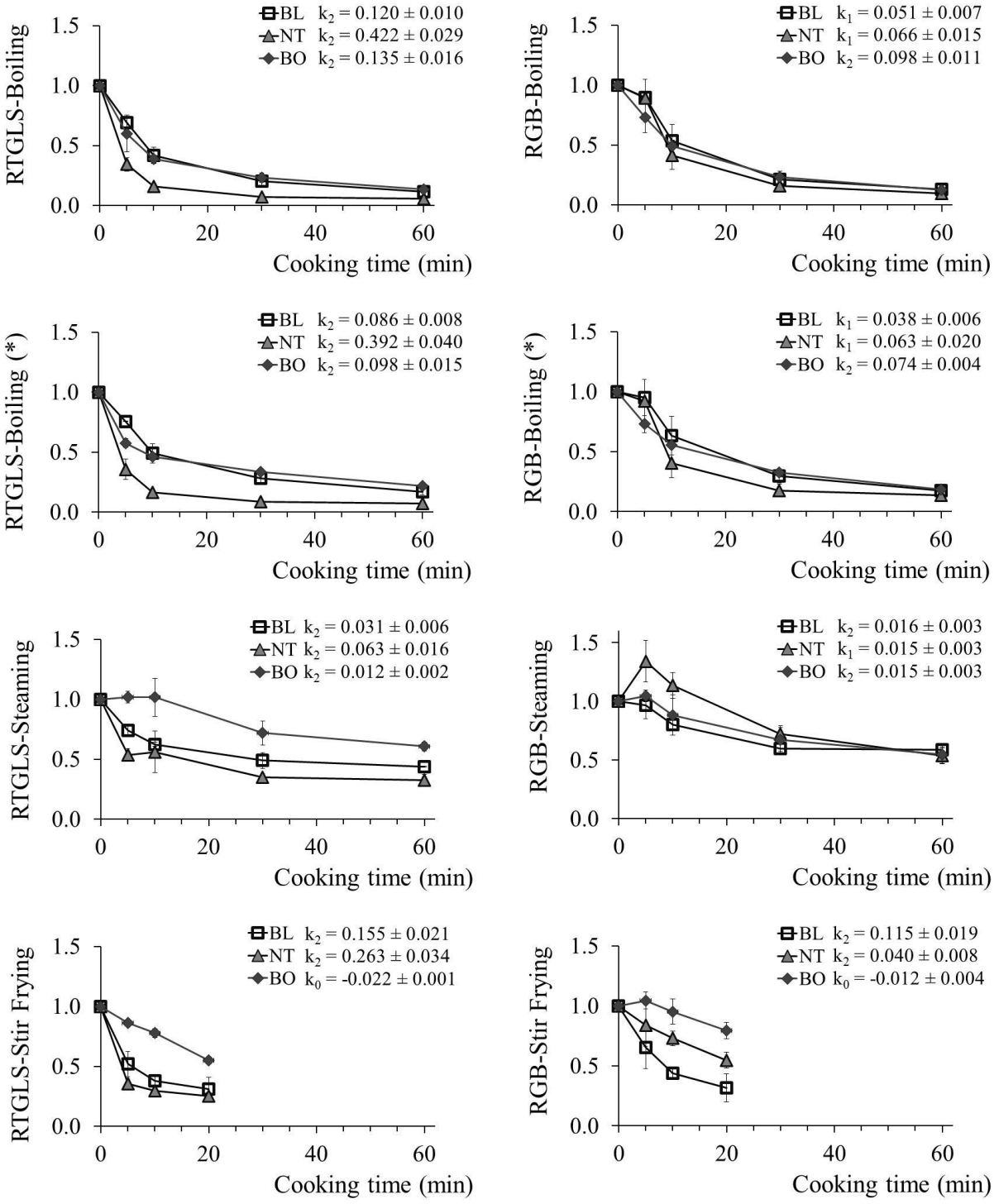

Figure 5.2.1. Relative total glucosinolate and glucobrassicin contents as affected by cooking method and time.

Legend:
RTGLS: total glucosinolate content relative to time zero; RGB: glucobrassicin content relative to time zero.
(*): sum of the content detected in boiled leaves and cooking water.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

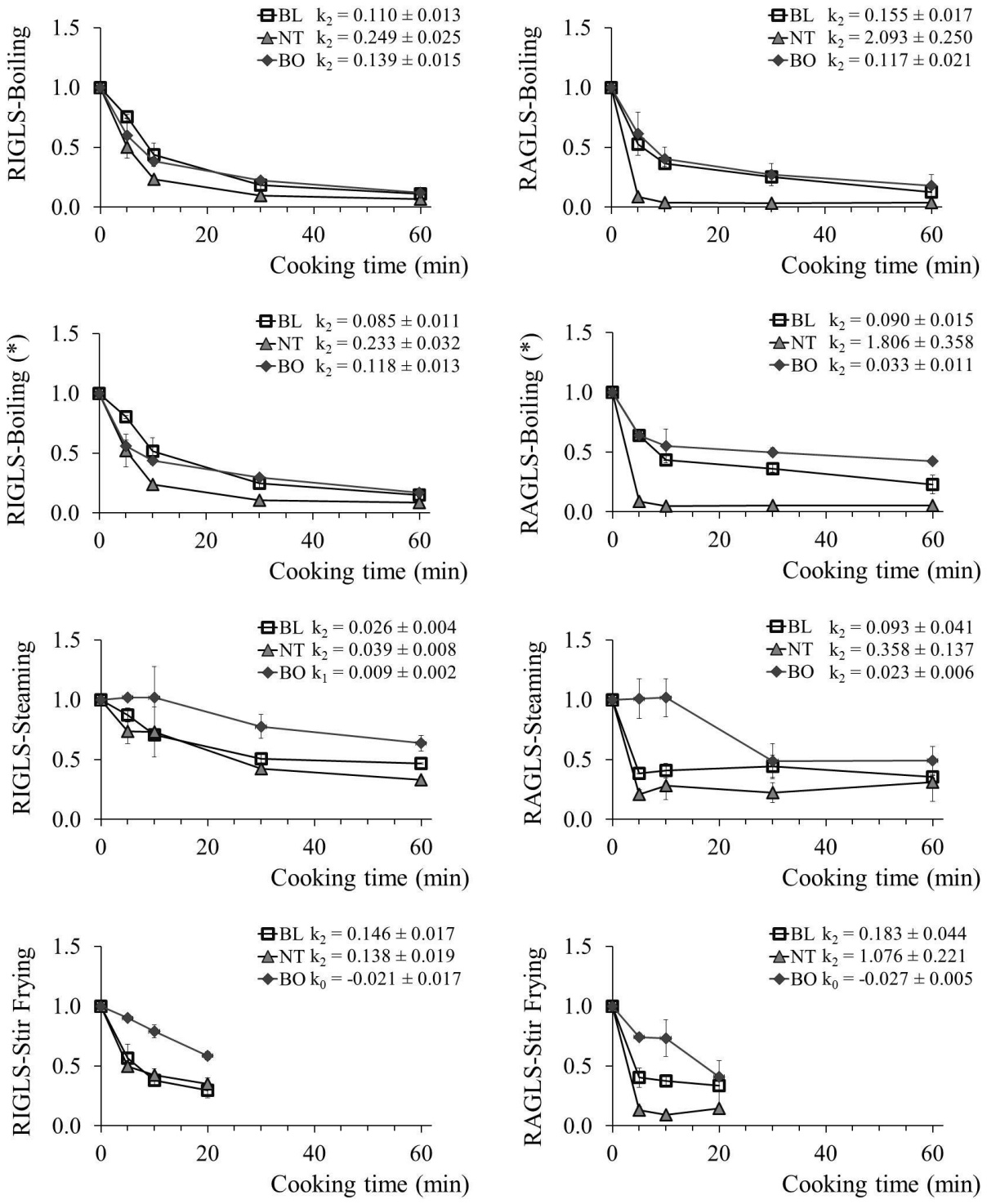

Figure 5.2.2. Relative indolic and aliphatic glucosinolate contents as affected by cooking method and time.

Legend:
RIGLS: indolic glucosinolate content relative to time zero; RAGLS: aliphatic glucosinolate content relative to time zero.
(*):sum of the content detected in boiled leaves and cooking water.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Previous works (Hennig *et al.*, 2012; Oliviero *et al.*, 2012) have described GLS thermal degradation as a first order kinetic reaction. In the present study, following Akaike selection procedure total GLS degradation during boiling followed a second order kinetic. In this case, GLS breakdown was faster in Nero di Toscana, as indicated by its significantly higher degradation kinetic constant with respect to the other two landraces (**Figure 5.2.1**).

An overall higher retention of glucosinolates was detected when cooking water was included (a lower k_d in all situations); in this case, the difference between Nero di Toscana and the other two landraces was even higher than what detected for leaves only. As a result, the relative GLS content of Nero di Toscana decreased to less than 20% its initial values after 10 minutes boiling, compared to about 50% retention of the two other landraces.

Steaming determined a quite well characterized pattern. For Boerenkool no apparent decrease of GLS content was detected during the first 10 minutes, with a subsequent overall slightly significant decrease; this is perhaps due to a combination of the effect of leaf structure and GLS type, determining an overall low degradation and better extractability, at shorter steaming times.

The pattern of the other two landraces was represented by a second order kinetic, with again higher constant for Nero di Toscana. Overall, steaming determined a substantial lower GLS loss than boiling.

Stir frying also determined a different GLS evolution, depending on landrace. In Boerenkool a moderate linear decrease was observed, whereas a second order kinetic represented GLS evolution of the other two landraces. Nero di Toscana showed the fastest degradation for the relative TGLS content, also for this cooking method, although the differences with Broccolo Lavagnino were less pronounced than for the other cooking methods. Indeed the degradation was substantially faster for aliphatic glucosinolates, whereas glucobrassicin degradation rate was faster in BL. Beside this, second order kinetic constant, and so GLS degradation, of stir frying were higher than for boiling in Broccolo Lavagnino and lower in Nero di Toscana making the overall difference between the two varieties non significant. Although these facts likely depend on differences of leaf structure, our data do not allow to identify the specific determinant traits.

The pattern of indolic and aliphatic GLS was rather different (**Figure 5.2.2**). Indole GLS followed trends similar to what explained for total GLS, with generally lower kinetic breakdown constants, except for Boerenkool during boiling, and lower differences between landraces; Nero di Toscana and Broccolo Lavagnino did not differ in stir frying. On the contrary, the degradation of aliphatic GLS appeared to occur quite rapidly during the first minutes of cooking, with very high kinetic degradation constants for Nero di Toscana, especially for boiling and stir frying. Indeed this landrace had higher content of aliphatic GLS, less stable to cooking: for example, after 5 minutes of

boiling glucoiberin was not anymore detectable in Nero di Toscana leaves, whereas sinigrin and glucoraphanin accounted, respectively, for 13 and 7% of their initial content. Low amounts of aliphatic GLS were detected in cooking water, giving lower recovery factors.

Glucobrassicin degradation pattern did not differ among varieties for boiling, although best fit was given by a first order kinetic for Broccolo Lavagnino and Nero di Toscana and by a second order for Boerenkool. Glucobrassicin degraded less rapidly in steamed Nero di Toscana with respect to the other two landraces, and with respect to Broccolo Lavagnino during stir frying.

Overall, steaming most retained GLS, whereas the difference between boiling and stir frying was strongly dependent on the characteristics of the variety.

It has been demonstrated that the food matrix plays an important role in GLS thermal degradation (Hennig *et al.*, 2013; Dekker *et al.*, 2009). Also in our study the three landraces had different retention of GLS during cooking. In this respect, phenotypical features could play a role. Overall, thinner leaves of Boerenkool were associated with lower degradation. Nero di Toscana had very crinkled leaves that could contribute to a larger surface, increasing the exposure to cooking water and heating; moreover its fleshy texture could lead to a more drastic collapse of the structure during cooking. In a previous study, Rosa and Heaney (1993) found differences of GLS losses between cultivars, attributing this fact to differences in leaf characteristics, such as thickness and waxiness.

Ascorbic acid. Total ascorbic acid showed a decreasing pattern, which was not fitted by a model, allowing consistent comparisons between landraces and cooking systems on the basis of kinetic parameters (**Figure 5.2.3**): in fact, although some significance of overall data fitting was obtained, the initial pattern of ascorbic acid, that remained almost constant during the first minutes of cooking, is not consistent with a monotonously decreasing function. Between cooking methods, the differences between varieties were rather low and generally not significant, within cooking times; Broccolo Lavagnino showed a slower initial decrease, determining an overall lower order of degradation kinetic. Overall, steaming allowed a higher retention at intermediate cooking times (10-20 min), whereas the differences with boiling were not significant after 60 minutes cooking.

This pattern of total ascorbic acid was generated by two rather different trends of DAA and AA. In fact, DAA decreased to only about 4-13% of initial DAA after five minutes cooking; this fact was clearly connected to its conversion into AA, as demonstrated by the strong increase of the latter after five minutes cooking and followed by degradation for longer cooking times. The consistency of this interpretation is confirmed by the fact that initial AA increase was particularly high in Boerenkool, in which DAA represented over 75% total ascorbic acid.

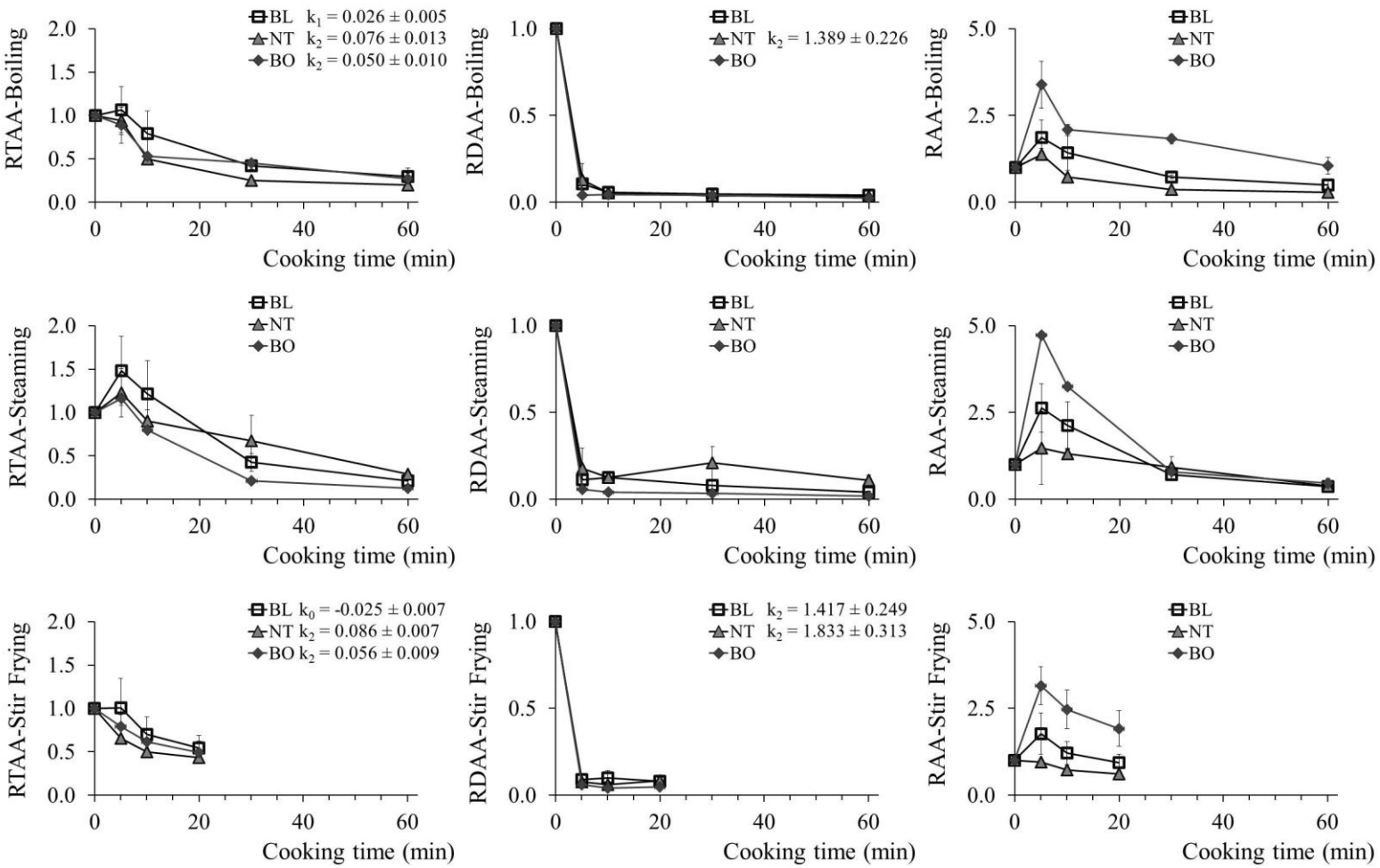

Figure 5.2.3. Relative total, dehydro- and ascorbic acid contents as affected by cooking method and time.

Legend: RTAA: total ascorbic acid content relative to time zero; RDAA: dehydro-ascorbic acid content relative to time zero; RAA: ascorbic acid content relative to time zero.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Indole-3-carbinol and ascorbigen formation. **Figure 5.2.4** reports the values of I3C and ABG conversion factors from glucobrassicin, obtained relating I3C or ABG molar content to the glucobrassicin molar content in the same sample. The conversion values obtained were quite low (from non-detectable to 23.5%).

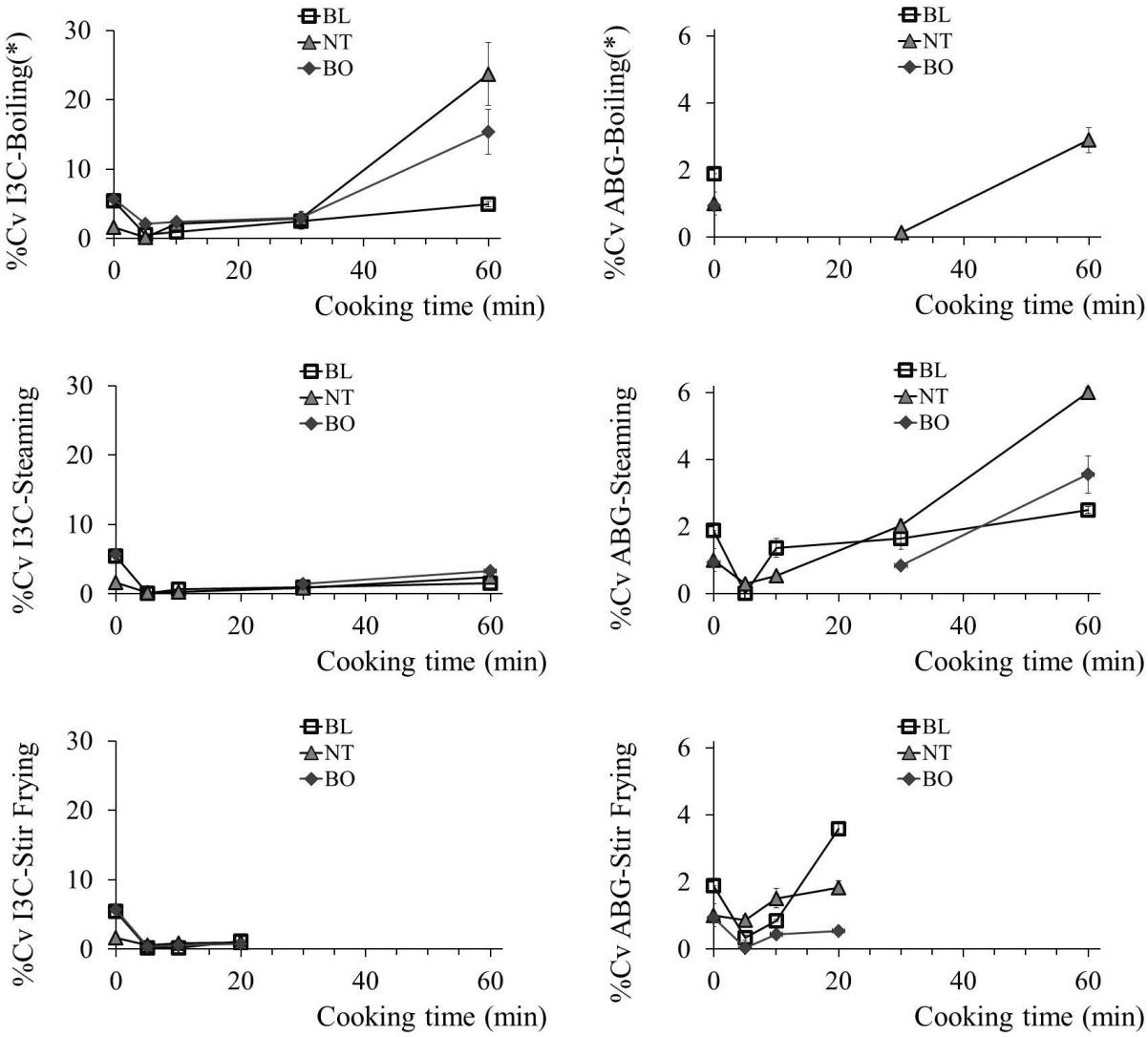

Figure 5.2.4. Conversion value percentage (%Cv) of glucobrassicin into indole-3-carbinol and ascorbigen as affected by cooking method and time.

Legend:
I3C: indole-3-carbinol; ABG: ascorbigen.
(*):sum of the content detected in boiled leaves and cooking water.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool

Indole-GLS derived compounds were not detectable in boiled leaves, independently on variety. I3C was however detected in cooking water. The absolute content of this compound does not indicate a clear pattern, apart from the highest values at 60 minutes boiling or steaming, since they derive from the balance of glucobrassicin conversion and external conditions. However, the pattern of conversion coefficient from glucobrassicin is much clearer. In boiling water, conversion linearly increased, with no significant differences among varieties, until 30 minutes cooking; after 60 minutes the conversion dramatically increased for Nero di Toscana and Boerenkool, whereas it maintained a linear pattern in Broccolo Lavagnino. Conversion was lower and linear, without a final increase in all varieties for steaming, with Broccolo Lavagnino having again the lowest increases as a function of cooking time. GB conversion into I3C was finally very low for stir fried kale leaves.

Also for ABG the absolute values of its content do not reveal a clear pattern, that is however detectable for conversion factors. Also in this case a higher increase of the conversion value was noticed for longer cooking times, especially for Nero di Toscana upon steaming and boiling, and Broccolo Lavagnino during stir frying.

The almost complete absence of ABG following boiling can be related to the findings of Ciska *et al.*, (2009) showing a decrease of ABG in fermented cabbage as a function of boiling time, mainly due to leaching, whereas I3C resulted less affected.

Carotenoids. Pigment profiles after cooking are represented in **Figure 5.2.5**. An asymptotic increase of total carotenes, β-carotene and lutein, for boiling, and of β-carotene for steaming was observed. In case of β-carotene, this increment was significantly higher for Broccolo Lavagnino and also for Boerenkool, in case of boiling. This pattern is likely due to a higher extractability of these compounds (Lemmens *et al.*, 2009). Dos Reis *et al*. (2015) also observed that steam processing resulted in an increase of broccoli lutein and β-carotene extractability.

Lutein content, on the contrary, linearly decreased in steamed Boerenkool and Broccolo Lavagnino leaves, to about 54-42% the content of raw leaves, whereas no changes were observed in Nero di Toscana.

Stir frying caused a marked decrease of carotenoid content, with special respect to β-carotene, reducing its content to 10-23% of that of raw leaves, independently on variety.

Partially in agreement with our results, Chang *et al*. (2013) found large variations of carotenoids content after cooking: after boiling they observed lutein increase and β-carotene decrease, whereas stir-frying determined mainly a decrease of both compounds.

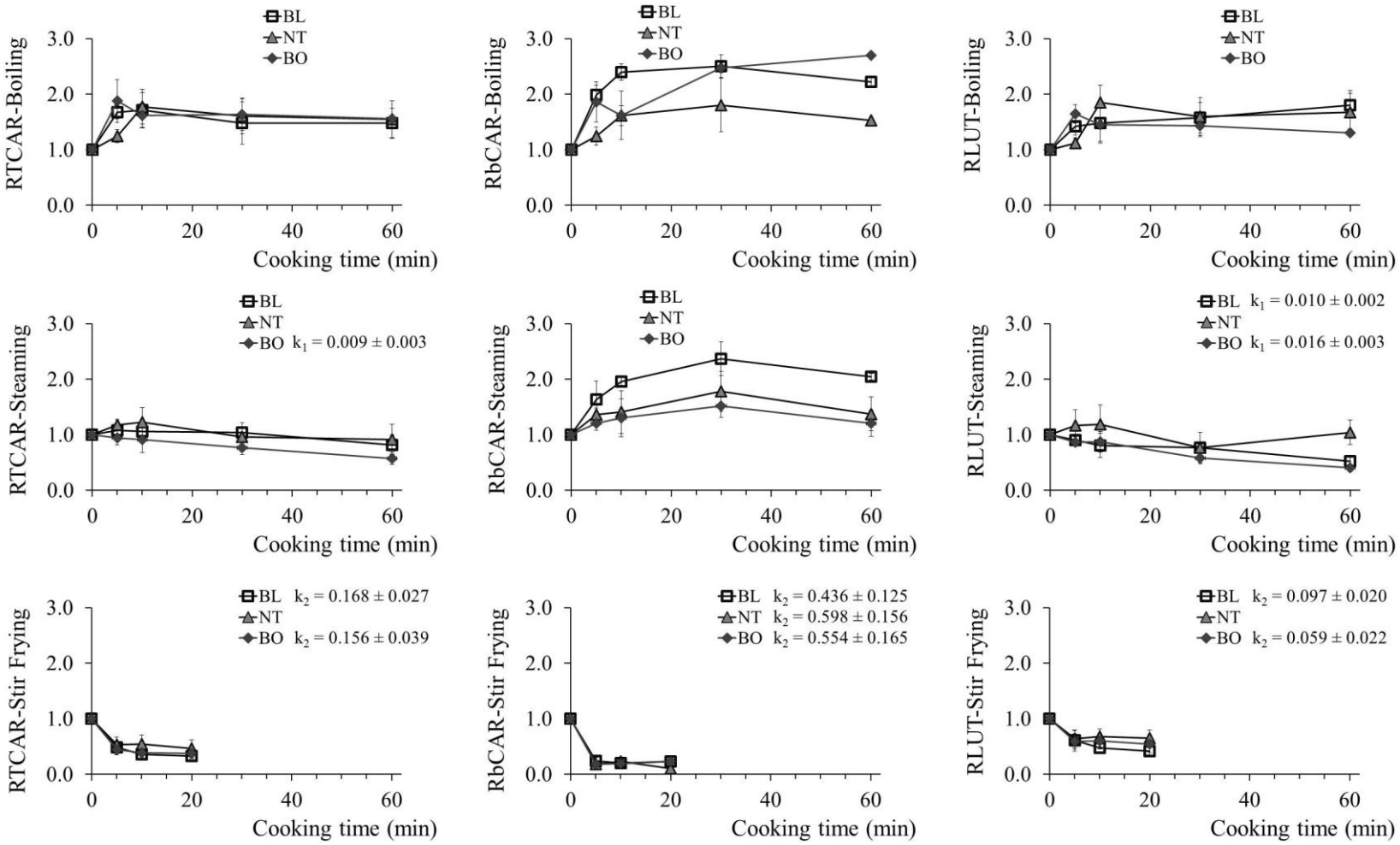

Figure 5.2.5. Relative contents of total carotenoids, β-carotene and lutein as affected by cooking method and time.

Legend: RTCAR: total carotenoid content relative to time zero; RbCAR: β-carotene content relative to time zero; RLUT: lutein content relative to time zero. BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

Different patterns of carotenoids after cooking are described; however in most cases, it was highlighted that water-cooking treatments better preserve these antioxidant compounds (Miglio *et al.*, 2008). Boiling and steaming seem to enhance carotenoids bioavailability, probably following cell wall structure disruption of the leaf matrix (Castenmiller *et al.*, 1999).

Total phenolic content (TPC) and antioxidant activity (TEAC). The trends observed for TPC and TEAC are shown in **Figure 5.2.6**. TPC loss from the leaf matrix during boiling followed a second order degradation kinetic, with only slightly lower constant for Broccolo Lavagnino.

However, high amounts of phenolic compounds were detected in boiling water, due to leaching; so when considering the sum of TPC in boiled leaves and cooking water no overall phenolic losses occurred for this cooking system. Steaming caused only a very limited linear TPC decrease, as indicated by the zero order kinetic.

Finally, stir frying determined a second order kinetic loss of phenolics, with constants somewhat lower with respect to what observed for boiling on the leaves only, and no differences among varieties.

The antioxidant activity relative to uncooked material followed different patterns. For boiled leaves and stir frying, its pattern was similar to that of TPC, with generally lower decrease rates. In boiling, the antioxidant activity remained indeed constant during the initial times of cooking. For steaming, an initial increase of relative antioxidant activity was detected, followed by a moderate decrease for longer cooking times, however resulting in an overall slightly significant linear decrease only for Nero di Toscana.

In case of boiled leaves plus cooking water, the pattern was substantially different from that observed on phenolics. In fact, a second order kinetic decrease represented it for all landraces, indicating that phenolics leached in water seemed to be subject to a substantial loss of antioxidant capacity, as represented in **Figure 5.2.7**. In fact the relation between TPC and antioxidant capacity, indicates a much higher antioxidant efficacy of phenolics retained in boiled leaves with respect to those leached in water. Besides this fact, the slope of the relation between TPC and antioxidant capacity was almost the same for raw and boiled leaves; however, boiled leaves reached comparable antioxidant effect at lower TPC concentration, supporting the idea of a lower antioxidant power of the phenolics leached in water.

Our results are in agreement with a previous work on red cabbage (Volden *et al.*, 2008), indicating a decrease of antioxidant activity following thermal treatment, except for steaming, confirming that this cooking way had a less marked effect on the compounds responsible of the antioxidant activity.

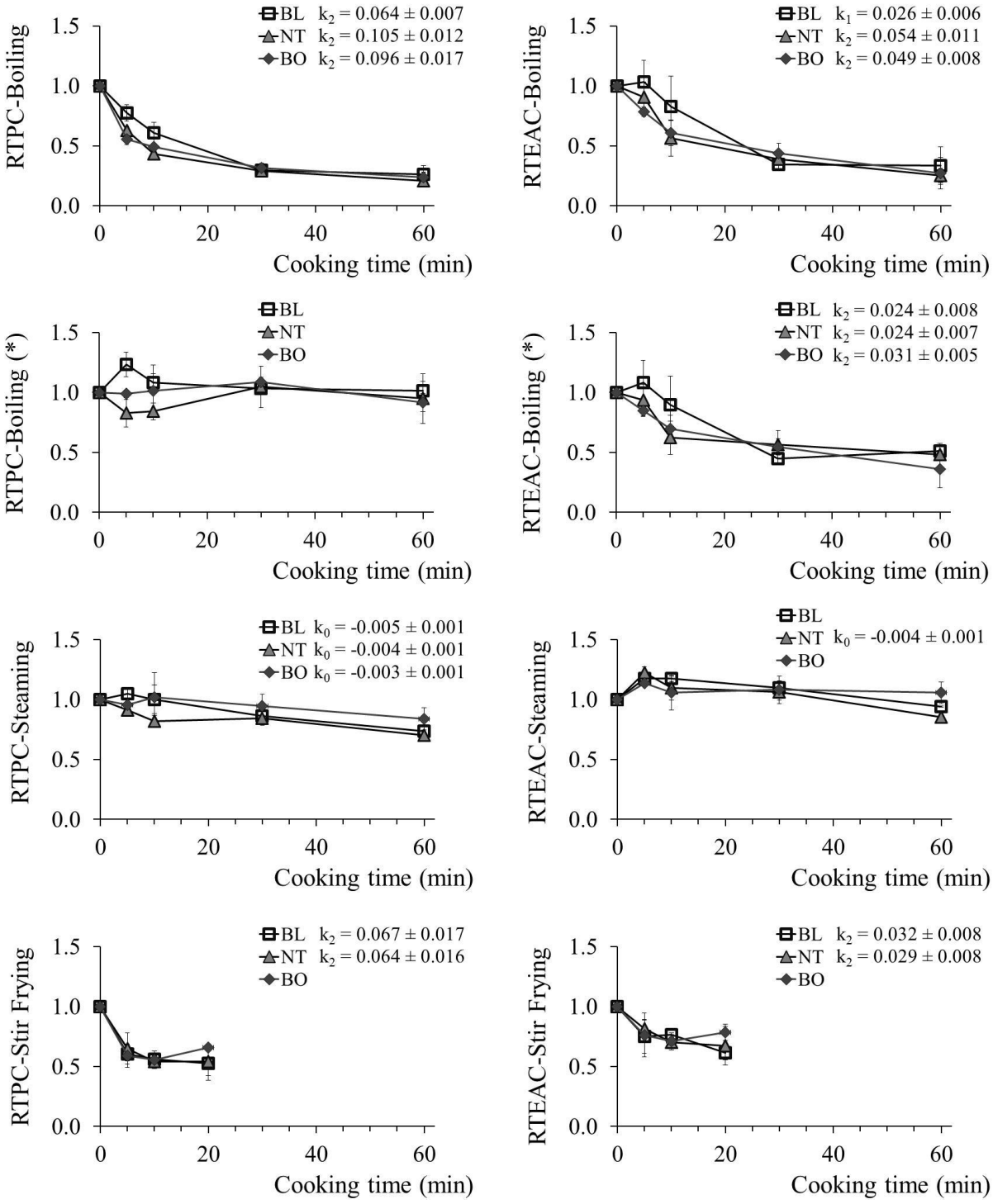

Figure 5.2.6. Relative total phenolic content and relative trolox equivalent antioxidant activity as affected by cooking method and time.

Legend:
RTPC: total phenolic content relative to time zero; RTEAC: trolox equivalent antioxidant activity relative to time zero.
(*):sum of the content detected in boiled leaves and cooking water.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

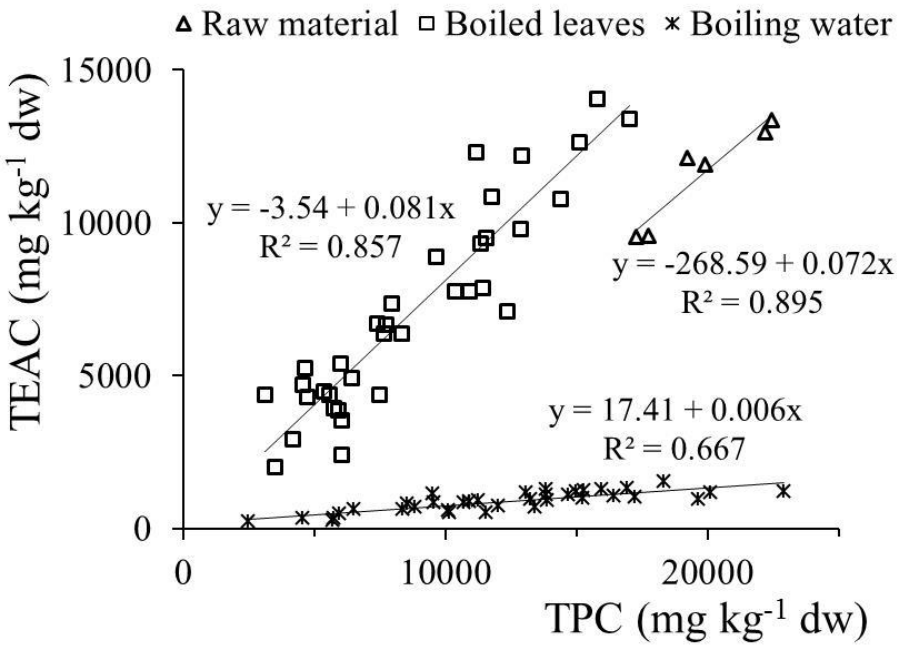

Figure 5.2.7. Correlation between antioxidant activity and total phenolic content of raw and boiled leaves, and cooking water.

Legend:
TPC: total phenolic content; TEAC: trolox equivalent antioxidant activity.

Colour traits. **Figure 5.2.8** represents the pattern of hue and saturation, that were chosen as better suitable traits to represent colour variation (Mc Guire, 1992), as affected by the experimental treatments.

Steaming and stir frying determined a more marked linear decrease of hue values, indicating a loss of green hue.

Colour saturation slightly increased during the first minutes of boiling; this trend can be connected to a change of surface-reflecting properties, as reported in the case of vegetable bleaching (Tijskens *et al.*, 2001); saturation then linearly decreased to slightly lower values than initial ones at longer boiling times. Saturation was not affected by steaming, whereas stir frying determined a decrease of colour saturation, represented by a second order degradation kinetic. This pattern, similar to the one detected on carotenoids, confirms that pigments are more sensitive to the more drastic thermal conditions occurring with stir frying.

Miglio *et al*. (2008) reported a decrease of broccoli florets *L** values in all cooking treatments used (boiling, steaming, frying). Moreover these authors found steamed and fried broccoli parts (stem and florets) to be less green with respect to the raw and the boiled products. The loss of greenness

observed in steaming was mainly due to the highest chlorophyll degradation, determined by a major exposure of kale leaves to air.

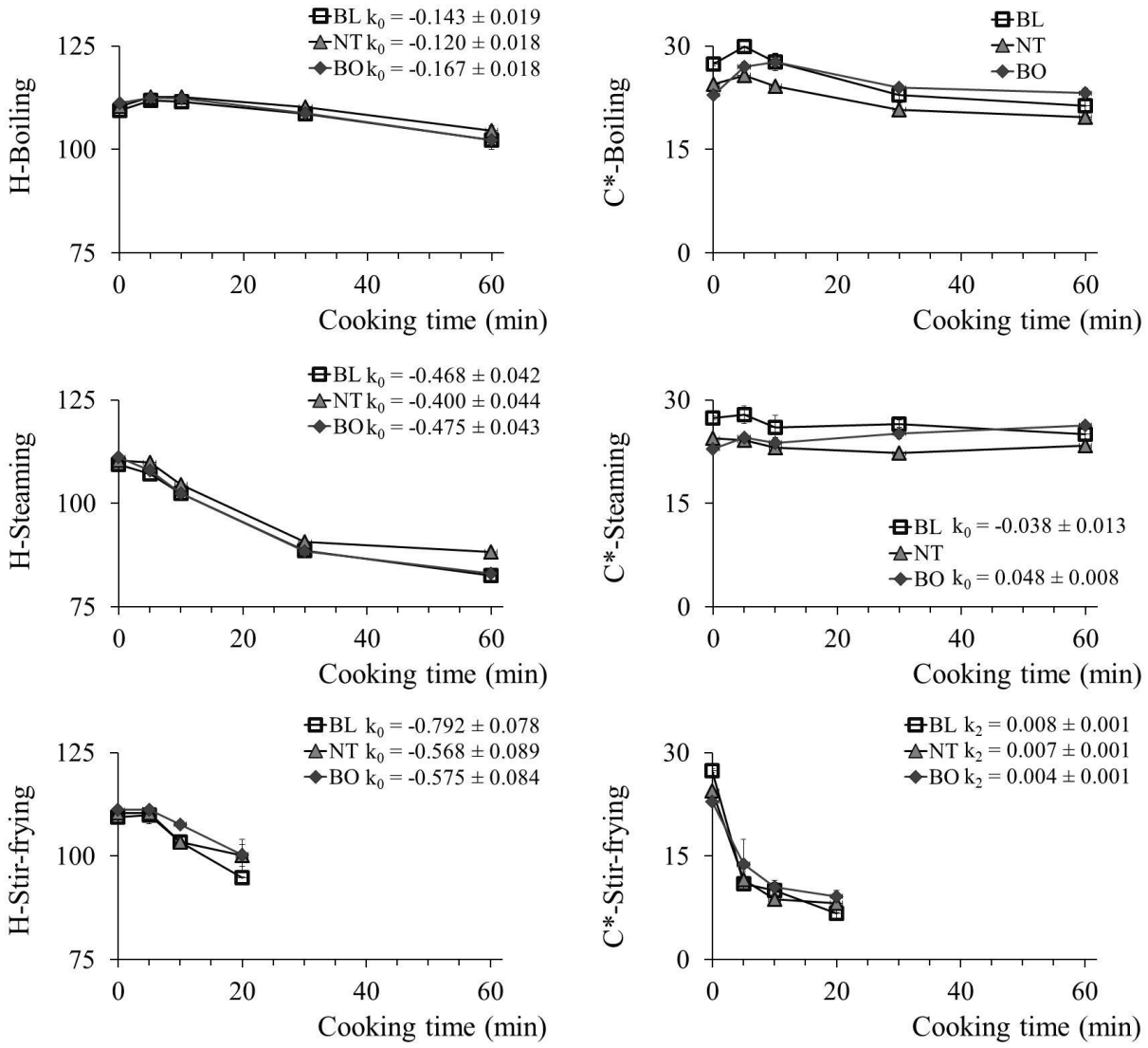

Figure 5.2.8. Colour traits as affected by cooking method and time.

Legend:
H: hue; C*: saturation.
BL: Broccolo Lavagnino; NT: Nero di Toscana; BO: Boerenkool.

CONCLUSIONS

This study was focused on kale, an ingredient of many traditional food preparations across Europe, that is also particularly rich of phytochemical compounds with potential health promoting properties, besides their impact on sensory quality.

The main aims of this work were the investigation of the effect of traditional cooking methods on key components, with special respect to boiling for long time or cooking at high temperature (stir frying), in comparison to the milder treatment of steaming, and the determination of kinetic constants. At the same time, differences among landraces were assessed.

Kale variety plays an important role in determining the fate of bioactive compounds, with special respect for glucosinolates. We could assume that phenotypical traits of kale leaves, such as shape, texture, composition of external layers, strictly related to the kale cultivar, could have a role in determining the phenomena of leaching and thermal damage. The kind of glucosinolate compound may however be involved. Nero di Toscana, showed the highest GLS contents in raw leaves, but, at the same time, was subject to the highest losses following thermal treatment.

Also the cooking method had a significant impact in the final content of bioactives. Carotenoids appeared to be the most stable compounds, but were subject to decay in stir frying. Total phenolic compounds and antioxidant activity were mainly affected by boiling (due in part to leaching) and stir-frying (major exposure to the oxygen associated with a higher temperature, due to the presence of oil). It is interesting to note that, total phenolics are apparently completely retained in traditional recipes in which the raw materials are boiled for long time, but the cooking water is conserved, as it occurs in some vegetable soups of the Italian tradition. However, the properties of phenolics may be subject to changes, as indicated by the different antioxidant capacity of phenolics in boiled leaves and cooking water of our experience.

Steaming showed the highest bioactive compound retention factors, while physical traits (green colour of the leaves) were mostly affected by this cooking method.

The content of putative health-promoting indole-derived compounds, indole-3-carbinol and ascorbigen, were very low in all thermal treated kale varieties, although these vegetables are very rich in their precursors. The low calculated conversion factors are probably caused by the inactivation of the myrosinase enzyme and the instability of these compounds during thermal treatment.

The results indicate that, although some ways of cooking are strictly inherent to traditional recipe preparation, some possibility of improving bioactive component retention may exist. The study of degradation kinetic allowed to calculate variety specific parameters, even if, the evolution of

phytochemicals hardly follows simple models in open systems such the ones used in this experience.

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Chapter 5.3

Broccoli glucosinolate degradation is reduced performing thermal treatment in binary systems with other food ingredients

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ABSTRACT

Glucosinolate (GL) stability has been widely studied in different *Brassica* species. However, the matrix effect determined by the presence of other ingredients occurred in many broccoli-based traditional recipes may affect GL thermal degradation. In this study, the matrix effect on GLs thermal degradation was investigated by means of binary systems containing broccoli and another ingredient such as potato, corn starch, lentils protein or onion. Data showed that in binary systems the GL degradation was lower compared to the only-broccoli system, in particular in the broccoli/onion systems. The kinetics of GL degradation in broccoli/onion systems at different ratios followed a second order model. Finally the possibility that the effect was related to the amount of flavonoids present in onions was ruled out by data obtained using broccoli/onion systems made with three onion varieties having different flavonoid content.

This study shows for the first time that the presence of other food ingredients can efficiently reduce GL thermal degradation. The protective effect of onion, often present in the traditional recipes of broccoli soups in many countries, point out that the interaction of different ingredients may not only improve the taste of a dish, but also the healthiness.

Keywords: glucosinolates; healthy cooking; thermal degradation; broccoli; model systems; kinetic.

INTRODUCTION

Vegetables of the *Brassicaceae* family such as broccoli, cabbage and Brussels sprouts are characterized by the presence of glucosinolates (GLs), a class of sulfur-containing bioactive compounds. Myrosinase (MYR) is the endogenous enzyme that catalyzes GL hydrolysis into isothiocyanates which have been extensively studied for their anticarcinogenic activities (Verkerk *et al.*, 2009; Moreno *et al.*, 2006; Stoewsand, 1995).

Broccoli (*Brassica oleracea* L. var. *italica*) contains relatively high levels of glucoraphanin which is the precursor of sulphoraphane, an isothiocyanate known to have a positive effect in the mammalian detoxification (phase 2) enzyme activity (Liang *et al.*, 2007; Farnham *et al.*, 2004;).

Cooking of *Brassica* vegetables, by steaming, stir-frying and microwaving, has been shown to differentially modulates GL retention (Sarvan *et al.*, 2014; Palermo *et al.*, 2013; Jones *et al.*, 2010). However all cooking procedures influence the final GL content by i) inactivating MYR, ii) changing the vegetable matrix (cell lysis) and forthcoming leaching in the cooking water, iii) promoting the thermal degradation of GLs.

Previous works have investigated the factors affecting thermal degradation of GLs in broccoli and other *Brassicas* such as temperature, time and type of cooking, water activity (a_w) (Sarvan *et al.*, 2014; Hanschen *et al.*, 2012; Hennig *et al.*, 2012; Oliviero *et al.*, 2012). Mathematical models have been developed to predict the kinetic of GL degradation upon cooking/processing (Sarvan *et al.*, 2014; Hennig *et al.*, 2012; Oerlemans *et al.*, 2006; Dekker *et al.*, 2000).

Dekker *et al.* (2009) showed that thermal degradation rate constants of specific GLs may vary in different vegetables, whereas Hennig *et al.* (2014) found differences in the glucoraphanin and glucobrassicin degradation rate constants even in different genetic lines of broccoli. Finally, Hanschen *et al.* (2012) carried out a very detailed study on the factors affecting allyl GL thermal degradation and concluded that the broccoli matrix decreases GL thermal stability compared to simplified aqueous model systems. The main outcome of these studies is that the physicochemical composition of the plant matrix can significantly contribute to the variability of GL thermal stability.

Brassica vegetables are normally consumed as part of a whole meal, in combination with other meal components. This means that other ingredients are used and mixed together with vegetables during food preparation. In this respect, the fate of GLs during preparation of *Brassica* vegetables, and specifically during cooking, may be affected by the overall meal composition.

The aim of this study was to evaluate the effect of added components on the kinetic of the GL thermal degradation in broccoli using specifically design binary model system. The effect of

different macromolecules (starch and protein) as well as the presence of onions rich in polysaccharides and polyphenols was tested.

MATERIALS AND METHODS

Materials

A batch of broccoli (*B. oleracea* var. *italica*), fresh onions (*Allium cepa* L.), potato and corn starches were purchased from a local supermarket (C1000, Wageningen, The Netherlands). Lentil protein isolates (55%) were from AGT Foods, (Canada).

Standards for GLs analysis: sinigrin hydrate (Fluka) and glucotropaeolin (Laboratory of Biochemistry, Plant Breeding and Acclimatization Institute at Radzikow, Błonie, Poland). For the quantification of MYR activity the calibration curve was done by using standard MYR enzyme (thioglucosidase from *Sinapis Alba*, Sigma-Aldrich). Standard for flavonoids (FLA) analysis: quercetin 3-glucoside and kaempferol (Fluka, UK).

Sample preparation

Onions were peeled, cut into smaller pieces, stored over night at -20°C and subsequently freeze-dried (Christ Alpha 1-4 LD Freeze Dryer, SciQuip Ltd, Newton, United Kingdom). Broccoli batch was heated in a microwave oven at 900 W for 5 min to inactivate MYR (Oliviero *et al.*, 2012). After cooling down on ice, the broccoli batch was frozen overnight at -20°C and subsequently freeze dried.

Freeze-dried material was ground to a fine powder by using a Waring blender (model 34BL99, Dynamics Corp. of America, New Hartford, CT, USA). Then freeze-dried material was stored at -20°C till further preparation.

Model mixtures were prepared by mixing appropriate amount of broccoli powder and other ingredient. Since a_w is known to have an effect on GL degradation, as already discussed in a previous work by Oliviero *et al.* (2012), different volumes of water were added to each system in order to obtain the same value of water activity (a_w, 0.99). a_w of each system was measured at 25°C using a LabMaster-a_w (Novasina Lachen, Switzerland).

Myrosinase activity determination

MYR activity was determined by a coupled enzymatic procedure, as described by Van Eylen *et al.* (2006). The MYR extraction was performed according to Oliviero *et al.* (2014). Briefly, 0.05 g of freeze-dried broccoli were mixed with 140 mL of potassium phosphate buffer (50 mM, pH 7), at 15°C overnight. The day after, upon centrifugation (2670 *g* for 10 min) the supernatants were filtered (folded filters Grade 595 1/2 - 4-7 μm, Whatman) to clarify the solution. Then the filtrated were concentrated by using filter tubes (Amicon Ultra-4 cut-off 30 kDa, Millipore). For the determination of the purified and concentrated enzyme a D-glucose enzyme kit (Enzyplus EZS 781+, Raisio Diagnostics, Finland) was used. MYR activity was determined by a spectrophotometer (Cary UV 50, Bergen op Zoom, The Netherlands) at 340 nm for 7 minutes.

Model systems heating

To test the effect of added ingredients on broccoli thermal degradation, four systems were prepared by adding to broccoli one of the following ingredients: potato starch, corn starch, lentil proteins, and freeze-dried onion. A system, containing only broccoli, was used as control. Two broccoli/other ingredient weight ratios were used, 1/9 and 1/1. Considering 2 g of model system:

- ✓ 2.3 mL of water were added to the broccoli and potato starch, corn starch, or lentil proteins systems, with a 1/9 weight ratio;
- ✓ 5 mL of water were added to the broccoli and potato starch, corn starch, or lentil proteins systems, with a 1/1 weight ratio;
- ✓ 4 mL of water were added to the broccoli/onion systems, with a 1/9 weight ratio;
- ✓ 7 mL of water were added to the broccoli/onion systems, with a 1/1 weight ratio;
- ✓ 8 mL of water were added to the broccoli control.

After mixing and adding water, each system was transferred into metal tubes with hermetic caps in which the temperature profile could be monitored with a thermocouple. The samples were heated in a heating block (Liebisch Labortechnik, England). Two temperatures were chosen for the heating studies, 90°C and 100°C.

The heating-up-time was around 4 min which was then considered as the starting time (time zero). Samples were collected after 0 and 300 min of heating. After heating, the samples were cooled in ice, extracted from the metallic tubes and frozen in liquid nitrogen. Once the samples were frozen, they were reduced to a fine powder using a mill (MM 400, Retsch, Germany), and analyzed for GL content.

To test the effect of onion amount, three model systems with different broccoli/onion ratios were used: 1/9, 3/7, and 1/1. Considering 2 g of model system:

- ✓ 4 mL of water were added to the 1/9 broccoli/onion weight ratio;
- ✓ 5.5 mL of water were added to the 3/7 broccoli/onion weight ratio;
- ✓ 7 mL of water were added to the 1/1 broccoli/onion weight ratio.

Again, the volume of water was chosen in order to obtain the same value of a_w in all the model systems evaluated.

Broccoli/onion systems were heated at 100°C (heating up time around 4 min). Samples were collected after heating, at different time between 0 and 300 min (0, 15, 30, 60, 120, and 300 min). After heating, the samples were treated as reported above and analyzed for GL and FLA contents.

Finally, three different onion varieties were tested; in particular yellow, white, and red onions were used to prepare model systems with broccoli, using only the 1/9 broccoli/onion weight ratio and addition of 4 mL of water. Temperature and times of heating were the same as reported in previous experiment. After heating, the samples were treated as reported above and analyzed for GL and FLA contents.

All the heating experiments were carried out in duplicate, and each duplicate consisted of two tubes, for a total of four replications for the same sample.

Glucosinolates extraction and analyses

The method already described by Oliviero *et al.* (2012) was used. Briefly, 1 g of sample was extracted using preheated methanol (incubated at 75°C for at least 60 min). 200 µL of 3 mM glucotropaeolin solution were added as internal standard and the samples were incubated in a water bath at 75°C for 20 min. After incubation, the samples were centrifuged, and the supernatants were collected and re-extracted twice with hot methanol (70%). The extracted GLs were desulphated and the separation was conducted using a LiChrospher® 100 RP-18 column 5µm (250 × 4.6 mm). Elution from the HPLC column was performed by a gradient of water (A) and acetonitrile (B), as follows: from 0 to 2 min, 0% B; from 2 to 7.5 min, 0-8% B; from 7.5 to 14 min, 8-25% B; from 14 to 18 min, 25% B; from 18 to 20 min, 25-0% B; from 20 to 25 min, 0% B as post-run. The flow rate was 1 mL min^{-1} and the injection volume was 20 µL. Detection was performed at ʎ = 229 nm.

GL recovery trials were carried out by spiking binary model systems with different amounts of a standard GL not present in that broccoli batch. The glucosinolate sinigrin was used as spike and was added as soon as the ingredients were mixed and subsequent heated as described previously.

Recovery evaluation was performed in triplicate and calculated as percentage, comparing the amount of sinigrin determined by HPLC and the known amount added before extraction. The internal standard for GL quantification was glucotropaeolin.
Recovery proofs were evaluated using two different amounts of sinigrin for each model system, in order to evaluate the possible effect of food matrix on GL extractability. Calculated recoveries ranged between 79 and 105%, with the lowest value obtained for the broccoli/onion model system.

Flavonoids extraction and analysis

FLA were analyzed using the same methanolic extract obtained from GL extraction. Previous works (D'Antuono *et al.*, 2008; Ferioli *et al.*, 2013) indicate that the extraction procedure with hot methanol was suitable for the extraction of both GLs and phenolics.
Separation was conducted using a Varian Polaris C18-A column 5µm (150 × 4.6 mm), with a Varian Chromsep guard column SS 10 × 3.0 mm and a particle size of 5 µm. The elution was carried out by HPLC in a gradient mode of trifluoroacetic acid in water, pH = 2.5 (A) and acetonitrile (B), as follows: from 0 to 20 min, 0-42% B, from 20 to 25 min, 42% B; from 25 to 30 min, 0% B. The flow rate was 1 mL min^{-1}, and the injection volume was 20 µL. Each chromatogram was recorded at 370 nm, and absorption spectra were recorded between 200 and 500 nm. FLA were quantified by external standard mode, constructing calibration curves of two compounds, quercetin 3-glucoside and kaempferol. Stock solutions were prepared in methanol and concentrations of diluted solutions were included in the range 6.25-100 mg L^{-1}.
The comparison with a more specific method applied to onions (Riggi *et al.*, 2013) confirmed the adequacy of our approach.

Statistics

The data of the three experiments have been initially processed by means of analysis of variance, taking into account the effects of the main factors and all their interactions. Methodological details of the statistical analysis are given as ESI. † All the statistical analyses were carried out by means of the SYSTAT® package, $p \leq 0.05$ was considered significant across the whole study, however in some cases also a $p \leq 0.01$, as level of significance, has been found.

RESULTS AND DISCUSSION

MYR activity and GLs in the broccoli batch

No residual MYR activity was detected after the microwaving pre-treatment.

Four GLs were identified and quantified: glucoraphanin (aliphatic GLs), glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin (indolic GLs).

Glucoraphanin was the most abundant GL in the broccoli batch used for this study, in agreement with previous results (Lewis *et al.*, 1991; Tian *et al.*, 2005). Average glucoraphanin content was 4.13 ± 0.78 μmol g^{-1} dry weight (dw), corresponding to 57.4% of total GLs. Among indolic GLs, neo-glucobrassicin was the most abundant compound (2.18 ± 0.45 μmol g^{-1}), representing 30.3% of total GLs.

GL thermal degradation in binary model systems

Four model systems, made of broccoli powder blended with other ingredient, were studied and compared with a control containing only broccoli powder. The second ingredient was chosen with the aim to formulate model systems that could mimic traditional domestic recipes. Two model systems included two starches different for their physical properties, namely from potato and corn (Singh *et al.*, 2003). In the third broccoli were mixed with protein isolated from lentils, while in the fourth system a freeze-dried onion powder was used. The results of the thermal treatments on total GLs and specifically on glucoraphanin concentration are shown in four panels of **Figure 5.3.1**. The higher order interaction (temperature × ratio × mixture) had significant effect on relative total GLs and glucoraphanin patterns. Total GL content in the five model systems ranged between 5.82-7.28 μmol g^{-1} broccoli (dw) at beginning of the holding phase (time zero), and 0.20-2.53 μmol g^{-1} broccoli (dw) after 300 minutes of heating.

The broccoli/added ingredients strongly affected the total GL retention. In the 1/1 mixtures, only onion, at 90°C had positive effect on retention, compared to the control. All the other combinations did not show any differences, at 90°C, or had lower retention, at 100°C, compared to the control. At the 1/9 ratio, all the systems showed a trend of increased GLs stability, significant for all mixtures at 90°C, and for lentil and onion only at 100°C.

The trend of glucoraphanin during heating was substantially similar to that of total GLs (**Figure 5.3.1**, bottom panels). The main differences, with respect to total GLs, were the higher relative retention of glucoraphanin and the stronger protective effect of the 1/9 mixtures. As for total GLs,

the strongest effect was found in the broccoli/onion mixture at 1/9 ratio, where approximately 45% and 53% of glucoraphanin could be recovered after heating treatment at 100 and 90°C. Good protective effect was also observed with lentils proteins, while a lower protective effect was obtained in the broccoli/potato or broccoli/corn starch systems which was in any case a significantly higher effect than in the control samples.

This suggests that the physicochemical structure of the matrix can play a significant role in determining the degradation rate of GLs and likely of other small molecular weight phytochemicals.

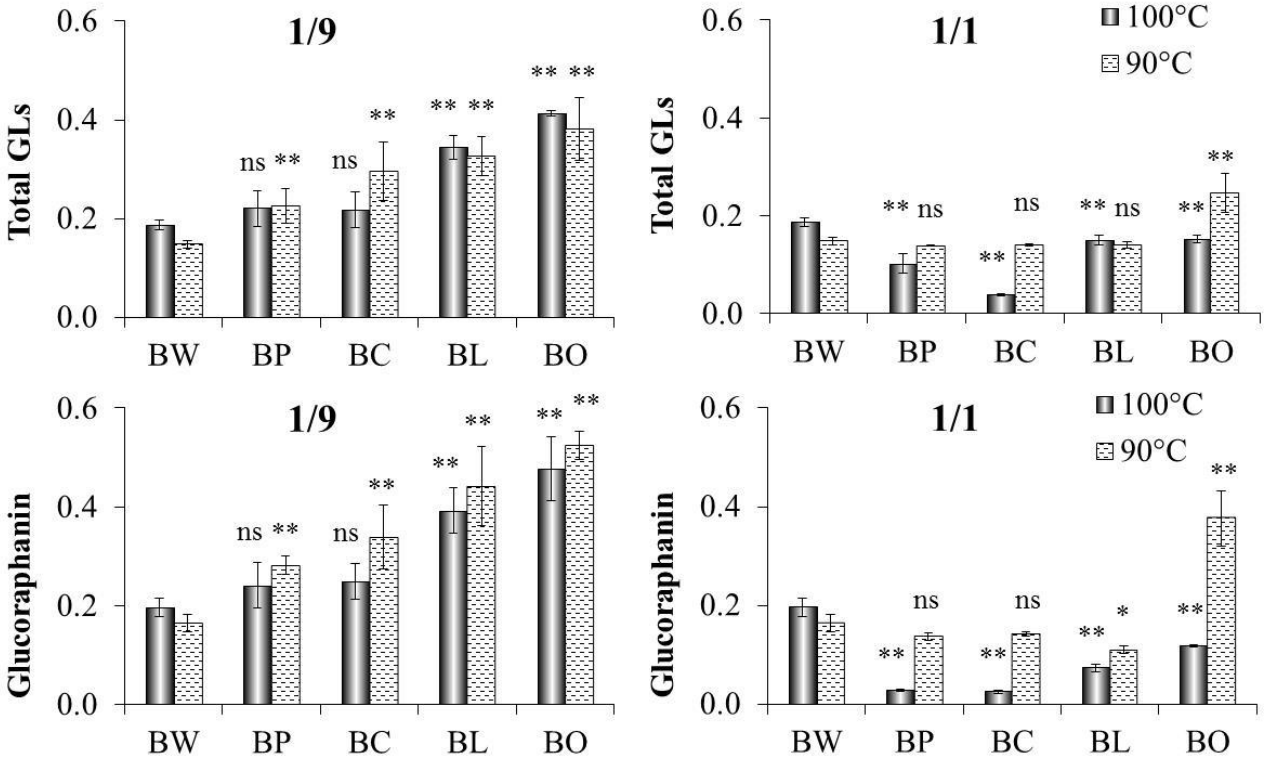

Figure 5.3.1. Total GL relative contents (with respect to initial content) after 300 minutes of heating, for different broccoli mixtures at 1/9 (top left panel) and 1/1 (top right panel) weight ratios. The two bottom panels showed the glucoraphanin relative contents (with respect to initial values in the corresponding samples). BW: broccoli, as control; BP: broccoli/potato starch; BC: broccoli/corn starch; BL: broccoli/lentil proteins; BO: broccoli/onion.[1]

[1] Symbols above columns indicate level of significance of the effect of different model systems compared to the broccoli only control (BW), according the Dunnett test, within each combination of temperature and ratio.

Significance: ******: $p \leq 0.01$; *****: $p \leq 0.05$; ns: non significant.

GL thermal degradation in presence of different amount of onion

The main results of the previous experiment is that the other component of broccoli-based systems can significantly prevent GL degradation. Onions proved to be particularly efficient especially at the 1/9 ratio. To obtain more information about this protective effect three broccoli/onion model systems, differing for their relative amounts (1/9, 3/7, and 1/1), were prepared, heated at 100°C and data were acquired between 0 and 300 minutes. **Table 5.3.1** reports total GL and glucoraphanin contents, at the various heating times and for the different broccoli to onion ratios.

Data showed that the higher the presence of onion the lower the GLs and glucoraphanin degradation with a substantial amount (up to 50%) of the total GLs still present after 1 hour of treatment with a 1/9 ratio. Also in this case, all the effects (heating time, broccoli to onion ratio and their interaction) were statistically significant and the data confirmed a second order kinetic relation, as represented in **Figure 5.3.2**, for the 1/9 broccoli/onion ratio.

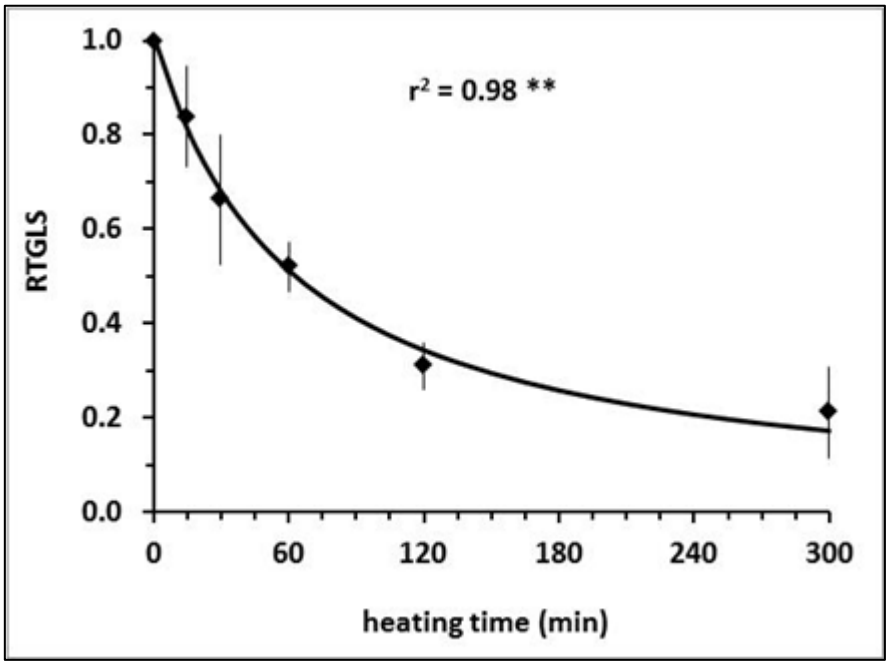

Figure 5.3.2. Example of data fitting by means of a second order kinetic equation: total GL content relative to initial time (RTGLS) as a function of heating time. Broccoli/onion model system at 1/9 ratio.

****** Correlation coefficient statistically significant at $p < 0.01$.

Table 5.3.1. Total GLs and glucoraphanin contents relative to time zero, as a function of broccoli/onion ratios.

Time of heating	**Broccoli/onion ratios***							
(min)	**1/9**	**3/7**	**1/1**	**C**	**1/9**	**3/7**	**1/1**	**C**
	Total GLs (relative content) ± SD**				**Glucoraphanin (relative content)** ± SD**			
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
15	0.83 ± 0.11	0.72 ± 0.17	0.80 ± 0.13	0.55 ± 0.08	0.81 ± 0.16	0.74 ± 0.15	0.78 ± 0.16	0.59 ± 0.10
30	0.66 ± 0.14	0.59 ± 0.14	0.59 ± 0.07	0.40 ± 0.05	0.60 ± 0.12	0.62 ± 0.12	0.61 ± 0.07	0.48 ± 0.07
60	0.52 ± 0.05	0.38 ± 0.05	0.23 ± 0.05	0.25 ± 0.05	0.52 ± 0.10	0.39 ±0.06	0.27 ± 0.06	0.29 ± 0.06
120	0.31 ± 0.05	0.35 ± 0.07	0.19 ± 0.02	0.15 ± 0.01	0.29 ± 0.06	0.37 ± 0.08	0.20 ± 0.03	0.19 ± 0.01
300	0.30 ± 0.08	0.15 ± 0.03	0.07 ± 0.02	0.05 ± 0.01	0.23 ± 0.04	0.16 ± 0.03	0.08 ± 0.02	0.08 ± 0.02
Kinetic equation parameters***								
RCi	1.01±0.04	0.99±0.05	1.05±0.05	1.01±0.02	0.98±0.02	0.99±0.02	1.04±0.04	0.99±0.04
k^2	0.016±0.002	0.021±0.003	0.030±0.003	0.038±0.002	0.015±0.002	0.020±0.002	0.029±0.003	0.038±0.003

***** Broccoli/onion ratio: 1/9, 3/7 and 1/1; broccoli only control: C.

****** Relative contents are referred to the correspondents initial contents, for each broccoli/onion system.

******* RCi: relative initial concentrations at time zero; k^2: second order rate kinetic constant.

The second order kinetic degradation rate parameters (k^2) for total GLs and glucoraphanin are reported at the bottom lines of **Table 5.3.1**. The rate constant (k^2) decreased as a function of onion content, demonstrating the protective effect of onion. The rate constants for total GLs and glucoraphanin degradation were not significantly different. This result was not in agreement with Hennig *et al.* (2012) who indicated that GL degradation followed a first order kinetic corresponding to a monomolecular degradation. This discrepancy suggests that the degradation of phytochemical components, particularly in the case of GLs in a mixed food matrix such as the one used in this study, can take place according to more complex patterns and are not only determined by the temperature/time combination.

GL thermal degradation in presence of different onion varieties

Onion are one of the richest sources of FLA (Slimestad *et al.*, 2007) and it may be that these compounds played a role in the GL degradation. Three model systems, in the ratio broccoli/onion 1/9, were prepared using red onion, yellow onion, and white onion. FLA profiles of the three onion varieties are reported in **Table 5.3.2**. Red onions resulted the richest source of this class of bioactives, with a total FLA content more than double than yellow and white onions. Details of modifications of FLA composition along heating of the model systems are provided as ESI (**Table 1S** ESI†). Results of the GL thermal degradation are reported in **Table 5.3.3** and confirmed that the addition of onion powder to broccoli significantly decreased GL degradation rate respect to the broccoli control only. Despite the huge differences of FLA content, the trends of GL degradation were not affected by onion varieties. No significant differences were detected between onion varieties, nor was the onion variety × time interaction significant. The second order kinetic degradation rate parameters (k^2), reported at the bottom of **Table 5.3.3**, confirm this finding. Interestingly, also FLA thermal stability was lower in the broccoli matrix compared to the onion matrix (**Table 1S** ESI†).

On the basis of this relation, the whole set of data was fitted by a generalized second order kinetic relation, in which the rate constant was expressed as a linear function of onion concentration. The overall data fitting was highly significant for both total GLs and glucoraphanin (see parameters of **Table 2S** ESI†), whereas an example of surface response for total GLs is represented in **Figure 5.3.3**. the variation of the rate equation (Dk) as a function of the added ingredient (onion in this case), although being ingredient specific, indicates this method as applicable to different combinations.

Table 5.3.2. FLA content, expressed as mg kg^{-1} of onion dry weight ± SD, for the 3 onion varieties used in the third heating experiment.

Compound*	**RT (min)**	**Onion variety****		
		YO	**WO**	**RO**
Q-3,7,4′-TGLC	8.8	8.2 ± 1.7	5.5 ± 1.3	23.7 ± 4.6
Q-7,4′-DGLC	11.4	52.0 ± 2.3	29.0 ± 4.3	81.5± 11.1
Q-3,4′-DGLC	12.3	1068.3 ± 22.5	978.7 ± 74.6	2492.6 ± 4.4
I-3,4′-DGLC	12.7	37.3 ± 1.6	32.0 ± 8.3	62.6 ± 5.4
Q-3-GLC	14.2	18.8 ± 0.2	46.6 ± 3.0	132.2 ± 4.0
Q-4-GLC	15.6	2125.8 ± 2.6	2163.0 ± 160.0	4818.6 ± 437.8
I-4-GLC	16.3	165.4 ± 3.6	255.1 ± 14.2	211.9 ± 0.8
Q	19.2	94.5 ± 0.1	131.1 ± 30.6	562.9 ± 103.6
K	22.1	2.1 ± 0.3	3.2 ± 0.7	9.3 ± 1.9
FLA		3572.3 ± 22.5	3644.2 ± 297.3	8395.3 ± 634.3

***** Q-3,7,4′-TGLC: quercetin 3,7,4′-triglucoside; Q-7,4′-DGLC: quercetin 7,4′-diglucoside; Q-3,4′-DGLC: quercetin 3,4′-diglucoside; I-3,4′-DGLC: isorhamnetin 3,4′-diglucoside; Q-3-GLC: quercetin 3-glucoside; Q-4-GLC: quercetin 4-glucoside; I-4-GLC: isorhamnetin 4-glucoside; Q: quercetin aglycone; K: kaempferol; FLA: sum of all the flavonoids identified.

****** YO, yellow onion; WO, white onion; RO, red onion.

Table 5.3.3. Total GLs and glucoraphanin contents relative to time zero, as a function of onion variety at 1/9 broccoli/onion ratio.

Time of heating	**Model systems (broccoli/onion varieties)***							
(min)	**YO**	**WO**	**RO**	**C**	**YO**	**WO**	**RO**	**C**
	Total GLs (relative content)**				**Glucoraphanin (relative content)****			
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
15	0.83 ± 0.11	0.73 ± 0.15	0.80 ± 0.05	0.55 ± 0.08	0.81 ± 0.16	0.87 ± 0.22	0.83 ± 0.11	0.59 ± 0.10
30	0.66 ± 0.14	0.58 ± 0.11	0.71 ± 0.12	0.40 ± 0.05	0.60 ± 0.12	0.71 ± 0.15	0.63 ± 0.08	0.48 ± 0.07
60	0.52 ± 0.05	0.46 ± 0.09	0.49 ± 0.10	0.25 ± 0.05	0.52 ± 0.10	0.55 ± 0.13	0.46 ± 0.09	0.29 ± 0.06
120	0.31 ± 0.05	0.20 ± 0.03	0.35 ± 0.03	0.15 ± 0.01	0.29 ± 0.06	0.24 ± 0.04	0.31 ± 0.05	0.19 ± 0.01
300	0.30 ± 0.08	0.17 ± 0.03	0.19 ± 0.01	0.05 ± 0.01	0.23 ± 0.04	0.19 ± 0.03	0.16 ± 0.01	0.08 ± 0.02
Kinetic equation parameters***								
RCi	1.01±0.04	1.04±0.08	0.98±0.04	1.01±0.02	0.98±0.05	1.04±0.09	1.01±0.04	0.99±0.03
k^2	0.016±0.002	0.016±0.003	0.018±0.002	0.038±0.002	0.015±0.002	0.015±0.004	0.018±0.002	0.038±0.003

***** YO, broccoli/yellow onion; WO, broccoli/white onion; RO, broccoli/red onion; C, broccoli control.

****** Relative contents are referred to the correspondents initial contents, for each broccoli/onion system.

******* RCi: relative initial concentrations at time zero; k^2: second order rate kinetic constant.

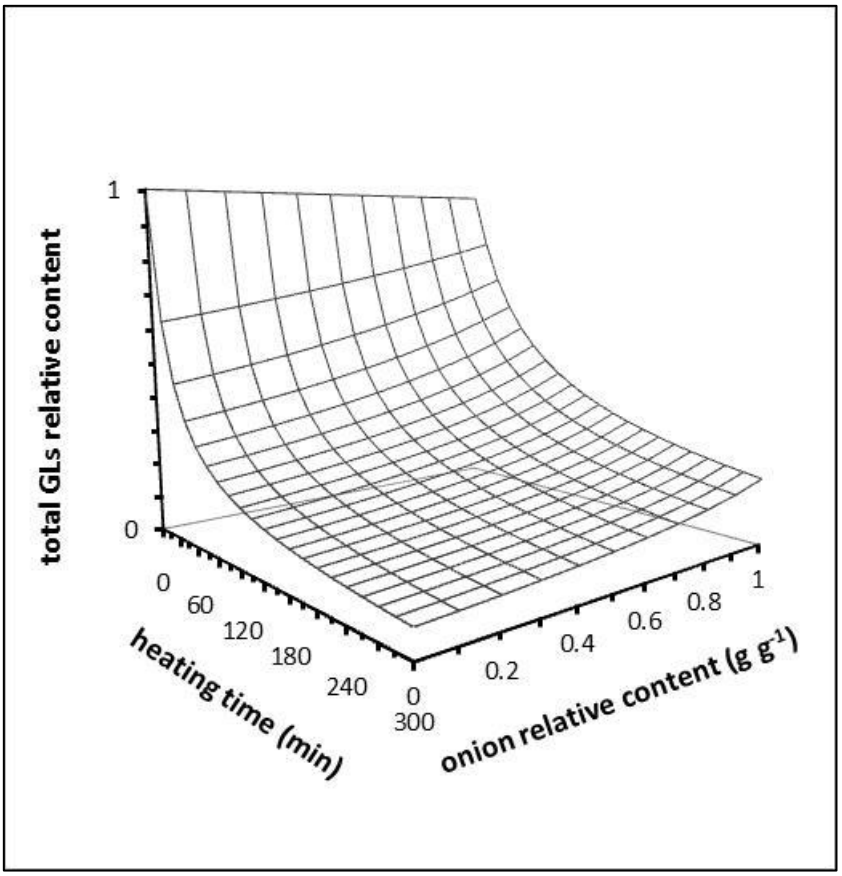

Figure 5.3.3. Surface response of total GL relative contents, as a function of heating time (T= 100°C) and relative onion content in the broccoli/onion model systems. Individual data points are not represented for a matter of clarity.

The finding that onion FLA concentration do not affect GL degradation rate can have different explanations. It has been reported that the addition of broccoli powder to aqueous solutions decreases the thermal stability of aliphatic ITC in model systems (Hanschen *et al.*, 2012).

This fact has been explained by the synergistic effect exerted by broccoli vitamin C on Fe(II) catalyzed GL degradation, likely by recycling of Fe(III) to Fe (II). Vitamin C and Iron content is lower in onion compared to broccoli (Cunningham *et al.*, 2001; Pennington and Fisher, 2010) which might have contributed to reduce the adverse effect of the vitamin C/iron system in broccoli/onion models. This would also explain why a lower GL degradation rate was observed in all the mixtures from experiment 1 at least at the ratio 1/9. Beside this effect, however, onion matrix provided extra protection to GL degradation through a variety of cell components. GLs were more stables in mixtures because of the dilution of the broccoli matrix rather than because of the presence of specific protective compounds in the onion matrix.

CONCLUSIONS

This study highlighted the importance of added ingredients on the thermal degradation behavior of GLs (in particular glucoraphanin) from broccoli. The employed model systems highlighted the potentiality of onions as broccoli-accompanying ingredient in all the experiments. On the other hand flavonoids may be degraded faster when onions were mixed with broccoli. Therefore, further studies are necessary to find the better trade off combinations and elucidate which components and which mechanisms are involved in thermal degradation of healthy phytochemical to deliver a clear message to consumers about the healthy cooking procedure and the ingredient combinations.

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ESI†: SUPPLEMENTARY MATERIAL

Materials and methods

Statistics

Experiment 1: four factor factorial, including the effects of: mixture (5 levels: broccoli only control, and the four combination of broccoli and added ingredient), heating temperature (two levels: 90 and 100°C), ingredient ratio (two levels: 1/1 and 1/9 broccoli/added ingredient), heating time (two levels: time zero, after 300 minutes). The data of total GLs and glucoraphanin were also transformed into relative amounts with respect to the initial content (time zero): in this case the data were processed as a three factor factorial, missing the time of heating effect.

Experiment 2: two factor factorial, including the effects of: mixture (four levels: broccoli only control and 1/1, 3/7, 1/9 broccoli/onion ratio) and heating time (six levels: 0, 15, 30, 60, 120, 300 min).

Experiment 3: two factor factorial, including the effects of: mixture (four levels: broccoli only control and 1/9 ratio with three different onion varieties) and heating time (six levels: as in experiment 2).

In experiment 1, the differences between the broccoli only control and the mixtures, within each of the other combinations of factors, have been tested by means of the Dunnett test. The differences between the other treatments have been tested by means of the Tukey HSD test. The kinetics of relative GLs and glucoraphanin variation have been analyzed and modeled according to the following procedure.

The data for which a series of time points was available, were used to preliminary explore the kind of kinetic model better fitting the data: a) the broccoli control and the three broccoli/onion weight ratios for experiment 2, and b) the broccoli control and the 1/9 ratio with three different onion varieties for experiment 3. Two procedures were adopted:

a) the trends of log-transformed or reciprocal-transformed relative (to time zero) GLs and glucoraphanin content against time were tested for deviation from linearity, to test the better fit of a first order and a second order kinetic equation, respectively; higher order models were not considered, since data showed that they would not have added any further improvement, besides being empirical;

b) the untransformed data relative (to time zero) GLs and glucoraphanin content were fitted by means of a) a first order; b) a second order; c) a n order kinetic equation, according to:

(1) $RC = RCi * e^{-k* t}$, first order;

(2) $RC = RCi / (1 + k^2 * t * RCi)$, second order;

(3) $RC = (RCi^{(1-n)} + (n-1) * k^n * t))^{(1 / (1-n))}$, n-order, where:

RC: relative (to time zero) GL content (either total GLs or glucoraphanin); RCi: initial (at time zero) RC; k, k^2, k^n: kinetic degradation constants for order 1, 2 and n relations, respectively; t: time from time zero; n: order of the n-order equation.

In equation 3, therefore, the order n was estimated as a parameter. As a consequence, equations 1 and 2 had two parameters, whereas equation 3, had 3 parameters.

The n-order model was compared to both the order 1 and 2 order kinetic equations by means of the corrected Akaike information criterion (Burnham and Anderson, 2002).

The calculated rate constants from the best kinetic model resulting from the selection procedure (a second order equation, as it will be further illustrated) were related to the concentration of the broccoli/onion systems, to check the nature and significance of this relation. The experiment 2 data were used for model building, by fitting them by means of a generalized second order kinetic model in which the rate constant was a linear function of onion relative concentration, according to the relation:

(4) $RC = RCi / (1 + ((Ik^2 + Dk * RCONC) * t * RCi))$,

in which RC, RCi, t, have the same meaning as in (2);

the second order rate constant of (2): k^2, is here expressed as a linear function of onion relative concentration:

(5) $k^2 = Ik^2 + Dk * RCONC$, where:

Ik^2, value of the rate constant, at 0 onion concentration (broccoli only);

Dk, rate of k^2 variation, function of onion relative concentration;

RCONC, relative onion concentration in the model system.

In this form, all the three parameters (RCi, RCONC and Ik^2) have a functional meaning.

Finally, data of experiments 1 and 3 were used as validation set for the relation obtained, for a total of 84 validation points, including individual replications. In particular: a) the data of the broccoli control and the broccoli/onion systems at 1/1 and 1/9 ratio, at 100°C heating temperatures, were used from experiment 1 (24 points); b) the data of the broccoli control and all combinations with three onion varieties (1/9 weight ratio), at different heating times, were used from experiment 3 (60 points).

The degradation kinetics of onion FLA was also assessed, including a zero-order kinetic, as suggested by the data pattern, in the form:

(6) $RCF = RCFi + k^0 * t$, where:

RCF: relative flavonoid content; RCFi: relative flavonoid content at time zero; k^0: zero-order kinetic degradation constant.

References

Burnham KP and Anderson DR, *Model selection and multi-model inference: a practical information theoretic approach*. Springer, New York, (2002).

Table 1S. Total FLA content, relative FLA content with respect to time zero, and percentage individual FLA, in broccoli/onion systems and their trends during heating.

				Flavonoid components*				
ratio**	**Time of heating (min)**	**FLA (mg kg^{-1})**	**relative FLA**	**Q-4-GLC**	**Q-3,4′-DGLC**	**Q**	**I-4-GLC**	**others**
						%		
1/9	0	3835 ± 248	1.00	62.5 ± 0.6	26.4 ± 0.7	3.1 ± 0.4	4.5 ± 0.1	3.5 ± 0.2
	15	3713 ± 272	0.97 ± 0.01	66.0 ± 0.4	21.1 ± 0.3	4.9 ± 0.2	4.2 ± 0.1	3.8 ± 0.2
	30	3876 ± 570	1.00 ± 0.10	71.6 ± 2.2	15.9 ± 2.8	6.2 ± 0.7	4.1 ± 0.1	3.7 ± 0.1
	60	3581 ± 197	0.93 ± 0.09	72.3 ± 4.3	13.7 ± 1.8	8.2 ± 1.7	4.2 ± 0.1	3.7 ± 0.2
	120	3168 ± 240	0.83 ± 0.02	67.4 ± 1.9	13.4 ± 2.8	11.4 ± 0.9	3.9 ± 0.2	4.0 ± 0.1
	300	2509 ± 98	0.65 ± 0.08	66.7 ± 9.3	12.8 ± 2.9	23.7 ± 1.5	3.6 ± 0.6	5.0 ± 0.9
k^0_{rfla}* **			-0.0012±0.0001					
3/7	0	3683 ± 305	1.00	62.5 ± 0.13	26.5 ± 0.2	3.1 ± 0.1	4.4 ± 0.1	3.5 ± 0.1
	15	3460 ± 284	0.94 ± 0.02	66.0 ± 1.68	21.5 ± 2.2	4.6 ± 0.6	4.1 ± 0.1	3.8 ± 0.1
	30	3469 ± 250	0.94 ± 0.05	68.5 ± 1.25	17.6 ± 1.2	6.0 ± 0.7	4.0 ± 0.2	3.9 ± 0.3
	60	3273 ± 245	0.89 ± 0.07	69.8 ± 2.54	14.5 ± 3.1	7.7 ± 1.0	4.0 ± 0.3	3.9 ± 0.3
	120	2968 ± 563	0.81 ± 0.14	68.9 ± 2.55	11.3 ± 2.9	13.8 ± 2.0	3.5 ± 0.1	4.0 ± 0.5
	300	2042 ± 147	0.55 ± 0.07	53.8 ± 0.99	14.5 ± 3.1	22.0 ± 5.1	2.3 ± 0.2	5.6 ± 0.8
k^0_{rfla}* **			-0.0014±0.0001					
1/1	0	3545 ± 280	1.00	63.1 ± 0.49	27.0 ± 0.4	3.0 ± 0.3	4.4 ± 0.2	3.7 ± 0.3
	15	3224 ± 146	0.91 ± 0.05	67.7 ± 1.18	21.6 ± 0.9	5.1 ± 0.8	4.0 ± 0.4	4.3 ± 0.6
	30	3453 ± 245	0.97 ± 0.05	72.2 ± 3.19	15.8 ± 1.3	5.9 ± 0.2	3.7 ± 0.2	3.9 ± 0.3
	60	2694 ± 338	0.76 ± 0.06	67.3 ± 3.54	19.0 ± 3.8	6.4 ± 1.6	3.4 ± 0.1	3.8 ± 0.3
	120	2327 ± 452	0.66 ± 0.08	65.3 ± 4.83	17.4 ± 3.5	11.4 ± 3.0	2.1 ± 0.1	4.9 ±1.0
	300	1607 ± 210	0.45 ± 0.08	52.5 ± 1.30	19.8 ± 3.8	20.7 ± 5.0	1.8 ± 0.1	5.4 ± 1.3
k^0_{rfla}* **			-0.0018±0.0002					

***** FLA: total flavonoids; RFLA: total flavonoids, relative to time zero; Q-4-GLC: quercetin 4-glucoside; Q-3,4′-DGLC: quercetin 3,4′-diglucoside; Q: quercetin aglycone; I-4-GLC: isorhamnetin 4-glucoside; others: other FLA components.

****** Broccoli/onion ratio: 1/9, 3/7 and 1/1.

******* **k^0_{rfla}**: zero order rate kinetic constant for flavonoids.

Table 2S. Calculated parameters for the overall relation between total GL and glucoraphanin contents, relative to time zero, time of heating and onion relative content of the model systems**.**

parameters*	**total GLs**	**glucoraphanin**
RCi	1.01±0.02	1.00 ± 0.02
Ik^2	0.040 ± 0.03	0.040 ± 0.03
Dk	-0.027 ± 0.04	-0.027 ± 0.05
overall r^2	0.93 **	0.91 **

Chapter 6

Asteraceae

Chapter 6.1

Identification and quantification of phenolic compounds in edible wild leafy vegetables by UHPLC-Orbitrap-MS

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ABSTRACT

Today wild leafy vegetables' knowledge and use as food is reduced; however the interest of consumers toward these species is growing and they are often associated to health promotion. In this study several wild species of the *Asteraceae* family and *Knautia integrifolia* were: 1) locally documented for their use in modern cuisine, 2) gathered in the wild, 3) and their content in phenolic compounds were determined by ultra-high-performance liquid chromatography coupled to Orbitrap high resolution mass spectrometry. Hydroxycinnamic acids resulted the most abundant compounds in all the species, except *Tragopogon pratensis*. 30 compounds were identified as flavonoids, mostly as glycosidic forms of luteolin, apigenin, kaempferol and quercetin. Three anthocyanins were detected, although they represent in most of cases less than 1% on the total phenolic content. Different phenolic profiles were observed between species, especially if the class of flavonoids is considered. Individual species may be of some interest for specific minor flavonoids.

Keywords: edible wild leafy vegetables; *Compositae*; *Knautia integrifolia*; phenolic compounds; Orbitrap

INTRODUCTION

Collecting edible wild herbs was a very common practice in the past, especially among rural families. Nowadays the consumption of wild vegetables as food has decreased, mostly due to the rapid development of agriculture and global supply chains. Only few people are able to recognize different species before and after flowering. However some of wild vegetables are still consumed and consumers' interest towards these species is growing (Sánchez-Mata *et al.*, 2011).

In particular several leafy vegetables of the *Asteraceae* family are generally gathered and used in raw salads (if harvested during early spring), or consumed after cooking (mainly boiling or stir frying). Among *Asteraceae* family, some species such as *Chondrilla juncea* L., *Cichorium intybus* L., *Hypochaeris radicata* L., *Sonchus asper* (L.) Hill, *Sonchus oleraceus* L., and *Taraxacum officinale* Weber. are well known and spread in Southern European countries (Guarrera, 2003; Leonti *et al.*, 2006). These species have been recently included in ethnobothanical studies which aimed to highlight consumers' perceptions about wild edible vegetables (Guarrera *et al.*, 2013).

The use as food of species of *Knautia integriflolia* (L.) Bertol. (*Dipsacaceae*) is very interesting in a limited area of Central Italy (mainly represented by Arezzo province, Tuscany) (Uncini Manganelli *et al.*, 2002). This wild plant, locally called "*gallinaccio*", typically grows along rivers and ditches for the presence of water and it is generally harvested during spring, between the end of March and the beginning of April. To our knowledge very few work has been carried out about chemical composition of *Knautia integrifolia*, especially in relation to the identification of bioactive compounds (Tonguç and Erbaş, 2012; Alankuş-Çalişkan *et al.*, 2004).

A study performed in some Mediterranean countries (Italy, Spain and Greece) concluded that wild gathered species, especially those belonging to the *Asteraceae* family, could represent a significant source of dietary phytochemicals (Leonti *et al.*, 2006). Previous studies highlighted the presence of several classes of bioactive compounds in leafy vegetables, such as carotenoids, tocopherols and ascorbic acid (Žnidarčič *et al.*, 2011; Santos *et al.*, 2012; Morales *et al.*, 2014). In relation to phenolic compounds various studies have been carried out on cultivated *Cichorium* types (Carazzone *et al.*, 2013; Innocenti *et al.*, 2005; Ferioli *et al.*, 2015). Total phenolic content in wild leafy vegetables has been mostly quantified by means of spectrophotometric techniques (Ranfa *et al.*, 2014; Abbasi *et al.*, 2015). The profile of these classes of phytochemicals in wild leafy vegetables has been less investigated (Wojdyło *et al.*, 2007), with special focus on caffeoyl derivatives (Fraisse *et al.*, 2011). In particular liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) has been scarcely applied to the determination of phenolic compounds in wild leafy vegetables. The use of HRMS technique can provide several advantages,

such as full scan acquisition mode and exact mass measurements, allowing a reliable identification of bioactive compounds (López-Gutiérrez *et al.*, 2014a; 2014b). Therefore, in addition to the most commonly phenolic compounds (such as hydroxycynnamic acids in case of *Asteraceae* family) other class of phenolics, like flavonoids, could be better identified.

The aim of this research has been the utilization of ultra-high-performance liquid chromatography (UHPLC) coupled to Orbitrap-MS to determine the phenolic profile and to quantify these phytochemicals in edible wild leafy vegetables. Minor compounds could be of great interest to characterize and to discriminate individual species.

MATERIALS AND METHODS

Chemicals and reagents

Standards of the following phenolic compounds were purchased from Extrasynthese (Genay, France): apigenin 7-*O*-glucoside, apigenin 6-*C*-glucoside (isovitexin), apigenin 8-*C*-glucoside (vitexin), isorhamnetin, kaempferol, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, luteolin, luteolin 4'-*O*-glucoside, luteolin 7-*O*-glucoside, luteolin 8-*C*-glucoside (orientin), quercetin 3-*O*-galactoside and quercetin 3-*O*-rutinoside. Caffeic acid, chlorogenic acid, ferulic acid, gallic acid, quercetin and quercetin 3-*O*-glucoside were supplied by Sigma-Aldrich (Madrid, Spain).

Individual stock solutions (with concentrations between 100 and 500 mg L^{-1}) were prepared by dissolving the standards in 10 mL of HPLC grade methanol; solutions were kept in amber vials and stored at -18°C, in the darkness. A multi-compound working solution (5 mg L^{-1}) was prepared by appropriate dilution of stock solutions with methanol:H_2O (80:20, *v/v*, pH 4 acidified with formic acid) and stored at -18°C in amber vial. From the multi-compound working solution, 6 diluted solutions were obtained (1, 0.5, 0.25, 0.1, 0.05, 0.01 mg L^{-1}).

Methanol (MeOH) LC-MS grade was purchased from Sigma-Aldrich; water and formic acid, both LC-MS grade, were obtained by Scharlau (Barcelon, Spain). Ammonium acetate (purity 97%) was supplied by Panreac (Barcelona, Spain). For accurate mass calibration, a mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (proteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulphate, taurocholic acid sodium salt hydrate and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo Fisher Scientific (Waltham, MA, USA) were used in the Orbitrap analyzer for calibration.

Millipore Millex-LG filters (0.20 µm, Millipore, Carrigtwohill, Ireland) were employed for filtration of extracts.

Apparatus

A Thermo Electron Corporation, Heto PowerDry LL3000 freeze-dryer (Thermo Fisher Scientific), a Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain), a vortex mixer WX from Velp Scientifica (Usmate, Italy), an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland), an ultrasonic from Elma (Singen, Germany) were used during samples' preparation and extraction procedure.

UHPLC-Orbitrap-MS analysis

Chromatographic analyses were carried out employing a Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA). A Waters (Milford, MA, USA) Acquity C18 column (2.1 × 100 mm, 1.7 µm particle size) was used for chromatographic separation.

A single-stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) was used for MS analyses, with the same conditions as reported by López-Gutiérrez *et al.* (2015). The mass spectra were acquired employing four alternating acquisition functions: full MS, ESI+, without fragmentation (higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25000 FWHM; scan time = 0.25 s; all-ion fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10000 FWHM; scan time = 0.15 s; full MS, ESI-, without fragmentation (HCD collision cell was switched off), mass resolving power = 25000 FWHM; scan time = 0.25 s; AIF, ESI-, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10000 FWHM; scan time = 0.15 s.

Mass range in the full scan mode was set at *m/z* 100-1000, whereas for MS/MS monitoring, it was at *m/z* 70-700. All the analyses were performed by using external calibration mode. Every day mass accuracy was checked by injecting a multi-compound standard solution, whereas every two weeks the analyzer was calibrated with mass accuracy standards. Data acquisition and processing were carried out using Xcalibur™ version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Qual and Quan browser. ICIS peak detection was applied, and ToxID™ 2.1.1, an automated compound screening software from Thermo Scientific, was used for screening purposes.

Samples

After a local documentation about their uses as food, wild leafy vegetables were collected during spring 2015. Two different areas of center-north Italy were selected for samples collection: Bertinoro, (province of Forlì-Cesena, Emilia-Romagna; lat. 44.15°, long. 12.13°, alt. 254 m), and Monterchi, (province of Arezzo, Tuscany; lat. 43.49°, long. 12.11°, alt. 310 m). A total of 13 wild leafy species were included in the experiment; a commercial variety of *Cichorium intybus* (Catalogna chicory), purchased in a local supermarket, was used as control. With regard to wild samples, 10 samples were gathered in Bertinoro and 5 in Monterchi (**Table 6.1.1**); all of them belong to the family of *Asteraceae*, except one from Monterchi, *Knautia integrifolia*, belonging to the family of *Dipsacaceae*.

The batch of samples is reported in **Table 6.1.1**, including the following species: *Reichardia picroides* (L.), *Chondrilla juncea* L., *Cichorium intybus* L., *Crepis vesicaria* L., *Crepis leontodontoides* All., *Crepis sancta* (L.) Babc, *Helminthia echioides* (L.), *Hypochaeris radicata* L., *Sonchus asper* (L.) Hill, *Sonchus oleraceus* L., *Taraxacum officinale* Weber, *Tragopogon pratensis* L., and *Knautia integrifolia* (L.) Bertol. Moreover wild samples of *Cichorium intybus* and *Helminthia echioides* were collected in both localities. Several vegetables belonging to *Crepis* or *Sonchus* species were included to highlight similarities or differences within species.

Table 6.1.1. List of edible wild leafy vegetables included in the experiment.

Tag	Species	Botanical family	Locality
CJb	*Chondrilla juncea*	*Asteraceae*	Bertinoro (Forlì-Cesena)
CIb	*Cichorium intybus*	*Asteraceae*	Bertinoro (Forlì-Cesena)
CSb	*Crepis sancta*	*Asteraceae*	Bertinoro (Forlì-Cesena)
CVb	*Crepis vesicaria*	*Asteraceae*	Bertinoro (Forlì-Cesena)
HEb	*Helminthia echioides*	*Asteraceae*	Bertinoro (Forlì-Cesena)
RPb	*Reichardia picroides*	*Asteraceae*	Bertinoro (Forlì-Cesena)
SAb	*Sonchus asper*	*Asteraceae*	Bertinoro (Forlì-Cesena)
SOb	*Sonchus oleraceus*	*Asteraceae*	Bertinoro (Forlì-Cesena)
TOb	*Taracum officinale*	*Asteraceae*	Bertinoro (Forlì-Cesena)
TPb	*Tragopogon pratensis*	*Asteraceae*	Bertinoro (Forlì-Cesena)
CIm	*Cichorium intybus*	*Asteraceae*	Monterchi (Arezzo)
CLm	*Crepis leontodontoides*	*Asteraceae*	Monterchi (Arezzo)
HEm	*Helminthia echioides*	*Asteraceae*	Monterchi (Arezzo)
HRm	*Hypochaeris radicata*	*Asteraceae*	Monterchi (Arezzo)
KIm	*Knautia integrifolia*	*Dipsacaceae*	Monterchi (Arezzo)
CIc	*Cichorium intybus*	*Asteraceae*	Commercial

After harvest the samples were immediately brought to the laboratory, washed and cleaned off damaged parts. The material was then frozen overnight and subsequently freeze-dried for 4 days. Freeze dried samples were stored at -18°C till further analyses.

Extraction procedure

The method already described by Alarcón-Flores *et al.* (2013) was used with some modifications. Thus, 150 mg of freeze-dried sample, exactly weighed, was extracted by sonication with 3 mL of methanol: H_2O (80:20, *v/v*), pH 4 (acidified with formic acid) for 10 min. The mixture was then centrifuged at 4136 *g* for 10 min and the supernatant fraction was filtered in a LC vial and used for injection into the UHPLC-Orbitrap-MS.

Chromatographic conditions

Elution from the UHPLC column was performed by a gradient of an aqueous solution of ammonium acetate 30 mM, pH 5 (eluent A) and methanol (eluent B), as follows: from 0 to 8 min, 5-30% B; from 8 to 13 min, 30-50% B; from 13 to 18 min, 50% B; from 18 to 47 min, 50-0% B; from 47 to 49 min, 0% B; from 49 to 53.3 min 0-5% B; from 53.3 to 58 min, 5% B as re-equilibration step.
Column temperature was held constant at 30°C; the flow rate was 0.2 mL min^{-1} and the injection volume was 10 µL.

Statistical analyses

The data were processed using analysis of variance (ANOVA), including species as experimental factor; for species common to both localities, locality and species × locality interaction were considered in a second step. Comparisons were carried out by means of the protected Fisher's LSD test. An overall view of data pattern was obtained by means of principal component analysis (PCA). Statistical analyses were carried out by means of the SYSTAT® package (San Jose, CA, USA).

RESULTS AND DISCUSSION

Database development

An already existing database was preliminarly used (López-Gutiérrez *et al.*, 2014a; 2015). The database was subsequently updated with information available in current literature related to species similar to the ones evaluated in this study. Therefore other typical phenolic compounds, commonly identified in wild leafy vegetables or even in cultivated forms, were included in the database, reporting their elemental composition, molecular ion and eventual characteristic fragments. Several compounds belonging to the class of hydroxycinnamic acids or glycosidic forms of luteolin, apigenin, quercetin and kaempferol were added (Granica *et al.*, 2015; Carazzone *et al.*, 2013; Abu-Reidah *et al.*, 2013). At the end of the revision, the database contained 47 phenolic compounds, among which 9 phenolic acids, 3 antocyanins, and 35 flavonoids, mostly as glycosidic forms (**Table 6.1.2**).

Individual standard solutions at 1 mg mL^{-1}, were injected in the system using the same conditions as reported in Materials and Methods. In this way a further characterization of analytes was carried out, highlighting important information for their identification, such as retention time (RT, min), ionization mode (ESI+ or ESI-) and characteristic fragment ions.

The gradient profile, used during chromatographic separation, was extended from 47.5 min (as reported by López-Gutiérrez *et al.*, 2014a) to 58 min. Chromatographic profile was changed in the initial part, from 0 to 30 min, adopting a less marked gradient, in order to achieve a better separation of the compounds that mostly eluted within this time. When a longer gradient profile is used, some isomeric and isobaric compounds could be separated, as for example: apigenin 6-*C*-glucoside (isovitexin) (RT, 19.38 min) and apigenin 8-*C*-glucoside (vitexin) (RT, 18.91 min) ($C_{21}H_{20}O_{10}$, *m/z* 431.09727); quercetin 3-*O*-glucoside (RT, 20.02 min) and quercetin 3-*O*-galactoside (hyperoside) (RT, 19.87 min) ($C_{21}H_{20}O_{12}$, *m/z* 463.08710); apigenin 7-*O*-neohesperidoside (rhoifolin) (RT, 20.68 min) and apigenin 7-*O*-rutinoside (isorhoifolin) (RT, 20.50 min) ($C_{27}H_{30}O_{14}$, *m/z* 579.17083); kaempferol 7-*O*-glucoside, (RT, 18.27 min), luteolin 8-*C*-glucoside (orientin) (RT, 18.34 min) and luteolin 6-*C*-glucoside (homoorientin) (RT, 18.48 min) ($C_{21}H_{20}O_{11}$, *m/z* 447.09219). On the other hand the separation of two isomeric compounds, kaempferol 3-*O*-glucoside and luteolin 4'-*O*-glucoside, ($C_{21}H_{20}O_{11}$, *m/z* 447.08993), was not possible since they eluted at the same RT of 21.07 min. Therefore, these compounds were quantified as their sum.

Table 6.1.2. Retention time and *m/z* ions selected for the identification and confirmation of the target compounds in the Orbitrap system.[a]

RT (min)	Compound Name	Elemental Composition	Polarity	Theoretical mass (*m/z*)	Mass error (ppm)	Fragment1 (*m/z*)	Fragment2 (*m/z*)	Fragment3 (*m/z*)
1.46	Caffeoyltartaric acid	$C_{13}H_{12}O_9$	ESI-	311.03976	-3.11	149.00840		
1.52	**Gallic acid**	$C_7H_6O_5$	ESI-	169.01425	-4.68	125.02442		
1.96	**5-*O*-caffeoylquinic acid (chlorogenic acid)**	$C_{16}H_{18}O_9$	ESI-	353.08671	-3.3	191.05449	179.03339	
2.23	Dicaffeoyltartaric acid	$C_{22}H_{18}O_{12}$	ESI-	473.07145	-2.92	311.03970	293.02914	179.03349
6.38	**Caffeic acid**	$C_9H_8O_4$	ESI-	179.03363	-3.7	135.04366		
13.66	5-*O*-feruloylquinic acid	$C_{17}H_{20}O_9$	ESI-	367.10236	3.49	191.05470	173.04396	
15.11	Quercetin hexose glucuronide	$C_{27}H_{28}O_{18}$	ESI-	639.11919	1.92	463.08609	301.03455	
15.24	**Ferulic acid**	$C_{10}H_{10}O_4$	ESI-	193.05063	-2.55	134.03733		
16.89	Luteolin diglucoside	$C_{27}H_{30}O_{16}$	ESI-	609.14501	1.18	285.03857		
17.18	Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	ESI-	515.11840	1.76	191.05381	353.08497	179.03271
17.20	*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	ESI-	337.09179	-4.51	191.05630	173.04480	163.0392
17.85	Luteolin 7-*O*-glucuronide	$C_{21}H_{18}O_{12}$	ESI-	461.06948	-2.43	285.03839	300.02576	
18.27	Kaempferol 7-*O*-glucoside	$C_{21}H_{20}O_{11}$	ESI-	447.09219	-4.29	327.04977	299.05505	257.04496
18.34	**Luteolin 8-*C*-glucoside (orientin)**	$C_{21}H_{20}O_{11}$	ESI-	447.09219	-4.09	327.04971	357.06007	297.04971
18.48	Luteolin 6-*C*-glucoside (homoorientin)	$C_{21}H_{20}O_{11}$	ESI-	447.09219	-4.09	327.04971		
18.72	Quercetin 3-*O*-glucuronide	$C_{21}H_{18}O_{13}$	ESI-	477.06637	-4.71	301.03303	178.99643	151.00157
18.73	Quercetin *O*-rhamnohexoside	$C_{27}H_{30}O_{16}$	ESI-	609.14501	-	463.08530	271.01862	179.03305
18.90	Apigenin 7-*O*-glucuronide	$C_{21}H_{18}O_{11}$	ESI+	447.09219	-1.42	271.05914	303.04892	
18.83	**Apigenin 8-*C*-glucoside (vitexin)**	$C_{21}H_{20}O_{10}$	ESI-	431.09727	1.95	311.05533	341.06540	283.06036
19.38	**Apigenin 6-*C*-glucoside (isovitexin)**	$C_{21}H_{20}O_{10}$	ESI-	431.09727	1.91	311.05522	341.06531	283.06011
19.46	Luteolin *O*-rutinoside	$C_{27}H_{30}O_{15}$	ESI-	593.15010	-4.65	285.03907	447.09128	300.02545
19.49	**Kaempferol 3-*O*-rutinoside**	$C_{27}H_{30}O_{15}$	ESI+	595.16575	-1.39	303.04878	287.0541	
19.53	Delphinidin 3-*O*-(6"-*O*-malonyl)-glucoside	$C_{24}H_{22}O_{15}$	ESI+	551.10059	-1.77	303.04813		

19.56	Quercetin malonyl glucoside	$C_{24}H_{22}O_{15}$	ESI-	549.08750	-4.39	300.02498	285.03801	
19.63	Kaempferol glucuronide	$C_{21}H_{18}O_{12}$	ESI-	461.06948	-2.43	285.03824	300.02505	257.04309
19.66	**Luteolin 7-*O*-glucoside**	$C_{21}H_{20}O_{11}$	ESI-	447.09219	-4.19	285.03931	269.04481	
19.87	**Quercetin 3-*O*-galactoside (hyperoside)**	$C_{21}H_{20}O_{12}$	ESI-	463.08710	-3.42	300.02737	271.02426	
19.90	Methyl quercetin glucuronide	$C_{22}H_{22}O_{13}$	ESI-	491.08202	-1.74	300.02603	315.04959	
19.92	**Quercetin 3-*O*-rutinoside (rutin)**	$C_{27}H_{30}O_{16}$	ESI-	609.14501	1.18	315.04959	302.0365	
19.99	Kaempferol malonyl glucoside	$C_{24}H_{22}O_{14}$	ESI-	533.09258	-0.6	285.03888	447.09070	130.08551
20.02	**Quercetin 3-*O*-glucoside**	$C_{21}H_{20}O_{12}$	ESI-	463.08710	-4.42	300.02509	302.03696	
20.19	Quercetin 3-*O*-arabinoside	$C_{20}H_{18}O_{11}$	ESI-	433.07654	2.47	300.02592	301.03278	
20.50	Apigenin 7-*O*-rutinoside (isorhoifolin)	$C_{27}H_{30}O_{14}$	ESI+	579.17083	-1.39	271.05913	433.11198	
20.65	**Apigenin 7-*O*-glucoside**	$C_{21}H_{20}O_{10}$	ESI+	433.11292	-1.29	271.05969	317.06506	
20.68	Apigenin 7-*O*-neohesperidoside (rhoifolin)	$C_{27}H_{30}O_{14}$	ESI+	579.17083	-2.31	271.05890		
20.81	Luteolin malonylhexose	$C_{24}H_{22}O_{14}$	ESI-	533.09258	-1.87	285.03851	429.08026	
20.81	Cyanidin 3-*O*-(6"-*O*-malonyl)-glucoside	$C_{24}H_{22}O_{14}$	ESI+	535.10823	-2.51	287.05410		
20.85	Kaempferol 7-*O*-(6"-*O*-malonyl)-glucoside	$C_{24}H_{22}O_{14}$	ESI+	535.10730	-1.51	287.05411	520.20654	367.12756
21.05	Cyanidin 3-*O*-glucoside	$C_{21}H_{20}O_{12}$	ESI+	449.10784	-1.71	287.05362	241.08467	
21.07	**Kaempferol 3-*O*-glucoside +Luteolin-4'-*O*-glucoside**	$C_{21}H_{20}O_{11}$	ESI-	447.09219	-4.49	284.03033	255.02762	
21.01	Isorhamnetin 3-*O*-glucoside	$C_{22}H_{22}O_{12}$	ESI-	477.10275	-1.1	315.04806	357.06024	
21.15	Kaempferol 3-*O*-rutinosid**e**	$C_{27}H_{30}O_{15}$	ESI-	593.15010	0.31	284.03264		
22.98	**Quercetin**	$C_{15}H_{10}O_7$	ESI-	301.03538	-1.7	151.00368		
23.97	**Luteolin**	$C_{15}H_{10}O_6$	ESI-	285.03936	-4.91	133.02834	151.00260	175.03898
26.68	**Apigenin**	$C_{15}H_{10}O_5$	ESI-	269.04445	-4.03	117.03303	149.02299	
27.41	**Isorhamnetin**	$C_{16}H_{12}O_7$	ESI-	315.05035	1.6	300.02682	271.02390	

[a] Compounds in bold are present in the multi-compound standard solution.

Data acquisition was performed using four acquisition functions, as reported in Material and Methods: positive and negative ionization modes were used to determine the abundance of the protonated $[M+H]^+$ or depronated $[M-H]^-$ molecular ions. Moreover, in order to evaluate the presence of characteristic fragments pseudo MS/MS experiments were performed applying AIF 30 eV in the HCD collision cell. In most of cases two or three characteristic fragments were monitored and used for identification and confirmation purposes. An example of chromatogram and corresponding mass spectra obtained for the fragment ions of two isomeric compounds is reported in **Figure 6.1.1**.

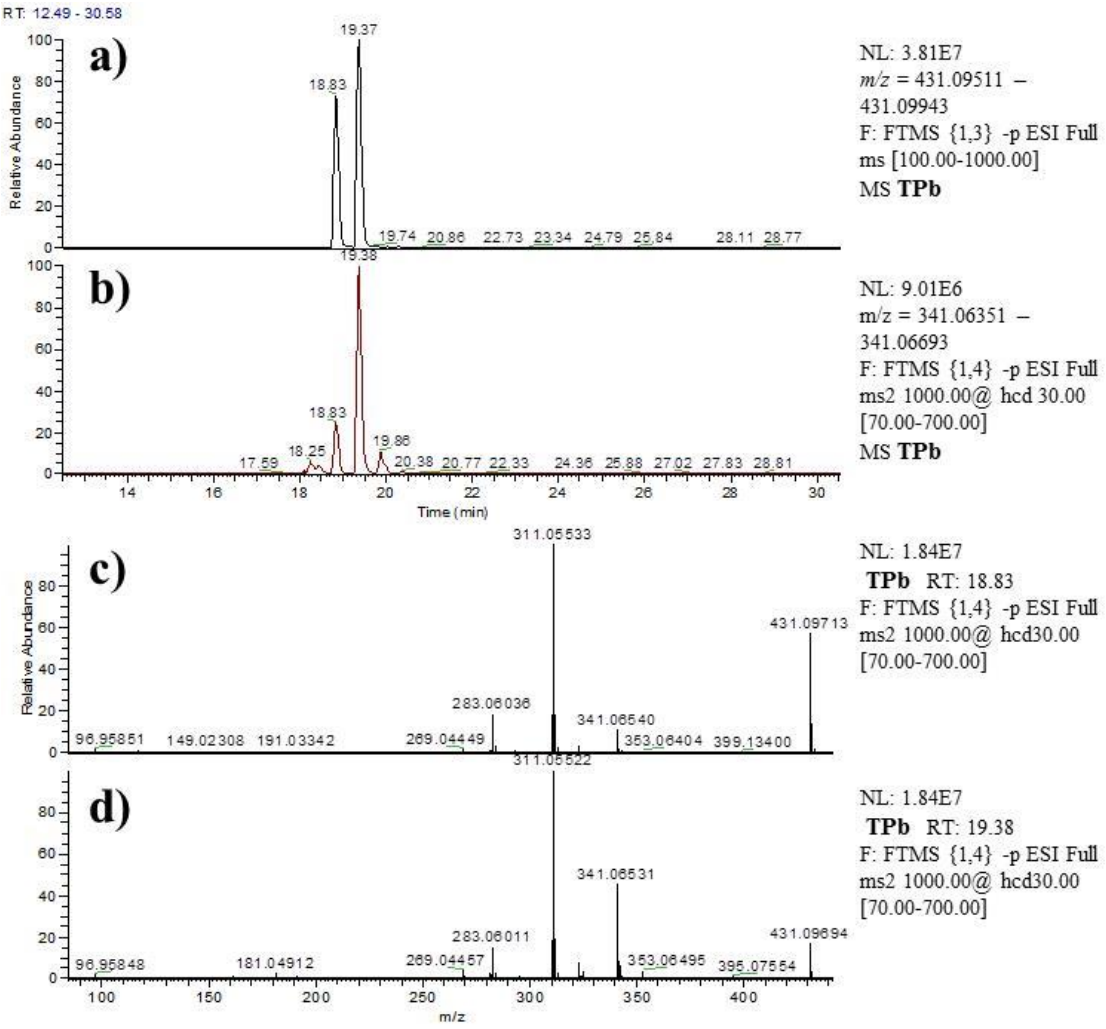

Figure 6.1.1. Characterization of two isomeric compounds, apigenin 8-*C*-glucoside and apigenin 6-*C*-glucoside, in Tragopogon pratensis (TPb) showing the: a) chromatogram for the molecular ion (*m/z* 431.09727); b) chromatogram for the fragment ion (*m/z* fragment 341.06522); c) e d) mass spectra of the two peaks detected in b), with RT 18.83 and 19.38 min.

All phenolic acids and most flavonoids present in the database showed a more abundant molecular ion when the negative ionization mode was used. Among flavonoids, only apigenin 7-*O*-glucuronide, apigenin 7-*O*-rutinoside, apigenin 7-*O*-glucoside, apigenin 7-*O*-neohesperidoside, kaempferol 3-*O*-rutinoside, and kaempferol 7-*O*-(6''-*O*-malonyl)-glucoside provided best signals when positive ionization mode was applied. Finally antocyanins were monitored in positive mode.

Target compounds were identified in the samples by using an automatic screening software, (ToxID^{TM}), with fixed searching criteria: RT ± 30 s and mass tolerance error ± 5 ppm. An excel spreadsheet compatible with the ToxID^{TM} software was developed containing characteristic information as molecular formula, RT, polarity, theoretical exact mass of molecular ion and fragments. The Excel file was saved as ".csv" file to be submitted to the software and to generate a ".pdf" report indicating, for each sample, the presence or absence of the phenolic compounds included in the database. The results obtained in the report take into account several factors, such as RT, mass tolerance and fragments. The risk of false positive was also diminished by the evaluation of the A+1 isotope peak, because the presence of ^{13}C. This is characteristic for those compounds with a high number of carbon atoms in the molecule (> 10).

Analysis of samples

A great variability on phenolic compounds content between samples was detected within a range of 1891-60830 mg kg^{-1} dw (**Figure 6.1.2**). The highest value was detected for *Knautia integrifolia*, followed by *Reichardia picroides* with 49316 mg kg^{-1} dw, whereas the lowest was obtained for *Sonchus asper*. Lower amounts were also found in *Sonchus oleraceus* and *Helminthia echioides* from Bertinoro, with 5596 and 7320 mg kg^{-1} dw, respectively. For the other samples, the total content of phenolic compounds ranged between 9488-28637 mg kg^{-1} dw.

A total of 40 phenolic compounds have been detected considering all the samples. In particular, 30 compounds belonging to flavonoids, 7 hydroxycinnamic acids, and 3 anthocyanins were identified. Relative contents for these classes of phenolics, expressed as mg mg^{-1} on the total phenolic content, are reported in **Table 6.1.3**.

Hydroxycinnamic acids

Hydroxycinnamic acids were the most abundant compounds representing over 85% of the total phenolics in most cases. The amount of this class of compounds ranged between 1388-53076 mg kg^{-1} dw (**Figure 6.1.2**), with the highest and the lowest amounts detected for *Knautia integrifolia* and *Sonchus asper* respectively. 98-99% of total phenolic compounds were quantified as hydroxycinnamic acids in *Crepis vesicaria*, *Crepis leontodontoides* and *Hypochaeris radicata*. Relative contents of individual compounds with respect to total hydroxycinnamic acid content, are reported in **Table 6.1.3**.

The following hydroxycinnamic acids were identified: monocaffeoyltartaric acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), dicaffeoyltartaric acid (chicoric acid), *p*-coumaroylquinic acid, caffeic acid, 5-*O*-feruloylquinic acid and dicaffeoylquinic acid.

These compounds have already been found in cultivated varieties of *Cichorium* (Papetti *et al.*, 2008; Ferioli *et al.*, 2015).

Most compounds were present in all the samples, except monocaffeoyltartaric acid, that was not detected in *Tragopogon pratensis*, *Crepis sancta* and *Hypochaeris radicata*, and caffeic acid, not detected in *Reichardia picroides*, *Sonchus asper* and *Cichorium intybus* from Bertinoro; lower amounts of this compound were also found in the other *Cichorium* samples.

5-*O*-caffeoylquinic acid was the most abundant compound in all the samples, except in *Helminthia echioides* from Bertinoro, *Crepis leontodontoides* and *Taraxacum officinale* in which dicaffeoyltartaric acid prevailed; very similar values for the two compounds were shown in *Crepis vesicaria*. The highest amounts of 5-*O*-caffeoylquinic acid were found in *Knautia integrifolia*, *Reicharda picroides* and *Hypochaeris radicata* with, respectively, 35591, 24461 and 17202 mg kg^{-1} dw; in all the other samples its concentration was less than 10000 mg kg^{-1} dw. *Knautia integrifolia* and *Reicharda picroides* also showed high contents of dicaffeoylquinic (15442 and 21282 mg kg^{-1} dw, respectively). Other hydroxycinnamic acids did not exceed 800 mg kg^{-1} dw.

Flavonoids

Flavonoids content ranged between 212-12598 mg kg^{-1} dw (**Figure 6.1.2**). In this case, the lowest value was observed for *Crepis vesicaria*, whereas the highest concentration was detected in *Tragopogon pratensis*; a significant high value was also obtained for *Knautia integrifolia*, with 7521 mg kg^{-1} dw. The percentage of flavonoids was slightly higher than the percentage of

hydroxycinnamic acids only in *Tragopogon pratensis*, 65.4% and 34.6%, respectively. High flavonoid relative contents were also observed in *Sonchus asper* and *Helminthia echioides* from Bertinoro, with 25.7% and 30.8%, respectively, of total phenolic content (**Table 6.1.3**).

Flavonoids were mostly represented by glycosidic forms. Only 2 aglycones, luteolin and apigenin, were identified and quantified in almost all samples, in fact luteolin was not found only in *Cichorium intybus* from Bertinoro and *Crepis vesicaria*, and apigenin was not detected in *Helminthia echioides* from Bertinoro, *Reichardia picroides* and *Taraxacum officinale*.

Some of the flavonoids were present in all or most samples, although others were characteristic of some species. In particular apigenin 8-*C*-glucoside and apigenin 6-*C*-glucoside were identified only in *Tragopogon pratensis*, quercetin 3-*O*-glucoside in *Sonchus oleraceus*, quercetin 3-*O*-arabinoside in *Crepis sancta*, and luteolin malonyl hexose in *Cichorium*.

Relative flavonoid contents, expressed as mg mg^{-1} on the total flavonoid content, are reported in **Table 6.1.3**.

Luteolin derivatives. Several compounds were identified as luteolin derivatives; the most abundant were luteolin 7-*O*-glucuronide, luteolin 7-*O*-glucoside, luteolin 8-*C*-glucoside, luteolin *O*-rutinoside and luteolin. The relative content of lutein derivatives ranged between 3.0-96.0% of total flavonoids (TFLc), with the lowest percentage in wild *Cichorium intybus* and the highest in *Taraxacum officinale*. The presence of several luteolin glycosides has been reported previously in *Taraxacum officinale* (Schütz *et al.*, 2005). *Crepis vesicaria*, *Tragopogon pratensis* and *Knautia integrifolia* showed relative high content in luteolin derivatives (80.2-94.0% of TFLc). Among identified compounds, the highest amounts were detected for luteolin 8-*C*-glucoside in *Tragopogon pratensis* and *Knautia integrifolia*, with 8344 and 6741 mg kg^{-1} dw (representing 66.2 and 89.6% of TFLc, respectively). Luteolin *O*-rutinoside was detected in all samples, except *Sonchus asper*, with relative amounts ranging between 0.1-73.6% TFLc, and the highest value for *Taraxacum officinale*. The highest relative content of luteolin 7-*O*-glucoside was found in *Crepis vesicaria* (54.2% of TFLc), whereas *Sonchus* species showed higher relative contents of luteolin 7-*O*-glucuronide, with 23.4-23.7% of TFLc.

Kaempferol derivatives. 5 compounds were identified as kaempferol derivatives: kaempferol 7-*O*-glucoside, kaempferol glucuronide, kaempferol malonyl glucoside, kaempferol 3-*O*-rutinoside, and kaempferol 3-*O*-glucoside that was quantified as a sum with luteolin 4'-*O*-glucoside. Relative content of kaempferol derivatives ranged between 0.1-86.2% of TFLc with the highest value for

Crepis sancta, showing high relative contents of kaempferol 7-*O*-glucoside (72.6% of TFLc) and kaempferol 3-*O*-rutinoside (13.1% of TFLc). High kaempferol derivatives values were also observed in *Reichardia picroides* (43.4% of TFLc), with 38.8% represented by the sum of kaempferol 3-*O*-glucoside and luteolin 4'-*O*-glucoside. In only 3 cases, belonging to *Cichorium intybus*, the fraction of kaempferol derivatives was higher than the fraction of luteolin derivatives. In this case, the most representative compound was kaempferol glucuronide (10.2-15.1% of TFLc). Heimler *et al.* (2009) also detected kaempferol glucuronide as the most abundant kaempferol derivative in *Cichorium intybus*

Apigenin derivatives. 7 apigenin derivatives were identified. The most abundant were apigenin glucuronide, apigenin 7-*O*-glucoside and apigenin. Relative content of apigenin derivatives ranged between 0.1-69.2% TFLc, with the highest value detected in *Sonchus asper* (69.2% TFLc) and for *Sonchus oleraceus* (38.3% TFLc); for both *Sonchus* species the most representative derivative was apigenin glucuronide, with 65.0 and 34.6% of TFLc respectively. Apigenin derivatives represented 10.2% of TFLc in *Reichardia picroides*. Apigenin 8-*C*-glucoside and apigenin 6-*C*-glucoside are characteristic flavonoids of *Tragopogon pratensis*, with 8.9 and 6.0% of TFLc respectively; very little amounts were detected in *Crepis leontodontoides* and *Knautia integrifolia*. In the other cases, apigenin derivatives were less than 3% of TFLc.

Quercetin derivatives. 10 flavonoids were identified as quercetin derivatives; quercetin 3-*O*-galactoside, quercetin malonyl glucoside, and quercetin 3-*O*-glucuronide were the most abundant compounds. The sum of all quercetin derivatives ranged between 0.2-82.1% of TFLc. The highest relative content was found for the commercial variety of *Cichorium intybus* and in both wild *Cichorium intybus* ranged between 75.8-79.1% TFLc. The high relative content detected in *Cichorium intybus* was mostly due to the presence of quercetin 3-*O*-glucuronide (41.5-58.9% of TFLc), quercetin malonyl glucoside (10.1-15.1% of TFLc) and quercetin 3-*O*-galactoside (7.3-14.4% of TFLc). Relative quercetin derivatives content of *Crepis leontodontoides* were 48.6% of TFLc, with prevailing composition in quercetin 3-*O*-rutinoside and quercetin *O*-rhamnohexoside. Small amounts of quercetin 3-*O*-glucoside were detected in *Sonchus oleraceus*, whereas quercetin 3-*O*-arabinoside was identified in *Crepis leontodontoides* and *Crepis sancta*.

Anthocyanins

Anthocyanins content (**Figure 6.1.2**) were very low (4-627 mg kg^{-1} dw), compared with both hydroxycinnamic acids and flavonoids, never exceeding 1% of total phenolics, except for *Cichorium intybus*, where the percentage ranged from 1.5 to 3.1%. Among the other species higher relative contents were shown by *Sonchus oleraceus* and *Sonchus asper*, with 0.9 and 0.5%, respectively (**Table 6.1.3**). Anthocyanins were absent in *Crepis leontodontoides*.

Three compounds were identified: cyanidin 3-*O*-(6''-*O*-malonyl)-glucoside, delphinidin 3-*O*-(6''-*O*-malonyl)-glucoside, cyanidin 3-*O*-glucoside. All them were present in *Cichorium intybus. Knautia integrifolia* only contained cyanidin 3-*O*-glucoside, whereas cyanidin 3-*O*-(6''-*O*-malonyl)-glucoside was only identified in *Crepis sancta* and *Hypochaeris radicata.* Delphinidin 3-*O*-(6''-*O*-malonyl)-glucoside was detected in *Crepis vesicaria* and *Tragopogon pratensis*.

Locality and species × locality effects on phenolic contents

Significant variability was detected among species for their content of phenolic compounds. Whereas locality and species × locality interaction in some cases were not significant (**Table 6.1.3**). However considering total phenolic, total hydroxycinnamic acid, total flavonoid and total anthocyanin relative contents also locality and species × locality interaction resulted significant. Common species from Monterchi showed significant higher hydroxycinnamic acid relative contents, mainly due to 5-O-caffeoylquinic acid, in fact the sample of *Helminthia echioides* from this locality showed a high relative content. On the contrary dicaffeoylquinic acid resulted the most abundant in *Crepis vesicaria* and *Cichorium intybus* from Bertinoro, with respect to the common species from Monterchi.

Species from Bertinoro showed a higher flavonoid relative content; this fact was mainly determined by *Crepis sancta* from Bertinoro that showed the highest relative contents for some kaempferol derivatives, in particular kampferol and kaempferol 7-O-glucoside. Moreover for *Helminthia echioides* from Bertinoro higher relative contents of luteolin O-rutinoside, quercetin 3-O-galactoside and methyl quercetin glucuronide were observed, with respect to the sample from Monterchi.

In all cases anthocyanin relative contents resulted higher for the samples from Bertinoro, mainly due to the contribute of *Cichorium* and *Crepis* species.

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Table 6.1.3. Relative phenolic contents, expressed as mg mg^{-1} dry weight, detected in the samples. The acronyms used for each sample are the same reported in **Table 6.1.1**.

Phenolic relative content (mg mg^{-1} dw)	**CIc**	**CIb**	**CIm**	**HEb**	**HEm**	**CSb**	**CVb**	**CLm**	**CJb**	**RPb**	**TOb**
Hydroxycinnamic acid relative content (mg mg^{-1} on total hydroxycinnamic acid content)											
Monocaffeoyltartaric acid	0.017	0.014	0.023	0.050	0.017	-	0.015	0.006	0.015	0.011	0.000
5-*O*-caffeoylquinic acid (chlorogenic acid)	0.555	0.525	0.587	0.269	0.463	0.468	0.355	0.347	0.614	0.524	0.242
Dicaffeoyl tartaric acid (chicoric acid)	0.097	0.133	0.195	0.649	0.413	0.328	0.353	0.488	0.151	0.001	0.674
p-coumaroylquinic acid	0.071	0.003	0.009	0.018	0.002	0.011	0.003	0.017	0.009	0.007	0.013
Caffeic acid	0.004	-	0.003	0.009	0.009	0.005	0.008	0.004	0.006	-	0.003
Feruloylquinic acid	0.050	0.013	0.035	0.002	0.006	0.007	0.009	0.004	0.018	0.001	0.002
Dicaffeoylquinic acid	0.205	0.312	0.148	0.003	0.089	0.181	0.258	0.135	0.186	0.456	0.066
Flavonoid relative content (mg mg^{-1} on total flavonoid content)											
Luteolin diglucoside	0.004	0.001	0.003	-	-	0.000	-	0.002	-	-	-
Luteolin 7-*O*-glucuronide	0.033	0.017	0.016	-	0.019	0.009	0.072	0.065	0.011	0.099	-
Kaempferol 7-*O*-glucoside	0.000	-	-	0.013	0.001	0.726	-	-	-	0.026	-
Luteolin-8-*C*-glucoside (orientin)	-	0.003	0.002	-	0.068	-	0.192	0.181	-	-	-
Quercetin 3-*O*-glucuronide	0.589	0.415	0.510	-	-	-	-	-	-	-	-
Quercetin *O*-rhamnohexoside	-	-	-	0.001	0.005	0.000	-	0.270	0.242	0.000	0.000
Apigenin glucuronide	0.005	0.011	0.009	0.001	0.001	0.001	-	0.001	0.002	0.102	-
Apigenin-8-*C*-glucoside (vitexin)	-	-	-	-	-	-	-	0.001	-	-	-
Apigenin-6-*C*-glucoside (isovitexin)	-	-	-	-	-	-	-	0.001	-	-	-
Luteolin *O*-rutinoside	0.003	0.001	0.001	0.536	0.325	0.000	0.135	0.115	0.527	0.209	0.736
Quercetin malonyl glucoside	0.114	0.151	0.101	0.007	0.017	0.002	0.036	0.006	0.022	0.000	0.016

Kaempferol glucuronide	0.102	0.120	0.151	-	0.085	-	0.009	-	-	0.001	-
Luteolin 7-*O*-glucoside	0.014	0.006	0.004	0.107	0.298	0.088	0.542	0.123	0.092	0.070	0.137
Quercetin 3-*O*-galactoside (hyperoside)	0.073	0.124	0.144	0.148	0.014	-	0.006	0.053	0.015	0.002	0.013
Methyl quercetin glucuronide	0.045	0.081	0.001	0.068	0.011	0.002	0.003	0.040	0.011	0.000	0.007
Quercetin 3-*O*-rutinoside (rutin)	-	0.020	0.003	0.002	0.001	-	-	0.102	-	-	0.003
Kaempferol malonyl glucoside	-	-	-	0.002	0.005	0.006	-	0.001	0.000	0.011	0.000
Apigenin-7-*O*-rutinoside (isorhoifolin)	-	-	-	0.001	-	0.000	0.001	0.000	0.001	0.004	-
Apigenin-7-*O*-glucoside	0.003	0.003	0.002	0.019	0.003	0.001	0.000	0.001	0.009	0.028	0.002
Apigenin-7-*O*-neohesperidoside (rhoifolin)	-	0.000	0.000	0.009	0.000	0.000	-	-	0.002	0.008	0.000
Luteolin malonyl hexose	0.001	0.001	0.002	-	-	-	-	-	-	-	-
Kaempferol 3-*O*-glucoside+ Luteolin 4'-*O*-glucoside	0.009	0.045	0.050	0.067	0.130	-	-	0.012	0.002	0.388	-
Kaempferol 3-*O*-rutinoside	0.002	-	0.000	0.013	0.007	0.131	-	-	0.062	0.008	0.001
Luteolin	0.002	-	0.000	0.006	0.007	0.031	-	0.011	0.001	0.044	0.085
Apigenin	0.002	0.001	0.001	-	0.002	0.001	0.000	0.005	0.001	-	-
Anthocyanin relative content											
(mg mg^{-1} anthocyanin relative content)											
Delphinidin 3-*O*-(6"-*O*-malonyl)-glucoside	0.566	0.232	0.295	0.719	0.781	-	1.000	-	0.753	0.852	0.088
Cyanidin 3-*O*-(6"-*O*-malonyl)-glucoside	0.404	0.427	0.521	0.281	0.219	1.000	-	-	0.247	0.148	0.912
Cyanidin 3-*O*-glucoside	0.031	0.341	0.184	-	-	-	-	-	-	-	-
Phenolic class relative content											
(mg mg^{-1} on total phenolic content)											
Sum of hydrocinnammic acid	0.907	0.878	0.870	0.691	0.964	0.806	0.987	0.981	0.856	0.946	0.896
Sum of flavonoids	0.083	0.091	0.115	0.308	0.034	0.194	0.013	0.019	0.143	0.054	0.104
Sum of anthocyanins	0.010	0.031	0.015	0.001	0.002	0.000	0.001	-	0.001	0.000	0.000

Continued

Phenolic relative content (mg mg^{-1} dw)	**TPb**	**SAb**	**SOb**	**HRm**	**KIm**	**Species** Sign.(LSD) [1]	**Locality** Sign.(LSD) [1]	**Locality × Species** Sign.(LSD) [1]
Hydroxycinnamic acid relative content (mg mg^{-1} on total hydroxycinnamic acid content)								
Monocaffeoyltartaric acid	-	0.210	0.045	-	0.014	** (0.005)	** (0.003)	ns
5-*O*-caffeoylquinic acid (chlorogenic acid)	0.896	0.662	0.514	0.643	0.671	** (0.057)	* (0.033)	** (0.057)
Dicaffeoyl tartaric acid (chicoric acid)	0.012	0.022	0.195	0.237	0.001	** (0.050)	* (0.029)	** (0.050)
p-coumaroylquinic acid	0.011	0.012	0.005	0.003	0.015	** (0.004)	* (0.002)	** (0.004)
Caffeic acid	0.001	-	0.023	0.004	0.001	** (0.002)	* (0.001)	ns
Feruloylquinic acid	0.012	0.011	0.009	0.001	0.008	** (0.005)	** (0.003)	** (0.005)
Dicaffeoylquinic acid	0.069	0.083	0.208	0.112	0.291	** (0.046)	** (0.027)	ns
Flavonoid relative content (mg mg^{-1} on total flavonoid content)								
Luteolin diglucoside	-	-	-	-	-	** (0.001)	** (0.001)	ns
Luteolin 7-*O*-glucuronide	-	0.237	0.234	0.005	-	** (0.021)	ns	ns
Kaempferol 7-*O*-glucoside	-	-	0.001	0.003	0.003	** (0.022)	** (0.013)	** (0.022)
Luteolin-8-*C*-glucoside (orientin)	0.662	-	-	-	0.896	** (0.021)	** (0.012)	** (0.021)
Quercetin 3-*O*-glucuronide	-	0.000	0.001	-	0.000	** (0.115)	ns	ns
Quercetin *O*-rhamnohexoside	0.001	-	-	0.003	0.001	** (0.001)	** (0.001)	** (0.001)0
Apigenin glucuronide	-	0.650	0.346	0.003	-	** (0.028)	ns	ns
Apigenin-8-*C*-glucoside (vitexin)	0.089	-	-	-	-	** (0.013)	** (0.008)	** (0.013)
Apigenin-6-*C*-glucoside (isovitexin)	0.060	-	-	-	0.015	** (0.002)	** (0.001)	** (0.002)
Luteolin *O*-rutinoside	0.135	-	0.002	0.013	0.001	** (0.028)	** (0.016)	** (0.028)
Quercetin malonyl glucoside	0.002	0.011	0.051	0.079	-	** (0.036)	ns	ns

Kaempferol glucuronide	0.000	-	0.003	0.027	0.000	** (0.009)	** (0.005)	** (0.009)
Luteolin 7-*O*-glucoside	0.004	0.029	0.239	0.376	0.004	** (0.028)	** (0.016)	** (0.028)
Quercetin 3-*O*-galactoside (hyperoside)	0.015	0.011	0.033	0.076	0.025	** (0.023)	ns	** (0.023)
Methyl quercetin glucuronide	0.008	0.006	0.020	0.046	0.014	** (0.009)	** (0.005)	** (0.009)
Quercetin 3-*O*-rutinoside (rutin)	0.001	-	0.005	0.001	-	** (0.019)	** (0.011)	** (0.019)
Kaempferol malonyl glucoside	-	0.000	0.018	0.018	0.000	** (0.006)	ns	** (0.006)
Apigenin-7-*O*-rutinoside (isorhoifolin)	-	0.001	0.001	-	-	** (0.000)	ns	ns
Apigenin-7-*O*-glucoside	0.000	0.019	0.025	0.014	0.000	** (0.005)	** (0.003)	** (0.005)
Apigenin-7-*O*-neohesperidoside (rhoifolin)	-	0.005	0.005	-	0.000	** (0.001)	** (0.001)	** (0.001)
Luteolin malonyl hexose	-	-	-	-	-	** (0.001)	** (0.000)	** (0.001)
Kaempferol 3-*O*-glucoside+ Luteolin 4'-*O*-glucoside	0.003	-	-	0.213	0.020	** (0.042)	ns	ns
Kaempferol 3-*O*-rutinoside	0.008	-	0.000	0.003	0.009	** (0.009)	** (0.005)	** (0.009)
Luteolin	0.001	0.014	0.009	0.113	0.002	** (0.013)	ns	ns
Apigenin	0.008	0.017	0.006	0.008	0.000	** (0.001)	** (0.001)	** (0.001)
Anthocyanin relative content								
(mg mg^{-1} anthocyanin relative content)								
Delphinidin 3-*O*-(6"-*O*-malonyl)-glucoside	1.000	0.845	0.289	-	-	** (0.041)	* (0.024)	** (0.041)
Cyanidin 3-*O*-(6"-*O*-malonyl)-glucoside	-	0.155	0.711	1.000	-	** (0.228)	** (0.131)	ns
Cyanidin 3-*O*-glucoside	-	-	-	-	1.000	** (0.042)	** (0.024)	** (0.042)
Phenolic class relative content								
(mg mg^{-1} on total phenolic content)								
Sum of hydrocinnammic acid	0.346	0.735	0.836	0.985	0.873	** (0.021)	** (0.012)	** (0.021)
Sum of flavonoids	0.654	0.257	0.155	0.015	0.123	** (0.021)	** (0.012)	** (0.021)
Sum of anthocyanins	0.000	0.009	0.010	0.000	0.004	** (0.002)	** (0.001)	** (0.002)

[1] Significance: *: $p \leq 0.05$; **: $p \leq 0.01$; ns: non significant; values in parenthesis: LSD, $p = 0.05$

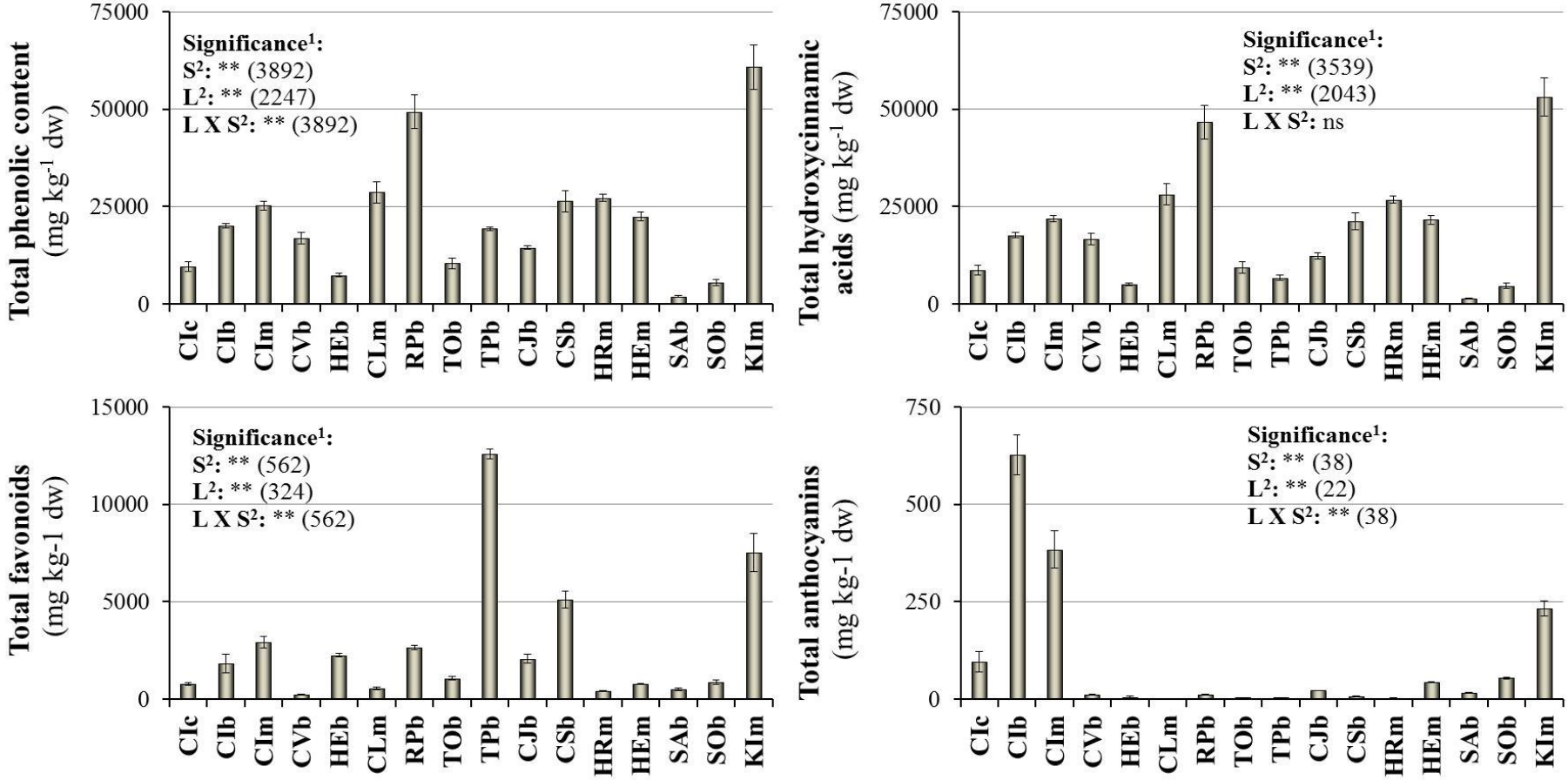

Figure 6.1.2. Total phenolic, hydroxycinnamic acid, flavonoid, and anthocyanin contents, expressed as mg kg^{-1} dw, in edible wild leafy vegetables. The acronyms used for each sample are the same reported in **Table 6.1.1**.

[1] Significance: **, $p \leq 0.01$; ns, non significant; values in parenthesis: LSD, $p = 0.05$.

[2] S: species; L: locality; L × S: locality × species interaction.

For what concern absolute contents (**Figure 6.1.2.**) we noticed, generally, higher phenolic contents for the sample from Monterchi. All the three species from Monterchi showed higher total phenolic absolute contents, with significant effects of locality and the interaction of species × locality. Also total hydroxycinnamic acid absolute contents were higher for the samples from Monterchi, however in this case the effect of the interaction resulted not significant. The same trend was found for total flavonoids when considered *Cichorium* species, whereas for the other samples a higher absolute content of flavonoids was calculated for *Crepis sancta* and *Helminthia echioides* from Bertinoro.
In case of total anthocyanins a significant higher absolute amount was detected for *Cichorium intybus* from Bertinoro

Tentative profiling on the basis of phenolic composition

Principal component analysis gave a further contribution to profile edible wild leafy vegetables as a function of phenolic composition. Three principal components were extracted, explaining 77.16% of the total variance, as reported in **Table 6.1.4**.

Table 6.1.4. Loadings of first three principal components (PC) on phenolic composition, and explained variance of individual PC.[1]

	Principal components		
	PC1	PC2	PC3
Total phenolic content	ns	ns	ns
Relative hydroxycinnamic acid content	**0.948****	ns	ns
Relative anthocyanin content	ns	**-0.875****	ns
Relative luteolin isomer content	-0.865*	ns	ns
Relative kaempferol isomer content	ns	ns	**0.870****
Relative apigenin isomer content	-0.765*	ns	-0.348*
Relative quercetin isomer content	ns	**-0.867****	ns
Explained total variation (%)	35.21	25.94	16.01

[1] *: Significant at $p \leq 0.05$; **: Significant at $p \leq 0.01$; ns: non significant**.**

Figure 6.1.3 reports the layouts of samples in PC space. PC1, explaining 35.21% of variance, was positively correlated to hydroxycinnamic acids relative content, and negatively to luteolin and apigenin isomers relative contents. PC1 discriminates *Tragopogon pratensis*, which is the only

species having higher flavonoids than hydroxycinnamic acids. PC2, explaining 25.94% of variance, was negatively correlated to the relative contents of anthocyanins and quercetin isomers. *Cichorium* species, showing higher contents of anthocyanins and quercetin isomers, were effectively separated from other samples by PC2. Moreover PC2 also slightly separated the group of *Sonchus oleraceus*, *Sonchus asper*, *Chondrilla juncea* and *Helminthia echioides* from Bertinoro, with lower total phenolic content. Finally PC3 explaining 16.01% of variance was positively correlated to kaempferol isomers relative content and negatively, with lower values, to apigenin isomers and hydroxycinnamic acids relative content. In this case *Crepis sancta*, with the highest kaempferol isomer relative content, was well separated. PC3 could also be used to discriminate, although with a less marked difference, *Sonchus asper* by the other samples, such as *Helminthia echioides* and *Chondrilla juncea* from Bertinoro.

CONCLUSIONS

Several species belonging to the family of *Asteraceae* together with *Knautia integrifolia* of the *Dipsacaceae* family were analyzed by means of LC-HRMS for the first time. The use of Orbitrap as HRMS contributed to a satisfactory detection of several minor compounds and their subsequent quantification.

This study represents a contribution to the investigation of bioactive compounds in edible wild leafy vegetables, mainly focused on their content in phenolic compounds.

According to the results, specific phenolic compounds fingerprints were detected for edible leafy vegetables. This fact was more evident when the class of flavonoids was considered: some compounds were identified and quantified only in specific samples, so they could be considered characteristic compounds for these species.

Several classes of phenolics were useful to discriminate *Cichorium* and other species. Based on phenolic composition, it was not possible to discriminate *Knautia integrifolia*, even if this species belong to another botanical family (*Dipsacaceae*).

Wild leafy vegetables could play an important role in diet biodiversity; in fact they could contribute to supply several minor compounds when they are used as food.

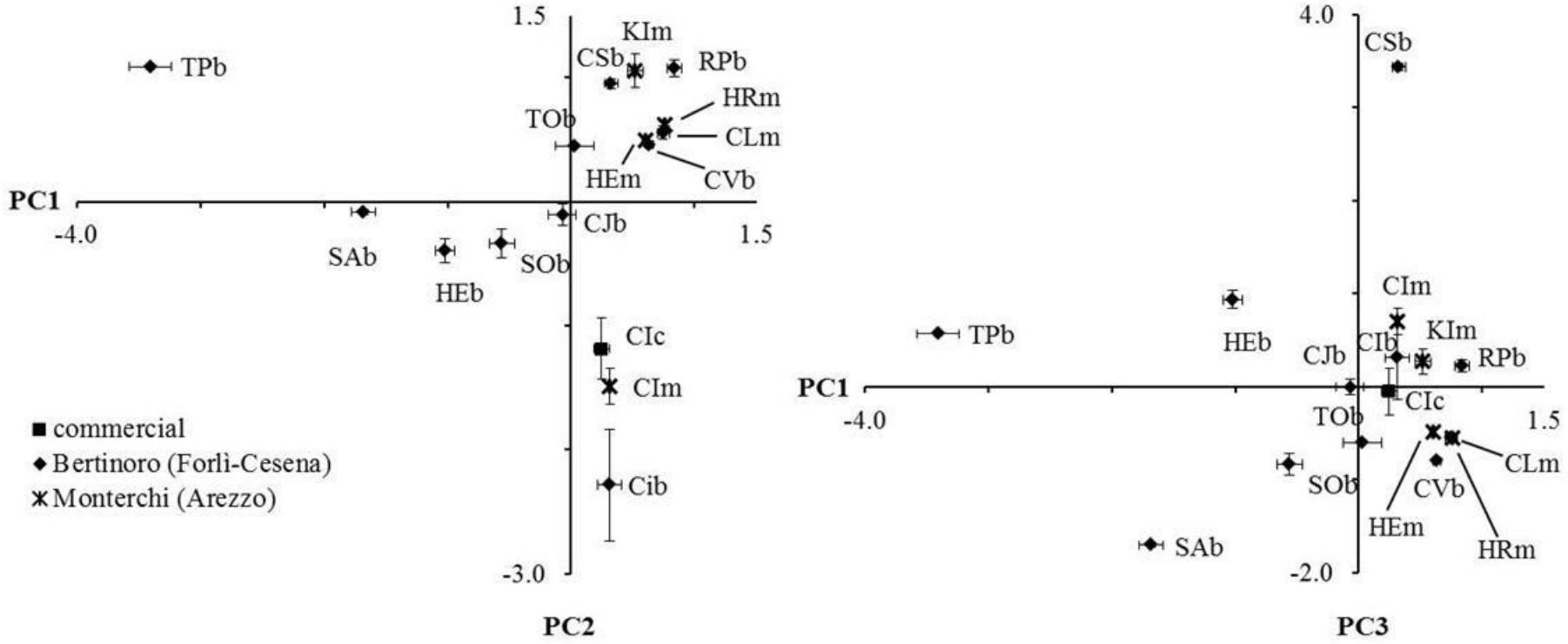

Figure 6.1.3. Layout of edible wild leafy vegetables species on the principal component space, as determined by principal component analysis of phenolic compounds. The acronyms used for each sample are the same reported in **Table 6.1.1**.

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Chapter 7

Conclusions

The aim of this thesis was the documentation and characterization of traditional crops and foods, following BaSeFood scheme and carry out experimental activity of local resource characterization according to three main lines: 1) local documentation, 2) plant raw material characterization and 3) evaluation of process' yield factors and phytochemical retention following cooking.
Hereafter the main findings, for each one of these parts, are reported and discussed.

7.1. Local documentation

The documentation of traditional crops and foods, in a selected area of north Tuscany (mainly represented by the province of Arezzo) was carried out following BaSeFood approach, defined as a bottom-up approach (**Chapter 3**). In this way we had a direct witness from local people, with first-hand experience of the values of traditional crops and foods. In particular, with this approach, we could highlight the strong link between traditional foods and local skills and knowledge. To transmit this aspect to modern urban consumers could represent one of the main challenges for a correct approach to traditional food appreciation. Qualitative interviews, carried out by means of open questions, allowed us, in a certain way, to not rule out any topic that could result interesting for an eventual experimental development of this thesis.
Documentation was also carried out in a way to detect, in the selected geographic area, plant raw materials that could fit the following characterization activity purposes. In particular, in the province of Arezzo, two kinds of leafy vegetables resulted interesting: kale (*Brassicaeae* family) and edible wild leafy vegetables (mainly belonging to *Asteraceae* family).
Kale is a traditional crop from the province of Arezzo, and Tuscany more in general. In the last years it became part of endangered crops, so we assisted to a slight expanding production trend, mainly due to the recovery of urban consumer interest about traditional crops and foods. Moreover kale is traditionally cooked and used to prepare soups (Ribollita Toscana).
Harvesting edible wild leafy vegetables resulted a very common practice for this area, during spring time. Their use is strictly related to knowledge in local contexts and to local availability. These vegetables can be used fresh to prepare salads, or even after cooking.
So, after documentation, these vegetables were taken into account for experimental activities.

Main results obtained from documentation showed, in particular, that these activities could represent a useful tool for two main reasons:
1) documentation could be very helpful for consumers that could discover or re-discover traditional foods; in fact in many cases in urban contexts the term “traditional” is perceived as a credence trait, so it need to be communicated from other parties. Moreover by re-introducing in their diet traditional foods, consumers could contribute to enhance the intake of some minor compounds, otherwise not provided;
2) documentation could be helpful also for stakeholders, as smallholder farmers and producers, who could use the results to promote their product, for process optimization and raw material selection.

7.2. Plant raw material characterization

Many traditional recipes contain a local crop as main ingredient. Traditional crops are generally characterized by high variability in morphological traits among local populations. The valorization of these crops requires, after the enhancement of their knowledge, the characterization of the within species variability, with particular attention to their content in phytochemicals. Traditional crops considered in the experimental part were: 1) primitive wheats (from a previous documentation activity carried out during BaSeFood project development), 2) kale and 3) edible leafy vegetables (from the activity of documentation carried out during the thesis).
Experimental activities highlighted that local populations are also characterized by high variability for what concern their content of phytochemicals. In our cases local accessions of primitive hulled wheats (mainly einkorn and emmer wheats) and kale were for the first time explored for their content in bioactive compounds. In particular cross-country comparisons were carried out (**Chapter 4.1** and **Chapter 5.1**). Also wild leafy vegetables represent a good source of phytochemicals (**Chapter 6.1**), in particular a great variability within species was observed for minor phenolic compounds, mostly represented by glycosidic forms of flavonoids. However their knowledge is nowadays very limited between younger generations, so documentation could have a role in covering this gap and transmit an appropriate information.
Experimental activities contributed to further knowledge of local populations bioactive composition and also these data could be exploited by food chain stakeholders.

7.3. Process' yield factors and phytochemical retention following cooking

The variability detected in local accessions is also important because it has been demonstrated that the chemical composition of plant matrix can contribute to determine the final retention after processing/cooking. So, based on their morphological traits and chemical composition, some local populations could result particularly interesting for specific purposes.
The analysis of the processing used to obtain traditional foods from raw materials, evaluating the technological chain effects on phytochemicals and comparing the different local-scale production flow charts, represented therefore a characterizing part of this work. Determination of yield factors during traditional processing and phytochemical compounds retention factors during cooking was useful in analyzing and highlighting critical points along the food processing/cooking chain. Experimental activities were mainly addressed to evaluate the fate of bioactive compounds during traditional food processing/cooking.

Hulled wheat products represent a good example of traditional product evolution as affected by simple technologies (**Chapter 4.2** and **Chapter 4.3**). In Italy the need to meet the growing market demand for whole kernels, determined the introduction of new machines for glume removal. The readapted two-step procedure, with minimal kernel breaking, gave the opportunity to obtain two kinds of products, crushed or peeled kernels, the latter preferred by consumers. The great flexibility assured by the modern plant may also promote the on-site preservation of local landraces, mostly associated to niche products in urban contexts. In some cases the recovery of emmer wheat production has resulted in the registration of PGI for the Garfagnana emmer (north Tuscany), and PDO for the Monteleone di Spoleto emmer (south Umbria). Moreover the resulting waste from pearling, mainly consisting of the bran part, showed the highest contents in all bioactives, representing an interesting by-product. However comparing traditional plants from Turkey and Armenia with the updated procedure adopted in Italy we found similar results for both yield factors and phytochemical retention of crushed kernel fractions. So, in this case, the introduction of new technologies has apparently little effect of final outputs of the processes, their efficacy being mostly connected to time and labour savings.

The preparation of traditional recipes in most case includes cooking as an important operation. Most commonly documented cooking ways resulted to be boiling and stir frying; these methods were compared with a more modern practice, steaming. Our results showed that cooking may either negatively or positively affect bioactive retention and potential availability. From **Chapter 5.2**,

following cooking, in many cases we observed a decrease in bioactive compounds, and this fact could be due to thermal degradation, as observed for vitamin C in kale, or their leaching following boiling in water, as for phenolic compounds, or hydophilic compounds in general; in case of glucosinolates both facts occurred. On the other hand, when considering carotenoids, we observed an increase of their concentration following boiling and steaming, due to a major extractability, mainly associated to a matrix effect.

In general higher decreases of phytochemicals were observed following boiling and stir frying, whereas for steaming lower losses were found, indicating this method as being more conservative method for phytochemical compounds retention. However during boiling, most losses were due to the leaching of phytochemicals in cooking water, so in case of soups they are not lost.

Consumers often perceive food processing as negative for the healthiness of vegetables, but more and more studies are showing that, when it is properly done, it may even enhance some nutritional qualities.

The use of kinetic models to evaluate bioactive content resulted useful to make prevision around the formation or degradation of compounds. Moreover kinetic models associated to relative bioactive content allowed to make comparison among different local populations and cooking ways.

Traditional foods, and foods in general, are very complex systems in which many variables occur and can have a role to determine changes during processing/cooking. In many cases traditional foods are prepared by using and mixing together more than one ingredient during their preparation. So other ingredients of a recipe could have an important role in the fate and thermal degradation of bioactive during cooking. This point is still poorly explored and further studies are necessary to better understand and elucidate which components and mechanisms are involved. The use of model systems, as reported in **Chapter 5.3**, represents a good starting point to investigate the effect of the complexity of food matrix on reaction kinetics.

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