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Cereals and pomegranate: insight into their phenols and polysaccharides

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Preamble

This PhD work started as a continuation of my master's thesis in pharmacy, with the title "Millet from Burkina Faso: evaluation of bioactive compounds before and after fermentation". Under the guidance of Professors Nadia Mulinacci and Marzia Innocenti I was involved in the NUTRATOSCAFRICA project, born with the aim of finding a correlation between daily-consumed African cereals and a greater protection against gastrointestinal diseases. Within the NUTRATOSCAFRICA project my research group was involved in evaluating the phenolic compounds in cereals before and after fermentation. Cereals represent the first chapter of my PhD thesis, with particular attention on modern and ancient wheat species, millet as a rediscovered crop and fermentation as a useful technique to improve the nutritional value of cereals.

The main goals in working with cereals have been:

- Optimization of extractive procedures to accurately estimate the phenolic amount in cereals, focusing attention on bound phenols. Our newly investigated approach proposes a single extraction step in acidic conditions.

- Evaluation of phenolic content in cereal samples before and after fermentation.

- Systematic comparison, in terms of phenolic compounds, between ancient and modern wheat species grown in the same agronomical condition.

- *In vitro* biological tests to evaluate the antidiabetic, prebiotic, and anti-inflammatory activity of our extracts.

The second chapter of my PhD thesis is focused on pomegranate. In particular, my work investigated phenolic and polysaccharides fractions. In this context, part of the project was carried out at the University of Aveiro, Portugal, in the last year of my PhD, thanks to a collaboration with Professor Manuel Coimbra. The aim was to improve my knowledge about polysaccharides.

The main goals regarding pomegranate have been:

- Evaluation of phenolic composition of both peel and arils of a new Spanish pomegranate cultivar, Purple Queen®, grown on different sediments dredged from the port of Livorno, Italy.

- Systematic comparison of the phenolic composition of fifteen different pomegranate varieties, grown in the same nursery.

- A focus on polysaccharides of three widespread pomegranate varieties. This work is still on going.

Chapter 1

Cereals

1.1 Introduction

Cereals, in the *Graminacee* family, are considered staple foods recognized as good sources of minerals, vitamins, fiber, essential fatty acids, and protein and greatly contribute to the nutritional balance of the world's population (Hussain *et al.,* 2019). Characterized by a great adaptability to different environments, easy preservation and transportability, cereals represent the most important crops in the world and are transformed into a range of products able to satisfy the habits and food traditions of different countries. In terms of consumption, cereals have a privileged position among other agricultural products. According to FAO (Food and Agriculture Organization), their production accounts for about 2800 million tons per year with the main recognized producing countries of Africa and Asia contributing 80% of global cereal production (Figure 1.1.1).
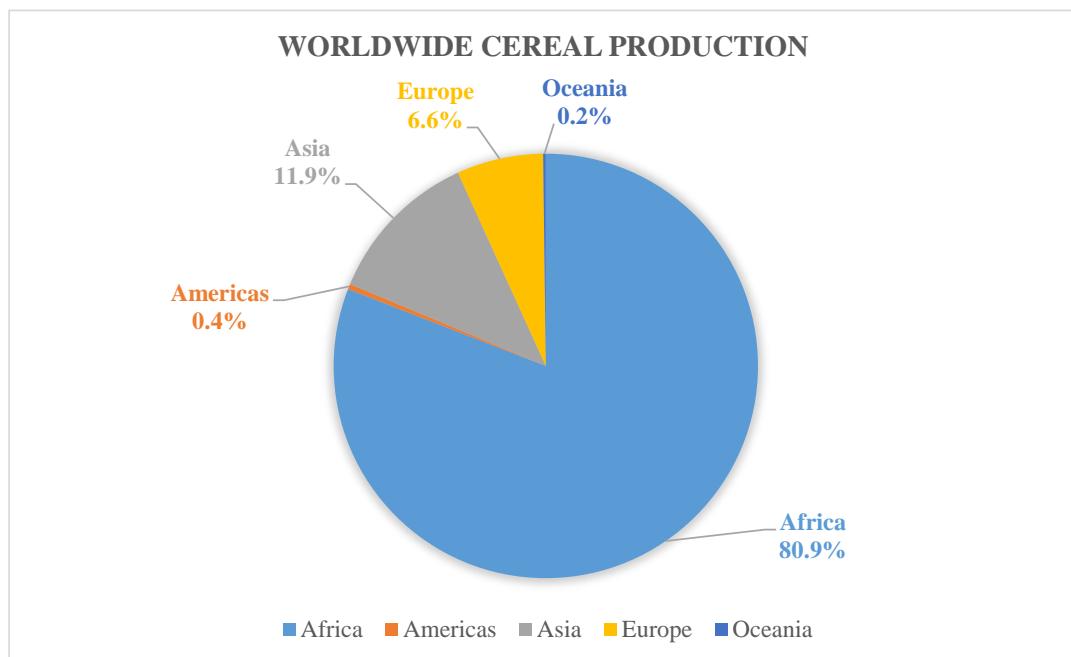

Figure 1.1.1 Distribution of cereals production worldwide (FAO, 2018).

Regular consumption of cereal grains, especially in their whole-grain form, has gained popularity and their related products have been regarded as ''healthy and functional foods'' because of their potential protection against life-style and diet-related disorders, such as obesity, diabetes, cardiovascular diseases and cancers (Masisi *et al.,* 2016). Although the general requirements of human diet are met mainly by wheat and other widely-grown grains, such as rice and maize, the growing emphasis of today on a healthy and balanced diet has created a demand for new, rediscovered crops or ancient cereal species (Ciesarová *et al.,* 2016).

1.1.1   Wheat: modern and ancient species

Wheat is considered a leading grain for consumption worldwide due to its nutraceutical profile, relatively easy harvesting, storage, transportation and processing as compared to other grains (Ciesarová *et al.,* 2016). Wheat production accounted for about 749 million tons in 2016: 40% distributed in Asia, with China as the main producer, 30% in Europe and 15% in America. Africa and Oceania are leading regions in the production of other cereal varieties (FAOSTAT, 2018). From a botanical point of view, wheat includes several species belonging to the genus *Triticum*. The best known species are *Triticum aestivum* L. and *Triticum durum* L.. The former, also known as "common wheat" or "soft wheat", is hexaploid wheat and constitutes 95% of the total production. Its flour is suitable for bread and bakery products. The remaining 5% includes *Triticum durum* L., tetraploid wheat also known as "durum wheat", which is traditionally used for pasta production (Dhanavath *et al.,* 2017; Dinu *et al.,* 2018). A further classification recognizes wheat as "ancient" or "modern" grains. In the last century, wheat breeding efforts concentrated on yield increases, typically using high-energy inputs in terms of fertilizers, herbicides, insecticides and fungicides to produce modern varieties characterized by genetic uniformity and adaptation to conventional agriculture (Dinu *et al.,* 2018; Arzani *et al.,* 2017). This agronomic trend is commonly referred as the Green Revolution, when Mendel's laws were

systematically applied to increase production and the technological quality of wheat grains. Compared to ancient varieties, the modern ones are characterized by several advantages such as higher yields, tolerance to environmental stresses, lower susceptibility to pathologies and insects, higher glutenin content leading to technological improvement in the quality of the bread and pasta. On the other hand, although there is no shared definition, it is generally accepted that ancient wheat has remained unchanged over the last hundred years. From a botanical point of view ancient grains include: einkorn (*T. monococcum* L.), a diploid wheat which is now cultivated in limited regions of the world; emmer (*T. turgidum* L.), a tetraploid wheat and spelt (*T. aestivum*), a hexaploid wheat. The latter two are considered the ancestors of *Triticum durum* L. and *Triticum aestivum* L., respectively (Figure 1.1.2) (Arzani *et al.,* 2017). In addition, there are several cultivars which have remained unchanged over the years: Russello, Senatore Cappelli, Timilia or Tumminia and Urria (*Triticum durum*), as well as Autonomia B, Frassineto, Gentil Rosso, Inallettabile, Maiorca, Sieve, Solina, and Verna (*Triticum aestivum*).
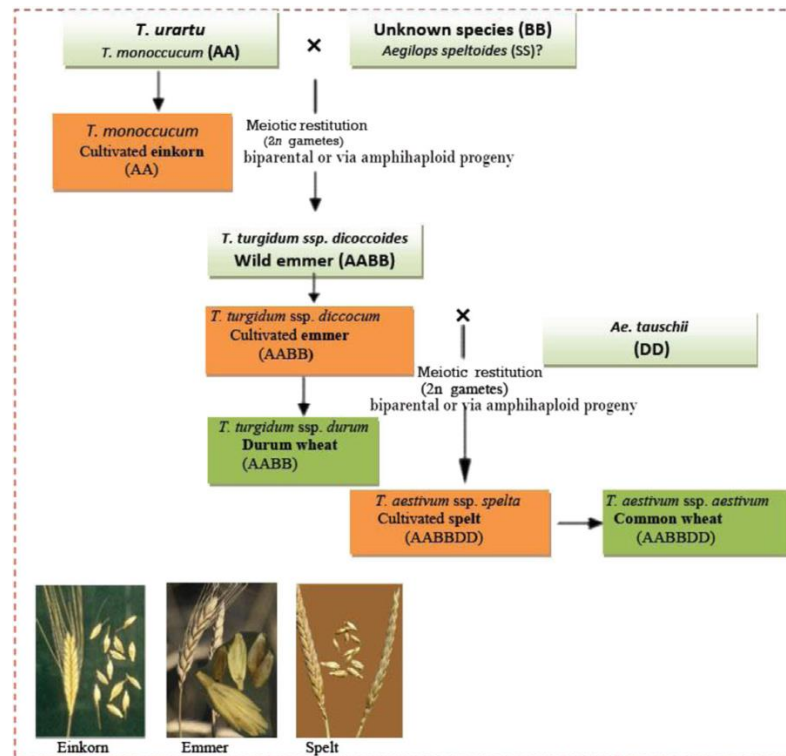
Figure 1.1.2 Phylogeny of *Triticum* species, including eikorn, spelt, durum and common wheat

(Arzani *et al.,* 2017).

The effects on wheat of the agronomic revolution concern not only environmental aspects, as yield and pest resistance, but also nutraceutical ones, linked to wheat caryopsis composition. In fact, intense breeding results in larger wheat grains with a higher percentage of several molecules such as starch, and a lower percentage of others, following the so called "yield dilution phenomenon" (Shewry *et al.,* 2015). Although a number of studies on the content of bioactive molecules in ancient and modern wheat have been published, definitive comparisons of these species grown together in randomised field plots are actually rare. The reason behind this, is that ancient wheat species are usually grown in organic, or traditional low-input farming systems, while modern wheat species are usually bred for high-input intensive systems (Hidalgo *et al.,* 2009; Dinu *et al.,* 2018).

Considering the starch composition, several studies highlighted lower amounts of total and resistant starch in old wheat species compared to modern ones. However, the content of the

slowly digested amylose in ancient varieties was significantly higher than amylopectin, decreasing both glucose and insulin post prandial levels (Brandolini *et al.,* 2011). When analyzing the lipid fraction, it was also observed that eikorn contains approximately 50% more lipids, with higher amount of monounsaturated fatty acids (MUFA) and approximately 21% less saturated fatty acids (SFA) (Hidalgo *et al.,* 2009). These values are associated with a reduction in total cholesterol, LDL cholesterol and triglycerides with a marked improvement in the lipid profile of diabetic patients or those with a high risk of cardiovascular diseases (Dinu *et al.,* 2018). With regard to the mineral content, it is now widely established that modern grains have lower amounts, especially in iron and zinc, than ancient grains (Erba *et al.,* 2011). The lack of these elements in populations of developing countries reinforces the need to support the development of local and diversified cereal crops rich in such micronutrients. Considering the fraction of tocopherols and carotenoids, ancient grains such as eikorn showed 8-10 times higher levels of lutein, the main carotenoid found in cereals, than modern grain and this contributes to increasing the antioxidant properties of the former (Ziegler *et al.,* 2015). Moreover, a recent study conducted by an Italian research group investigated the phenolic fraction in both ancient and modern grains grown in the same Italian field. The results underline a greater quantity of phenolic compounds in the ancient varieties (average of 882 μg/g) compared to the modern ones (average of 662 μg/ g) (Gotti *et al.,* 2018).
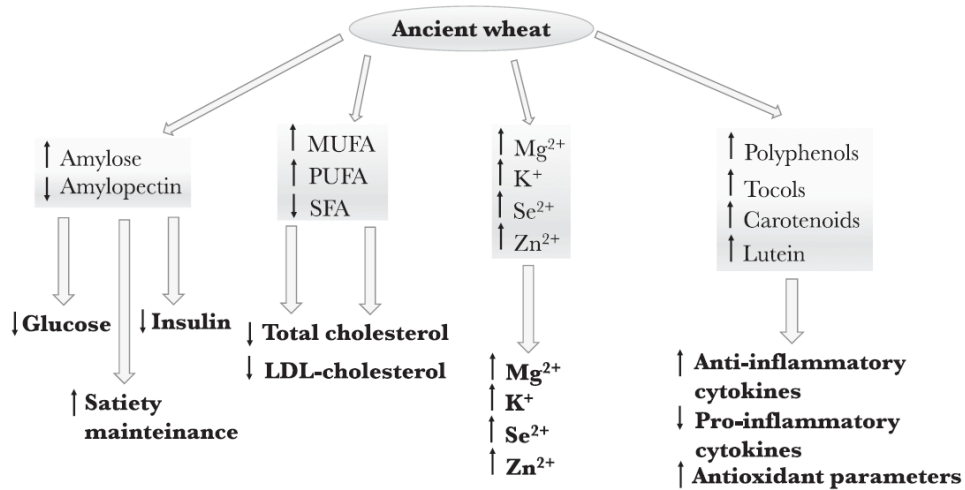
Figure 1.1.3 Beneficial properties of ancient wheat species.

(Arzani *et al.,* 2017)

Concerning *in vivo* studies, in the last few years only five and thirteen studies on animals and humans, respectively, have been published (Dinu *et al.,* 2018). Using rat models, the capability of ancient cultivars to ameliorate the antioxidant and anti-inflammatory parameters in blood plasma and hepatic tissue, with respect to modern wheat species, has been highlighted (Carnevali *et al.,* 2014; Benedetti *et al.,* 2012). Furthermore, the effects of ancient and modern wheat diets on plasma lipid profile and acute glycaemic response on Zucker diabetic fatty rats were evaluated. The results pointed to a less pronounced disease development in rats fed with ancient wheat (Thorup *et al.,* 2014).

Some of the *in vivo* studies on humans investigated the functionality of ancient wheat on oxidative stress and pro inflammatory markers, highlighting beneficial properties linked to the assumption of old varieties instead of modern ones (Dinu *et al.,* 2018). Furthermore, replacing modern durum and common wheat with old varieties could induce a decrease in the so called adverse reaction linked to the consumption of wheat, as wheat allergies and wheat intolerance (Békés *et al.,* 2017). For instance, significantly reduced irritable bowel syndrome (IBS) symptoms were observed by replacing modern durum and common wheat with Kamut® (Sofi *et*

al., 2014). Moreover, a lower secretion of pro-inflammatory cytokine CXCL10, responsible of non-celiac gluten sensitivity (NCGS), was assessed when replacing common wheat with the old durum "Senatore Cappelli", selected by Nazzareno Strampelli in 1915, and Kamut® (Valerii *et al.,* 2015).

Keeping in line with the state of art, it is possible to say that further studies comparing old and modern species grown in the same field need to be carried out, with attention to caryopsis chemical characterization and human intervention trials (Hidalgo *et al.,* 2009; Dinu *et al.,* 2018).

1.1.2 Millet

Millet is a generic term that refers to various small grains belonging to the *Poaceae* family, growing easily in areas of the world with a temperate, tropical, or subtropical climate. Different millet species are present worldwide: *Pennisetum glaucum*, *Eleusine coracana*, *Panicum sumatrense*, *Echinocloa crus-galli*, *Panicum miliaceum*, *Setaria italica* (Figure 1.1.4).

Figure 1.1.4 *Pennisetum glaucum*, *Eleusine coracana*, *Panicum miliaceum*, *Echinocloa crus-galli*, *Panicum sumatrense*, (from left to right).

In comparison to others, this cereal offers the advantages of multiple uses and resistance to drought, allowing it to be grown almost everywhere (Ragaee, 2006; Amadou *et al.,* 2013). Despite being considered a minor cereal in Western countries, it is a main crop, together with barley, spelt and kamut, and a staple food in some regions of Africa and Asia, ranking as the sixth most important crop worldwide (Chandr, 2013). Millet contains high levels of proteins, minerals and vitamins that confer it a higher nutritional value than non-millet cereals. Its proteins are reported to possess significant levels of essential amino acids. Compared to other cereals, millet is rich in slowly digestible starch (SDS) and resistant starch (RS) important in the prevention of diseases related to type-II diabetes, obesity and coronary heart disease due to its resistance to digestion, resulting in a slow release of glucose into the blood stream and reducing postprandial glycaemic and insulinemic responses (Muthamilarasan *et al.,* 2015). The advantages linked to the daily consumption of millet grains have recently been reported in the literature. A 2010 study showed that a typical diet of a child in Burkina Faso, based on cereals and legumes, contributes to a reduced incidence of gastrointestinal diseases, mainly by modulating the intestinal microbiota composition, maximizing energy intake from fibres and protecting from inflammation and non-infectious colonic diseases. In particular, the gut microbiota of fifteen healthy children aged 1-6 years living in a village in rural Africa was compared to the gut microbiota of western European children of the same age. None of the children had taken antibiotics or probiotics in the six months before the sampling dates. The results revealed that the different dietary habits could affect bacterial lignages, for example the ratio between *Firmicutes* and *Bacteroidetes phyla*. *Firmicutes* were, in fact, twice as abundant in European children. The ratio of *Firmicutes* to *Bacteroidetes* can be considered a useful obesity biomarker: its proportion decreased with weight loss. It was therefore reasonable to surmise that the increase in F/B ratio in European children, probably driven by their high-calorie diet, might predispose them to future obesity. In the same study it was also

demonstrated that short chain fatty acids, whose protective role against gut inflammation is well proven, were greater in BF children because of a unique bacterial community in African children able to use xylene and xylose to produce SCFA. The presence of short chain fatty acids possibly helps children of Burkina Faso to resist the establishment of some intestinal microbes such as *Esterichia* and *Salmonella* (De Filippo *et al.*, 2010). A lower incidence of type II diabetes was also pointed out in Chinese populations that consumed this cereal daily (Montonen *et al.*, 2003; Zhen *et al.*, 2015). One of the possible mechanisms of action behind this effect is the inhibition of pancreatic α-amylase and intestinal glucosidase by millet's phenolic fraction (Etxeberria *et al.*, 2015). It has been noted recently that fractions of millet particularly rich in phenolic compounds, such as hull and bran, have a higher activity in inhibiting these enzymes, compared to fractions from the decorticated cereal (Pradeep *et al.*, 2018). Furthermore, millet is increasingly appreciated in western countries as an ingredient for gluten-free foods, a growing field in the food industry due to the growing number of celiacs (approx. 3% of world population) whose nutritional needs are not fully satisfied by existing products (Muthamilarasan *et al.*, 2015;. Xiang *et al.*, 2019). In light of all these peculiarities. this cereal has recently been called "nutritious millet" or "nutritious cereal".

1.1.3 Phenolic composition of cereals

Phenolic compounds are secondary metabolites of plants and are known to protect against pests and pathogens, but also to contribute to color and sensory characteristics. In the human diet, they provide health benefits associated with a reduced risk of chronic diseases caused by reactive oxygen species, mainly thanks to their antioxidant properties (Hung, 2016; Varga *et al.,* 2018). Typically in cereals, phenolic compounds are present in free and bound forms, the latter linked to cell wall constituents such as polysaccharides, proteins, lignin and cutin in the outer layers of caryopses (Alves *et al.,* 2016; Arranz *et al.,* 2010). The literature reports that insoluble phenols are the major contributors to the total antioxidant capacity of cereals and need to be released through hydrolysis procedures (to break the linkage between phenols and cell wall) during the extractive step. Despite their abundance in plants, phenolic compounds are characterized by poor bioavailability (below 90%) because they are scarcely absorbed in the small intestine. However, they are carried in the colon by fiber and hydrolysis performed by the gut microbiota increases the *in situ* release of phenolic compounds and consequently the production of their metabolites (González-Aguilar *et al.,* 2017; Tuohy *et al.,* 2012). These metabolites are more easily absorbed and have better properties than the precursors (Chiou *et al.,* 2014; Rebello *et al.,* 2014).

The main classes of phenolic compounds in cereals are phenolic acids and flavonoids (Shahidi *et al.,* 2013). Flavonoids are generally present in their *O* or *C* glycosylated forms, created by the attachment of a sugar substituent to a hydroxyl group during *in planta* flavonoid synthesis. Even though *C* glycosyl flavonoids are a less understood subclass of secondary plant metabolites in comparison to their more common *O* glycosyl cousins, they are largely expressed in cereal caryopsis (Courts *et al.,* 2015). Some of the previously identified flavonoids in wheat grains are schaftoside and its isomer isoschaftoside; vitexin, apigenin and luteolin were identified both in wheat and millet (Sanak *et al.,* 2016) (Figure 1.1.5).
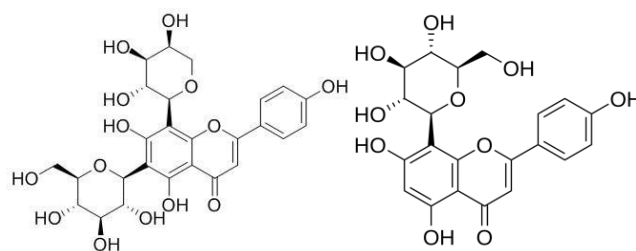
Figure 1.1.5 Chemical structure of schaftoside (left) and vitexin (right)

Phenolic acids include a large class of molecules derived from benzoic acid (C6-C1) or from cinnamic acid (C6-C3). In cereals, the main benzoic derivatives are *p*-hydroxybenzoic, gallic, vanillic and syringic acid (Figure 1.1.6). The generally more abundant cinnamic derivatives include ferulic, *p*-coumaric, caffeic, synapic and chlorogenic acids (Shahidi *et al.,* 2016).
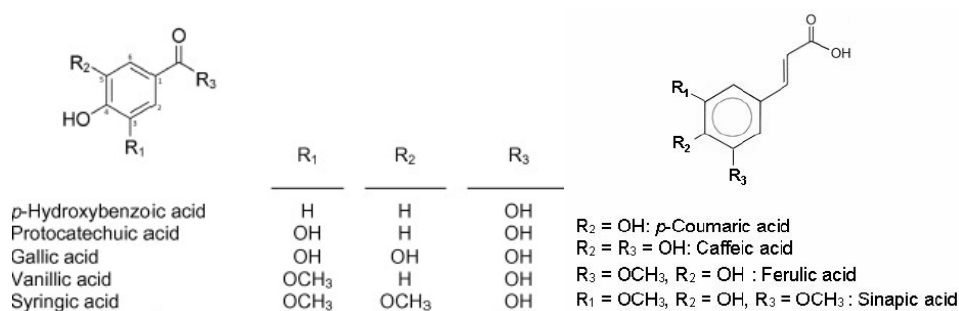
Figure 1.1.6 Hydroxybenzoic and cinnamic derivatives of cereals

Reviewing some literature data about total phenolic acids content of modern and old wheat species, it emerged that the ancient ones presented higher amounts of phenolic compounds, ranging from 766.2 to 1004 µg/g compared to 541.6 to 729.8 µg/g in modern wheat. It is worth noting that all the wheat species analysed and summarized in Table 1.1.1 presented cinnamic (TCC) derivatives as approximately 95% of total phenolic content (TPC). Among these, ferulic acid was identified as the main phenolic compound, representing approximately 90% of cinnamic derivatives and 80% of all phenolic acids.

Modern wheat	TBC	TCC	AF	TPC	References
Triticum aestivum	46.1 (8.5% di TPC)	495.5 (91.5% di TPC)	398.8 (80.5% di TCC 73.6% di TPC)	541.6	Li *et al.,* 2008
Triticum durum	42.6 (7.5% di TPC)	527.9 (92.5% di TPC)	403.3 (76.4% di TCC 70.6% di TPC)	570.5	Li *et al.,* 2008
Mieti	24.4 (3.7% di TPC)	638.8 (96.3% di TPC)	574.2 (89.8% di TCC 86.5% di TPC)	663.2	Gotti *et al.,* 2018
Bolero	32.4 (4.4% di TPC)	697.4 (95.6% di TPC)	618.5 (88.7% di TCC 84.7% di TPC)	729.8	Gotti *et al.,* 2018
Ancient wheat					
Andriolo	41.8 (4.7% di TPC)	848.4 (95.3% di TPC)	772.4 (91% di TCC 86.8% di TPC)	890.2	Gotti *et al.,* 2018
Frassineto	43.1 (5.6% di TPC)	723.2 (94.4% di TPC)	670.1 (92.6% di TCC 87.4% di TPC)	766.2	Gotti *et al.,* 2018
Gentil Rosso	53.4 (5.2% di TPC)	950.6 (94.7% di TPC)	862.3 (90.7% di TCC 85.8% di TPC)	1004	Gotti *et al.,* 2018
Inalettabile	37.4 (4.6% di TPC)	784.7 (95.4% di TPC)	711.3 (90.6% di TCC 86.5% di TPC)	822.2	Gotti *et al.,* 2018
Verna	48.2 (5% di TPC)	910.2 (95% di TPC)	817.5 (89.8% di TCC 85.3% di TPC)	958.4	Gotti *et al.,* 2018

Table 1.1.1 Total phenolic content in different ancient and modern wheat species. **TBC** (Total Benzoic Content), **TCC** (Total Cinnamic Content), **AF** (ferulic acid content), **TPC** (Total Phenolic Content). Data are expressed in µg/g.

With regard to the phenolic composition of different millet varieties, the total content of phenolic acids ranged from 367.5 to 3772.6 µg/g with higher percentages for Kodo and Proso, and lower percentages for Foxtail and Little millet (Table 1.1.2). As for wheat grains, the predominant phenolic fraction is represented by the cinnamic acids (TCC) which make up approximately 88% of the total phenolic content. Ferulic acid (approx. 46% of TCC) and *p*-coumaric acid (approx. 36% of TCC) are the main hydroxycinnamic derivatives. It is

noteworthy that among the single varieties, lower percentages of ferulic acid are compensated by higher values of its biosynthetic precursor recognized as *p*-coumaric acid (Pradeep *et al.,* 2017).

Millet varieties	TBC	TCC	AF	p-CA	TPC	References
Kodo	343 (9% di TPC)	3429.6 (91% di TPC)	2209 (64% di TCC 58.5% di TPC)	802 (23% di TCC 21% di TPC)	3772.6	Shahidi *et al.,* 2013
Finger	156.2 (27% di TPC)	416.5 (73% di TPC)	358.4 (86% di TCC 63% di TPC)	41.4 (10% di TCC 7% di TPC)	572.7	Shahidi *et al.,* 2013
Foxtail	174.2 (8.5% di TPC)	1870.5 (91.5% di TPC)	856.5 (46% di TCC 42% di TPC)	942.7 (50.5% di TCC 46% di TPC)	2044.7	Shahidi *et al.,* 2013
Foxtail	27.8 (5% di TPC)	484.2 (95% di TPC)	135.2 (28% di TCC 26% di TPC)	85.1 (18% di TCC 17% di TPC)	512	Pradeep *et al.,* 2018
Foxtail	53 (14% di TPC)	314.5 (86% di TCC)	100 (32% di TCC 27% di TPC)	125 (40% di TCC 34% di TPC)	367.5	Pradeep *et al.,* 2018
Proso	378.4 (15.5% di TPC)	2065.2 (84.5% di TPC)	444.6 (21.5% di TCC 18% di TPC)	1235.2 (60% di TCC 50% di TPC)	2443.6	Shahidi *et al.,* 2013
Little	269.3 (15% di TPC)	1526.6 (85% di TPC)	355.3 (23% di TCC 20% di TPC)	1085.2 (71% di TCC 60% di TPC)	1795.9	Shahidi *et al.,* 2013
Little	43.7 (9% di TPC)	422 (91% di TPC)	128.3 (30% di TCC 27.5% di TPC)	175 (41.5% di TCC 37.5% di TPC)	465.7	Pradeep *et al.,* 2018

Table 1.1.2 Total phenolic content in different millet varieties.
TBC (Total Benzoic Content), **TCC** (Total Cinnamic Content), **AF** (ferulic acid content), **p-CA** (para coumaric acid), **TPC** (Total Phenolic Content). Data are expressed in µg/g.

The higher percentage of phenolic compounds within old wheat species and millet varieties could actually be related to different aspects such as inter- and intra-matrix genotypic differences, plant-development environment interactions and also from the efficiency of the extraction procedures. The extraction process is one of the crucial steps for recovery and accurate estimation of phenolic compounds in cereals. Different parameters, mainly related to

the concentration of the extractive solvent, temperature of the process and time of extraction, should be taken into account. The choice of extractive solvent is generally based on the chemical nature and polarity of the compound to be extracted according to the *"similia similibus solvuntur"* principle. Phenolic compounds are water-soluble molecules; therefore, the optimal solvents in which they are extracted from the matrix are aqueous solutions or hydro-alcoholic solutions (Acosta-Estrada *et al.,* 2014). When we talk about the extraction of phenolic compounds from cereal matrices, it is necessary to distinguish between extraction procedures for the recovery of free and bound phenols. Free phenols, present in the form of monomers in the cytoplasm and in the cellular organelles, are easily extracted with aqueous solutions of the most common polar solvents: ethanol, methanol, acetone (Stalikas, 2007). Bound phenols, instead, represent almost 70% of the total phenolic amounts and are linked to the macromolecular constituents of the cell wall and need to be released through hydrolysis under alkaline or acidic conditions (Acosta-Estrada *et al.,* 2014). Observing the previous work in greater depth, the data concerning the procedures to recover phenolic compounds in cereals are very controversial, without a specific method of extraction. The approaches proposed for the recovery of bound phenols, in both wheat and millet, preferred the use of sodium hydroxide ranging from 2 to 10 M at room temperature, otherwise the acidic conditions are reported to induce hydroxycinnamic acid degradation (Kim *et al.,* 2006; Verma *et al.,* 2009; Acosta-Estrada *et al.,* 2014). Some hydrolytic procedures proposed in the literature regarding cereals are collected in Table 1.1.3.

Basic hydrolisis	Extractive ratio (g/mL)	References
NaOH 2M 1h	1/15	Adom *et al.*, 2003
NaOH 2M 4h	1/40	Kim *et al.*, 2006
NaOH 2 M 4h	0.25/1	Li *et al.*, 2008
NaOH 2 M 4h	1/75	Verma *et al.*, 2009
NaOH 2 M 4h	0.2 /1.5	Arranz *et al.*, 2010
NaOH 10 M 16h	1/15	Dinelli *et al.*, 2011
NaOH 10 M 16h	1/15	Dinelli *et al.*, 2013
NaOH 4 M 4h	0.5/15	Brandolini *et al.*, 2013
NaOH 4 M 4h	0.5/15	Volkan *et al.*, 2015
NaOH 4 M 4h	0.5/15	Hidalgo *et al.*, 2017
Acid hydrolisis	**Extractive ratio (g/mL)**	**References**
HCl 2 M 1h	0.2/0.4	Gao & Mazza 1994
HCl 6 M 4h	1/75	Verma *et al.*, 2009
MeOH/H$_2$SO$_4$ 9:1 v/v 20 h	0.2/2	Arranz *et al.*, 2010

Table 1.1.3 Basic and acidic procedures proposed in the literature regarding cereals.

1.1.3   Fermentation to improve the nutritional value of cereals

Fermentation has been traditionally used for ages as a cost-effective and low-energy preservation process to produce indigenous fermented foods with improved nutritional, health and sensorial qualities (Wang *et al.,* 2014; Adebiyi *et al.,* 2017). In this context, the fermentation of cereal is an economically sustainable, ancient technique capable of increasing the concentration of beneficial substances, making proteins more available, reducing anti-nutritional components, providing food preservation, and improving food texture and shelf-life through the action of several microorganisms (Hur *et al.,* 2014; Nout, 2009; Terefe, 2016; Ganguly *et al.,* 2019). Cereal-based fermented products are an important part of diets in several African, south Asian, and Middle Eastern countries, constituting almost one-third of consumed foods. Low cost and need for low-tech home-made procedures accessible to poorer rural societies are two of the main advantages of fermentation (Oguntoyinbo *et al.,* 2015; Prakash, 2016). In developing countries, fermentation of cereals is mainly used as a way to increase the shelf life, to improve conservation and safety. During the fermentation process, the use of specific microorganisms leads to the production of antimicrobial compounds (such as bacteriocins and antibiotics), hydrogen peroxide and other substances with antifungal effects (Chilton *et al.,* 2015). Today's growing emphasis on a healthy and balanced diet has created a demand for new functional foods. Mimicking the Asian and African tradition, novel fermented cereals could represent a promising trend in the production of new healthy foods (Ciesarová *et al.,* 2016). In recent years, there has been renewed interest in fermented cereals in Europe, especially for the supposed health benefits linked to these foods (Terefe, 2016). First of all, pH reduction during the fermentation process optimizes the activity of the endogenous phytase, leading to a significant reduction of a large part of phytic acid, one of the main antinutritional factors present in cereals (Marco *et al.,* 2017). Furthermore, prolonged fermentation times reduce oligosaccharides, disaccharides, monosaccharides and fermenting polyols (FODMAPS).

The reduction of the FODMAPS content in fermented cereals increases the tolerance towards these compounds of patients with irritable bowel syndrome (Laatikainen *et al.,* 2016; Ziegler *et al.,* 2016). Moreover, much epidemiological evidence has highlighted how the consumption of fermented-cereal foods, taken daily in high doses, is associated with the reduced risk of cardiovascular disease, diabetes and cancer (Rahman *et al.,* 2017). One of the reasons for this preventive effect has been associated with the presence of antioxidant molecules capable of reducing *in vivo* the amount of free radicals (Colombo *et al.,* 2008). These natural antioxidants, such as phenolic compounds, are mainly present in bound forms so they need to be released, mainly with hydrolytic treatment at laboratory scale. On the other hand, cereal fermentation seems to be a suitable technique able to enhance the release of bound phenols before consumption and to induce the production of a greater amount of free phenolic compounds with respect to the unfermented substrate (Simirgiotis *et al.,* 2013). This may be due to breakage of the link between bound phenols and the cell wall, but also to the induced synthesis of bioactive compounds determined by microbial communities used during the fermentation process (Hur *et al.,* 2014; Dey *et al.,* 2016). The temperature of the process and the selected microorganisms are recognized as playing crucial roles in increasing the antioxidant power. Looking to the future, with the current growing interest by consumers in everything perceived as natural and healthy, the outlook consists of creating unique flavors, textures, nutritional profiles, and health benefits while maintaining 100% natural products via fermentation (Terefe 2016).

1.2 Results on millet and wheat phenolic composition

1.2.1 Optimized hydrolytic methods by response surface methodology to accurately estimate the phenols in cereal by HPLC-DAD: the case of millet.

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Abstract

Extraction of free and bound phenols from millet in acidic and basic hydrolytic conditions were compared for the first time. Acidic hydrolysis was able to extract the highest amount of total phenolic compounds (up to 178 mg/100g) while the basic hydrolysis underestimates the phenolic concentration. Our findings pointed out for the first time that methyl ferulate is naturally present as bound phenol in millet. Response Surface Methodology was then applied to both acidic and basic hydrolytic extractive conditions: the acidic procedure, optimized in terms of extractive time and temperature and concentration of the acidic mean, gave the best results, allowing definition of Method Operable Design Region and quantitation of the total amount of phenols in millet samples in a single extractive step. This optimized method is suitable for further accurate investigations of the typical phenols of the numerous varieties of this recently re-discovered minor cereal.
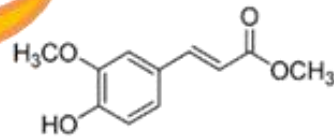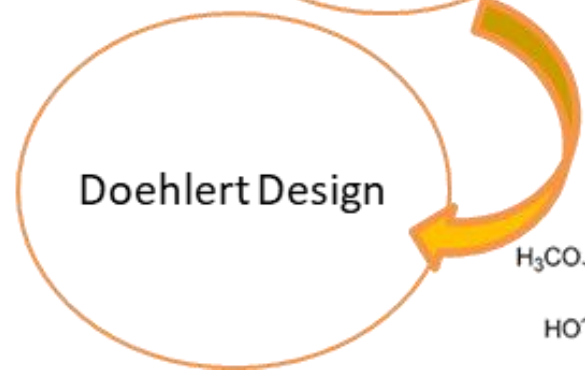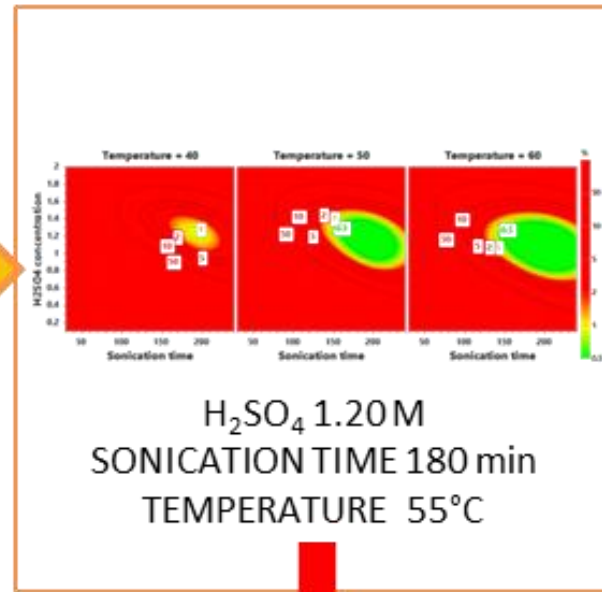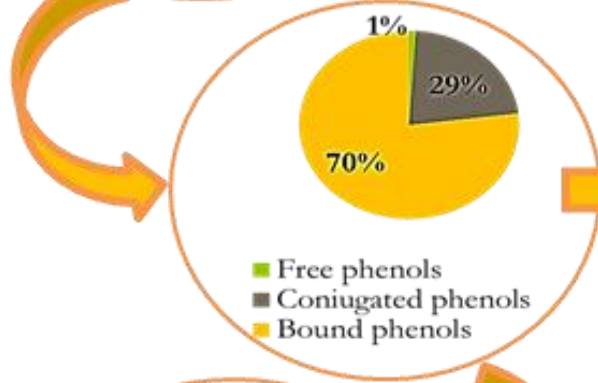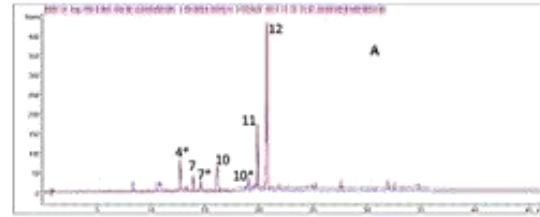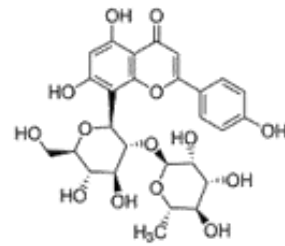
1.2.1.1 State of the art and aims of the work

Millet belongs to the *Poaceae* family and grows easily in areas of the world with a temperate, tropical, or subtropical climate. This cereal, compared to others, offers the advantages of multiple uses and resistance to drought, allowing it to be grown almost everywhere (Ragaee, 2006; Amadou *et al.,* 2013). Typically in cereals, the phenolic compounds are present in free and bound forms, the latter linked to cell wall constituents such as polysaccharides, proteins, lignin and cutin in the outer layers of caryopses (Alves *et al.,* 2016; Arranz *et al.,* 2010). The literature points out that insoluble phenols are the major contributors to the total antioxidant capacity of cereals that need to be released through hydrolysis procedures (to break the linkage between phenols and cell wall) during the extractive step. Hydrolysis conditions can significantly affect the total amount and profile of recovered phenolic compounds. It has been reported that in cereals, the values of phenolic content are lower after extraction in acidic compared to basic conditions, and thus acidic conditions could lead to a degradation of the hydroxycinnamic acids (Kim *et al.,* 2006; Verma *et al.,* 2009; Acosta-Estrada *et al.,* 2014). Nevertheless, despite this assertion, a systematic comparison between acidic and basic hydrolysis has not yet been reported for cereals, and particularly not for millet.

To optimize the experimental conditions for basic and acidic hydrolysis, Response Surface Methodology (RSM) has been applied (Lewis *et al.,* 1999). RSM makes it possible to obtain predictive maps of the responses throughout the selected experimental domain, to discover the possible interactions between factors, and to identify the zone where the responses are simultaneously optimized. Furthermore, the multivariate optimization strategy led to the definition of the method operable design region (MODR) (Deidda *et al.,* 2018); MODR is a multidimensional region of the

experimental domain where the desired quality of the performances is achieved, maintaining the risk of error under a selected threshold, and is defined according to the analytical Quality by Design (QbD) strategy (Orlandini *et al.,* 2013). The QbD approach has recently been introduced in the pharmaceutical field as an innovative quality paradigm, but it has up to now only two examples of applications in the field of food analysis, limited to the optimization of chromatographic methods (Silva *et al.,* 2014; Ancillotti *et al.,* 2018).

The aim of the present research was to define the best conditions for a complete recovery of all the phenolic components of millet, mainly cinnamic derivatives and flavonoidic molecules. Toward this aim, some batches of millet from Burkina Faso were preliminarily studied for their phenolic composition by a chromatographic fractionation and subsequently investigated by mass spectrometry (MS). Regarding the quantitative recovery, hydrolytic methods, both in basic and acidic solutions, were initially tested. After this screening, the RSM was applied, both for basic and acidic hydrolyses, to propose a simple and efficient extractive procedure suitable for a complete recovery of all the phenolic components. Furthermore, in the case of acidic hydrolysis the method operable design region (MODR) was also defined. To our knowledge, this is the first time this approach in cereals, and particularly in millet, has been described.

1.2.1.2 Materials and methods

Samples and reagents

Two batches (MP-1 and MP-2) of millet from Burkina Faso were purchased from African local market, according to the Nutratoscafrica project.

All solvents were of analytical HPLC grade from Sigma Aldrich (St. Louis, Missouri, USA). Ultrapure water was obtained by the Milli-Q-system (Millipore SA, Molsheim, France). Sulfuric acid (95.0-98.0%) and sodium hydroxide ($\geq$ 98) were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Ferulic acid standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Standards of vitexin and vitexin 2''-*O*-rhamnoside were purchased from Extrasynthese (Genay, France).

Extraction of phenolic compounds

Free phenolic compounds. Millet was milled using a laboratory miller, and the obtained whole flour was defatted twice with hexane (1:10 (w/v) ratio) and kept on a mechanical shaker for 1 h.

Two g of the defatted flour were suspended in 20 mL of acidic MeOH (1% HCl) and, after 30 minutes of sonication (DK Sonic, 42 KHz), stirred by a magnetic stirrer for about 12 h. The supernatant was separated by centrifugation (5000 rpm, 10 min), the residue re-suspended in 25 mL of the extractive mixture, sonicated for 30 minutes, and stirred for 2 h. The two supernatants were collected and brought up to a volume of 50 mL in a flask. A further set of experiments was performed using as extractive mixture EtOH:H_2O 7:3 v/v (HCOOH, 1%) and then applying the same steps described above.

Bound phenolic compounds. The two batches (MP-1, MP-2) were both treated according to the method BF reported by Balli *et al.,*(2019). Briefly, 1 g of defatted

flour was suspended in 25 mL of MeOH:H$_2$O 7:3 v/v (0.1 M NaOH); the solution was sonicated 1 h at 60° C, than CH$_3$COOH was added until pH close to 6.5-7.0; the sample was centrifuged at 5,000 rpm for 10 minutes. The MP-2, due to its higher content in bound phenols, was selected for testing different hydrolysis methods both in basic and acidic media. The applied methods, BS, BK and A were previously applied by Balli *et al.,* 2019 to study a fermented wheat. Method BS only differs from method BF for the stronger basic conditions (4M NaOH). Method BK was applied on the residue of the extraction of free phenols extracting 1 g of such residue with 40 mL of NaOH 2 M, and stirring the solution at room temperature for 4 hours.

The hydrolysis was also performed in acidic conditions applying method A: 1 g of defatted flour was extracted with 25 mL of MeOH:H$_2$SO$_4$ 9:1 v/v; the solution was sonicated 2 h at 60° C and centrifuged at 5.000 rpm for 10 minutes.

HPLC-DAD analysis of phenolic extracts

All the phenolic extracts were analysed using a HP 1200L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA) after removing suspended solids by centrifugation at 14.000 rpm, for 10 min. Firstly, a Poroshell 120, EC-C18 (150 x 3 mm, 2.7 μm, Agilent, USA) column was used for preliminary comparison between the two batches of millet. Successively, a Raptor ARC-18 column (150 x 3 mm, 5 μm, Restek USA) was used for all the other analysis. The solvents were the same used for the semipreparative HPLC. The following gradient method was applied in the two columns: solvent A varied from 0.10 to 10% in 5 min, from 10 to 15% in 5 min, from 15 to 30% in 10 min, from 30 to 35% in 5 min, from 35 to 40% in 3 min, from 40 to 45% in 3 min, from 45 to 100% in 11 min and then was kept in these conditions for 5 min. Total time of analysis 47 min, equilibration

time 5 min, flow rate 0.4 mL/min with the Poroshell column and 0.8 mL/min with the Raptor column. Injection volume 10 µL. The chromatograms were recorded at 280 nm, 330 nm, 350 nm.

Fractionation of MP-1 by semipreparative HPLC-DAD

About 1 g of millet flour was extracted in 50 mL of acidic MeOH (1% HCl v/v), then filtered and the solution recovered and concentrated up to few mL. This extract was used for the next fractionation by semipreparative HPLC-DAD, with a system HP series 1050 and a Polaris RP-C18 Ether column (250×10 mm, 5µm, Varian, Germany). Elution was performed at a flow rate of 4 mL/min with CH_3CN (solvent A) and H_2O (pH 3.2, formic acid, solvent B). Solvent A varied from 0.10 to 10% in 10 min, from 10% to 15% in 10 min, from 15% to 30% in 10 min, from 30% to 100% in 5 min and then 10 min at 100% A; total time of analysis 45 min, equilibration time, 10 min; injection volume of sample 100 µL; a total of 10 fractions were recovered.

MS analysis

The HPLC-DAD-MS analysis of the phenolic extracts were performed using the same column and chromatographic conditions described in the HPLC-DAD section. HPLC-DAD-MS system was from Waters and composed by 2695 HPLC, 2996 DAD and 4 micro MS equipped with Zspray ESI source. The ESI interface parameters were capillary 2.90 kV, cone 64 V in the first 18 minutes and 30 V till the end of the analysis, source temperature 120°C, desolvation temperature 350°C, cone gas flow 19 (L/Hr), desolvation gas flow 350 (L/Hr). Data were acquired in negative ion mode from 110 m/z to 1000 m/z.

The isolated fractions by semipreparative HPLC were analysed without applying a chromatographic separation but by direct infusion in MS and MS^n on a LTQ (Thermo Scientific, Germany) ion trap mass spectrometer. Each fraction was taken to dryness by evaporation under vacuum and re-suspended in a CH_3CN/H_2O mixture, containing 0.1% formic acid. This solution was infused by syringe into the ESI interface of the instrument. Sheath and auxiliary gas flow rates were 10 and 2 (arbitrary units), respectively; capillary voltage and tube lens voltages, as the collision energy and wideband activation voltage in MS^n experiments, were optimized for each compound of interest during the infusion. The mass spectrometer was calibrated with the standard mixture indicated by the producer immediately before the acquisition of the samples, both in positive and in negative ion mode.

Quantitation of phenolic acids and flavonoids by HPLC-DAD

The phenolic acids were quantified using a five-point calibration curve with ferulic acid as external standard (purity $\geq$ 99%) at 330 nm, linearity range 0-0.21 µg (R^2=1.0). The content of flavonoid aglycones was determined using a five-point calibration curve with vitexin as external standard (purity $\geq$ 95%) at 350 nm, linearity range 0-1.23 µg (R^2=1.0); vitexin 2"-*O*-rhamnoside (purity $\geq$ 95%) at 350 nm, linearity range 0-0.67 µg, (R^2=1.0) was selected to quantify the glycosylated flavonoids.

Response Surface Methodology

Response surface methodology was carried out on the second batch of millet (MP-2) with the support of Modde 10 software package (Modde 10 reference), which was purchased from S-IN (Vicenza, Italy). For the investigation of both acidic and basic hydrolysis, a Doehlert Design was selected. In the case of basic hydrolysis, sonication

time was studied at five levels (range 60-120 min), NaOH concentration at seven levels (range 0.10 M - 4.00 M), and temperature at three levels (range 20-60 °C). For acidic hydrolysis, H_2SO_4 concentration was investigated at five levels (range 0.1-2.0 M), sonication time at seven levels (range 30-240 min), while temperature at three levels (20-40-60 °C). The planned experiments were run in a randomized order and data treatment was performed. The software was used to calculate the hypothesized quadratic models, to perform ANOVA, and to find the MODR by means of risk failure map, setting the risk of failure threshold to 1% (corresponding to 99% probability).

Proximate analysis

A Soxhlet extraction was used to gravimetrically determine the fat content in MP-2 sample, according to ISS protocol (1996/34). The protein content was determined by the Kjeldal method, with N*6.25 (N= total nitrogen). Dietary fiber (soluble and insoluble) was assessed according to AOAC Method 991.43 (Determination of soluble, insoluble and total dietary fiber in foods and food products, final approval 1991).

Statistic methods

Each experiment was performed in triplicate and results were expressed as mean ± SD using EXCEL software (version 2013) in-house routines. One-way ANOVA and F-test ($p < 0.05$) by Microsoft Excel statistical software and Fisher's LSD (DSAASTAT software v. 1.1, Onofri, Pisa, 2007) were used for pointing out significant differences between quantitative data.

Modde 10 software package (Modde 10 reference) was purchased from S-IN (Vicenza, Italy) and was used to generate the Doehlert Design used for RSM, to perform data analyses and to find the MODR by means of risk failure maps.

1.2.1.3 Results and Discussion

The two samples of small millet from Burkina Faso (MP1-MP2) were morphologically similar in terms of shape, size, color of the caryopsis and well represent typical samples found in the local markets and consumed by the local population. To better characterize their composition in terms of phenolic compounds, a fractionation by semipreparative HPLC was first applied. The defatted flours of the two samples were preliminary characterized in terms of free and bound phenols and the richest one regarding bound phenols was selected for further investigations and to optimize the phenolic recovery also using RSM.

Characterization of phenols by HPLC-DAD-MS and MS^n

A preliminary investigation was performed on MP-1 and MP-2 millet samples to obtain chromatographic profiles regarding their content in flavonoids and cinnamic acids as free components; this step allowed to recognize MP-1 as the richest sample. This latter one was selected to apply a fractionation by semipreparative HPLC-DAD. Analysing by direct infusion in HPLC-MS and MS^n the ten fractions recovered from the semipreparative HPLC a group of *C*-glycosylated flavonoids (**3**, **4** and **7**), one *O*-glycosylated flavonoid (**6**), and five cinnamic derivatives (**8**-**11** and **12**) were detected (Table 1.2.1.1).

Analytes	λ_{max} (nm)	[M-H]$^-$	Fragment ions (m/z)	Identified compounds
1	327	468	332,306,289,161	N^1,N^4-dicaffeoylspermidine
3	270,349	609	489,429,357,327	luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside
4	268,334	593	503,473,413,327,299	vicenin II
6	268,334	577	413,293	vitexin-2"-*O*-rhamnoside
7	268,336	431	311,283	vitexin
8	287sh,323	339	324,307,193,175	ferulic acid rhamnoside
9	287sh,323	339	324,307,193,175	ferulic acid rhamnoside isomer
10	287sh,323	193	178,161,134	isoferulic acid
11	290sh,310	177	162,145,118	methyl hydroxycinnamate
12	300sh,324	207	192	methyl ferulate

Table 1.2.1.1 Identified compounds in millet sample MP-1 according to UV-Vis and

mass spectra

Compound **1** was identified as N^1,N^4-dicaffeoylspermidin: the MS^2 spectrum showed a

base peak at 332 m/z due to the loss of 162 Da and two other peaks at 289 m/z and 161

m/z. This fragmentation suggested the presence of a caffeoyl group linked to an amide

portion, as already described by (Kang *et al.,* 2016) for sorghum grains. The mass

spectra in negative ionization for compound **3** (Fig. 1.2.1.1) showed the deprotonated

molecular ion at 609 m/z. The MS^2 of this species produced the fragment at 489 m/z

[M-H-120]$^-$, a typical loss of *C*-glycosyl flavonoids, corresponding to cross-ring

cleavages in the sugar moiety (Iswaldi *et al.,* 2011).
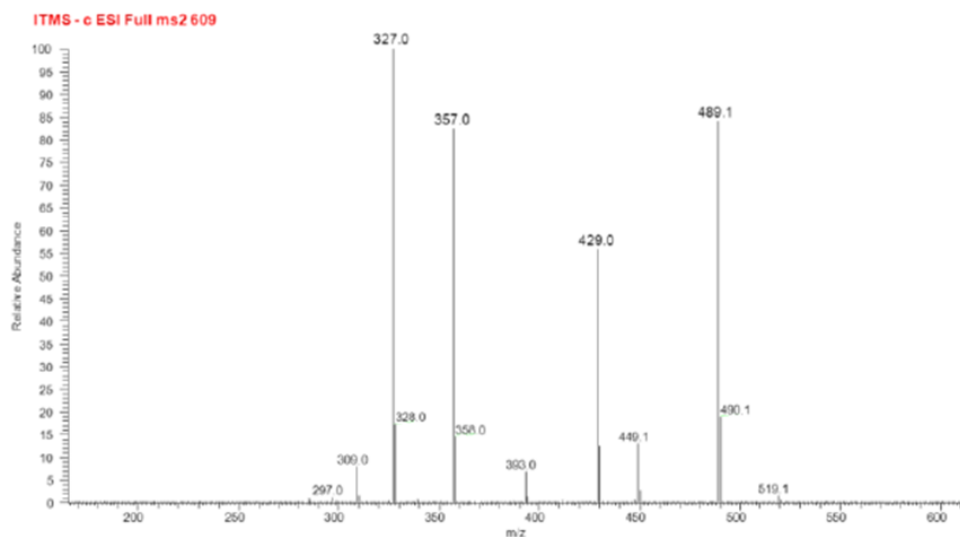
Figure 1.2.1.1 MS^2 spectrum of the deprotonated molecular ion at 609 m/z of the compound **3**:

luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside

The ion species at 327 m/z obtained also from MS^3 (data not shown) was linked to the loss of 162 Da typical of hexoses (Simirgiotis *et al.,* 2013). In light of these findings, compound **3** was identified as luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside. Compound **4**, with a deprotonated molecular ion at 593 m/z, showed a MS^2 spectrum with three ion species at 503 m/z [M-H-90]$^-$, 473 m/z [M-H-120]$^-$, and 413 m/z [M-H-180]$^-$ and, according to literature (Chandr, 2013; Silva *et al.,* 2014) it was identified as vicenin II. Compound **6** was identified as vitexin 2"-*O*-rhamnoside, an *O*-glycosylated flavonoid, with the deprotonated molecular ion at 577 m/z and the species at 413 m/z and 293 m/z attributable to the loss of 164 and 120 Da, respectively (Figure 1.2.1.2) as previously highlighted (Silva *et al.,* 2014; Wu *et al.,* 2012; Dinelli *et al.,* 2011).
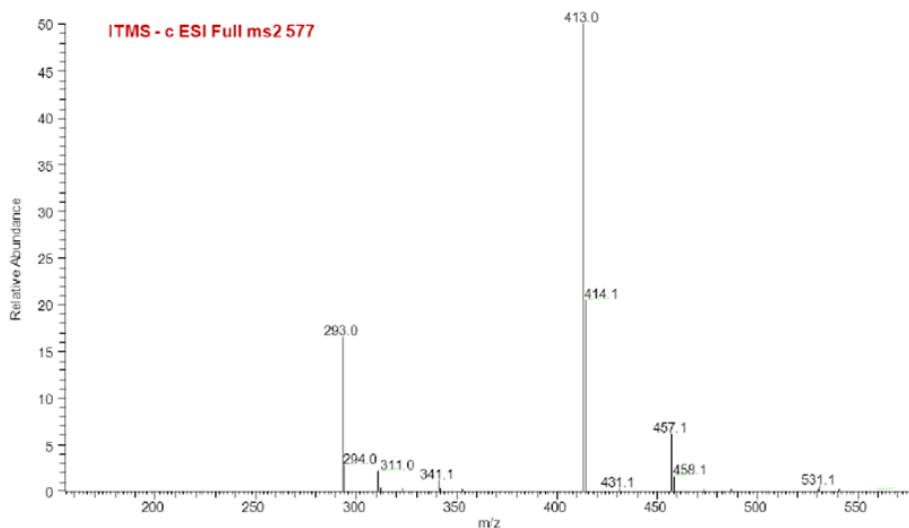
Figure 1.2.1.2 MS^2 spectrum of compound **6** vitexin 2"-*O*-rhamnoside

Compound **7** with a deprotonated molecular ion at 431 m/z and a typical fragmentation of a *C*-glycosylated with a loss of 120 Da (311 m/z) was identified as vitexin. With regard to the cynnamoyl derivatives, the isobaric compounds **8** and **9** at 339 m/z were identified as two ferulic acid rhamnoside isomers. The fragmentation in MS^2, highlighting three ion species at 324 m/z [M-H-15]$^-$, 193 [M-H-146]$^-$ and 174 m/z [M-H-164]$^-$ with different relative abundances, was in agreement with the hypothesized structure (Figure 1.2.1.3).
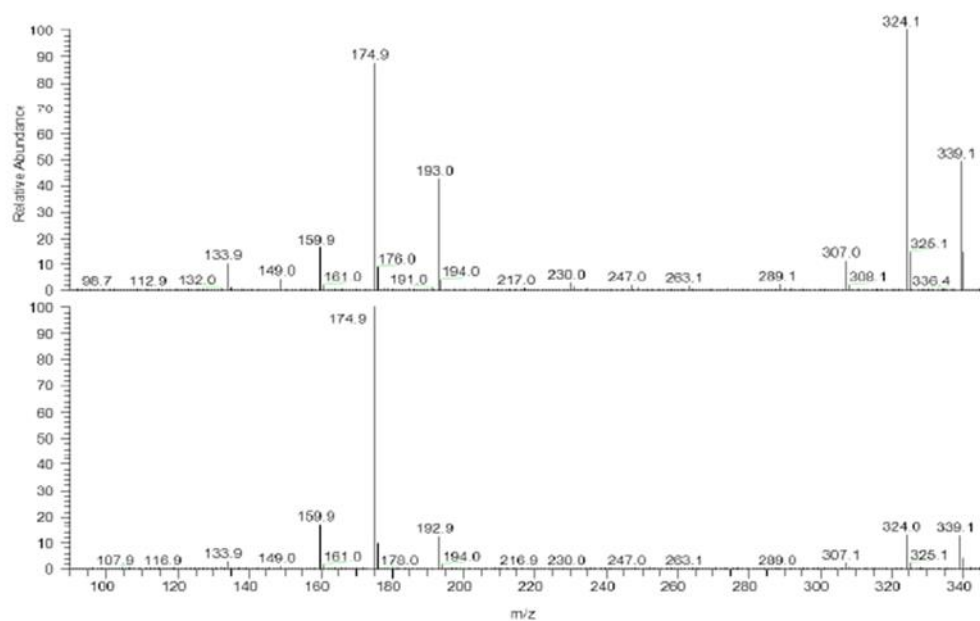
Figure 1.2.1.3 MS^2 spectra of compounds **8** and **9** (ferulic acid rhamnoside isomers).

Compound **10** was identified as isoferulic acid with a deprotonated molecular ion at 193 m/z and ion species at 178 m/z, 161 m/z and 134 m/z (Li *et al.,* 2003). Compound **11** showed a deprotonated molecular ion at 177 m/z and was identified as methyl hydroxycinnamate (Figure 1.2.1.4).
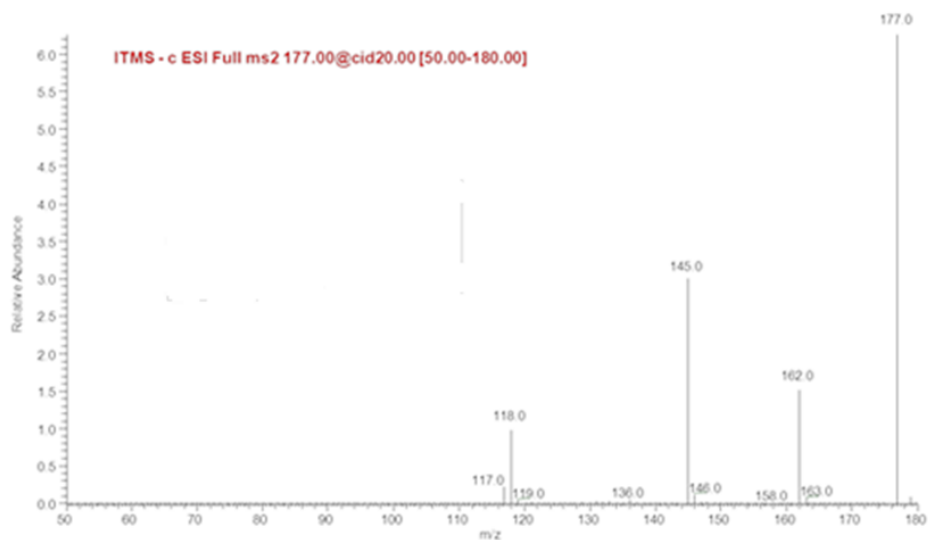
Figure 1.2.1.4 MS^2 spectrum of compound **11** as methyl hydroxycinnamate

Compound **12** was initially identified in millet as methyl ferulate in agreement with a deprotonated molecular ion at 207 m/z and the ion at 192 m/z attributable to the loss of a methyl group (Figure 1.2.1.5). Compounds **11** and **12** were tentatively identified as cinnamic ester derivatives detected in millet for the first time, to the best of our knowledge, but already reported as components of cereals according to literature (Nyström *et al.,* 2005; Karamać *et al.,* 2005).
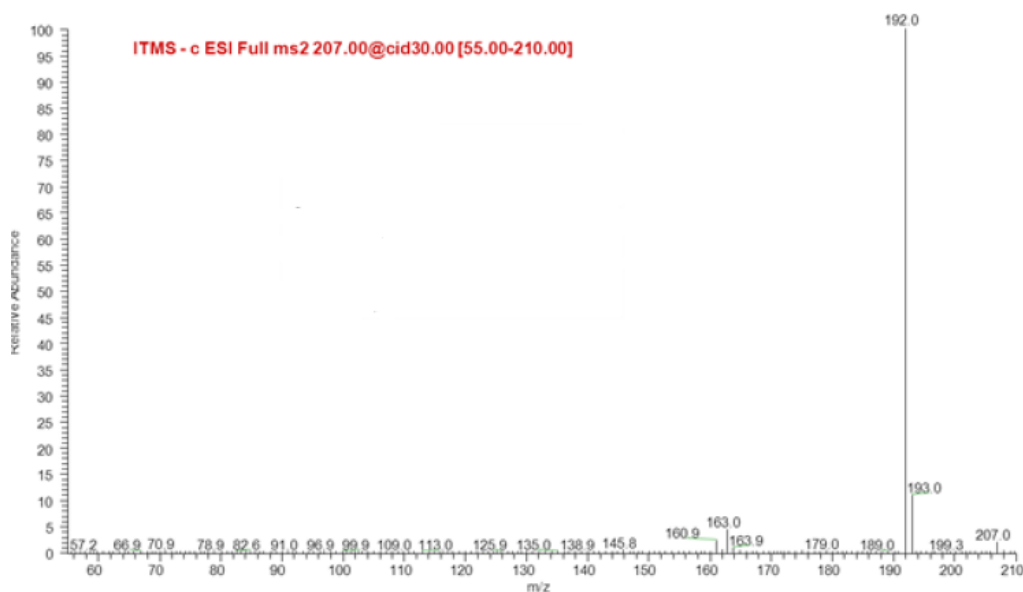
Figure 1.2.1.5 MS[2] spectrum of compound **12** as methyl ferulate

Free and bound phenol distribution

Manifold methods have been used for the extraction of free phenols in cereals, and specifically for millet varieties (Chandr, 2013; Pradeep *et al.,* 2018; Xiang *et al.,* 2019). According to the preliminary work of Banerjee *et al.,* (2012) in which the extraction power of different solvents was compared, acidified MeOH was selected as the best solvent to recover this fraction. Moreover, our preliminary tests, using also an acidified EtOH:H$_2$O 7:3 v/v mixture, confirmed the acidified MeOH as the most suitable solvent to recover the free phenolic compounds (data not shown). The HPLC-DAD profiles of the free phenols obtained from the two batches of millet were similar (Figure 1.2.1.6 a), with only small differences in the relative abundance of a few analytes (**3** and **4**).

Applying method BF, the HPLC-DAD profiles of the two extracted batches showed some differences in the composition of bound phenols (Figure 1.2.1.6 b).
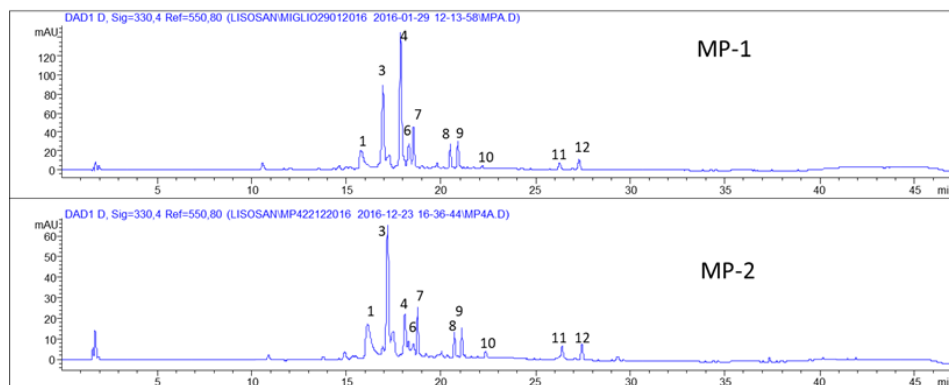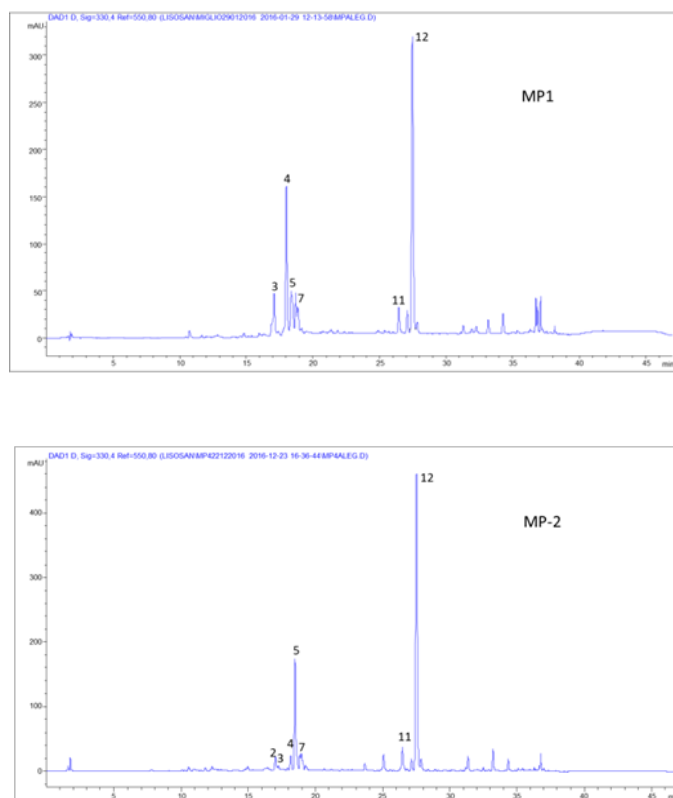
Figure 1.2.1.6 Chromatographic profiles at 330 nm of **a**) free and **b**) total phenols of MP-1 and MP-2 (Poroshell column). Compounds **1**, N^1,N^4-dicaffeoyl-spermidin; **2**, *p*-coumaric acid; **3**, luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside; **4**, vicenin II; **5**, ferulic acid; **6**, vitexin 2''-*O*-rhamnoside; **7**, vitexin; **8**, ferulic acid rhamnoside; **9**, ferulic acid rhamnoside isomer; **10**, isoferulic acid; **11**, methyl hydroxycinnamate; **12**, methyl ferulate.

Vitexin 2"-*O*-rhamnoside (**6**) was not detected in the two batches; *p*-coumaric acid, which is reported as the most abundant phenolic acid in millet (Pradeep *et al.,* 2018), was not present in sample MP-1 (Figure 1.2.1.6 b). On the other hand, methyl ferulate (**12**) was identified for the first time in both the millet samples. How this molecule is related to the extractive procedure will be discussed later in this article.

Quantitative evaluation of free and bound phenols

The quantitative results for the two batches are reported in Table 1.2.1.2. Although the total phenols obtained after basic hydrolysis were higher than the free ones in both millet samples, this result was not confirmed for some of the compounds. Samples MP-1 and MP-2 showed a total content of free phenols of 127.4 mg/100g and 63.3 mg/100g, respectively, with approximately 90% of flavonoids. Although the highest content of total phenols was found in MP-1, MP-2 was characterized by the highest amount of the bound fraction (60.4 mg/100g), calculated by the difference between total and free phenols.

		Extraction of free phenols		Extraction of total phenols	
Analytes	Compounds	MP-1	MP-2	MP-1	MP-2
1	N^1,N^4-dicaffeoyl-spermidin	6.8	7.1	nd	nd
2	p-coumaric acid	nd	nd	nd	2.3
3	luteolin-(7-O-glucopyranosyl)-8-C-glucopyranoside	44.3	35.3	17.3	3.2
4	vicenin II	44.7	7.6	45.2	6.7
5	ferulic acid	nd	nd	16.5	34.2
6	vitexin 2"-O-rhamnoside	12.9	3.0	nd	nd
7	vitexin	9.1	4.6	9.4	4.9
8	ferulic acid rhamnoside	2.5	1.3	nd	nd
9	ferulic acid rhamnoside isomer	3.2	1.6	nd	nd
10	isoferulic acid	1.1	0.5	nd	nd
11	methyl hydroxycinnamate	1.1	1.2	4.4	5.3
12	methyl ferulate	1.7	1.1	56.5	67.1
Total		127.4	63.3	149.3	123.7

Table 1.2.1.2 Free and total phenolic compounds in the two batches of millet. Total phenols were recovered applying BF method. Data are the mean of three independent extractions and are expressed as mg/100 g of dry weight. RSD <1%. nd, not detected.

In the subsequent step, we applied different extractive procedures, both in basic and acidic conditions, to identify the most suitable one for an exhaustive recovery of phenols. To this aim, we selected sample MP-2 due to its higher amount of bound phenols. The same batch was also analysed regarding its nutritional composition: a total fiber amount of 10.8 % (of which 88% was insoluble fiber), 8.3% of total proteins and 1.2% of ash were found.

Optimization of the extractive methods for total phenols

Regarding bound phenols, almost all the literature about millet reports the application of basic hydrolyses with NaOH 1-2 M, usually at room temperature, similarly to what proposed for the other cereals (Table 1.1.3). At the same time, acidic hydrolysis was reported as not suitable because it induces a degradation of hydroxycinnamic and hydroxybenzoic acids (Adom *et al.,* 2002; Arranz *et al.,* 2010; Dinelli *et al.,* 2011). Nevertheless, in the literature data reporting the effect of acidic procedures on the stability of phenolic compounds during their extraction from cereals are scant and not

supported by sufficient experimental data. For this reason, in this investigation a set of different extractive procedures (both in acidic and basic conditions) were systematically applied to sample MP-2 and the effectiveness of these procedures was compared in terms of total phenols recovered and applied extraction times.

For the analysis of the extracts, we selected the Raptor column, which guaranteed a better chromatographic resolution particularly for compounds **5** and **6**, identified as ferulic acid and vitexin 2"-*O*-rhamnoside, respectively. These peaks were co-eluted using the Poroshell column (Fig. 1.2.1.7), while the Raptor column made it possible to maintain the same resolution for the other analytes and to reduce the total analysis time.
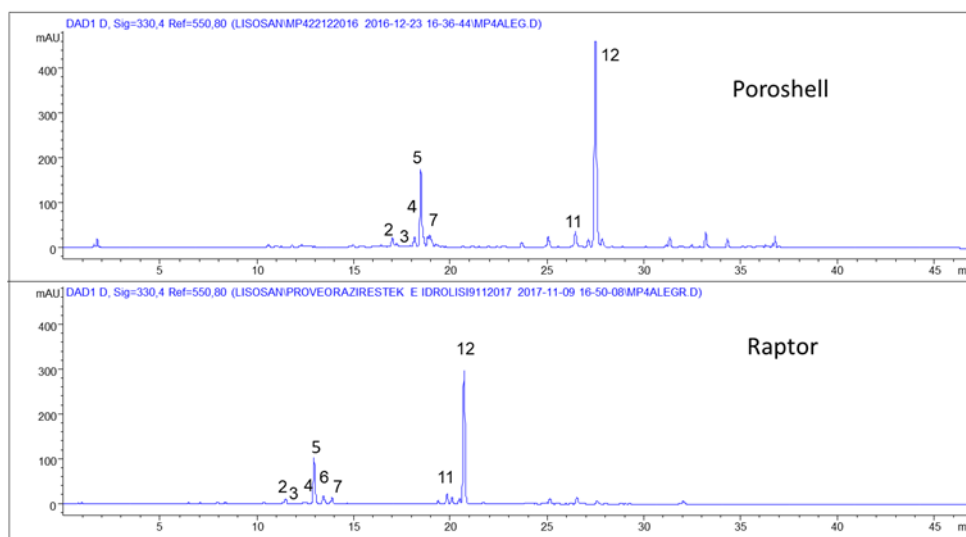
Figure 1.2.1.7 Chromatographic profiles at 330 nm of the bound phenols from method BF using the Poroshell and Raptor columns. Compounds **2**, *p*-coumaric acid; **3**, luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside; **4**, vicenin II; **5**, ferulic acid; **6**, vitexin 2"-*O*-rhamnoside; **7**, vitexin; **11**, methyl hydroxycinnamate; **12**, methyl ferulate.

As for the basic hydrolysis, a methanol solution with 4 M NaOH (method BS) was applied and compared with the softer conditions of method BF (0.1 M NaOH). The former procedure induced a partial degradation of the phenolic compounds in millet, higher than that observed applying method BF, demonstrating that the use of NaOH 4 M leads to full disappearance of methyl ferulate, completely converted into ferulic acid. On the contrary, no differences in flavonoid composition were observed (Figure 1.2.1.8).
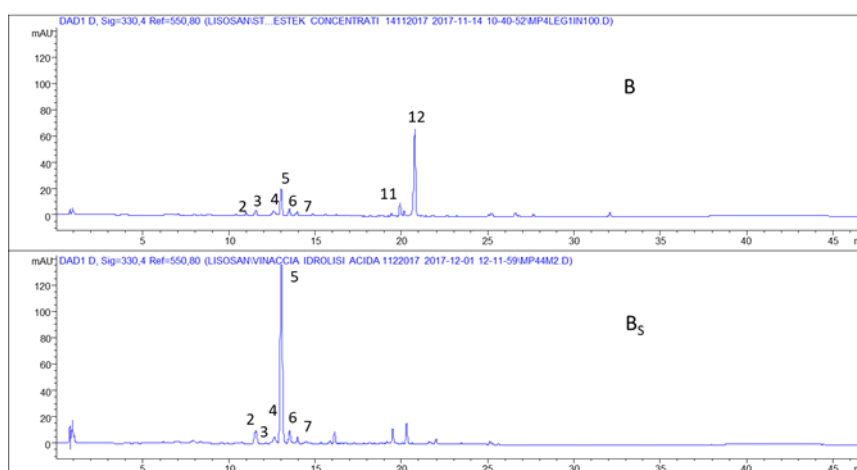
Figure 1.2.1.8 Chromatographic profiles of total phenols extracts from MP-2 with BF and BS methods. Compounds **2,** *p*-coumaric acid; **3,** luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside; **4,** vicenin II; **5,** ferulic acid; **6,** vitexin 2"-*O*-rhamnoside; **7,** vitexin; **11,** methyl hydroxycinnamate; **12,** methyl ferulate.

At the same time, to investigate the effects of acidic hydrolysis, MP-2 was also extracted in methanol with H_2SO_4 1%, as previously applied (Arranz *et al.,* 2010); the extraction was carried out with the aid of ultrasounds to obtain the phenolic profile shown in Figure 1.2.1.9.

The chromatograms obtained from basic hydrolysis with NaOH 0.1 M revealed two main compounds: ferulic acid (**5**) and methyl ferulate (**12**), while those from acidic hydrolysis (Figure 1.2.1.9) revealed only methyl ferulate (**12**), previously found in rice (Tanaka *et al.,* 1964) but never identified in millet to date. In order to exclude that methyl ferulate was an artefact of the extraction process (due to the simultaneous presence of ferulic acid and methanol), softer basic conditions (NaOH 0.1 M in methanol) were tested on a solution of ferulic acid as pure standard: no formation of methyl ferulate was observed, allowing us to conclude that this ester is naturally present in bound form in millet. The absence of methyl ferulate is attributable to the strong basic conditions applied in the previous studies, which induced the breaking of the ester bond.
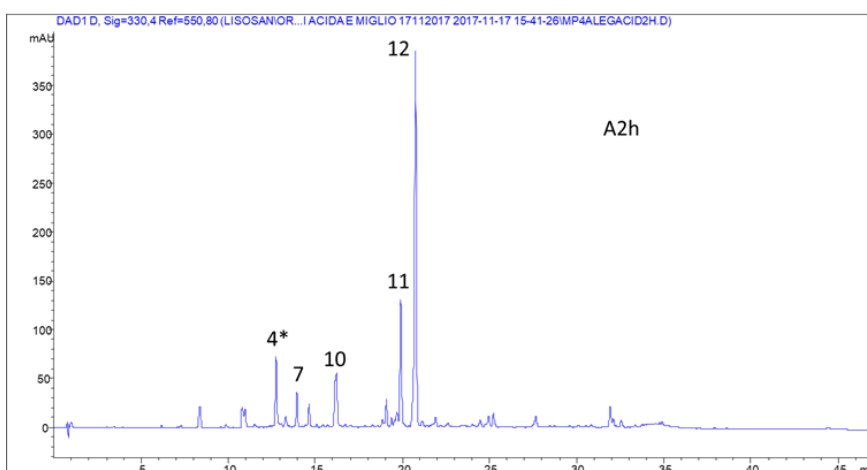
Figure 1.2.1.9 Chromatographic profile at 330 nm of bound phenols from method A of MP-2 (on the Raptor column). Compounds **4***, luteolin 8-*C*-glucopyranoside, **7**, vitexin; **10**, isoferulic acid; **11,** methyl hydroxycinnamate; **12,** methyl ferulate

The previously proposed method BK (Kim *et al.,* 2006) was then tested to verify if a unique extractive step could be sufficient to recover free and bound phenols. This procedure was applied on the solid residue recovered from the extraction of free phenols; the results from the HPLC-DAD analyses highlighted the absence of the flavonoids, thus confirming that these molecules were only present in free form (Figure 1.2.1.10).
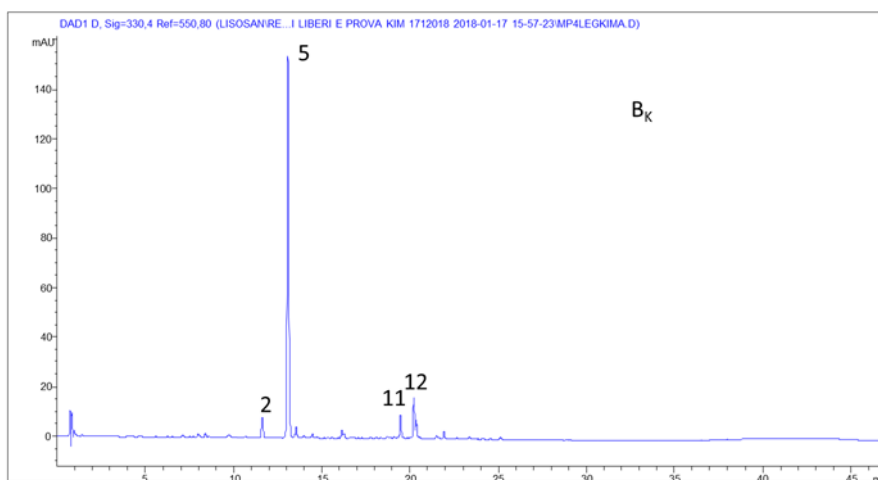
Figure 1.2.1.10 Chromatographic profile (Raptor column) at 330 nm: bound phenols from method BK applied to MP-2. Compounds **2,** *p*-coumaric acid; **5**, ferulic acid; **11,** methyl hydroxy cinnamate; **12,** methyl ferulate.

Furthermore, the basic hydrolyses applied on the flour (methods BF and BS) modified the flavonoid content.Luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside (**3**) strongly decreased (as much as 88%) with respect to the amounts found in the free phenolic extract (Table 1.2.1.3). On the contrary, vicenin II (**4**) and vitexin 2"-*O*-rhamnoside (**6**) remained unaltered during basic hydrolysis but, analogously to compound **3**, both disappeared after acidic hydrolysis. The HPLC-DAD-MS analyses confirmed that the acidic condition did not alter the distribution of the phenols, with only the exception of

two *O*-glycosylated flavonoids that were hydrolysed in the corresponding aglycones. Indeed, regarding vitexin 2"-*O*-rhamnoside (**6**), acidic hydrolysis increased the concentration of the aglycone, vitexin (**7**). In addition to these changes, acidic hydrolysis also revealed one new flavonoid, compound **4***, with the same UV-Vis spectrum of compound **3** and a MS spectrum with an ion at 447 m/z. Based on these findings, it was identified as luteolin 8-*C*-glucopyranoside, and the complete hydrolysis of compound **3** was also confirmed by the quantitative findings.

Overall, in terms of recovery of total phenols, the tested methods gave different results. The strong basic hydrolysis (method BS) led to the degradation of methyl ferulate (**12**) and to a minimum recovery of phenols (Table 1.2.1.3), while the acidic procedure (method A) gave the highest recovery of total phenols with a value of 163.8 mg/100g. Differently from hydrochloric acid (Gao *et al.,* 1994), the use of sulfuric acid to hydrolyse the phenolic compounds in other matrices different from cereals, has been successfully applied (Bellumori *et al.,* 2019).

It has been reported that, in several varieties of millet, ferulic acid is the most abundant molecule among the phenolic acids, reaching values close to 86% of the total phenols. Our findings with a strong basic hydrolysis (method BS) were perfectly in agreement with the literature (ferulic acid 87% of total phenolic acids), but by choosing milder basic conditions (method BF) ferulic acid was not the main compound (only 23% of the total phenols) because methyl ferulate, revealed for the first time, was not hydrolysed in these conditions. This finding allowed us to ascertain that methyl ferulate is the most abundant molecule among phenolic acids and their derivatives (70%). Starting from these results, RSM was applied to optimize the extractive yields, working both in basic and acidic media.

Analytes	Compounds	BF	BS	A
2	*p*-coumaric acid	3.9 a	7.9 b	nd
3	luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyranoside	1.8 a	2.1 a	nd
4	vicenin II	5.4 a	12.7 b	nd
4*	luteolin-8-*C*-glucopyranoside	nd	nd	40.8
5	ferulic acid	23.1 a	75.5 b	nd
6	vitexin 2"-*O*-rhamnoside	11.6 a	12.7 a	nd
7	vitexin	7.5 b	4.6 a	15.7 c
10	isoferulic acid	nd	nd	12.7
11	methyl hydroxycinnamate	5.5 a	nd	9.1 b
12	methyl ferulate	69.7 a	nd	85.5 b
Total		**128.5**	**115.5**	**163.8**

Table 1.2.1.3 Concentrations of phenolic compounds from different extractive procedures applied on MP-2 sample. Data are the mean of three independent extractions (RSD <1%) and are expressed as mg/100 g of dry weight. In each raw, different letters indicate significant differences at p < 0.05. nd, not detected.

Response surface methodology (RSM) for basic extraction

In order to evaluate the possibility of increasing the recovery values obtained by basic hydrolysis, the experimental parameters were investigated in greater depth with the aid of RSM to identify the zone where a selected target value of recovery of total phenols could be obtained with a probability of 99%.

The considered factors were sonication time, NaOH concentration and temperature, while the studied responses were the total recovery of bound phenols and the recovery of compound **5**, ferulic acid. This latter was selected in order to have detailed information on the effects of the basic hydrolysis on the possible degradation of compound **12**, methyl ferulate. A quadratic polynomial model, with linear, quadratic and interaction terms, was postulated to link the factors to the responses, according to the following:

$$y=\beta_0+\beta_1 x_1+\beta_2 x_2+\beta_3 x_3+\beta_{11} x_1^2+\beta_{22} x_2^2+\beta_{33} x_3^2+\beta_{12} x_1 x_2+\beta_{13} x_1 x_3+\beta_{23} x_2 x_3+\varepsilon$$

where y is the experimental response, xi are the independent variables, β0 is the intercept, βi are the true coefficients, and ε is the experimental error.

A Doehlert Design (Lewis, 1999; Ancillotti *et al.,* 2018) was generated to estimate the coefficients of the model and the related experimental plan is reported in Table 1.2.1.4, including three replicate experiments at the center of the experimental domain in order to estimate the experimental variance. Sonication time was studied at five levels (range 60-120 min), NaOH concentration was studied at seven levels (range 0.10 M - 4.00 M), and temperature was studied at three levels (range 20-60 °C).

Exp. no.	Sonication time (min)	NaOH concentration (M)	Temperature (°C)	Total phenolic compounds (mg/100g)	5 Ferulic acid (mg/100g)
1	150	2.05	40	127.0	82.4
2	120	4.00	40	125.7	81.9
3	120	2.70	60	124.4	80.7
4	30	2.05	40	130.6	81.4
5	60	0.10	40	118.4	11.2
6	60	1.40	20	124.7	66.5
7	120	0.10	40	129.4	21.7
8	120	1.40	20	129.4	81.3
9	90	3.35	20	118.3	74.3
10	60	4.00	40	129.4	79.4
11	60	2.70	60	130.9	82.2
12	90	0.75	60	129.8	83.3
13	90	2.05	40	124.5	80.3
14	90	2.05	40	128.7	83.8
15	90	2.05	40	122.7	78.7

Table 1.2.1.4 Doehlert Design for response surface methodology in the study of basic hydrolysis

The calculated models were refined by deleting selected not significant terms in order to improve their quality. According to ANOVA results, the model for the total recovery of phenolic compounds was valid but not significant, evidencing that no one of the investigated factors exerted a significant influence on this response. On the other hand,

the model for the recovery of compound **5** was found to be significant, but not valid. Thus, even if the goodness of prediction (Q^2=0.498) of this model was acceptable, the model could be used only to give a description of the trend of the response, without using it in a predictive way.

The contour plots reporting NaOH concentration vs. sonication time at three different values of temperature (20-40-60 °C) are reported in Fig. 1.2.1.11a for the total bound phenols and Fig. 1.2.11b for ferulic acid.
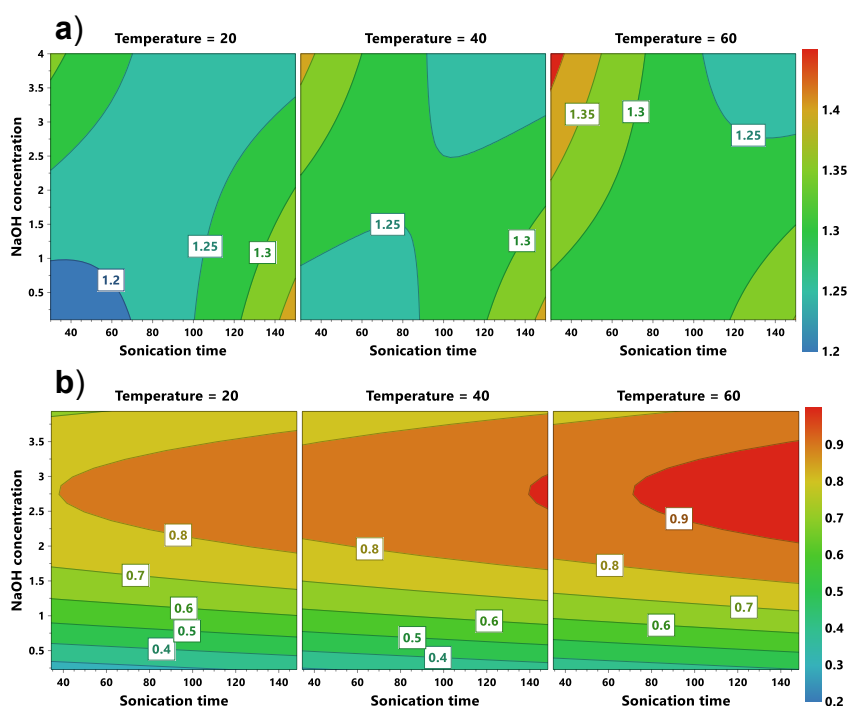
Fig. 1.2.1.11 Contour plots obtained by plotting NaOH concentration (0.1-4 M) *vs.* sonication time (60-150 min) at three levels of temperature (20 °C, 40 °C and 60 °C). (**a**) Total recovery of phenolic compounds; (**b**) recovery of compound **5** ferulic acid.

By examining the plot in Fig. 1.2.1.11a it appears that the maximum value of total phenolic compounds which could be achieved is about 140 mg/100 g, thus much lower than the value which could be obtained by using acidic hydrolysis (Table 1.2.1.5). As regards compound **5** (Fig. 1.2.1.11b), the zone where this response was maximized

corresponded at high temperature, high sonication time and medium-high concentration, thus confirming that stronger basic conditions could actually lead to the degradation of **12** methyl ferulate and thus to an increased amount of compound **5**.

RSM for acidic extraction and method operable design region

In the case of acidic hydrolysis, the selected factors were H_2SO_4 concentration, sonication time and temperature, and the considered response was, again, the total recovery of bound phenols. Similarly to the RSM study of basic hydrolysis, a quadratic polynomial model with linear, quadratic and interaction terms was postulated, and the Doehlert Design was chosen to estimate its coefficients. In any case, for acidic hydrolysis the experimental domain for reagent concentration was narrower and the one for sonication time was wider with respect to those considered for the basic conditions. Hence, in this case H_2SO_4 concentration was investigated at five levels (range 0.1-2.0 M) and sonication time at seven levels (range 30-240 min), while three levels of temperature were chosen (20-40-60 °C). The experimental plan is shown in detail in Table 1.2.1.5.

Exp. no.	H$_2$SO$_4$ concentration (M)	Sonication time (min)	Temperature (°C)	Total phenolic compounds (mg/100g)
1	2.000	135	40	164.0
2	1.525	240	40	165.9
3	1.525	170	60	171.0
4	0.100	135	40	86.5
5	0.575	30	40	99.0
6	0.575	100	20	112.9
7	1.525	30	40	145.7
8	1.525	100	20	154.6
9	1.050	205	20	158.2
10	0.575	240	40	166.7
11	0.575	170	60	168.3
12	1.050	65	60	157.7
13	1.050	135	40	165.0
14	1.050	135	40	162.8
15	1.050	135	40	174.0

Table 1.2.1.5 Doehlert Design for response surface methodology in the study of acidic hydrolysis

The model for the total recovery of phenolic compounds was refined, leading to very good results in terms of goodness of fit (R^2=0.963) and goodness of prediction (Q^2=0.701), and it was demonstrated as significant and valid in terms of ANOVA.

The contour plots are reported in Figure 1.2.1.12 and show that the maximization of the response was obtained by setting high levels of sonication time, medium-high levels of H$_2$SO$_4$ concentration, and high levels of temperature. Both a quadratic effect of H$_2$SO$_4$ concentration and a negative interaction between H$_2$SO$_4$ concentration and sonication time were highlighted.

a)
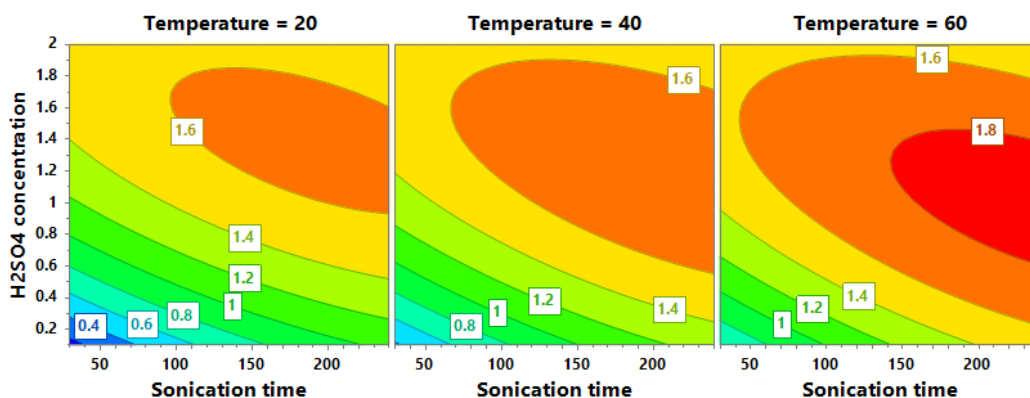
b)
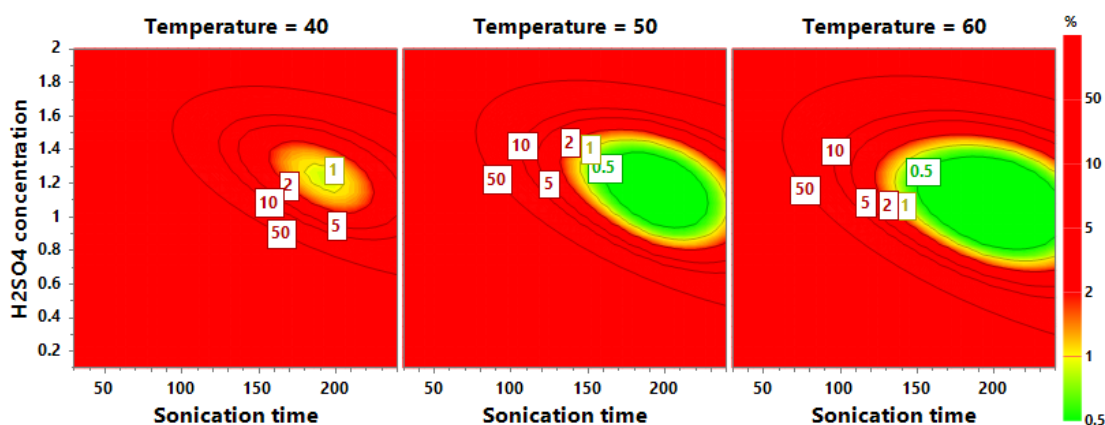
Figure 1.2.1.12. a) Contour plots for the response total recovery of phenolic compounds, obtained by plotting H_2SO_4 concentration (0.1-2 M) vs. sonication time (30-240 min) at three levels of temperature (20 °C, 40 °C and 60 °C). **b**) Probability maps obtained by plotting H_2SO_4 concentration (1.113 M) vs. sonication time (212min) at three levels of temperature (40 °C, 50 °C and 60 °C). The MODR is green and included within the line corresponding to 1% risk of having a total recovery of phenolic compounds lower than 165 mg/100g

The target value for the total recovery of phenolic compounds was set as 165 mg/100 g and the zone where the predicted value of the response is equal or higher to the target corresponds to the green zone depicted in the sweet spot plots presented in Figure 1.2.12b. The green zone becomes wider when moving from low to high levels of temperature.

However, the sweet spot region is calculated on the basis of the model but cannot give any guarantee that the response will fulfil the requirement ($\geq$165 mg/100 g) with high probability. As a matter of fact, to define the MODR it is necessary to consider model uncertainty because the MODR is a set of experimental conditions where the criteria meet acceptance limits with a selected probability (Deidda *et al.,* 2018). In this case, calculation of the MODR was performed by MODDE® 10 software by using Monte-Carlo, setting the desired probability as 99% and expanding the factor ranges from an optimum set-point to the widest possible range where the prediction for the total recovery of phenolic compounds is equal or higher to the target limit of 165 mg/100 g. The starting set-point was the following: H_2SO_4 concentration, 1.113 M; sonication time, 212 min; temperature, 55 °C. The resulting probability maps are represented in Figure 1.2.12b and the MODR was defined as the zone included within the line corresponding to a risk of failure equal to 1%, corresponding to probability $\pi \geq 99\%$.

The resulting MODR was the following: H_2SO_4 concentration, 0.827-1.398 M; sonication time, 163-240 min; temperature, 40-60°C. The MODR was validated by testing verification points at its edges and verifying the prediction of the model. Inside the MODR every combination of the factor values can be selected as working points (Orlandini *et al.,* 2013) and in this case the selection was made taking into consideration that saving time in terms of sonication could lead to practical advantages for the analyst. Hence, the final optimized conditions were: H_2SO_4 1.20 M; sonication time of 180 min; temperature 55 °C. When applying these conditions (n=3, α/2=0.025), the measured recovery of the total phenols reached a concentration of 178±2 mg/100 g, twenty percent higher with respect to the best result obtained in a basic media.

The validated method, attained by applying RSM and MODR, allowed recovery of the free and bound forms in only one extractive step, the detection of methyl ferulate for

the first time and confirmation that the acidic hydrolysis is the suitable procedure to guarantee the highest recovery of the total phenols from millet and presumably from cereals, differently from that in the literature to date.

Finally, the validated method in acidic media was tested on five not decorticated millet samples which differed a lot in colour (yellow, green and reddish), shape and size of the caryopsis. Furthermore, to confirm the goodness of the method in the recovery of total phenols, a comparison was made on these samples by applying an acid procedure with few modifications (Table 1.2.1.6). The obtained results confirmed the selected procedure by RSM and MODR as suitable to guarantee the maximum phenolic recovery also working on millet samples with very different morphological characters.

	Total phenols mg/100g	
Samples	*Method A_1	Method A_{RSM}
M1	55.8 a	69.0 b
M2	45.1 a	82.8 b
M3	89.5 a	90.8 b
M4	106.8 a	135.0 b
M5	88.6 a	100.3 b

Table 1.2.1.6 Total phenolic recovered after two different acidic hydrolysis on different millet samples. In each raw, different letters indicate significant differences at $p < 0.05$

*Method A_1 differs from the optimized method (Method A_{RSM}) as follow: sonication time, 135 min; temperature of sonication, 40°C; and molarity, 1.05 M.

M1-M5 are not decorticated millet samples collected from the same geographical area (Cesa-Arezzo, Italy) with different color and shape.

1.2.1.4 Conclusions

This is the first report to focus on the comparison between acid and basic hydrolytic conditions in recovering the bound phenols of millet. Despite the literature data, the applied acidic hydrolysis was able to extract the highest amount of total phenolic compounds while, according to our results, the basic hydrolysis underestimates the phenolic concentration.

For the first time methyl ferulate was shown to be naturally present as bound phenol in millet, while the absence of this molecule in the previous works is attributable to the strong basic conditions usually applied and responsible of the breaking of the ester bond.

The best acidic procedure was defined and validated by RSM and MODR. The novelty of the work can be related to several aspects: a) a comparison with different extractive procedures in basic and acidic media suitable for millet but easily applicable also to other cereals; b) the use of RSM to define the best one-step extraction for recovering free and bound phenols; c) the identification of an acidic hydrolysis as extractive procedure faster and easier in comparison of those proposed by the literature to date.

1.2.2 Phenolic composition of ancient and modern wheat species

from Tuscany and Campania

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Abstract

A big challenge for our century has to do with discovering new foods, including a re-discovering of new cereal species, with greater health effects. Ancient grains are defined as species that have remained unchanged over the years and they are gaining growing scientific interest thanks to their healthier nutritional profile with respect to modern ones. The phenolic composition of ten wheat species from Tuscany (eight old and two modern grains) and eleven wheat species from Campania (nine old and two modern grains) was evaluated; Tuscan grains were grown under two types of densities. Different hydrolysis strategies were preliminarily performed, both in acidic and basic media, on the Tuscan samples. The best extractive procedure with H_2SO_4 (1.20 M, sonication time of 180 minutes and temperature of 55° C) was then applied to all Campania wheat grains. The results showed that the ancient species, cultivated at a higher density, were characterized by greater yields per unit area, highlighting that it is possible to increase agronomic yields without losing phenolic compounds. All the modern and old species from Tuscany and Campania showed similar phenolic content, ranging from 0.67 to 1.14 mg/g and from 0.72 to 0.85 mg/g in Tuscan and Campania samples, respectively. No significant differences were observed in terms of phenolic composition among old and modern species.

1.2.2.1 State of the art and aims of the work

Food products derived from cereal grains constitute a major part of the daily diet and wheat is considered the most important cereal crop worldwide (Shewry *et al.,* 2015).

Today, most wheat species are hybrids created from ancient wheat over the last 100 to 150 years. Cereal varieties developed in recent years have been oriented toward promoting crop yields, reducing height and timing of crop maturation, increasing proteins content and technological characteristics (Adom *et al.,* 2002). Although these modern wheat varieties have positive properties in terms of production, compared with the original ancient wheat, little attention has been given to the nutritional value of the ancient species (Dinu *et al.,* 2018). In this context, establishing the amount of phenolic compounds in ancient and modern wheat varieties can be valuable in order to select specific cereal grains suitable for the production of health-promoting staple food (Gotti *et al.,* 2018).

The aim of this work was to shed light on the phenolic content of different wheat species from Tuscany cultivated under two types of densities and species from Campania. To avoid environmental interference, both ancient and modern wheat samples, were grown in the same field and harvested in the same period of the year, dividing the wheats into Tuscan and Campania groups.

1.2.2.2. Materials and methods

Samples and reagents

A total of 21 samples were analysed. Eight ancient and two modern wheat species were purchased in 2018 from Cesa, Arezzo, Italy, and were cultivated under two densities: density 1= 250 m^2, density 2= 350 m^2. Nine old wheat species and three modern ones were cultivated in 2019 in Caselle in Pittari, Salerno, Italy. The ancient and modern varieties were as follows. Ancient varieties from Tuscany: Gentil Rosso (GRd1; GRd2); Verna (VRd1; VRd2); Frassineto (FRd1;Frd2); Bianconostrale (BNd1; BNd2); Inalettabile (ILd1; ILd2); Andriolo (ADd1;ADd2); Sieve (SVd1; SVd2) and Gentil Bianco (GBd1;GBd2). Modern varieties from Tuscany: Control (COd1; COd2) and Bologna (BOd1; BOd2) Ancient varieties from Campania: Carosella 1 (CAR1), Carosella 2 (CAR2), Russulidda (RUS), Ianculidda 1 (IA1), Ianculidda 2 (IA2), Annibale (AN), Saragolia Rossa (SR), Cappelli (CA). Modern varieties from Campania: Ambrogio (AM) Aureo (AU) and Bologna (BO) (Table 1.2.2.1). All solvents were of analytical HPLC grade from Sigma Aldrich (St. Louis, Missouri, USA). Ultrapure water was obtained by the Milli-Q-system (Millipore SA, Molsheim, France). Sulfuric acid (95.0-98.0%) and sodium hydroxide ($\geq$ 98) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Ferulic acid standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Standards of schaftoside was purchased from Extrasynthese (Genay, France).

	Ancient wheat								Modern wheat	
	Gentil Rosso	Verna	Frassineto	Bianconostrale	Inallettabile	Andriolo	Sieve	Gentil Bianco	Control	Bologna
Density 1	GRd1	VRd1	FRd1	BNd1	ILd1	ADd1	SVd1	GBd1	COd1	BOd1
Density 2	GRd2	VRd2	FRd2	BNd2	ILd2	ADd2	SVd2	GBd2	COd2	BOd2

b)

Ancient wheat								Modern wheat		
Carosella 1	Carosella 2	Russulidda	Ianculidda 1	Ianculidda 2	Annibale	Saragolla Rossa	Cappelli	Ambrogio	Aureo	Bologna
CAR1	CAR2	RUS	IA1	IA2	AN	SR	CA	AM	AU	BO

Table 1.2.2.1. Ancient and modern wheat varieties from Tuscany (**a**) and Campania (**b**).

Extraction of phenolic compounds

Free phenols. The Tuscan samples Gentil Rosso (GRd1), Bianconostrale (BNd2), Verna (VRd2), and Gentil Bianco (GBd2) were treated, according to the method reported by Balli *et al*. (2019), for the extraction of free phenolic compounds. Briefly, two g of the defatted flour were suspended in 20 mL of acidic MeOH and, after 30 minutes of sonication (DK Sonic, 42 KHz), stirred by a magnetic stirrer for about 12 h. The supernatant was separated by centrifugation (5000 rpm, 10 min), the residue re-suspended in 25 mL of the extractive mixture, sonicated for 30 minutes, and stirred for 2 h. The two supernatants were collected and brought up to a volume of 50 mL in a flask.

Bound phenols: basic hydrolysis by NaOH. 1 g of the residue obtained after free phenols extraction was suspended in 25 mL of $MeOH:H_2O$ 7:3 v/v (0.1 M NaOH); the solution was sonicated for 1 h at 60 °C, then CH_3COOH was added until pH reached 6.5-7.0; the sample was centrifuged at 5,000 rpm for 10 min.

Total phenols: acidic hydrolyses by H_2SO_4. All the wheat samples from Tuscany were preliminarily treated with an acidic hydrolysis (Method A). Briefly 1 g of defatted flour was suspended in 25 mL of $MeOH^+$ (1.05 M H_2SO_4), the solution was sonicated for 135 min at 40 °C. The sample was then centrifuged at 5,000 rpm for 10 min. Furthermore, all the density two samples from Tuscany (GRd2, FRd2, BOd2, BNd2, ILd2, ADd2, VRd2, SVd1, GBd2 and COd2) and all the wheat species from Campania were selected to perform the optimized acidic hydrolysis (Method Arsm) proposed by Balli *et al*., (2020) for millet.

HPLC-DAD analysis of phenolic extracts

All the phenolic extracts were analyzed using a HP 1200L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA) after removing suspended solids by centrifugation at 14,000 rpm for 10 min with a Raptor ARC-18 column (150 x 3 mm, 5 µm, Restek USA). The gradient method was the same proposed by Balli *et al*., for millet samples (2020).

Quantitation of phenolic acids and flavonoids by HPLC-DAD

Phenolic acids were quantified using a five-point calibration curve with ferulic acid as external standard (purity ≥ 99%) at 330 nm, linearity range 0 - 0.21 µg (R^2=1.0). The content of flavonoid was determined using a five-point calibration curve with schaftoside standard (purity ≥ 95%) at 350 nm, linearity range 0-0.79 µg, (R^2=0.999).

Statistical methods

Each experiment was performed in triplicate and results were expressed as mean ± SD using EXCEL software (version 2013) in-house routines. One-way ANOVA and *F*-test (p < 0.05) by Microsoft Excel statistical software and Fisher's LSD (DSAASTAT software v. 1.1, Onofri, Pisa, 2007) were used to identify significant differences between quantitative data.

1.2.2.3 Results and Discussion

Characterization of phenols by HPLC-DAD analysis

The HPLC-DAD profiles of modern and ancient wheat species, from Tuscany and Campania, resulted superimposable with a total of eleven compounds: two flavonoids (compounds **1** and **2**) and eight cinnamic derivatives (compounds **3**-**11**) (Figure 1.2.2.1).
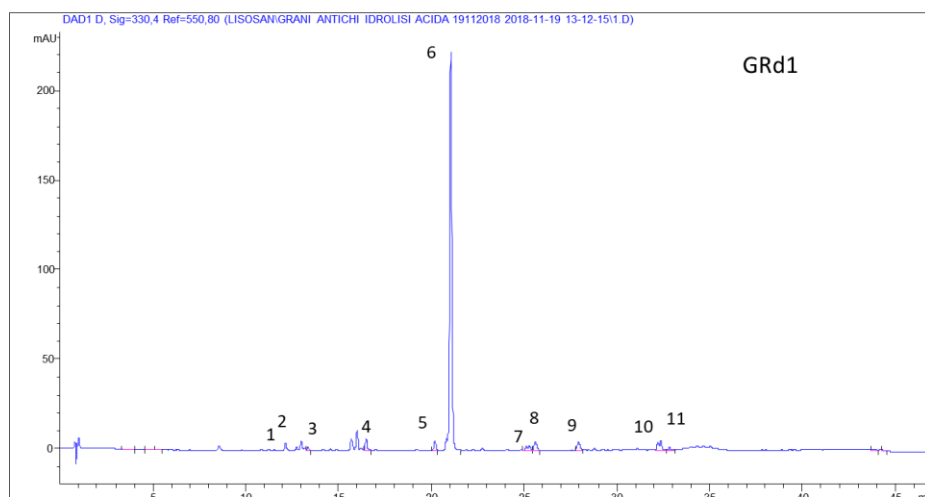
Figure 1.2.2.1 HPLC-DAD profile at 330 nm of total phenolic extract of Gentil Rosso

grown at density one (GRd1).

All the compounds were tentatively identified by their retention time, UV-Vis spectra and

the previous published data on a fermented wheat Italian dietary supplement (Balli *et al.,*

2019). The identified compounds are listed in Table 1.2.2.2.

Analytes	**λ max (nm)**	**Identified compounds**
1	327	isoschaftoside
2	270,349	schaftoside
3	287sh,323	ferulic acid
4	287sh,323	cin. derivative
5	290sh,310	methyl hydroxycinnamate
6	300sh,324	methyl ferulate
7	287sh,323	cin. derivative
8	287sh,323	cin. derivative
9	287sh,323	cin. derivative
10	287sh,323	cin. derivative
11	287sh,323	cin. derivative

Table 1.2.2.2 Phenolic compounds identified in wheat species and their λ max

Total phenolic content

One of the main limitations that disincentives the cultivation of ancient grains is related to their reduced yield per unit area, with respect to modern species (Cooper, 2015). In this context an intensive treatment (density 2) was performed to verify if the agricultural conditions could influence the plants' grown and the plants' phenols production. All the Tuscan samples were firstly treated with acidic hydrolysis (Method A) for the recovery of the total phenols in a single extractive step. The quantitative evaluations, for both the densities, are reported in Figure 1.2.2.2.
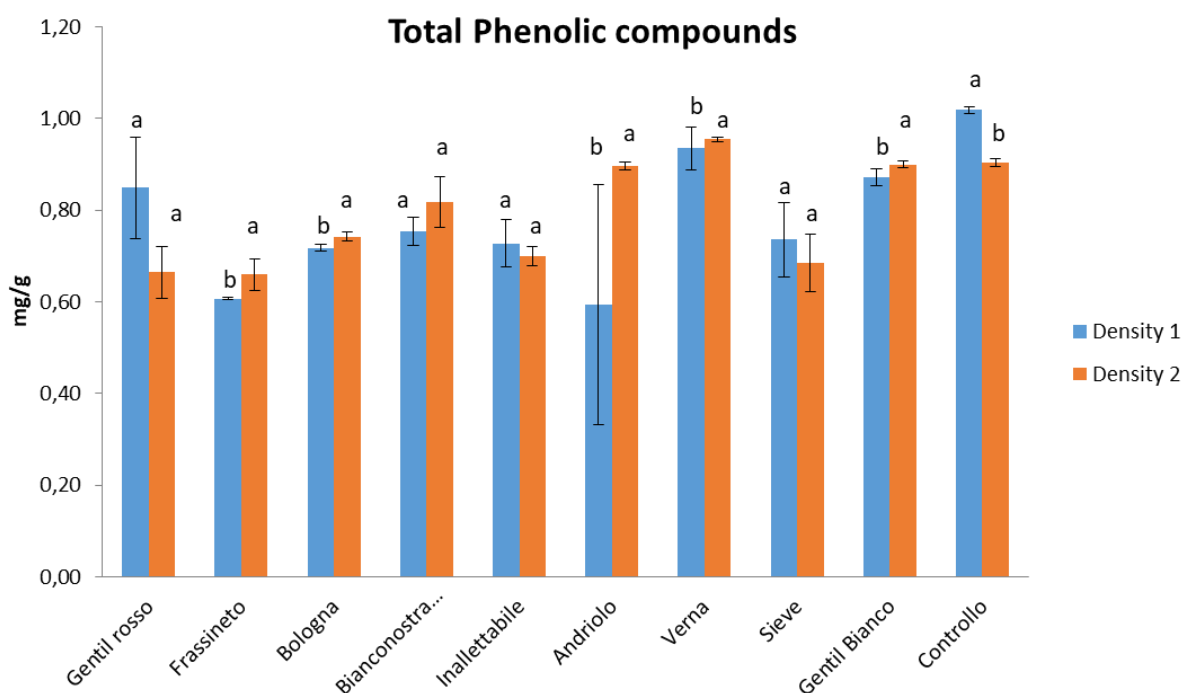
Figure 1.2.2.2 Total phenolic content in Tuscan wheat species for both densities. The data are expressed in mg/g as a mean of a triplicate. Different letters indicate significant differences at $p < 0.05$.

The total phenolic amounts ranged from 0.66 mg/g to 0.99 mg/g with higher values for Gentil Rosso, Andriolo and Verna from density 2, and Control from density 1. Significant

differences in the phenolic amount were observed between the two density types for some of the species. The results highlighted the greater amount of total phenols in most of the samples grown with higher rate of plants per m^2 (density 2), confirming, a certain capability of plants to increase phenolic compound expression in stressful conditions (Sarker *et al.,* 2018). On the other hand, no significant differences were observed in the total phenolic amount of ancient (Gentil Rosso, Frassineto, Bianconostrale, Inalettabile, Andriolo, Verna, Sieve and Gentil Bianco) and modern (Bologna and Control) wheats. This result is in agreement with other recent studies in literature in which the phenolic composition of ancient and modern species was compared, highlighting a similar content (Dinu *et al.,* 2018; Schewry *et al.,* 2018). According to Balli *et al.*, (2020), the hydrolytic method (Method Arsm), optimized by Response Surface Methodology for millet, was performed on a pool of selected samples from Tuscany (GRd1, FRd2, BOd2, BNd2, ILd1, ADd2, VRd2, SVd1, GBd2 and COd1). The total phenolic amounts recovered after the two acidic hydrolyses are compared in Figure 1.2.2.3.
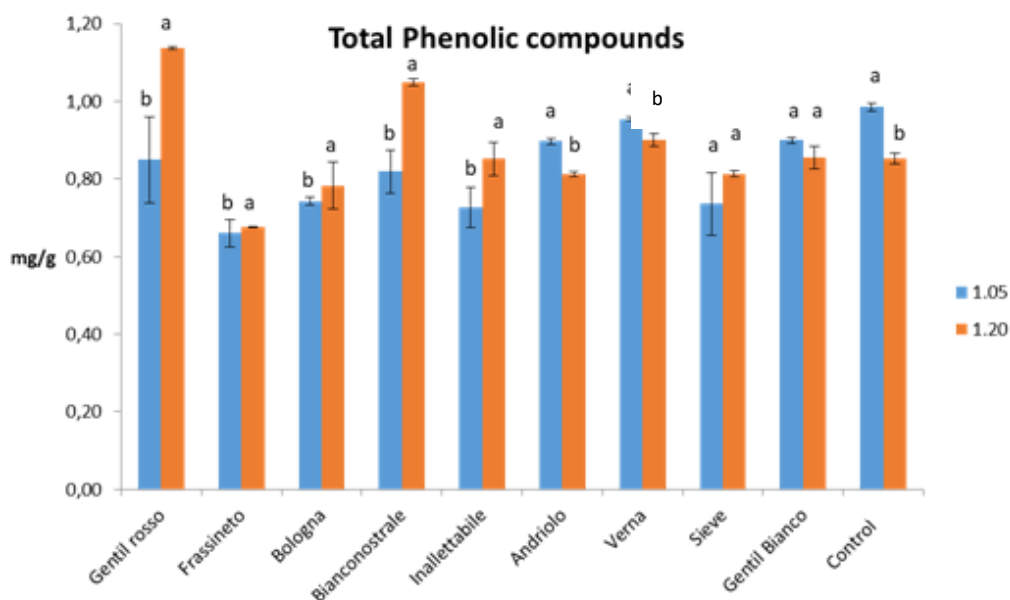
Figure 1.2.2.3 Total phenolic content after acidic hydrolyses by H$_2$SO$_4$ (1.05 and 1.20 M)
Data are expressed as a mean of a triplicate in mg/g. Different letters indicate significant differences at $p < 0.05$.

The results highlighted that the total phenolic compounds recovered with the optimized procedure (1.20 M) were significantly higher in almost all the samples, with few exceptions (Andriolo, Verna and Control).

The low amounts of flavonoids, ranging from 2% to 5 % of total phenols, are in agreement with the literature on wheat and not determined by the hydrolytic procedures. According to Balli *et al*., (2020), no degradation of *C*-glycolsylated flavonoids was observed applying the acidic hydrolysis by sulphuric acid. Indeed, through the extraction of free phenols we were able to confirm that flavonoids were natively present in lower amounts in these wheat samples and did not diminish after acidic hydrolysis (Figure 1.2.2.4).
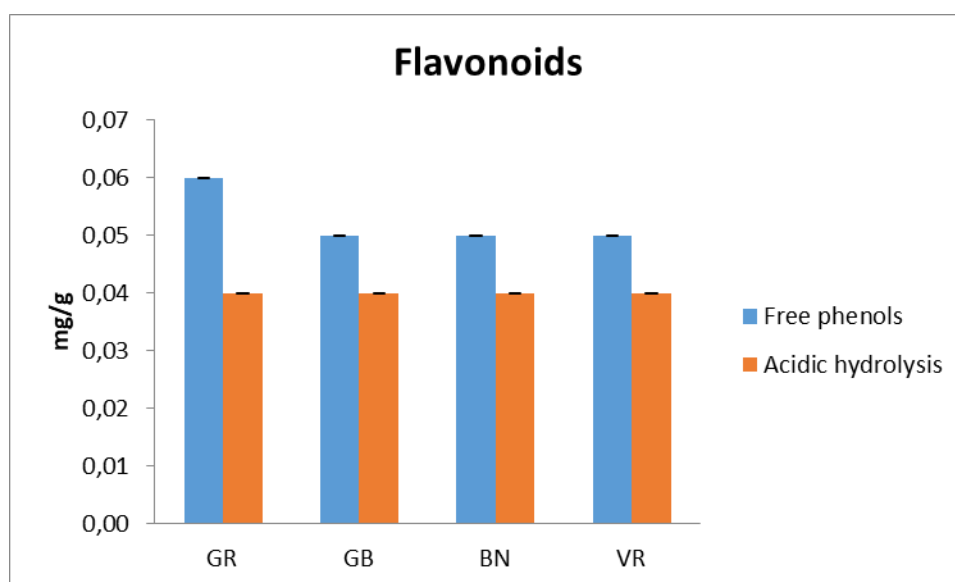
Figure 1.2.2.4 Flavonoids content after free phenols and acidic hydrolysis 1.20 M.

Data are expressed in mg/g as a mean of triplicate.

Within phenolic compounds, flavonoids are those more sensitive to the agronomic and environmental conditions, for example drought stress and excess of light, which could influence their biosynthesis in plants (Rozema *et al.,* 1997; Tattini *et al.,* 2004).

Because basic hydrolysis is the most common method proposed so far for the extraction of bound phenolic compounds in cereals, some wheat samples from Tuscany (VRd2, GBd2, GRd1, BNd2) were also treated in alkaline conditions. It is generally carried out with sodium hydroxide ranging from 1 to 4 M and may require extraction times of several hours (Dinelli *et al.,* 2011; Acosta-Estrada *et al.,* 2014).

Comparing the total phenolic amounts obtained after basic procedure (free+bound compounds) and acidic hydrolyses, the results confirmed the ability of acidic conditions, in a single extractive step, to increase the percentage of recovered phenols (Figure 1.2.2.5).
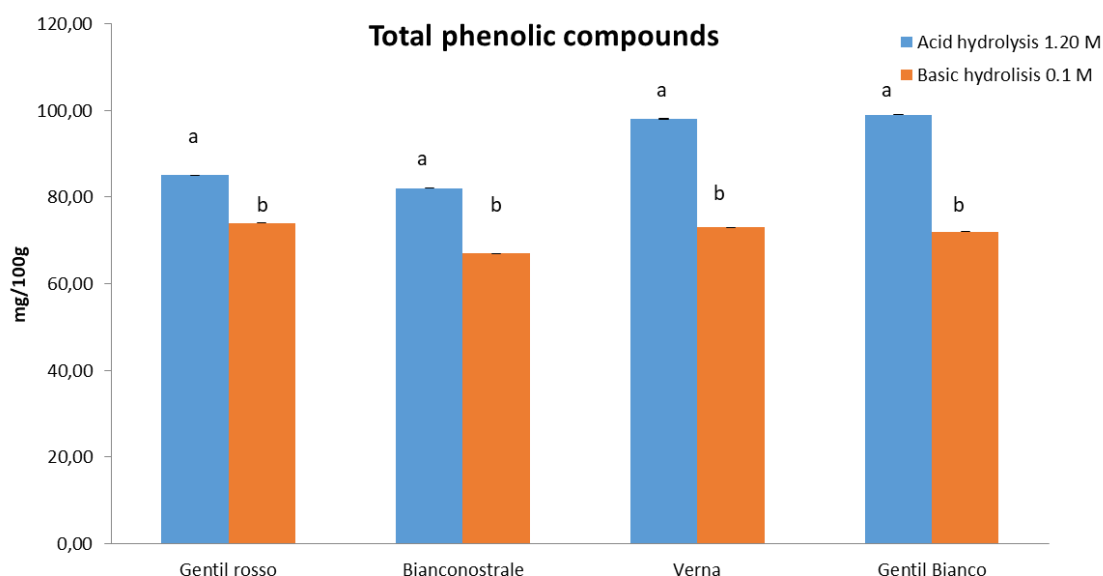
Figure 1.2.2.5 Total phenolic compounds recovered after acidic hydrolysis (1.20 M) and basic hydrolysis (0.1M). Data are expressed in mg/g as a mean of triplicate.

In light of these results, the optimized acidic procedure was also performed on the Campania wheat samples. The total phenolic compounds in the optimized procedure ranged from 0.72 to 0.85 mg/g and from 0.67 to 1.14 mg/g in Tuscan and Campania wheat species, respectively. Our values were in the same range as those obtained by Gotti *et al*.,

(2018) and those collected in the EU Healthgrain project (Poutanen *et al.,* 2008). Moreover, ancient and modern wheat species presented comparable results in terms of recovered total phenols.

The distribution and the total phenolic content of Tuscan and Campania samples are described in Table 1.2.2.3.

a)

Analytes 330 nm	GR	VR	FR	Ancient wheat BN	IL	AD	SV	GB	Modern wheat CO	BO
3	0.01	0.01	0.01	0.02	0.00	0.00	0.01	0.01	0.02	0.01
4	0.03	0.03	0.02	0.02	0.02	0.02	0.03	0.03	0.02	0.03
5	0.03	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.01	0.02
6	0.81	0.68	0.47	0.59	0.64	0.63	0.64	0.64	0.67	0.57
7	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
8	0.04	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.03	0.02
9	0.05	0.03	0.03	0.04	0.03	0.03	0.03	0.03	0.02	0.03
10	0.05	0.04	0.04	0.07	0.04	0.04	0.04	0.04	0.03	0.04
11	0.03	0.01	0.01	0.03	0.01	0.01	0.01	0.01	0.00	0.02
Total cinnamics	1.10	0.87	0.63	0.83	0.79	0.77	0.82	0.82	0.83	0.75
Analytes 350 nm										
1	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.02
2	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.02
Total flavonoids	0.04	0.03	0.04	0.02	0.02	0.04	0.03	0.03	0.02	0.04

b)

Analytes 330 nm	CAR1	CAR2	RUS	IA1	Ancient wheat IA2	AN	SR	CA	Modern wheat AM	AU	BO
3	0.01	0.01	0.01	0.02	0.02	0.01	-	0.01	0.01	0.00	0.01
4	0.02	0.03	0.03	0.03	0.03	0.02	0.01	0.01	0.02	0.01	0.03
5	0.02	0.02	0.03	0.02	0.02	0.02	0.01	0.02	0.02	0.01	0.02
6	0.60	0.61	0.60	0.64	0.58	0.67	0.41	0.56	0.64	0.53	0.63
7	0.01	0.01	-	0.00	-	0.00	-	0.01	0.01	0.02	0.00
8	0.01	0.00	-	-		0.01	-	0.01	-	0.01	-
9	0.05	0.04	0.04	0.05	-	0.04	0.03	0.06	0.03	0.05	0.03
10	0.02	0.02	0.01	0.01	0.02	0.02	0.01	0.03	0.01	0.02	0.02
11	0.03	0.02	0.02	0.03	0.02	0.02	0.02	0.03	0.02	0.03	0.02
Total cinnamics	0.75	0.75	0.74	0.79	0.69	0.81	0.50	0.75	0.76	0.68	0.77
Analytes 350 nm											
2	0.04	0.06	0.04	0.04	0.05	0.04	0.06	0.07	0.02	0.04	0.01
Total flavonoids	0.04	0.06	0.04	0.04	0.05	0.04	0.06	0.07	0.02	0.04	0.01

Table 1.2.2.3 Phenolic content by acidic hydrolysis in all wheat species from Tuscany (**a**) and from Campania (**b**). Data are expressed in mg/g as a mean of triplicate. RSD<5%

1.2.2.4 Conclusions

Currently, despite the rediscovered interest in ancient wheat, only few literature studies propose an accurate comparison between ancient and modern wheat, excluding environmental interferences. This work aimed to compare the phenolic content of different modern and old species from Tuscany and Campania by analysing samples grown in the same field.

With regard to the extractive procedures, the optimized acidic hydrolysis based on only a single step of extraction resulted the most suitable for the recovery of total phenolic compounds. The alkaline hydrolysis usually applied to recover the bound phenols from cereals underestimated the total amount. All the wheat samples presented similar phenolic profiles. Cinnamic derivatives represented the larger class of phenols, instead, the *C*-glycosylated flavonoids, schaftoside and its isomer, were natively lower. Total phenolic content ranged from 0.67 to 1.14 mg/g and from 0.72 to 0.85 mg/g in Tuscan and Campania samples respectively. No significant differences were observed among ancient and modern species despite some reported data in the literature.

1.3 Results of fermentation on millet and wheat

1.3.1   Does Fermentation really increase the phenolic amount in cereals?

A study on millet.

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Abstract

Millet is underutilized in Europe, with great advantages compare to other common cereals
as high proteins, minerals and vitamins levels, its ability to grown everywhere and the
absence of gluten that make it suitable for patient with celiac disease. In Asia and Africa
millet is mainly eaten as fermented, and its consume leads to beneficial properties on
human health. In this context, functional foods based on fermented millet could represent
new trend in European market. Three millet batches were compared in terms of free and
bound phenols by HPLC-DAD-MS. The richest one in terms of bound phenols was
selected for testing a basic (0.1 M NaOH) and an acidic (1.2 M H_2SO_4) hydrolysis with
149.3 and 193.6 mg/100g of phenols recovered, respectively. The ability of fermentation,
with yeast and *lactobacilli*, in increasing the phenolic compounds, was verified.
Fermentation increased the cinnamic acids and flavonoids (approx. 30%), mechanically
trapped into fiber. Vitexin and vitexin-2-*O*-rhamnoside, significantly higher in the
fermented millet, inhibited PTP1B enzyme overexpressed in type two diabetes of approx.
30%. The phenolic extract from fermented millet, demonstrated a higher antioxidant
protection on human erythrocytes by the Cellular Antioxidant Activity in Red Blood Cells
(CAA-RBC).
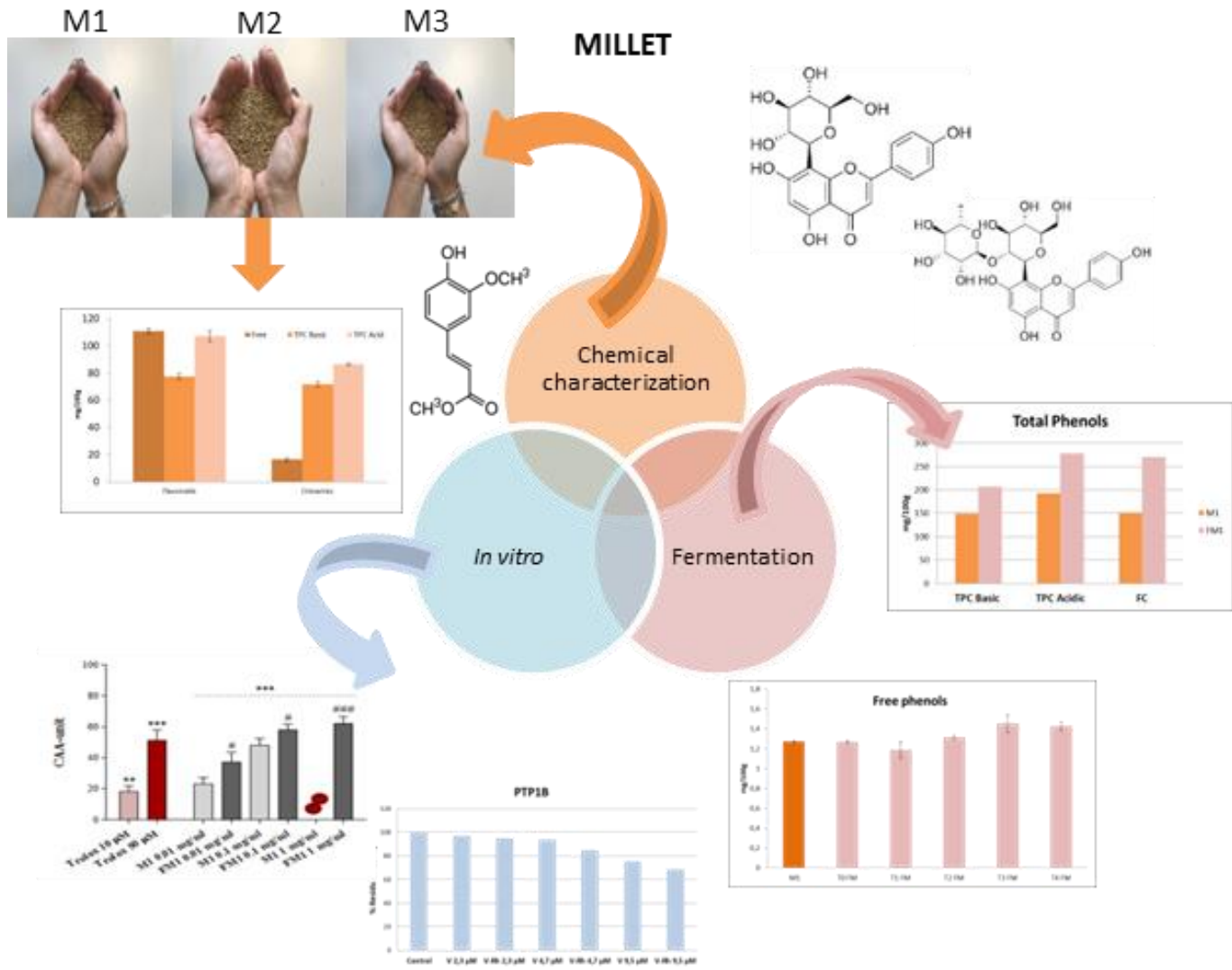
1.3.1.1 State of the art and aims of the work

Gluten free cereals have gained special consideration not only for their nutritional suitability for celiac, but also for their properties linked to the presence of many phytochemicals able to act as preventive factors against several human (Collar, 2019; Wei *et al.,* 2018). For millet the research related to the formulation of new gluten free foods is a challenging topic. Despite the potential advantages of millet consumption, this cereal is poorly utilized in Europe for human nutrition because of the scarce economic and technological support provided till now (Dias-martins *et al.,* 2018). On the contrary, in African and Asian tradition, millet is considered a staple crop and approximately 90% of world production is destined for human consumption in form of fermented cereal (Chandrasekara *et al.,* 2011). In particular, fermentation is reported to increase the bio-conversion of phenolic compounds from their linked or conjugated forms to their free ones, resulting in an increasing concentration of the phenolic component with greater antioxidant power (Saharan *et al.,* 2017). In this context the objective of this work was to verify how fermentation processes, performed using a commercial mixture of yeast and *lactobacilli* for bread production, influenced the phenolic profile in millet samples. The ability of vitexin and vitexin 2"-*O*-rhamnoside, present in millet samples in inhibiting the PTP1B enzyme overexpressed in type two diabetes, was investigated, and the *ex vivo* Cellular Antioxidant Activity (CAA-RBC) in Red Blood Cells was assessed for fermented and unfermented samples. The work aims to propose a simple method to improve the nutritional value of millet, a cereal not commonly used in Europe, paving the way for the design of new fermented products based on millet.

1.3.1.2 Materials and Methods

Samples and reagents

Three batches of millet namely M1, M2 and M3, were purchased from Burkina Faso's local markets. All solvents used were of analytical HPLC grade from Sigma Aldrich (St. Louis, Missouri, USA). Water was ultrapure (Milli-Q®), ferulic acid (purity ≥ 99%), vitexin and vitexin 2"-*O*-rhamnoside (purity ≥ 95%) were from Extrasynthese (Genay, France). Phosphate buffer saline (PBS) tablets, 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Fluka-Sigma-Aldrich, Inc. (St. Louis, MO).

Fermentation processes

The fermentation was performed on the M1 sample (FM1) at the CNR of Pisa using a natural sourdough purchased from a bakery, constituted by a mixture of *lactobacilli* and yeast strains in a ratio of approx. 100:1. Millet flour was obtained by grinding millet grains using a laboratory miller. Water was added to moisten the mix, and then selected microbial starter cultures were inoculated to initiate fermentation. Once the product was fermented, it was dried. The fermentation temperature was maintained around 38° C, while the pH reached value of 4. Different withdrawals were performed at time 0 (T0) and during the process, that is after 24 h (T1), 48 h (T2), 72 h (T3) and 96 h (T4) of fermentation.

Extraction of phenolic compounds

Free phenols. The flour obtained from three milled samples (M1, M2, M3) was defatted twice with hexane (1:10 (w/v)) and kept on a mechanical shaker for 1 hour. The samples were treated according to Balli *et al.,* 2019. Briefly, 2 g of defatted flour was suspended in

20 mL of acidic MeOH (1% HCl) and, after 30 minutes of sonication, stirred by a magnetic stirrer for about 12 h. The supernatant was separated by centrifugation (5000 rpm, 10 min), the residue re-suspended in 25 mL of the extractive mixture, sonicated for 30 minutes, and stirred for 2 h. The two supernatants were collected and taken to a volume of 50 mL. The extractive procedure applied to the fermented samples has been the same already used for other fermented products as reported by Balli *et al.,* (2019). Briefly, 250 mg of fermented flour of M1 was extracted with ethanol/water 80:20 v/v under magnetic stirring in ultrasound bath for about 15 minutes. The extract was centrifuged at 5000 rpm for 10 minutes, 10 mL of the supernatant were evaporated to dryness and the residue re-dissolved in 1.5 mL of acidified H_2O (1% HCOOH).

Total phenols. The flour of the three millet samples, and the fermented sample from M1 (FM1) taken after 96 hours, were treated with the following acidic and basic hydrolytic procedures. The M1 and FM1 samples were treated with an acidic hydrolysis for the recovery of total phenols by applying only a single extractive step according to Balli *et al.,* 2020: 1 g of defatted flour was suspended in 25 mL of $MeOH^+$ (1.20 M H_2SO_4), the solution was sonicated 180 min at 55°C. The sample was then centrifuged at 5000 rpm for 10 minutes (Acidic hydrolysis). The basic hydrolysis was carried out by the aid of ultrasounds as previously described (Balli *et al.,* 2019): 1 g of sample was treated with 25 mL of NaOH 0.1 M in MeOH/H_2O 7:3 v/v and sonicated (40 MHz) for 1 h at 60°C; the pH was neutralized with acetic acid, the suspension centrifuged at 5000 rpm for 10 minutes and the supernatant was recovered.

Analytical HPLC-DAD

The millet extracts recovered after centrifugation, were analyzed using a HP 1200L liquid chromatography equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA); the column was a Poroshell 120, EC-C18 (150x3 mm, 2.7 μm, Agilent, USA). The solvents for the mobile phase were (A) CH_3CN and (B) 0.1% formic acid/water; the multi-step linear solvent gradient was the same proposed by Balli *et al.,* (2020).

MS analysis

The HPLC-DAD-MS analysis of the phenolic extracts were performed according to Balli *et al.,* (2020). HPLC-DAD-MS system was from Waters and composed by 2695 HPLC, 2996 DAD and 4 micro MS equipped with Zspray ESI source. The ESI interface parameters were capillary 2.90 kV, cone 64 V in the first 18 minutes and 30 V till the end of the analysis, source temperature 120°C, desolvation temperature 350°C, cone gas flow 19 (L/Hr), desolvation gas flow 350 (L/Hr). Data were acquired in negative ion mode from 110 m/z to 1000 m/z.

Quantitative determination of phenolic acids and flavonoids

The phenolic acids were evaluated using a five-point calibration curve of ferulic acid at 330 nm, (R^2=1, linearity range 0-0.21 μg); the content of flavonoid aglycones was determined using a five-point calibration curve with vitexin (purity ≥ 95%) at 350 nm, linearity range 0-21 μg (R^2=1.0); vitexin 2"-*O*-rhamnoside (purity ≥ 95%) at 350 nm, linearity range 0-0.11 μg, (R^2=1.0) was selected to quantify the glycosylated flavonoids.

Folin-Ciocalteu reducing capacity

Polyphenols, estimated as Folin-Ciocalteu (FC) reducing capacity, were determined as reported by Domenici *et al.,* (2015) and expressed as mg /100g, dry weight (DW).

Inhibition test on PTP1B enzyme

The two flavonoids vitexin and vitexin 2"-*O*-rhamnoside were tested on PTP1B enzyme in a concentration of 0.205 mg/mL and 0.111 mg/mL, respectively. Enzymatic assays were carried out using human recombinant PTP1B and p-nitrophenylphosphate (pNPP) as reference substrate. According to Paoli *et al.,* (2013), the pNPP (2.5 mM final concentration) was dissolved in sodium β,β-dimethyl glutarate buffer (75 mM, pH 7.0), containing 1mM EDTA and 1 mM dithiothreitol. Reactions were started by addition of aliquots of the enzyme and stopped by adding 2 ml of KOH 0.2 M. The released p-nitrophenolate was quantified by reading absorbance of the final solution at 400 nm (ε = 18,000 M-1 cm-1). The net hydrolysis rate was determined subtracting the value of spontaneous hydrolysis rate of pNPP from each sample. The inhibitory power of vitexin standards was tested adding different amount of extracts: 5, 10 and 20 μL/mL for vitexin and 12, 25 and 50 μL/mL for vitexin 2"-*O*-rhamnoside. The percentage of inhibition was calculated normalizing the absorbance values obtained for assays carried out in the presence of inhibitor versus the control test. All the results were expressed as a mean of three independent experiments.

Ex vivo cellular antioxidant activity (CAA-RBC) assay in red blood cells

Human blood samples from healthy volunteers were collected in ethylenediaminetetraacetic acid (EDTA)-treated tubes and centrifuged for 10 minutes at 2300 xg at 4°C. Plasma and buffy coat were discarded and erythrocytes were washed twice

with PBS pH 7.4. Fermented and unfermented millet samples were extracted with 10% DMSO in distilled water according to Gabriele *et al.,* (2018). The antioxidant activity of millet extract was tested at three different concentrations (0.01, 0.1 and 1 mg/mL) on human erythrocytes as described by Frassinetti *et al.,* (2015). Trolox was used as a reference standard. The fluorescence was read at 485 nm excitation and 535 nm emission by using a Victor TM X3 Multilabel Plate Reader (Waltham, MA, US) and each value was expressed using the following formula: CAA unit = $100-(\int SA / \int CA) \times 100$, where $\int SA$ is the integrated area of the sample curve and $\int CA$ is the integrated area of the control curve.

Statistical analysis

Each experiment was performed in triplicate, and the results were expressed as the mean values $\pm$ SD; the EXCEL software in-house routines was applied. Differences between fermented and unfermented millet effects on human erythrocytes were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test and by unpaired t-test. A p-value lower than 0.05 was considered as statistically significant.

Proximate analysis

Dietary fiber (soluble and insoluble) was determined according to AOAC Method 991.43 (Determination of soluble, insoluble and total dietary fiber in foods and food products, final approval 1991).

1.3.1.3 Results and Discussion

Different batches of millet were compared in terms of free and total phenols, and the richest one was selected for fermentation. The effect of a fermentation process using a mix of yeast and *lactobacilli* on the phenolic content of millet was studied. The choice of this fermentation mixture, commonly used for bread making, can guarantee a better availability and reproducibility of the process, and constitutes a cheap way to improve the nutritional value of staple foods.

Phenolic characterization of non-fermented flours

The HPLC profiles obtained from the extraction of free phenols from the three analyzed batches are very similar from a qualitative point of view (Figure 1.3.1.1): compounds **3**; **4**; **6**; **7** have been recognized as flavonoids (**f**), while the compounds **1**; **5**; **8-12** as cinnamic (**c**) derivatives.
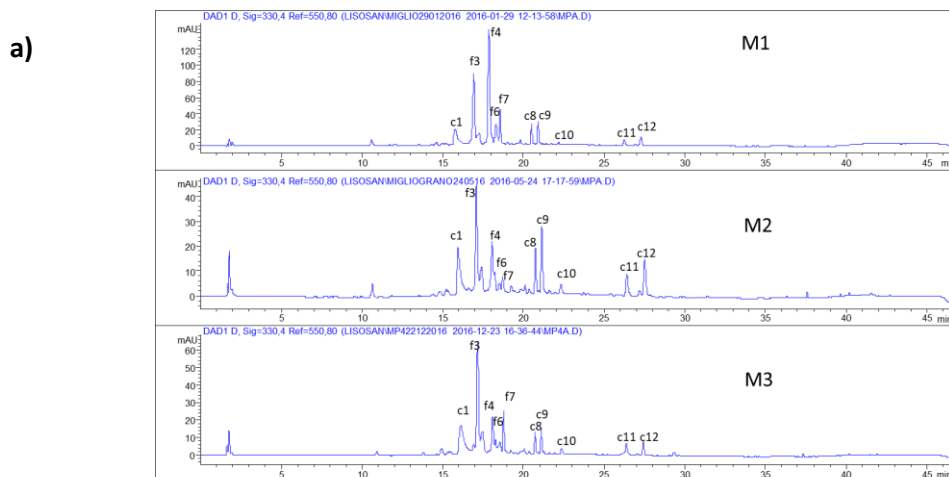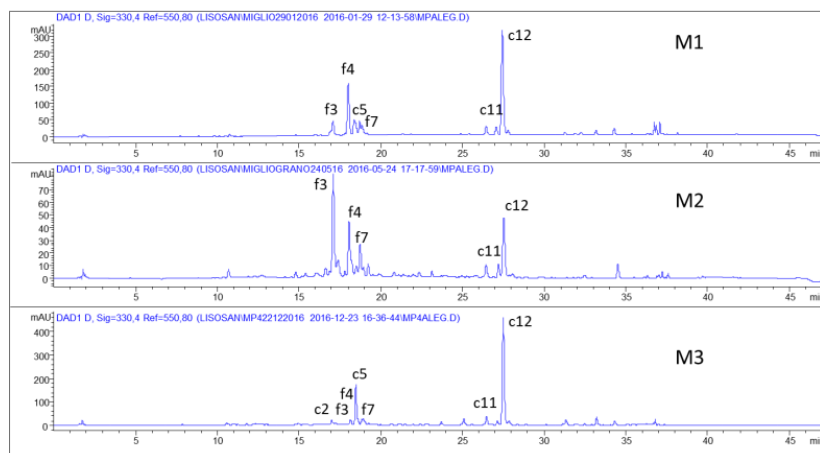
Figure 1.3.1.1 Chromatographic profiles at 330 nm of the free (**a**) and total phenols obtained after basic hydrolysis (**b**) of the millet batches (M1,M2,M3): c1, N^1,N^4-dicaffeoyl-spermidin; **c2**, *p*-coumaric acid; **f3**, luteolin (7-*O*-glucopyranosyl) 8-*C*-glucopyranoside; **f4**, vicenin II; **c5**, ferulic acid; **f6**, vitexin 2"-*O*-rhamnoside; **f7**, vitexin; **c8**, ferulic acid rhamnoside; **c9**, ferulic acid rhamnoside isomer; **c10**, isoferulic acid; **c11**, methyl hydroxycinnamate; **c12**, methyl ferulate

All these molecules have been already identified in millet (Balli *et al.,* 2020).

Analytes	[M-H]⁻	Identified compounds
c1	468	N^1,N^4-dicaffeoylspermidin
c8	339	ferulic acid rhamnoside
c9	339	ferulic acid rhamnoside isomer
c10	193	isoferulic acid
c11	177	methylhydroxycinnamate
c12	192	methyl ferulate
f3	609	luteolin-(7-*O*-glucopyranosyl)-8-*C*-glucopyiranoside
f4	593	vicenin II
f6	577	vitexin 2"-*O*-rhamnoside
f7	431	vitexin

Table 1.3.1.1 Identified compounds in millet samples

From a quantitative point of view, M1 showed a total of free phenols of 127.4 mg/100g, 87% of which are flavonoids; M2 showed a total of 48.2 mg/100g with 68% of flavonoids and M3 showed a total of 64.0 mg/100g with 83% of flavonoids (Table1.3.1.2).

	Free phenols			Total phenols (basic hydrolysis)		
Analytes	M1	M2	M3	M1	M2	M3
1	6.8[a]	7.4[a]	7.1[a]	-	-	-
2	-	-	-	-	-	2.3
5	-	-	-	16.5[b]	-	34.2[a]
8	2.5[a]	1.9[a]	1.3[a]	-	-	-
9	3.2[a]	1.6[b]	1.6[b]	-	-	-
10	1.1[a]	1.1[a]	0.5[b]	-	-	-
11	1.1[a]	1.3[a]	1.2[a]	4.4[b]	-	5.3[a]
12	1.7[a]	2.0[a]	1.1[b]	56.5[b]	40.8[c]	67.1[a]
3	44.3[a]	22.0[c]	35.3[b]	17.3[a]	13.9[b]	3.2[c]
4	44.7[a]	7.4[b]	7.6[b]	45.2[a]	8.6[b]	6.7[c]
6	12.9[a]	1.5[c]	3.0[b]	-	-	-
7	9.1[a]	2.0[c]	4.6[b]	9.4[a]	3.2[c]	4.9[b]
TCC	16.4[a]	15.3[b]	12.8[c]	77.4[b]	40.8[c]	108.9[a]
TFC	111[a]	32.9[c]	53.2[b]	71.9[a]	22.7[b]	14.8[c]
TPC	127.4[a]	48.2[c]	64.0[b]	149.3[a]	66.5[c]	123.7[b]

Table 1.3.1.2 The data are a mean of three independent extractions expressed as mg/100 g of dry weight. TCC: total cinnamic content; TFC: total flavonoids content; TPC: total phenolic content; RSD < 5%

Extraction of total phenols was carried out on the whole defatted flour with a basic hydrolysis using NaOH 0.1 M. The applied hydrolytic conditions were milder than those proposed in the literature (Chandrasekara *et al.,* 2010; Zhen *et al.,* 2015). Chromatographic profiles showed differences in relative abundances among the three batches, while regarding cinnamic derivatives, compound **c5** identified as ferulic acid, was only present in M1 and M3 batches (Figure 1.3.1.1b). Table 1.3.1.2 shows that M1 resulted the richest in terms of total phenols (149.3 mg/100g), in terms of bound phenols, calculated by difference (59.7 mg/100g), M3 was the richest one. The M1 sample was also treated with an acidic hydrolysis optimized on millet sample by Response Surface Methodology

according to Balli *et al.,* (2020). The acidic hydrolysis guaranteed a higher amount of total phenols recovered, with respect to the basic one (193.6 mg/100g instead of 149.3 mg/100g). Furthermore, a comparison between cinnamic acids and flavonoids yield in M1 sample, after acidic, basic hydrolysis and free phenols extraction, was carried out (Figure 1.3.1.2).
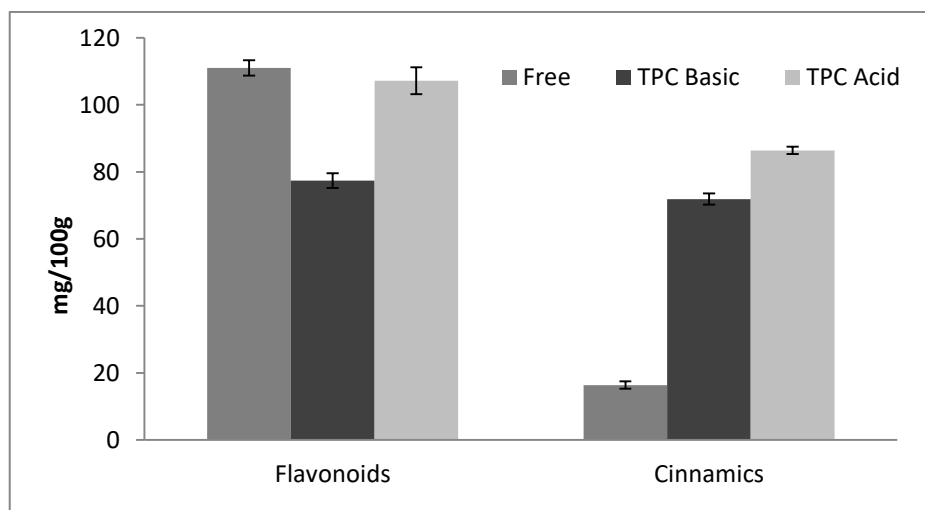
Figure 1.3.1.2 Total phenolic content (TPC) expressed as total flavonoid and cinnamic derivatives in M1 sample after acidic and basic hydrolysis. The data are expressed as a mean of three independent extraction in mg/100g of dry weight

Our results confirmed that flavonoids in millet are almost completely present in free forms, pointing out a higher recovery in acidic condition with respect to the basic hydrolysis. Analogously, the acidic hydrolysis guaranteed a higher release of cinnamic derivatives bound to cell wall structures.

Phenolic characterization of fermented flours

Millet is usually decorticated before consumption to improve its edible properties. This practice leads to reduction in some nutrients (minerals, fiber, and antioxidants as phenolic compounds) and anti-nutrients (phytates, tannins), both located in the peripheral parts of the grains (pericarp and aleurone layer) (Saleh *et al.,* 2013). In this context, fermentation

can be applied in order to consume the whole non-decorticated flour avoiding losing part of functional components. Controversial data about the effectiveness of fermentation in increasing phenolic content are present in the literature till now. Some studies reported that fermentation is able to increase the bio-conversion of phenolic compounds from their linked or conjugated to their free forms (Dey *et al.,* 2016), other works reported a decrease in the total phenolic content attributable to the rearrangement of phenolic structures after self-polymerization in acidic condition induced by fermentation (Adebiyi *et al.,* 2017; Taylor *et al.,* 2014). It's noteworthy that fermentation leads to the production of various unknown compounds extracted with phenols that can interfere with an unspecific assay like Folin Ciocalteau, predominantly used in the literature for the quantification of total phenolic compounds in cereal samples. In this context, the use of HPLC-DAD and of pure standards for quantification of phenolic compounds before and after fermentation, represents a more accurate method to estimate their real content. Herein, the extractions of both free and bound phenols were performed in order to verify whether fermentation is able to guarantee an almost complete release of the bound forms linked to the cellular structures, as cellulose or lignin. The M1 sample, the richest one in terms of total phenolic compounds content, was selected to evaluate the effect of fermentation. Different fermentation times were performed in order to study the evolution of the phenolic profile: at time 0, after 24, 48, 72 and 96 hours (T0, T1, T2, T3 and T4 respectively). HPLC-DAD profiles of free phenols from fermented matrix showed a lower number of peaks compared to those obtained from non-fermented sample: in particular, only flavonoids were detected (Figure 1.3.1.3).

Figure 1.3.1.3 Chromatographic profiles at 350 nm of the fermented millet (FM1): **f3**, luteolin (7-*O*-glucopyranosyl) 8-*C*-glucopyranoside; **f4**, vicenin II; **f6**, vitexin 2"-*O*-rhamnoside; **f7**, vitexin.
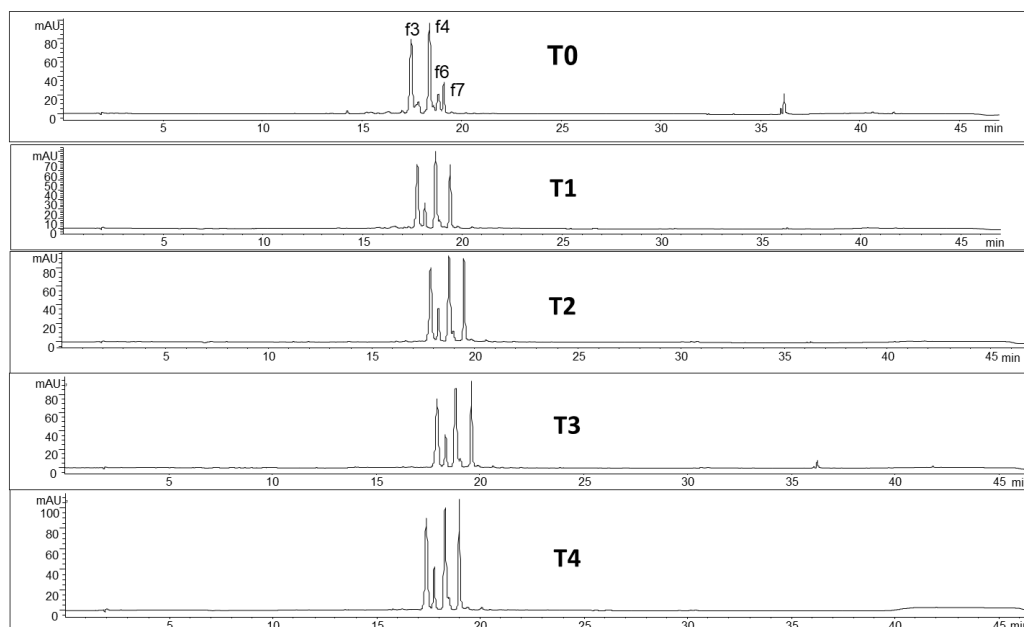
The total content of free phenols changed during fermentation, from 126.9 mg/100g at T0 to a maximum of 145.3 mg/100 g at T3; the value during the following 24 h (T4) resulted almost unchanged (Figure 1.3.1.4).
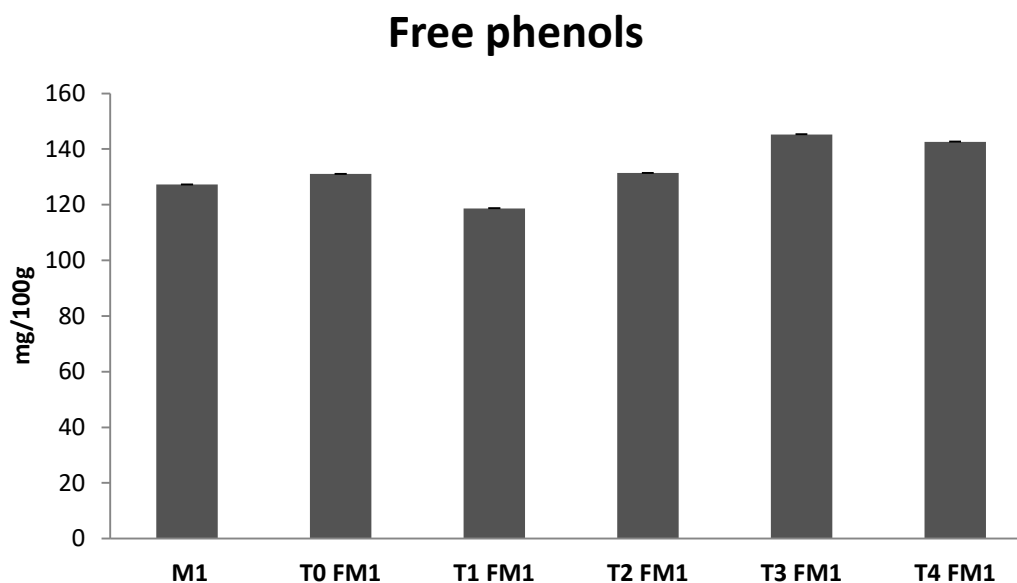
Free phenols

Figure 1.3.1.4 Free phenols content determined by HPLC-DAD in unfermented (M1) and fermented (FM1) millet flour at different withdrawals (T0-T4). Data are expressed in mg/100 g of dry weight as a mean of three different extractions.

The Folin Ciocalteu assay, pointed out for FM1-T3 a significant higher level of total phenols than M1 sample (270.1 ± 34.2 vs 150.3 ± 8.1 mg GAE/100g DW, $p < 0.05$). These results demonstrated a positive improvement of the millet flour in terms of phytochemicals content following the fermentation process (Table 1.3.1.3). For a more in deep characterization, the basic hydrolysis was applied on the sample collected at T3 (72 h of fermentation) for the chromatographic evaluation of total phenols. The corresponding HPLC-DAD profile was very similar to the non-fermented sample, showing methyl ferulate (**c12**) as the predominant compound (data not shown). From a quantitative point of view, the basic hydrolysis on FM1-T3, allowed extracting a higher amount of phenols (206.2 mg/100g) compared to unfermented sample (149.3 mg/100g).

The optimized acidic hydrolysis was then performed on FM1-T3 confirming the capability of this method to recover the highest amount of total phenols (278.3 mg/100 g) as already observed also for the unfermented matrix.

	Total phenols- Method B (mg/100g)	Total phenols-Method A (mg/100g)	FC reducing capacity (mg/100g)
M1	149.3±3.2*	193.6±4.0*	150.3 ± 8.1*
FM1-T3	206.2±0.8*	278.3±1.8*	270.1 ± 34.2*

Table 1.3.1.3 Total phenolic content estimated after basic hydrolysis (left column), acidic hydrolysis (central column) and as Folin-Ciocalteu (FC) reducing capacity, in the unfermented (M1) and fermented (FM1-T3) flours. Unpaired t-test for comparing millet flours with the respective fermented flour; *$p < 0.05$.

Our results pointed out that the three methods in Table 1.3.1.3 were suitable to highlight the differences in phenolic content after fermentation. Nevertheless, in order to not underestimate the phenolic amount in cereal grains the application of an acidic hydrolysis is suggested. The higher total phenolic values in fermented sample could be attributable to the action of microorganisms by using the part of the fiber for their grown, release the phenolic compounds "mechanically trapped" in these structures. According to this hypothesis, the evaluation of soluble fiber was also done. This latter, in M1 sample was 2 g/100g but after fermentation strongly decreased up to <0.10 g/100 g. It's noteworthy from our results that fermentation can be a useful pretreatment to release phenolic compounds from the soluble fiber where they are mechanically trapped, inducing an increase up to 35%. To verify this latter result, another batch of millet (M2) was fermented using the

same mixture of yeast and *lactobacilli*, and evaluated at time 0 (T0), 72 h (T3) and 96h (T4). The results highlighted again an increase of 36% in the total phenolic content passing from 66.5 mg/100g to 90.7 mg/100g for the unfermented and fermented matrix respectively (Figure 1.3.1.5). Higher phenolic contents in fermented flours could be associated to several health properties.
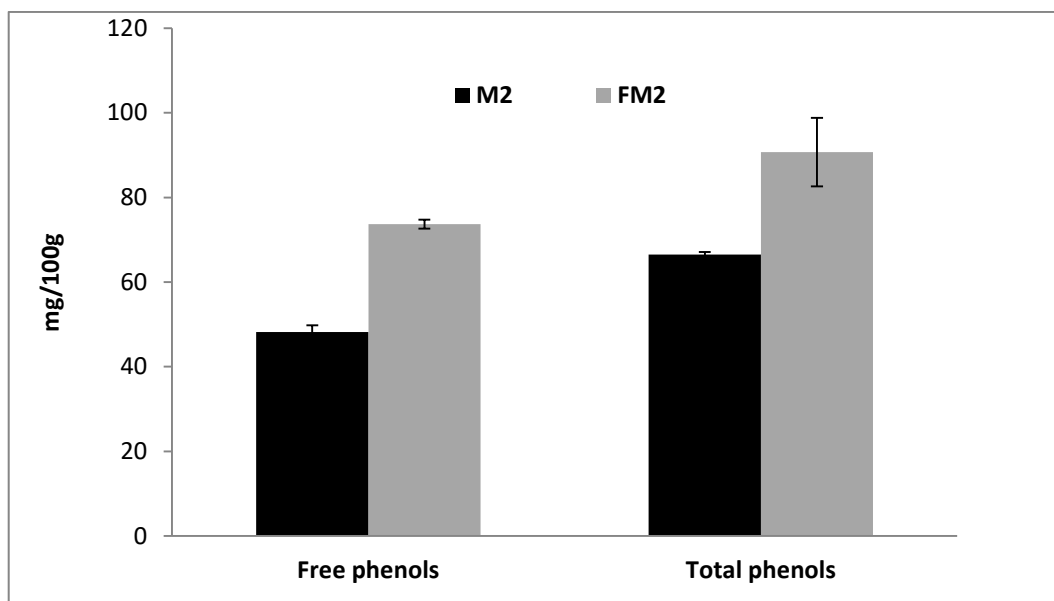
Figure 1.3.1.5 Free and Total phenolic amount before and after fermentation in M2 sample. The fermented sample was withdrawn after 72 hours of fermentation. Data are expressed in mg/100g as a mean of three independent extraction.

Inhibition test on PTP1B enzyme

The two standards, vitexin and vitexin 2"-*O*-rhamnoside, were evaluated *in vitro* for their inhibitory effect on PTP1B, known as a negative regulator of insulin receptors. The choice of these flavonoids was based on the inhibitory activity already showed by this class of molecules on PTP1B enzyme (Jiang *et al.,* 2012). On the opposite, the cinnamic derivatives that resulted higher in millet, were unable to inhibit the enzyme (Adisakwattana *et al.,* 2013). Our results pointed out that vitexin and vitexin 2"-*O*-rhamnoside were not

able to inhibit the enzyme in concentrations of 2.3 μM and 4.7 μM respectively, but an inhibition of 25% and 32% was pointed out for both these flavonoids, at 9.5 μM (Figure 1.3.1.6).
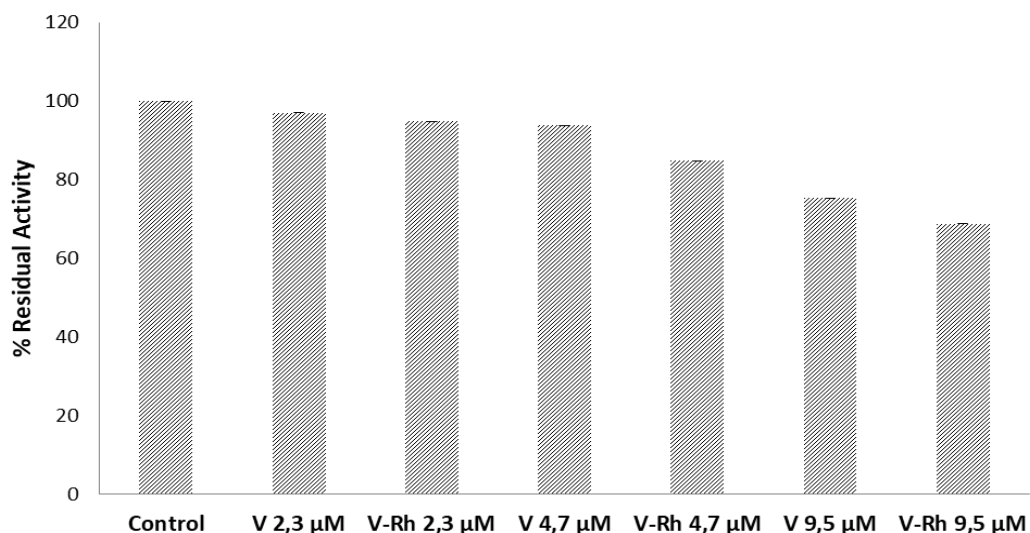
Figure 1.3.1.6 *In vitro* activity (as %) of PTP1B enzyme after a treatment with vitexin (V) and vitexin 2"-*O*-rhamnoside (V-Rh), both tested at three concentration values:
2.3 μM, 4.7 μM and 9.5 μM.

The concentration of the two flavonoids resulted higher in the fermented extracts (FM1) with respect to the unfermented millet (MF1). In particular, after the recovery of total phenols in acidic condition, vitexin increased from 45 mg/100g in MF1 to 70 mg/100g in FM1 (55% higher). Analogously, vitexin 2"-*O*-rhamnoside was 20 mg/100g in MF1 and 32 mg/100 g in FM1 (60% higher). In light of these findings, we can affirm that a daily consume of fermented millet containing higher amount of flavonoids with respect to the unfermented flour, could help to reduce the incidence of type two diabetes by the inhibition of PTP1B enzyme, overexpressed in these subjects. Further analyses will be conducted to better understand if the entire fermented extract is able to inhibit the PTP1B through a synergic action between the single components of the fermented flour.

Ex vivo cellular antioxidant activity on human erythrocytes

Erythrocytes play a key role in the body in terms of anti-inflammatory and antioxidant protection and represent a powerful tool to assess the radicals scavenging activity of many natural compounds (Caddeo *et al.,* 2018). In this study, the biological effects of millet, before and after the fermentation process, was evaluated on an *ex vivo* model of human erythrocytes under oxidative condition using the CAA-RBC assay (Figure 1.3.1.7).
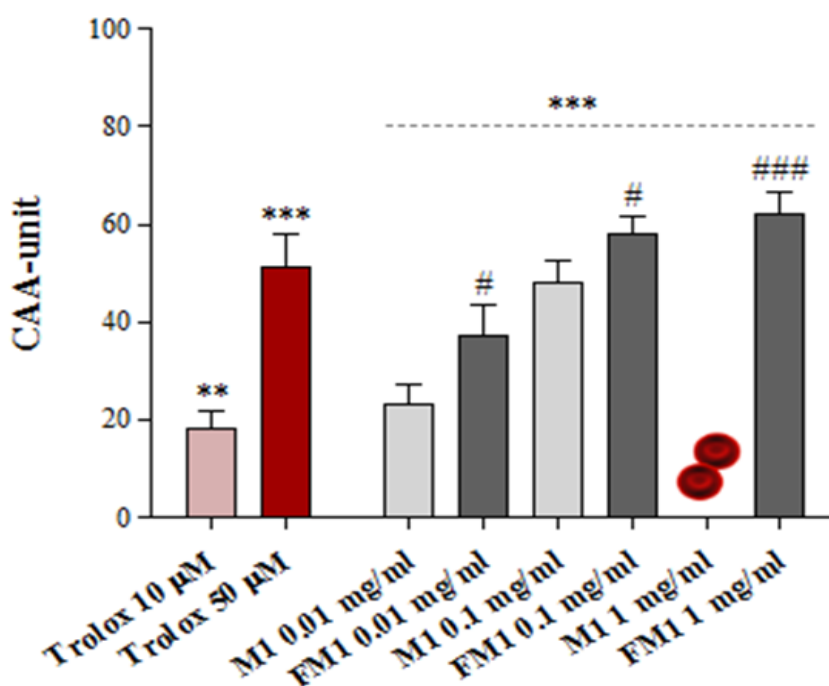
Figure 1.3.1.7 Effects of unfermented (M1) and fermented (FM1) millet extracts at different concentrations (0.01, 0.1 and 1 mg/mL) on the cellular antioxidant activity (CAA) of human erythrocytes under oxidative conditions. Trolox was used as a reference standard. One-way ANOVA with Dunnett's multiple comparison test: *significantly different from CNT, AAPH treated cells (CAA = 0), **$p \leq 0.01$, ***$p \leq 0.001$. Unpaired t-test: #significantly different from the respective non-fermented flour, #$p \leq 0.05$, ##$p \leq 0.01$, ###$p \leq 0.001$.

In this assay, following 1-hour pretreatment with millet extracts (0.01, 0.1 and 1 mg/mL), human erythrocytes were exposed to an oxidative insult induced by the thermal

decomposition of AAPH in peroxyl radicals. Our results demonstrated a good and dose-dependent antioxidant protection of human erythrocytes from all tested samples with the exclusion of millet flour at the highest tested concentration (1 mg/mL), that induced erythrocytes' hemolysis (Figure 1.3.1.7). The fermented millet obtained after 72 hours of fermentation (FM1) and unfermented millet (M1) extracts significantly raised the cellular antioxidant activity of human erythrocytes compared to control, that refers to only AAPH-exposed cells (CAA = 0; **p < 0.01), with CAA values comparable to or higher than Trolox 10 and 50 μM, used as reference standard. Besides, millet fermented extract exhibited, at all tested doses, significantly higher antioxidant protection than the co-respective unfermented ones (#p<0.05).

1.3.1.4 Conclusions

The work aimed to increase the knowledge on the effect of fermentation on millet. According to our results, the fermentation carried out using a mixture of natural sourdough mainly constituted by *lactobacilli*, increased the total amount of phenolic compounds in millet of approx. 30%, presumably releasing those mechanically trapped into the aliquot of soluble and fermentescible fiber. Indeed, soluble fiber can be used by *lactobacilli* and yeasts for their grown. Acidic hydrolysis compared to the basic one, guaranteed a higher recovery of total cinnamic and flavonoids both from fermented and unfermented samples. Both these classes of phenols resulted higher in the fermented millet with respect to the unfermented one, particularly the flavonoids recovery resulted consistently higher in the fermented flour, with increases over fifty percent. Fermentation time could last 72 hours because extend the process until 96 hours did not increase the total phenolic content in millet. The flavonoids of millet, showed a partial inhibition of PTP1B enzyme, overexpressed in diabetes, suggesting that a daily intake of fermented millet can contribute

to reduce the expression of type 2 diabetes. The results of this study also showed a good and dose-dependent antioxidant protection of human erythrocytes exerted by both unfermented and fermented millet samples. According to the phytonutrients enrichment determined by the chemical analyses, sourdough fermentation significantly raised the *ex vivo* antioxidant activities, higher in fermented millet flour with respect to the unfermented one. Today it is reasonable to conclude that the total phenolic content in cereals is underestimated due to various factors, such as the application of unsuitable extraction methods based on alkaline hydrolysis, but also for the mechanical entrapment of these molecules by the fibers. Fermented millet with a higher phenolic content, which leads to their higher bioavailability, could be used as new food, also suitable for the feeding of celiac.

1.3.2 Study on a Fermented Whole Wheat: Phenolic Content, Activity on PTP1B enzyme and *In Vitro* Prebiotic Properties

Diletta Balli, Maria Bellumori, Paolo Paoli, Giuseppe Pieraccini, Monica Di Paola, Carlotta De Filippo, Diana Di Gioia, Nadia Mulinacci[*] and Marzia Innocenti

*corresponding author

Abstract

 Fermented cereals, staple foods in Asia and Africa, are recently receiving a growing interest in Western countries. The object of this work is the characterization of a fermented wheat used as a food ingredient and dietary supplement. To this aim, the phenolic composition, the activity on protein tyrosine phosphatase 1B (PTP1B), an enzyme overexpressed in type-II diabetes, the *in vitro* prebiotic properties on *Lactobacillus reuteri* and the microbial composition were investigated. Basic and acidic hydrolysis were tested for an exhaustive recovery of bound phenols: the acidic hydrolysis gave the best yields. Methyl ferulate and neocarlinoside were identified for the first time in wheat. The inhibitory activity of the extracts of several batches were investigated on PTP1B enzyme. The product was not able to inhibit the enzyme, however, for the first time, a complete inhibition was observed for schaftoside, a major *C*-flavonoid of wheat. The microbial composition was assessed identifying *Lactobacillus, Enterococcus*, and *Pediococcus* as the main bacterial species. The fermented wheat was a suitable substrate for the growth of *L. reuteri,* recognized for its health properties in the human gut. The proposed method for phenols is easier compared to those based on strong basic hydrolysis; our results assessed the bound phenols as the major fraction, differently from that suggested by the literature for fermented cereals.

Fermented wheat

CHEMICAL CHARACHERIZATION

Basic and acidic hydrolysis
New phenolic compounds
Bound phenols as the mayor fraction

carlinoside

methylferulate
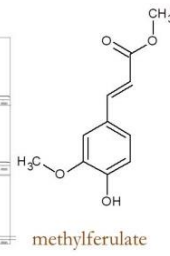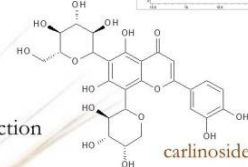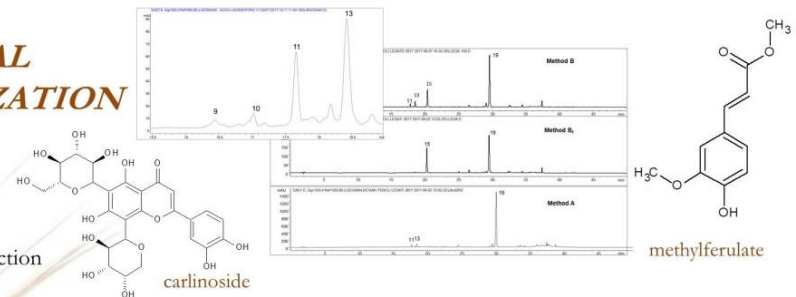
IN VITRO ASSAYS

Prebiotic effect on *Lactobacillus reuteri*
Inhibitory power of schaftoside on PTP1B, overexpressed
in type-II diabetes

Microbial composition: *Lactobacillus, Enterococcus* and *Pediococcus*
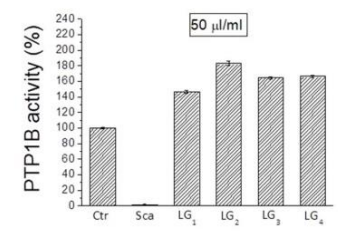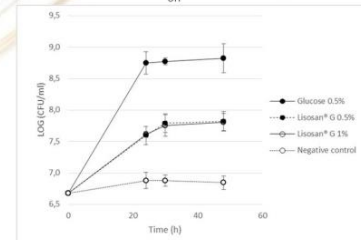
Diletta Balli, Maria Bellumori, Paolo Paoli, Giuseppe Pieraccini, Monica Di Paola, Carlotta De Filippo,
Diana Di Gioia, Nadia Mulinacci, Marzia Innocenti

1.3.2.1 State of the art and aims of the work

A fermented product from wheat (*Triticum aestivum*), used as food ingredient and food supplement, is Lisosan® G. The product is obtained as follows: the grounded wholegrain is mixed with water and sourdough in order to initiate the fermenting process. After three days the product is recovered and dried. Recently, Lisosan® G has been object of *in vivo* and *ex vivo* studies, but few data are available on its composition. A protective activity against the toxicity of cisplatin was observed on Wistar rats fed with different dosages of Lisosan® G (Longo *et al.,* 2011). The protective effects of Lisosan® G on human microvascular endothelial cells incubated with oxidized LDL and Lisosan® G were evaluated, at different concentrations of the product observing a decrease of some inflammatory markers (Lubrano *et al.,* 2012). The study of Lucchesi *et al.,* (2014) focused on the effects of Lisosan® G as an antioxidant for human endothelial progenitor cells exposed to oxidative stress. These cells, treated with the product before the incubation with hydrogen peroxide, increased the cell viability and adhesion, and decreased cellular senescence. The liver was a further target of studies on Lisosan® G: treatment with Lisosan® G on primary cultures of rat hepatocytes in presence of hydrogen peroxide, induced the inactivation of NF-KB transcription factor involved in oxidative damage and an up-regulation of Nrf2 responsible for cytoprotection by inducing detoxifying enzymes (La Marca *et al.,* 2013). Despite the studies focused on the evaluation of some health properties, no data concerning the phenolic content, the microbiological composition, the prebiotic effect and the activity on Protein Tyrosine Phosphatase 1B (PTP1B) enzyme are available for this wholegrain fermented product. PTP1B enzyme is recognized as an important target as negative regulator of insulin and leptin receptor signaling pathways (Verma *et al.,* 2017). The present work was aimed: (i) to investigate the chemical composition of Lisosan® G in terms of free, bound, and total phenolic compounds,

optimizing the extraction method; (ii) to investigate the ability of several batches of Lisosan® G and of schaftoside to inhibit the PTP1B enzyme; (iii) to assess the microbial composition of Lisosan® G by sequencing of the bacterial 16 rRNA gene; and (iv) to evaluate the prebiotic effect on the growth of a common human probiotic species *Lactobacillus reuteri*, a widely distributed species recognized for its health properties in the human gut.

1.3.2.2 Materials and Methods

Samples and Reagents

Four batches of Lisosan® G (LG_1, LG_2, LG_3, LG_4), a food supplement obtained from lysed fine bran and germ of organic wheat grains (*Triticum aestivum*), were provided by Agrisan Srl Company, Larciano (Pistoia, Italy). All solvents used were of analytical HPLC grade from Sigma Aldrich (St. Louis, MI, USA). Water was ultrapure (Milli-Q®, Merck Millipore, Darmstad, Germany), ferulic acid (purity $\geq$ 99%), and apigenin (purity $\geq$ 95%) were purchased from Extrasynthese (Genay, France).

Extraction of Phenolic Compounds

Free phenols. Lisosan® G, LG_1, (250 mg) was extracted with $EtOH/H_2O$ 80:20 *v/v* under magnetic stirring in ultrasonic bath for about 15 min. The extract was centrifuged at 5000 rpm for 10 min. Ten mL of the supernatant were evaporated to dryness and the residue re-dissolved in 1.5 mL of acidified H_2O (1% HCOOH). The same extraction was performed with H_2O acidified with 1% HCOOH.

Bound and total phenols. An acidic hydrolysis (method A) and three different basic hydrolyses (B_F, B and B_S methods) were applied on the fermented wheat as reported below. All the hydrolyses were carried out by the help of an ultrasonic bath (40 MHz). Method B_F: 1 g of the sample was extracted with 25 mL of NaOH 0.1 M in $MeOH/H_2O$

7:3 *v/v*, sonicated for 1 h at 60 °C. The pH was adjusted to neutrality with acetic acid and the solution centrifuged at 5000 rpm for 10 min to recover the supernatant. The same procedure was applied to the solid residue recovered after the extraction of free phenols. Method B was the same of method B_F with only a difference on the solvent-dry sample ratio:1 g of Lisosan® G was extracted in 100 mL NaOH 0.1 M in MeOH/H_2O 7:3 *v/v*. Method B_S: 1 g of the sample was extracted in 25 mL of MeOH/$H_2$0 7:3 *v/v* with NaOH 4 M, sonicated for 1 h at 60 °C. The pH was adjusted to neutrality with CH_3COOH and the solution diluted to a final volume of 50 mL with MeOH/$H_2$0 7:3 *v/v*; the sample was then centrifuged at 5000 rpm for 10 min to recover the supernatant. Method A was an acidic hydrolysis performed on the four batches (LG_1–LG_4) using the mixture MeOH/H_2SO_4 9:1 *v/v*: 1 g of Lisosan® G was extracted in 25 mL, sonicated for 2 h at 60 °C, and then centrifuged at 5000 rpm for 10 min to recover the supernatant.

Fractionation by Semipreparative HPLC

About 700 mg of Lisosan® G (LG_1) were almost completely dissolved in 150 mL of HCOOH (1%) under magnetic stirring for 15 min at 60 °C. After centrifugation (5000 rpm, 10 min), the supernatant was dried under vacuum and re-dissolved in 6 mL of distilled water obtaining the total Lisosan® G extract. This extract was fractionated by semipreparative HPLC using a Hewlett Packard 1050 series and a Polaris RP-C18 Ether column (250 × 10 mm, 5 μm, Varian, Germany); 12 fractions were recovered after 30 injections of 100 μL. Elution was carried out at a flow rate of 4 mL min^{-1} with CH_3CN as solvent A and H_2O (0.1% HCOOH) as solvent B. A linear elution gradient was employed: solvent A was increased from 0% to 10% in 10 min, from 10% to 15% in 10 min, from 15% to 30% in 10 min, from 30% to 100% in 5 min with a final plateau of 10 min. Total time of analysis was 45 min, equilibration time 10 min. The collection was carried out monitoring the chromatogram at 280 nm up to 15 min, successively 350 nm was the

wavelength selected to detect and recover the flavonoids. The fractions were dried, re-dissolved in 1 mL of water, and controlled by analytical HPLC-DAD and MS^n analysis.

Inhibition Test on PTP1B Enzyme

All four batches were treated as follow: the maximum amount of Lisosan$^®$ G (250 mg) was dissolved in 50 mL of acidified H_2O (1% HCOOH), after a magnetic stirring of 15 min. The extracts were then evaporated to dryness and re-dissolved in 2 mL of water. These extracts (LG_1–LG_4), the pure schaftoside (at concentration 2.7 µM), and the pure myricetin (at concentration 0.5 µM) were evaluated as inhibitors of the enzyme PTP1B. Furthermore, the IC_{50} was also determined for the schaftoside. Enzymatic assays were carried out using human recombinant PTP1B and *p*-nitrophenylphosphate (pNPP) as reference substrate. According to Paoli et al. (2013), the pNPP (2.5 mM final concentration) was dissolved in sodium β,β-dimethyl glutarate buffer (75 mM, pH 7.0), containing 1 mM EDTA and 1 mM dithiothreitol; this solution was used as control (Ctr). Reactions were started by addition of aliquots of the enzyme and stopped by adding 2 mL of KOH 0.2 M. The released p-nitrophenolate was quantified by reading absorbance of the final solution at 400 nm (ε = 18,000 M^{-1} cm^{-1}). The net hydrolysis rate was determined subtracting the value of spontaneous hydrolysis rate of pNPP from each sample. The inhibitory power of LG extracts and schaftoside standard was tested adding different amount of extracts (10 and 50 µL/mL) for each assay. Then, the percentage of inhibition was calculated normalizing the absorbance values obtained for assays carried out in the presence of inhibitor versus the control test. All the results were expressed as a mean of three independent experiments.

Bacteriological Analysis

In order to exclude the presence of bacterial contaminants of food specimens, such as coliforms and staphylococci, LG$_1$ batch was tested in McConkey III medium (Oxoid Basingstone, UK; for the detection of coliform, bacilli, *Salmonella* and *Shigella* species) and in Mannitol salt medium (Oxoid Basingstone, UK; for detection of presumptive pathogenic staphylococci). Lisosan® G (500 mg) were directly plated on the two different media and, after incubation for 24–48 h, presence of potential microbial contaminants were evaluated.

16. S Ribosomial RNA Gene Amplicons Preparation and Illumine MISEQ and Data Analysis

By LG$_1$ batch, library of 16S rRNA gene amplicons was prepared by IGA Technology Services (Udine, Italy) through amplification of the V3–V4 hypervariable region. The standard protocol was followed according to the 16S metagenomic sequencing library preparation guide from Illumina (Part #15044223 Rev. B; https://support.illumina.com/, San Diego, CA). Pooled V3–V4 amplicon libraries were sequenced using the Illumina MiSeq platform. Sequence data are available at European Bioinformatics Insitute-EMBL-EBI database, under the accession number PRJEB30414. Reads (total number 126.669) were further processed using the MICCA pipeline (version 1.6, ttp://compmetagen.github.io/micca/, San Diego, CA), as reported by Di Paola *et al.,* (2018). A total of 104.277 Operational Taxonomic Units (OTUs) were assigned by clustering the sequences with a threshold of 97% pair-wise identity. OTU tables for each taxonomic level were created. To deep at species level sequence alignment using Basic Local Alignment Search Tool nucleotide (BLASTn) software (San Diego, CA) in the National Center for Biotechnology Information (NCBI) database was performed. The

highest percentage of identity (query cover 100–99% and identity 99%). Expectation value (*E*-value) was used to select significant BLAST hits, keeping only outcomes with the lowest *E*-value (minimal *E*-value of 10^{-3}).

Test on Lactobacillus reuteri

The strain used was obtained from the German Collection of Microorganisms and Cell Cultures (*L. reuteri* DSM 17938). The lyophilized strain was re-vitalized in the Man Rogosa Sharpe (MRS) medium (Oxoid, Basingstone, UK) supplemented with 0.05% cysteine and incubated in anaerobic chamber at 37 °C for 24 h. The MRS medium composition was modified to perform the growth experiment with Lisosan® G in order to eliminate glucose and reduce the concentration of potential growth factors, as described [b]Khatib *et al.,* (2017). The modified medium is referred to as m-MRS. The prebiotic activity was evaluated using Lisosan® G, LG_1 batch, at 0.5% and 1% (*w/v*) in m-MRS. A positive growth control was performed using m-MRS with 0.5% (*w/v*) glucose and a negative control in m-MRS with no added carbon source. The medium containing Lisosan® G as potential carbon source was prepared as follows: the m-MRS ingredients were weighed and the medium autoclaved at 120 °C for 15 min. Lisosan® G was then added, the solution stirred at 80 °C and then autoclaved again at 102 °C for 10 min. The strain was grown overnight in MRS, centrifuged, washed in saline (0.9% NaCl), and re-suspended in saline to obtain an absorbance of 0.7 mAu at 600 nm. This suspension was used to inoculate at 2% (*v/v*) the flasks containing the m-MRS medium plus Lisosan® G or glucose or the negative control with no carbon source. The tubes were incubated at 37 °C in anaerobic conditions for 48 h and a 1 mL culture was sampled from each flask, serially diluted, and inoculated on MRS agar plates for viable bacterial counts at pre-established times (0, 24, 30, and 48 h of incubation). Upon incubation, the number of colonies,

corresponding to the number of viable cells, were counted and expressed as CFU mL^{-1}. The number was transformed into a log10 value (log CFU mL^{-1}).

Analytical HPLC-DAD

The extracts from Lisosan$^®$ G were centrifuged (5000 rpm, 10 min) and analyzed using a HP 1200L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA) using a Poroshell 120, EC-C18 column (150 × 3 mm, 2.7 μm, Agilent, USA). The solvents for the mobile phase were (A) CH$_3$CN and (B) 0.1% formic acid/water; the multi-step linear solvent gradient was: 0–5 min 0–10% A; 5–10 min 10–15% A; 10–20 min 15–30% A; 20–25 min 30–35% A; 25–28 min 35–40% A; 28–31 min 40–45% A; 31–42 min 100% A; 42–47 min 100–0% A; equilibration time 5 min; flow rate 0.4 mL min^{-1}; injection volume 10 μL. The following wavelengths were simultaneously selected: 240 nm, 280 nm, 330 nm, 350 nm.

MS Analysis of Lisosan$^®$ G fractions

The isolated fractions by semipreparative HPLC were analyzed by direct infusion in ESI-HRMS and MS^n on a LTQ-Orbitrap (Thermo Scientific, Bremen, Germany). Each fraction was taken to dryness by evaporation under vacuum and re-suspended in a CH$_3$CN/H$_2$O mixture, containing 0.1% formic acid. This solution was infused by syringe into the ESI interface of the instrument. Sheath and auxiliary gas flow rates were 10 and 2 (arbitrary units), respectively; capillary voltage and tube lens voltages, as the collision energy and wideband activation voltage in MS^n experiments, were optimized for each compound of interest during the infusion. The mass spectrometer was calibrated with the standard mixture indicated by the producer immediately before the acquisition of the samples, both in positive and in negative ion mode.

Quantitative Determination of Phenolic Acids and Flavonoids

The phenolic acids were evaluated using a five-point calibration curve of ferulic acid at 330 nm, ($R^2 = 1$, linearity range 0–0.21 µg), while the flavonoid content was determined using a five-point calibration curve of apigenin at 350 nm ($R^2 = 0.999$, linearity range 0–0.80 µg).

Statistical Analysis

Each experiment was performed in triplicate, and the results were expressed as the mean values ± SD; the EXCEL software (version 2013, Microsoft Corporation, WA, USA) in-house routines were applied. Significance in the prebiotic properties experiment was calculated within each evaluation time with a *t*-test, using the MEANS procedure (SAS). Statistical analysis of data from PTP1B was performed using the Student t-tests, using OriginPro 2018 (OriginLab Corporation, Northampton, MA 01060 USA http://www.originlab.com). The differences between the groups were considered significant when $p < 0.05$.

1.3.2.3 Results and Discussion

The phenolic molecules in the fermented wheat were studied as free, bound, and total phenols by applying different extractive procedures, some of them already suggested for wheat. The first aim was to verify if the fermentation process increases the free phenols as suggested by literature (Hur *et al.,* 2014) diminishing the bound fraction. In order to study better the minor components, Lisosan® G was fractionated by semipreparative HPLC and the content of several phenolic fractions was determined by chromatographic and MS analysis. After the identification of the main phenolic constituents and the optimization of the extractive and analytical procedures, several batches were investigated. The ability of

four batches of Lisosan® G and schaftoside standard to inhibit the PTP1B enzyme was successively evaluated *in vitro*. Microbial composition was also assessed. The potential prebiotic properties of Lisosan® G were tested on *L. reuteri* DSM 17938, a probiotic strain with demonstrated potential beneficial effects in treating and preventing human diseases.

Fractionation by Semipreparative HPLC

Regarding the free phenols, the hydroalcoholic mixture (ethanol /H_2O 8:2, *v/v*) and acidic water showed comparable results, confirmed by the chromatographic profiles (data not shown). Consequently, the aqueous extract was preferred as reference sample for the semipreparative HPLC. According to Figure 1.3.2.1a and Table 1.3.2.1, some analytes detected at 280 nm (compounds **1**–**8**) were recovered; while at 350 nm *C*-flavonoids (**9**–**11** and **13**), ferulic acid (**15**), and some unknown phenols were recorded (**16**–**18**).

a)
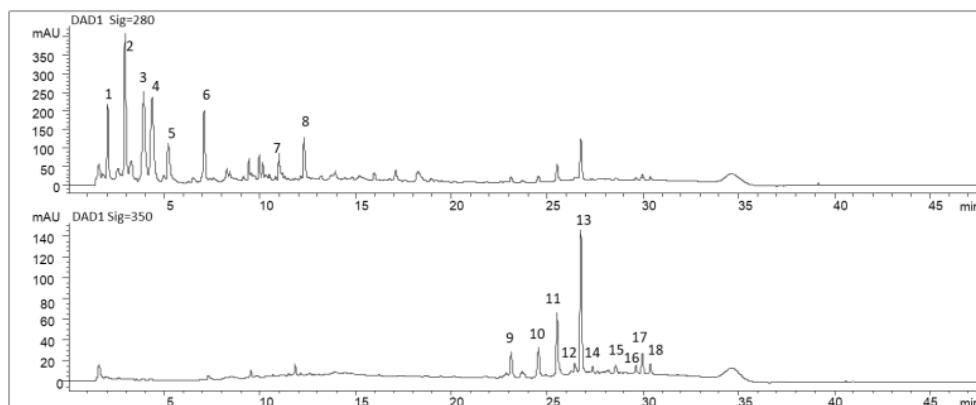
b)
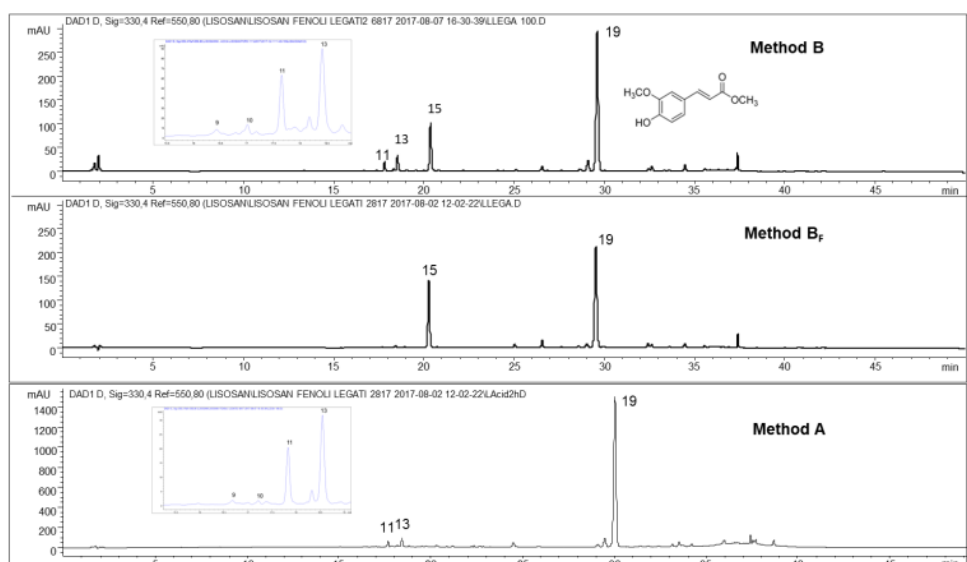
Figure 1.3.2.1 (**a**) Chromatographic profiles at 280 and 350 nm of the aqueous extract of Lisosan®
G on the Poroshell column, obtained applying the same gradient elution used for the
semipreparative HPLC. Compounds **1**–**8**, unknowns; **9** and **10**, neocarlinoside or its isobars,
isocarlinoside/carlinoside; **11**, isoschaftoside; **13**, schaftoside; **15**, ferulic acid; **16**–**18** unknowns;
and (**b**) comparison of the HPLC profiles at 330 nm obtained for bound phenols with methods B
and A on the whole flour, method B_F on the residue from free phenol extraction; **9** and **10**,
neocarlinoside/isocarlinoside/carlinoside; **11**, isoschaftoside; **13**, schaftoside; **15**, ferulic acid; **19**,
methyl ferulate and its chemical formula.

As expected in a fermented wheat, the presence of several free amino acids was confirmed by HRMS^n analyses in positive ion mode, in the more polar fractions (compounds **1**–**8**), the compound **8** was identified as tryptophan (205 *m/z*) in co-presence with valine (118 *m/z*), proline (116 *m/z*) and leucine (132 *m/z*), while the other analytes (**1**–**7**) to date remained unknown. The main detected phenols were cinnamic derivatives and flavonoids, as shown in Figure 1.3.2.1 a,b. From experimental data collected on the fractions by HRMS^n and from the results in the literature on wheat (Dinelli *et al.,* 2011; Leoncini *et al.,* 2012; Rahman *et al.,* 2017), it was possible to identify a group of *C*-glycosylated flavonoids (**9**–**11** and **13**) present only in two of the fractions from semipreparative HPLC (Figure 1.3.2.2).
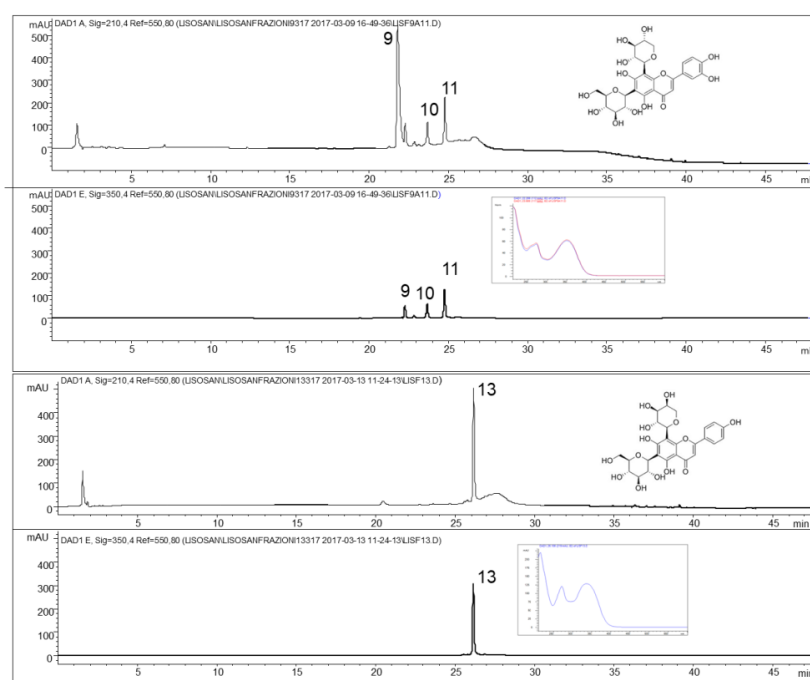
Figure 1.3.2.2 Chromatographic profiles at 210 nm and 350 nm of the two fractions containing the *C*-glycosylated flavonoids recovered by semi-preparative HPLC; **9** and **10**, neocarlinoside/isocarlinoside/carlinoside; **11**, isoschaftoside; **13**, schaftoside (UV–VIS spectra of peaks **9** and **13** and chemical formula of carlinoside and schaftoside are also shown).

The profiles at 210 nm, a non-selective wavelength, confirm the exclusive presence of flavonoid compounds. The mass spectra of compounds **9** and **10** (Figure 1.3.3.3) showed the $[M - H]^-$ ion at 579 m/z in negative ionization, and similar UV–VIS spectra. The MS/MS experiments on 579 m/z showed the loss of water (561 m/z) while the two fragment ions at 489 and 459 m/z are consistent with the loss of 90 and 120 mass units, respectively. According to Iswaldi *et al.,* (2011), these losses correspond to cross-ring cleavages in the sugar moiety of *C*-glycosilated flavonoids. In light of these findings, compounds **9** and **10** were tentatively identified as carlinoside, isocarlinoside, and/or neocarlinoside. These isobaric forms have only been reported once in wheat Rahman *et al.,* (2017), as a plant response associated to drought tolerance. Compounds **11** and **13**, with empirical formula $C_{26}H_{28}O_{14}$, exhibited the same deprotonated ion at 563 m/z. The ion species at 473 m/z and 443 m/z, obtained by MS/MS dissociation of the $[M−H]^-$ ion, showed again the losses of 90 and 120 mass units, respectively. From MS^3 experiments it was possible to confirm that the common fragment ion at 353 m/z is originated from the 563 m/z (after two successive losses of 120 and 90 mass units). According to literature (Colombo *et al.,* 2008; Simirgiotis *et al.,* 2013), it was possible to identify **11** as isoschaftoside and **13** as schaftoside.
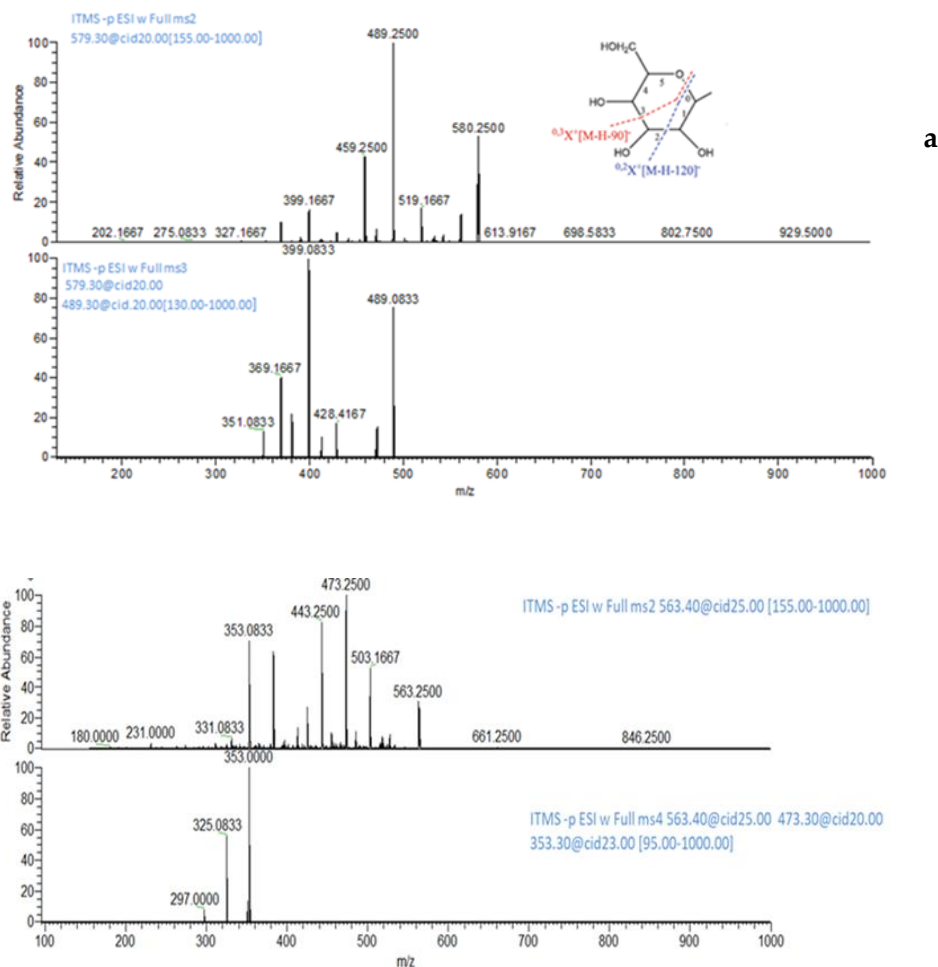
Figure 1.3.2.3 MS^2 and MS^3 spectra (**a**) of carlinoside/isocarlinoside/neocarlinoside (**10**) and MS^2 and MS^4 spectra (**b**) of isoschaftoside (**11**). All MS^n spectra were recorded at the optimised collision energy using wideband activation. The fragmentation of the glycosidic moiety, originating the losses of 90 and 120 mass units, is also shown.

The spectral data in positive and negative ion mode for the pool of *C*-glycosylated flavonoids are reported in Table 1.3.2.1. Compound **15** was identified as ferulic acid as also confirmed by the MS^2 spectrum of its deprotonated molecular ion showing the species at 178 *m/z*, corresponding to the loss of a methyl group, and 134 *m/z* from the successive loss of carbon dioxide (59 mass units).

		Positive Ion MS^n		Negative Ion MS^n	
Analytes	C-glycosylated flavonoids	[M–H]$^+$	fragment ions	[M–H]$^-$	fragment ions
9	Carlinoside/ Neocarlinoside/ Isocarlinoside	581	563; 545; 527; 509	579	489; 399
10	Carlinoside/ Neocarlinoside/ Isocarlinoside	581	563; 545; 527; 509	579	489; 399
11	Isoschaftoside	565	547; 529; 511; 427; 349	563	473; 353; 325
13	Schaftoside	565	547; 529; 511; 427; 349	563	473; 353; 325

Table 1.3.2.1 *C*-glycosylated flavonoids detected in Lisosan® G by MS^n experiments

Extraction of Free, Bound, and Total Phenols

The free phenols in the first batch of Lisosan® G were 38 mg/100 g, a comparable amount to those reported by other authors for wheat, in which the concentrations were lower than 20 mg/100 g (Dinelli *et al.,* 2011; Brandolini *et al.,* 2013). Regarding the bound forms, it was invesitgated whether the fermentation process can induce a release of the bound phenols. To recover this fraction, almost all the available studies on cereals reported the use of strong basic hydrolysis with NaOH (from 2 M to 10 M), usually at room temperature; the acidic hydrolysis was reported as not suitable, due to the degradation of hydroxycinnamic and benzoic acids (Dinelli *et al.,* 2011, Adom *et al.,* 2002). Nevertheless, we observed that few data are available on the effects of different basic or acidic procedures on the chemical stability of phenols during their extraction from cereals. Consequently, with the aim of selecting the best method to effectively recover the phenolic fraction, a methanol solution with 4 M NaOH (Method B$_S$) was firstly tested and compared with a softer condition with 0.1 M NaOH (Method B). HPLC-DAD analysis highlighted that the former procedure induced a partial degradation of the phenolic compounds when

compared with the weaker basic hydrolysis: the use of 4 M NaOH led to the degradation of methyl ferulate converted in ferulic acid, and of compounds **9** and **10** (Table 1.3.2.2).

At the same time, Lisosan® G was also treated according to Arranz *et al*., (2010) to better investigate the effects of the acidic hydrolysis on the phenolic fraction. The chromatographic profiles of the sample after basic hydrolysis with 0.1 M NaOH present two main compounds: ferulic acid (**15**) and methyl ferulate (**19**), while the acidic hydrolysis (Figure 1.3.2.1b) shows only the presence of methyl ferulate (**19**), a compound previously detected in rice Tanaka *et al.,* (1964), but to date never reported in wheat. Presumably, the absence of methyl ferulate in literature could be attributable to the applied strong basic hydrolysis (from 2 M to 10 M) causing the formation of ferulic acid by hydrolysis of the ester bond. On the other hand, the use of a basic medium but in a weaker condition could avoid this reaction. To verify this hypothesis, pure ferulic acid was treated in the same way of Lisosan® G with Method B (0.1 M NaOH in methanol); as expected, the formation of methyl ferulate was not observed and it was possible to conclude that this ester is not an artefact of the extraction process, but is naturally present in Lisosan® G. Observing the profile obtained from the basic extraction (Method B_F) of the solid residue of Lisosan® G remaining after recovery of the free phenols, it was possible to assess that *C*-glycosylated flavonoids (**9–13**) were only present in free form (Figure 1.3.2.1b). In terms of extraction efficiency, the methods tested for the recovery of bound phenols gave different results. The strong basic hydrolysis (Method B_S) led to a partial degradation of the compounds of interest (some minor *C*-flavonoids) and reduced the total phenolic recovery, in comparison to the other applied methods (Table 1.3.2.2a). A strong increase in solvent/dry sample ratio (from method B_F 1:25 *w/v*, to method B, 1:100 *w/v*) guaranteed a better recovery (up to 60% higher) in terms of total phenols (Table 1.3.2.2a).The basic hydrolysis (B) and the acidic procedure (A) applied to LG_1 batch were the more

advantageous, giving the highest yields in total phenols, with similar values of 279 mg/100 g and 265 mg/100 g, respectively. It is worth noting that the acidic hydrolysis (Table 1.3.2.2b, column LG_1) maintained almost the same amount of the C-glycosylated flavonoids (compounds **9**–**11** and **13**) extracted with the simple procedure for free phenols (Table 1.3.2.2a, column FP). The acidic hydrolysis was chosen as a reliable method to compare the four Lisosan® G batches for the following reasons: i) the quantitative data from the methods A were almost the same obtained applying the basic procedure B, but in the latter case applying a four times higher extractive ratio, ii) the phenolic compounds are chemically stable in acidic media differently to what can happen in the basic media, in which the more hydroxylated flavonoids can go toward a partial degradation, and iii) the same acid procedure applied to other cereals (data not shown) gave again the highest recovery in terms of total phenols. The first batch (LG_1) was the richest in terms of total phenolic content; the LG_2 and LG_3 resulted very similar otherwise the LG_4 was the poorest (Table 1.3.2.2b). These variable amounts of the phenolic components is in agreement with what expected for a natural product with a complex composition. Overall, in light with the findings in Table 1.3.2.2, it was possible to conclude that this type of fermentation is not able to increase the release of the bound phenolic fraction that remains higher than 80%, more or less the same amount measured in unfermented wheat (Brandolini *et al.,* 2013).

(a) Free (FP) and Total Phenols (B$_s$, B$_f$ and B) from Basic Hydrolyses in mg/100 g				
Compounds	FP	B$_s$	B$_F$ (on whole flour)	B
Carlinoside/isocarlinoside/ neocarlinoside (**9**)	6	-	4	2
Carlinoside/isocarlinoside/ neocarlinoside (**10**)	5	-	3	2
Isoschaftoside (**11**)	7	6	7	13
Schaftoside (**13**)	17	12	15	22
Ferulic Acid (**15**)	3	223	12	48
Methyl Ferulate (**19**)	-	-	70	178
Total ferulates	3	223	82	226
Total phenols	38	241	111	265
(b) Total Phenols Obtained Applying the Acidic Hydrolysis mg/100g				
Compounds	LG$_1$	LG$_2$	LG$_3$	LG$_4$
Carlinoside/isocarlinoside/ neocarlinoside (**9**)	2	2	2	2
Carlinoside/isocarlinoside/ neocarlinoside (**10**)	3	2	3	3
Isoschaftoside (**11**)	10	6	6	6
Schaftoside (**13**)	17	10	9	10
Ferulic Acid (**15**)	2	2	3	2
Methyl Ferulate (**19**)	245	197	208	158
Total ferulates	247	199	211	160
Total phenols	279	219	231	181

Table 1.3.2.2 Concentration of the phenolic compounds identified in Lisosan[®] G applying different extraction methods and evaluated through HPLC-DAD by suitable external standards. (**a**) free phenols (**FP**), and total phenols determined after basic hydrolyses (methods **B$_S$**, **B$_F$**, **B**) applied to Lisosan[®] G first batch (LG$_1$); (**b**) total phenols determined after acidic hydrolysis (method **A**) applied to the four batches of Lisosan[®] G (**LG$_1$**; **LG$_2$**; **LG$_3$**; **LG$_4$**). The data are a mean of three independent extractions expressed as mg/100 g dry product.

The relative standard deviation (RSD) was below 4% for all the detected phenol

Inhibition of PTP1B

The four batches of Lisosan[®] G and the schaftoside pure standard were in vitro evaluated for their inhibitory effect on PTP1B, known as a negative regulator of insulin receptors. The aqueous extracts (LG$_1$–LG$_4$) at a final concentration of 125 mg/mL and schaftoside, as main flavonoid, at a final concentration of 0.15 mg/mL, were tested adding different amount of extracts (Figure 1.3.2.4).

(a)

(b)
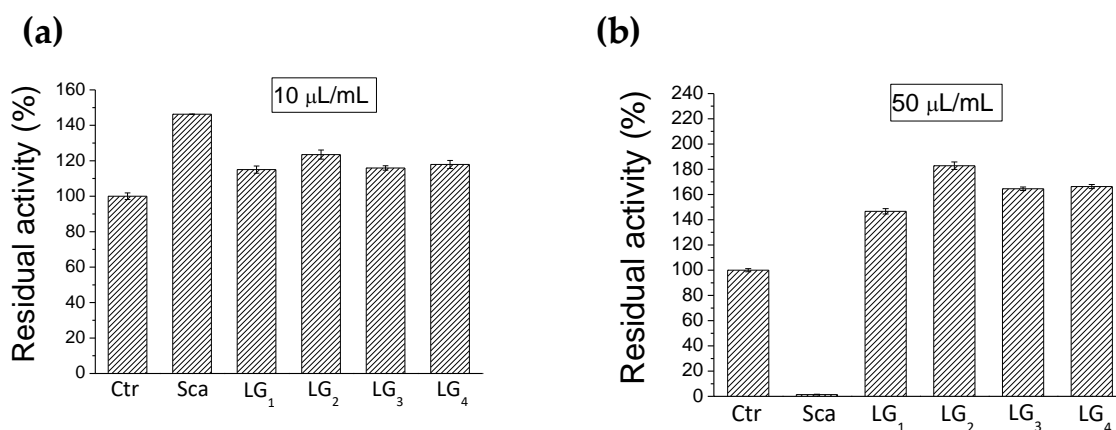
Figure 1.3.2.4 Residual activity of PTP1B enzyme (in vitro %); schaftoside standard (Sca) and Lisosan[®] G extracts (LG$_1$–LG$_4$) were tested at two concentrations: (**a**) 10 µL/mL corresponding to 2.7 µM for schaftoside and 1.25 mg/mL for Lisosan[®] G extracts; (**b**) 50 µL/mL corresponding to 13.5 µM for schaftoside and 6.25 mg/mL for Lisosan[®] G extracts. Ctrl for control (see experimental section).

The choice of testing schaftoside was also due to the inhibitory activity already showed by flavonoids on PTP1B enzyme as reported by literature (Jiang *et al.,* 2012). The results pointed out that the whole extracts and the schaftoside were not able to inhibit the enzyme in diluted (10 µL/mL) and in concentrate (50 µL/mL) solutions. On the contrary, pure schaftoside showed a complete inhibition of the enzyme in a concentration of 13.5 µM (Figure 1.3.2.4b) and an IC$_{50}$ value of 6.4 µM (Figure 1.3.2.5a). Considering that the extracts, containing the corresponding amount of schaftoside, resulted not active in the

enzymatic assay, we could speculate that other compounds present in Lisosan® G could act as antagonists of schaftoside, presumably hindering its interaction with PTP1B enzyme. This hypothesis was verified by using a myricetin standard added to the LG_1 and LG_2 extracts. The choice of myricetin was due to the fact that this flavonol previously showed an IC_{50} of 0.47 μM on PTP1B enzyme (data not shown). Analogously to what observed for the schaftoside, the results in Figure 1.3.2.5 pointed out that the addition of myricetin in the Lisosan® G extracts reduced the inhibitory activity of the molecule alone. In light with these findings, we can conclude that Lisosan® G contains unknown molecules that impede the interaction with the enzyme. These results agrees with recent evidence that confirmed the presence of different allosteric sites on the enzyme surface involved in the regulation of the enzyme activity (Hjortness *et al.,* 2018). Furthermore, it was possible to affirm that the cinnamic components alone were not responsible for such higher inhibitory activity; in fact from literature, ferulic acid in high concentration (100 μM) only exerted a weak inhibition (15%) on PTP1B (Adisakwattana *et al.,* 2013). Further analyses will be conducted to better define the inhibitory mechanism of schaftoside and the relationship with the applied dose.

a)
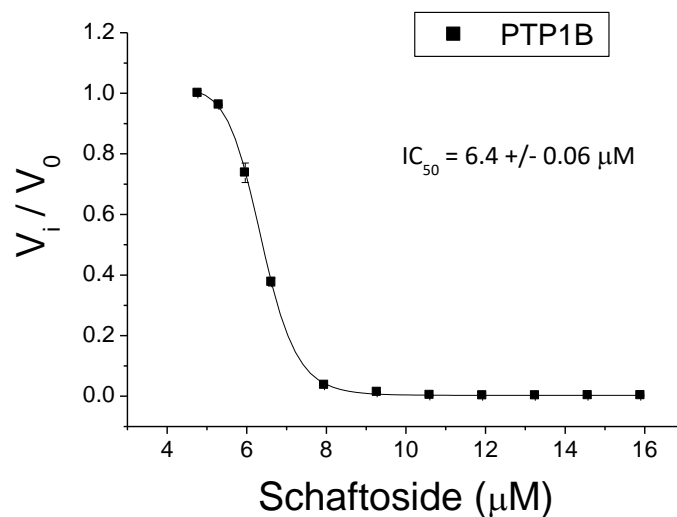
b)
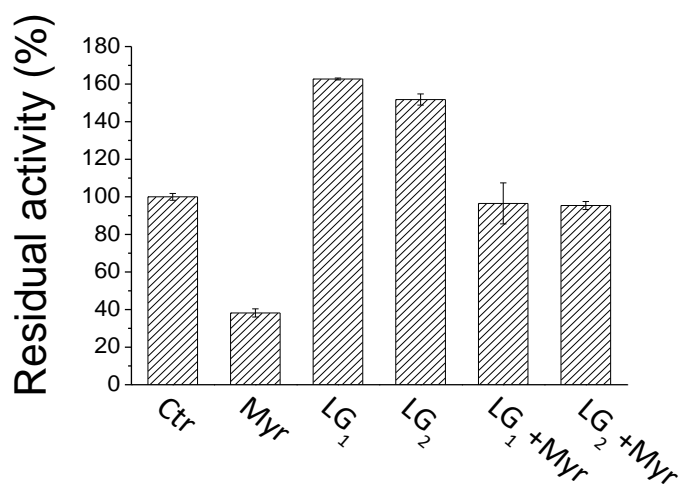
Figure 1.3.2.5 (**a**) IC_{50} of schaftoside. (**b**) Residual activity of PTP1B enzyme (in vitro %) of: Ctrl for control (see experimental section), myricetin standard (Myr), Lisosan® G extracts alone (LG$_1$–LG$_2$), and added with myricetin (LG$_1$+Myr; LG$_2$+Myr). The extracts were tested at 10 µL/mL corresponding to 0.5 µM for myricetin and 1.25 mg/mL for Lisosan® G extracts.

Microbial Composition of Lisosan® G

Bacteriological analysis firstly excluded the presence of microbial contaminants typical of food specimens, such as coliforms and staphylococci (Figure 1.3.2.6).
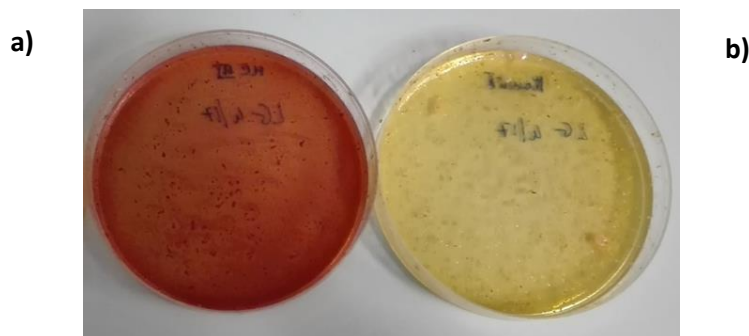
Figure 1.3.2.6 Lisosan® G (LG$_1$) was tested for presence of coliforms and staphylococci in (**a**) McConkey III agar medium and (**b**) mannitol salt agar medium, respectively. A total of 500 mg of Lisosan® G was plated. No Colony Forming Unit (CFU) were observed. In (B) the medium turned pink to yellow due to the low pH of the fermented product.

Since the microbial composition of Lisosan® G is unknown, quantification of specific bacterial species of interest by Real-Time PCR or q-PCR was not applied. *Next-generation sequencing* was chosen as suitable tool to perform the qualitative characterization of bacterial composition. The 16S rRNA gene sequencing was applied to identify the microbial composition of Lisosan® G (Table 1.3.2.3) with *Lactobacillus* (45.4%)*, Enterococcus* (28%), and *Pediococcus* (17%) as the main bacterial genera.

Phylum	Class	Order	Family	Genus	N. Reads	Amount (%)
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	47,344	45.4%
Firmicutes	Bacilli	Lactobacillales	*Enterococcaceae*	*Enterococcus*	29,195	28.0%
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Pediococcus*	17,711	17.0%
Firmicutes	Bacilli	Lactobacillales	Unclassified	Unclassified	3863	3.7%
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	Unclassified	3391	3.3%
Firmicutes	Bacilli	Lactobacillales	*Streptococcaceae*	*Lactococcus*	1218	1.2%
Unclassified		Unclassified	Unclassified	Unclassified	918	0.9%
				Others	637	0.6%
				Total	104,277	

Table 1.3.2.3 Microbial composition of Lisosan® G (LG$_1$). Number of sequenced reads and amount (%) of identified taxa were reported.

By BLAST alignment we found that the best hits for species identification (99% of identity) were *Lactobacillus fermentum*, *Enterococcus faecium*, and *Pediococcus acidiliacti*. Generally, numerous fermenting bacteria, especially lactic acid bacteria (LAB), have been identified in sourdoughs, including *Lactobacillus* and *Pediococcus spp*. In addition to acidification, the proteolytic activity of LAB allows release of compounds which can promote growth or inhibition and metabolic activities of other microorganisms, as well as organoleptic characteristics. *Enterococcus* genus includes known probiotic strains and was considered a good candidate for co-culture in food fermentation processes (Vuyst *et al.,* 2003).

Activity on Lactobacillus reuteri

Lactobacillus reuteri is a common component of the microbiota of human and animal intestine and it is widely used in probiotic formulations targeted to infants and adults to reduce the incidence and severity of diarrhea, prevent colics and necrotic enterocolitis, and maintain a functional mucosal barrier (Urbańska *et al.,* 2014). As shown in Figure 1.3.2.7, Lisosan® G was capable of stimulating the growth of *L. reuteri* DSM 17,938 by more than 1 Log (from 6.7 to 7.8) after 48 h of incubation; even if growth was significantly lower (*p* < 0.05) with respect to that on glucose. The ability to increase the growth of this

microorganism is certainly of interest for this fermented matrix characterized by the presence of probiotic strains and phenolic compounds potentially able to exert growth inhibition of harmful bacteria. The reason for the lack of difference in growth potential when Lisosan® G was tested at different concentrations (0.5 and 1%) may also be related to the copresence of different molecules and a broad spectrum of action of this complex product. The beneficial effects of Lisosan® G on human health have already been demonstrated, but prebiotic effects have not been described yet.
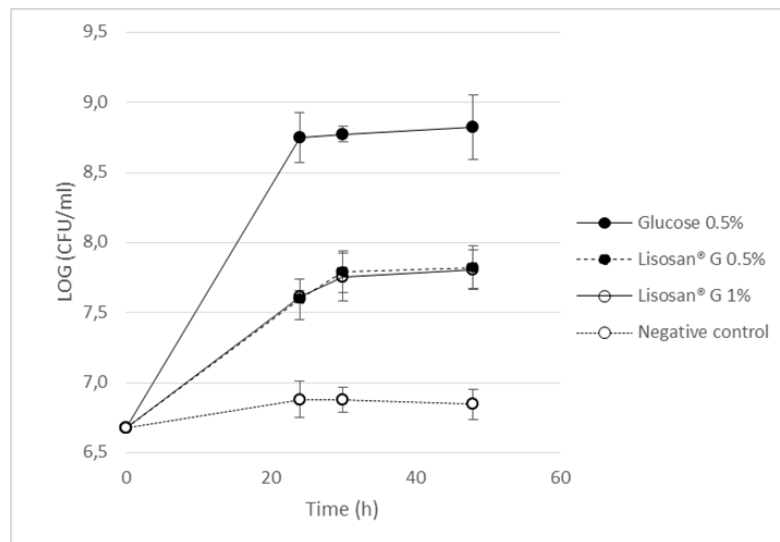
Figure 1.3.2.7 Ability of Lisosan® G (LG₁) tested at different concentration (0.5 and 1%) in stimulating the growth of *L.reuteri*.

1.3.2.4 Conclusions

This work improves knowledge on the composition and properties of this fermented grain, and it is the first report to focus on the content of free and bound phenolic compounds in Lisosan® G. Contrary to what reported in literature, the bound phenols remained high (more than 80%), although Lisosan® G was obtained after a fermentation process, usually described as able to increase hydrolytic processes and to strongly reduce the aliquot of bound phenols in cereals. Again, despite some data in the literature, the acidic hydrolysis was able to extract the highest amount of cinnamic derivatives, without degradation of the pool of *C*-glycosylated flavonoids. It was demonstrated that applying both soft basic and acidic hydrolysis on the whole flour it was possible to recover free and bound phenols through only one extractive step with higher yields compared to those obtained with the stronger basic hydrolysis suggested in the literature to date. Regarding the microbial characterization, it showed the presence of bacterial genera with fermentative capability such as *Lactobacillus*, *Pediococcus*, and *Enterococcus,* generally recognized as safe and used in the production of fermented food. The proteolytic activity of these bacteria can contribute to the release of compounds, such as phenols, growth of beneficial bacteria as *L. reuteri*, and inhibition of harmful bacteria. For the first time, a prebiotic effect on *L. reuteri* strain, widely used in probiotic formulation targeted to infants, was highlighted. This fermented wheat resulted not able to inhibit the PTP1B enzyme *in vitro*, however, the pure schaftoside, a main *C*-flavonoid of Lisosan® G, showed a strong inhibitory activity. Schaftoside was tested for the first time on PTP1B enzyme and was active as inhibitor at μM concentration (13.5 μM). Our findings open new perspectives to investigate on the role played by this *C*-glycosylated flavonoid and its analogous, typically present in wheat and in other cereals. Further studies are desirable to clarify the mechanism linked to the action of schaftoside and to test also other similar *C*-flavonoids on PTP1B enzyme. The

International Diabetes Federation announced that the global population of diabetics in 2015 was close to 400 million, and this number could rise to 600 million in 2040. In this context, research on new functional foods that can help stem the onset of this disease in the near future is recognized as strategic. Overall, our results provide further insights on the nutraceutical potential of this fermented food, whose beneficial effects were previously demonstrated by recent *in vivo* and *ex vivo* experiments.

Chapter 2

Pomegranate

2.1 Introduction

Pomegranate (*Punica granatum* L.) belongs to the *Punicaceae* family, is classified among the top seven fruits with the highest beneficial properties for human health (Sreekumar *et al.,* 2014; Pereira *et al.,* 2016). Its scientific name derives from the Latin *pomuni* (apple) *granatus* (grainy) meaning seeded apple. Pomegranate is native to central Asia, notably Iran, but since the tree is highly adaptive to a wide range of climates and soil conditions, it is grown in many different geographical regions including the Mediterranean, Asia, and California (Holland *et al.,* 2009). The pomegranate and its usage are deeply embedded in human history and its use, as food and medical remedy, has been found in many ancient human cultures. Despite this fact, cultivation of pomegranate has always been restricted and it has generally been considered a minor crop. The pomegranate tree requires a long, hot and dry season in order to produce good yield of high-quality fruit (Hussein *et al.,* 2018). Pomegranate fruit is a fleshy berry (6.25 to 12.5 cm wide with a weight range of 200-650 g) with varying color from reddish yellow to green with reddish zones. Its skin covers white, spongy membranes that form a number of cells, each packed full of angular seeds contained in a juicy pulp sac called arils having bittersweet flavor and color ranging from intense ruby to white (Hussein *et al.,* 2018). Differences are established according to potential market and consumer preference, taking into account important parameters such as fruit size, husk and aril colors, seed hardness, maturity, juice content, acidity, sweetness and astringency. The most common pomegranate variants and regions where they are recognized are: "Wonderful" throughout Europe, "Molar de Elche/Valenciana" in Spain,

"Ahmar/Aswad/Halva" in Iraq, "Mangulati" in Saudi Arabia, "Red Loufani/Ras el Bahgl" in Israel and Palestine, and "Apaseo/Apaseo tardía" in Mexico (Hussein *et al.,* 2018).

Figure 2.1.1 Pomegranate varieties

Nowadays scientific interest in the chemical characterization of pomegranate fruits is focused not only on the edible parts (arils), but also on the inedible ones such as the peel. Pomegranate peel represents 40-50% of the whole fresh fruit weight and is the main by-product obtained after juice extraction. The peel contains high percentages of water (around 70–75%) and high organic matter contents (around 96% of total solids), but it is also a good source of phenolic compounds (10–20%), polysaccharides (10–15%) and sugars (30–35%) (Pereira *et al.,* 2016). Under European regulations these characteristics mean that pomegranate waste should not be disposed in landfills as it presents a significant risk to local watercourses and leads to uncontrolled greenhouse gas production The use of such residues for production of multiple value-added products will promote innovation in agro waste bio-refineries and serve the agro-processing sector by making it more resource efficient and sustainable, enhancing agribusiness opportunities and supporting rural livelihoods (Talekar *et al.,* 2018; Zhai *et al.,* 2018).

2.1.1 Phenolic composition

Pomegranate fruits are a good source of phenolic compounds such as phenolic acids (hydroxycinnamic and hydroxybenzoic acids), hydrolysable tannins (ellagitannins, gallotannins) and flavonoids, all present both in the arils and peel at different percentages (Singh *et al.,* 2018). The major phenolic compounds identified in pomegranate are gallic acid (**a**), caffeic acid (**b**), chlorogenic acid (**c**), ellagic acid (**d**) (phenolic acids), apigenin (**e**), quercetin (**f**), pelargonidin (**g**), cyanidin (**h**) (flavonoids), punicalin (**i**), punicalagin (**j**), granatin A (**k**) and granatin B (**l**) (ellagitannins) (Figure 2.1.2).
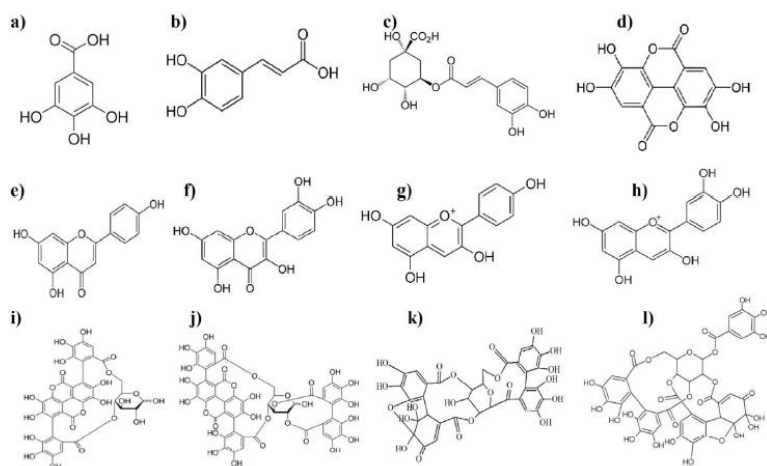
Figure 2.1.2 Chemical structures of the major phenolic compounds identified in pomegranate peel: gallic acid (**a**), caffeic acid (**b**), chlorogenic acid (**c**), ellagic acid (**d**), apigenin (**e**), quercetin (**f**), pelargonidin (**g**), cyanidin (**h**), punicalin (**i**), punicalagin (**j**), granatin A (**k**) and granatin B (**l**).

Phenolic composition usually varies among pomegranate cultivars depending on environmental conditions, for example geographic location, fruit stage of maturity, and peel and aril color. For this reason, there is a wide range in terms of phenolic content among different pomegranate varieties, both from a qualitative and quantitative point of view (Table 2.2.1).

Table 2.1.1 Total phenolic, flavonoid, anthocyanin and hydrolysable tannins reported in pomegranate peel from different regions (Singh *et al.,* 2018)

Table 1
Total phenolic, flavonoid, anthocyanin and hydrolysable tannins reported in PoP from different regions.

Source	Extract	Phenolic content	Flavonoid content	Anthocyanin content	Hydrolysable tannins content	Main identified compounds	References
Tunisian PoPx (4 ecotypes)	ACE	157.06–304.60 mg GAE/g	10.28–15.46 mg QE/g	6.84–54.51 mg C3G/100 g	144.96–292.23 mg GAE/g	Ellagitannins (punicalagin and punicalagin derivatives), cyanidin and pelargonidin derivatives	Abid et al. (2017)
Saudi Arabian PoPx	WME	262.5 mg GAE/g	–	–	–	–	Al-Zoreky (2009)
Iranian PoPx (9 cultivars)	ME	98.24–250.13 mg GAE/g	18.61–36.40 mg CE/g	–	–	–	Ardekani et al. (2011)
Indian PoPx (Ruby variety)	ME	841.5 mg GAE/g	–	–	–	Gallic, ellagic, chlorogenic, caffeic, syringic, p-coumaric and cinnamic acids, catechol, quercetin, kaempferol and apigenin	Arun et al. (2017)
Egyptian PoP powder	ME	10.96 mg GAE/g	–	–	–	–	Ashoush et al. (2013)
Turkish PoPx	PWE	264.3 mg TAE/g	13.0 mg CE/g		262.7 mg TAE/g	Gallic and ellagic acids, punicalagin A, punicalagin B	Çam and Hışıl (2010)
Turkish PoPx	ME	331.28 mg GAE/g	–	–	–	Ellagic, gallic, p-hydroxybenzoic, caffeic, chlorogenic, p-coumaric and ferulic acids	Dikmen et al. (2011)
Iranian PoPx	ME	147.2 mg GAE/g	1.17%	–	–	–	Doostan et al. (2017)
Tunisian PoPx (6 ecotypes)	ME	84.89–109.7 mg GAE/g	44.83–56.46 mg RE/g	–	111.23–140.55 mg TAE/g	–	Elfalleh et al. (2009)
Tunisian PoPx (6 ecotypes)	ME	–	–	63.76–84.31 mg C3G/g	111.23–140.55 mg TAE/g	Ellagic, gallic, caffeic and p-coumaric acids	Elfalleh et al. (2011)
Tunisian PoPx	AE	53.65 mg GAE/g DW	21.03 mg RE/g	51.02 mg C3G/g	62.71 mg TAE/g	–	Elfalleh et al. (2012)
	ME	85.60 mg GAE/g DW	51.52 mg RE/g	102.20 mg C3G/g	139.63 mg TAE/g		
Tunisian PoPx	ME	85.60 mg GAE/g DW	51.52 mg RE/g DW	102.2 mg C3G/g DW	139.63 mg TAE/g DW		Elfalleh et al. (2012)
South African PoPx (seven cultivars)	ME	179.3–295.5 mg GAE/g DW	97.8–121.1 mg CE/g DW	58.5–322.2 µg C3G/g DW	326.0–783.6 µg GAE/g DW	Ellagic, gallic and p-hydroxybenzoic acids, delphinidin and cyanidin 3,5-diglucosides, catechin, epicatechin and rutin	Fawole et al. (2012)
Peruvian PoPx	ME	101.8 g GAE/kg DW	–	447.1 mg/kg DW	44 g/kg DW	Phenolic acids, anthocyanins, gallotannins, ellagitannins, gallagyl esters and dihydroflavonol	Fischer et al. (2011)
Algerian PoPx	AE	4.41 mg TAE/ml	–	–	–	–	Gharzouli et al. (1999)
Turkish PoPx (Four cultivars)	AE	1775.4–3547.8 mg GAE/L	–	–	–	–	Gözlekçi et al. (2011)
Indian PoPx	AE	161.25 mg CE/g DW	7.57 mg CE/g DW	–	–	–	Kanatt et al. (2010)
Pakistan PoPx (Four varieties)	ME	255.35–289.40 mg GAE/g	50.86–58.63 mg RE/g			Punicalagin	Khalil et al. (2017)
Taiwan PoPx	AE	471.0 µg GAE/mg	257.0 µg CE/mg	–	–	Punicalagin , punicalin, strictinin A, and granatin B	Lee et al. (2010)
Chinese PoPx	MEAW	249.4 mg TAE/g	59.1 mg RE/g	–	–	–	Li et al. (2006)
Indian PoPx (Ganesh variety)	AE	435 mg TAE/g	–	–	–	–	Malviya, Jha, and Hettiarachchy (2014)
	WEE	297.5 mg TAE/g					
Iranian PoPx (21 accessions)	ME	82.0–230.4 mg GAE/g	–	–	–	Gallic, ellagic, caffeic, p-coumaric and vanillic acids, quercetin	Mansour et al. (2013)
Italian PoPx	EAE	3.750 mmol GAE/g	0.881 mmol RE/g	32.68 µmol C3G/g	–	Punicalagin and Ellagic acid	Masci et al (2016)
	AE	1.227 mmol GAE/g	0.387 mmol RE/g	41.36 µmol C3G/g			
	EE	1.503 mmol GAE/g	0.471 mmol RE/g	28.45 µmol C3G/g			
Egyptian PoPx	ME	124.34 mg GAE/g	59.44 mg QE/g	–	–	–	Moneim (2012)
Brazilian PoPx (cv. Wonderful)	WME	21.25 mg GAE/g	7.60 mg CE/g	–	–	Punicalagin	Morzelle et al. (2016)
Thailand PoPx	HWE	166.83 mg GAE/100 g	–	–	–	–	Nuamsetti et al. (2012)
	EE	152.65 mg GAE/100 g					
	AE	85.48 mg GAE/100 g					
Turkish PoPx (4 varieties)	AE	126.11–157.93 µg GAE/mg	9.44–13.25 µg QE/mg	5.89–22.03 µg C3G/mg	86.92–129.41 µg TAE/mg	–	Orak et al. (2012)
	ME	168.04–212.48 µg GAE/mg	14.37–20.52 µg QE/mg	7.45–29.35 µg C3G/mg	124.10–183.18 µg TAE/mg		
	EE	132.31–160.82 µg GAE/mg	12.83–17.27 µg QE/mg	7.93–19.54 µg C3G/mg	120.97–149.10 µg TAE/mg		
Israelian PoPx	AE	1.25 mg QE/l	–	–	–	Gallagic, ellagic and gallic acids punicalagin	Orgil et al. (2014)
Italian PoPx	EE	3019 mg GAE/l	–	–	–	Gallagic and ellagic acid derivatives: ellagic acid hexose, ellagic acid pentose, ellagic acid deoxyhexose, punicalin, pedunculagin	Pagliarulo et al. (2016)

Despite pomegranate consumption is mainly in the form of fresh fruits or juices, several studies reported in literature demonstrated that the highest amount of phenolic compounds is actually present in pomegranate peel (mesocarp+exocarp) (Fischer *et al.,* 2011). Indeed, pomegranate peel possesses significant free radical scavenging, anti-microbial, antiatherogenic and antimutagenic properties and it is reported to produce ameliorating effects against many critical maladies. Unfortunately, functional foods containing pomegranate peel are not in general well accepted by consumers because of their relatively reduced sensory features (Akhtar *et al.,* 2015). Considering pomegranate phenolic compounds as beneficial properties on human health, it is necessary to underline that only 5-10% of the total ingested phenols get absorbed in the small intestine. Among these, phenolic acids are maximally absorbed in the small intestine, while ellagitannins and flavonoids are reduced into smaller molecules or metabolized by gut microbiota (Santhakumar *et al.,* 2018). Urolithins from ellagitannins and ellagic acid are considered the more interesting pomegranate metabolites produced by gut microbiota (Tom *et al.,* 2017).
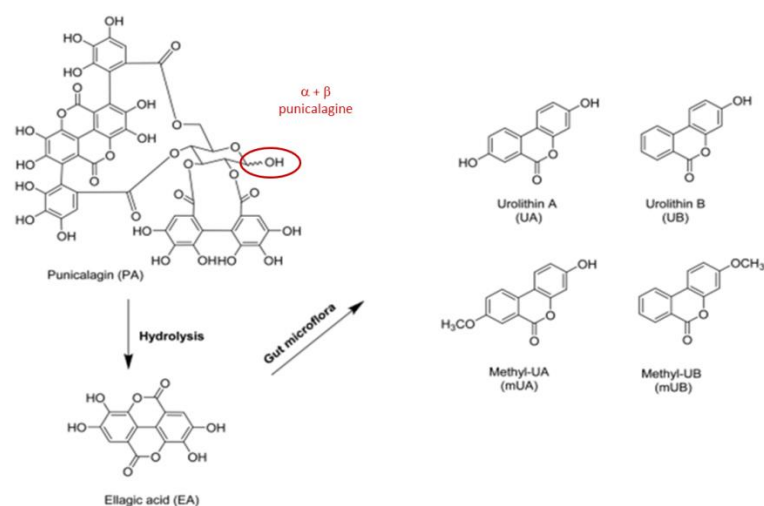
Figure 2.1.3 Punicalagin metabolism

Recently, human subjects have been categorized into three urolithins phenotypes or metabotypes, depending on the qualitative and quantitative proportions of urolithins produced: metabotype A, B and 0. This inter-individual variability, associated with differences in intestinal microbiota, might contribute to the large individual variation observed in the health benefits associated to urolithins (Tom *et al.,* 2017). Urolithins' ability to inhibit cancer cell proliferation on a range of human cell lines representing colon, kidney and liver models has been highlighted (Varghese *et al.,* 2017; Núñez-Sánchez *et al.,* 2016). Significantly higher anti-proliferative cancer activities (about 10 fold) against breast (MCF-7) and prostate (LNCaP) cancer cell lines were observed in peel of fifteen Israeli pomegranate varieties in comparison to the edible tissues of fruit as a result of higher levels of ellagitannins, punicalagin, ellagic and gallic acids (Orgil *et al.,* 2014). Furthermore, a mixture of ellagitannins and polysaccharides in the form of a decoction demonstrated the ability to counteract initial, intermediate and late stages of colon carcinogenesis in rats highlighting a synergic effect of both main pomegranate classes of molecules, suggesting a possible use of the decoction in primary and secondary prevention of human colon cancer (Tortora *et al.,* 2018). In addition, urolithins were demonstrated to be valid antinflammatory agents, reducing the key molecules involved in the regulation of inflammation (Tom *et al.,* 2017). The capability of pomegranate phenols and metabolites in reducing the oxidative stress that induced degenerative diseases such as Alzheimer's dementia has also been recently pointed out. Punicalagin was demonstrated *in vitro* and *in vivo* to reduce the oxidative state and concentration of oxidized homocysteine and advanced glycation end-products recognized as risk factors for Alzheimer's disease. Oral administration of punicalagins in mice also inhibits NF-kB activation and neuroinflammatory response in the lipopolysaccharide-induced brain (Kim *et al.,* 2017). Pomegranate powder and extract have enormous medical potential: they have been

observed to be effective against diabetes. The crude methanol extract of pomegranate peel demonstrated promising antidiabetic activity which might be related to α-glucosidase inhibition and an enhanced uptake of glucose. Oral administration in normal and diabetic rats reduced the concentration of glucose, triglycerides, cholesterol, LDL cholesterol, and very low density lipoprotein (VLDL) cholesterol and raised high density lipoprotein (HDL) cholesterol and haemoglobin levels (Radhika *et al.,* 2011). Moreover, it has been demonstrated that pomegranate powder might be used as skin repairing and whitening agent as it inhibited UV-induced skin pigmentation in brownish guinea pigs by inhibiting the proliferation of melanocytes (Hayouni *et al.,* 2011). Pomegranate powder is also a good source of antimicrobial compounds against *Streptococcus mutans*, *S. sanguis*, *S. mitis* and yeast (*Candida albicans*) commonly found in oral infections (Vasconcelos *et al.,* 2006). The health benefits of pomegranate's phenolic compounds are summarized in Table 2.1.2

Beneficial effects	References
Inhibits Skin Tumorigenesis	Afaq, Saleem, Krueger, Reed, and Mukhtar (2005)
Memory enhancing effect	Adiga et al. (2010)
Anti-cestodal activity	Al-Megrin (2016)
prevention and treatment of giardiasis	Al-Megrin (2017)
Antimicrobial activity	Al-Zoreky (2009)
Beneficial for treatment of glaucoma and diuresis	Satomi et al. (1993)
Manage type 2 diabetes and associated complications	Arun et al. (2017)
Hepatoprotective effects	Ashoush et al. (2013), Chidambara Murthy et al. (2002), Shishavan et al. (2017)
Wound healing potential	Hayouni et al. (2011), Chidambara Murthy, Reddy, Veigas, and Murthy (2004)
Promising drug candidate to treat prostate cancer	Deng et al. (2017)
Antiproliferative and apoptotoic effects on breast cancer cells	Dikmen et al. (2011)
Anti-inflammatory activity	Lee et al. (2010), Hollebeeck et al. (2012)
Antifungal activity against dermatophytes	Foss et al. (2014), Stojanović et al. (2017)
Protective against stomach injury and ulcer	Gharzouli et al. (1999)
Inhibits tumor proliferation in cervical cancer	Guo et al. (2016)
Suppressive effects on human breast cancer cells	Kim et al. (2002)
Blocks thyroid cancer growth and metastasis	Li et al. (2016)
Prevent age-related hearing loss	Liu et al. (2017)
Anti-tumor effects on human prostate cancer cells	Ma et al. (2015)
Prevents oral candidiasis	Madugula et al. (2017), César de Souza Vasconcelos, Sampaio, Sampaio, and Higino (2003)
Antiproliferative activity	Masci et al. (2016), Orgil et al. (2014), Shirode et al. (2014)
Antiulcerogenic effects	Moghaddam et al. (2013)
Chemopreventive activities against breast and colon cancer	Moreira et al. (2017)
Antimutagenic properties	Negi and Jayaprakasha (2003), Zahin et al. (2010)
Antiinflammatory and antiallergic activity	Panichayupakaranant et al. (2010)
Antidiabetic potential	Parmar and Kar (2007)
Antidiabetic and hypolipidemic activity	Radhika et al. (2011)
Antidiarrheal activity	Qnais et al. (2007)
Beneficial in treating knee osteoarthritis	Rafraf et al. (2016)
Immunomodulatory activities	Ross et al. (2001), Stojanović et al. (2017)
Antidiabetic and hypolipedimic activity	Salwe et al. (2015)
Inhibits cancer cell proliferation and induces apoptosis	Song et al. (2016)
Prevents autoimmune disorders	Stojanović et al. (2017)
Prevents liver fibrosis	Toklu et al. (2007)
Effective skin whitening agent	Yoshimura et al. (2005)

Table 2.1.2 Health properties of pomegranate peel (Singh *et al.,* 2018).

2.1.2 Polysaccharides composition

Pomegranate peel is a good source of polysaccharides, mainly pectin, with amounts ranging from 10% to 12% of fruit dry weight ([b]Khatib *et al.,* 2017). Knowledge about the composition and structure of polysaccharides of pomegranate fruits is limited to date, despite the relevance and economic importance of this raw material; most of the published articles are focused on phenolic compound distribution and their healthy properties (Shakhmatov *et al.,* 2019). Pectin polysaccharides are vital structural components of plant cell walls, associated with other polysaccharides such as cellulose and hemicellulose (Dranca *et al.,* 2018). Pectin is mainly present in the primary cell wall and in the middle lamella of plants and is one of the gelling agents added to food products to achieve desired texture or consistency, particularly in the manufacturing of jam and jelly. As hydrocolloid molecules, they are widely used in food, cosmetic and pharmaceutical industries (Talekar *et al.,* 2018). The diverse structural and macromolecular properties of pectins, their composition of neutral sugars and molecular weight are strictly dependent on their source. The chain structure of pectins mainly consists of α-(1-4)-D-galacturonic acid units forming long homogalacturonic chains interspersed by rhamnogalacturonan sections, where rhamnose and galacturonic acid residues alternate. Neutral sugar units are attached to the backbone and concentrated in highly branched ''hairy'' regions (Figure 2.1.3). Galacturonic acid comprises approximately 70% of pectin, and all the pectic polysaccharides can be divided into four main structures: homogalacturonan with a linear chain of 25-100 units of D-galacturonic acid linked with α(1→ 4) bonds; xylogalacturonan, a homogalacturonan substituted at *O*-3 with xylose (the 3-linked xylose has occasionally been found to be further substituted at *O*-4 with an additional linked xylose); rhamnogalacturonan I, containing a backbone of the disaccharide repeat [-α-*D*-GalA-1,2-α-L-Rha-1-4-]*n* with 20 and 80% of side chains, in the rhamnosyl residues, containing

individual, linear, or branched α-L-Araf and β-D-Galp residues; rhamnogalacturonan II, with an HG backbone of at least eight (and most probably more) 1,4-linked α-D-GalA residues decorated with side branches (α–d) consisting of 12 different types of sugars in over 20 different linkages (Mohnen *et al.,* 2008).
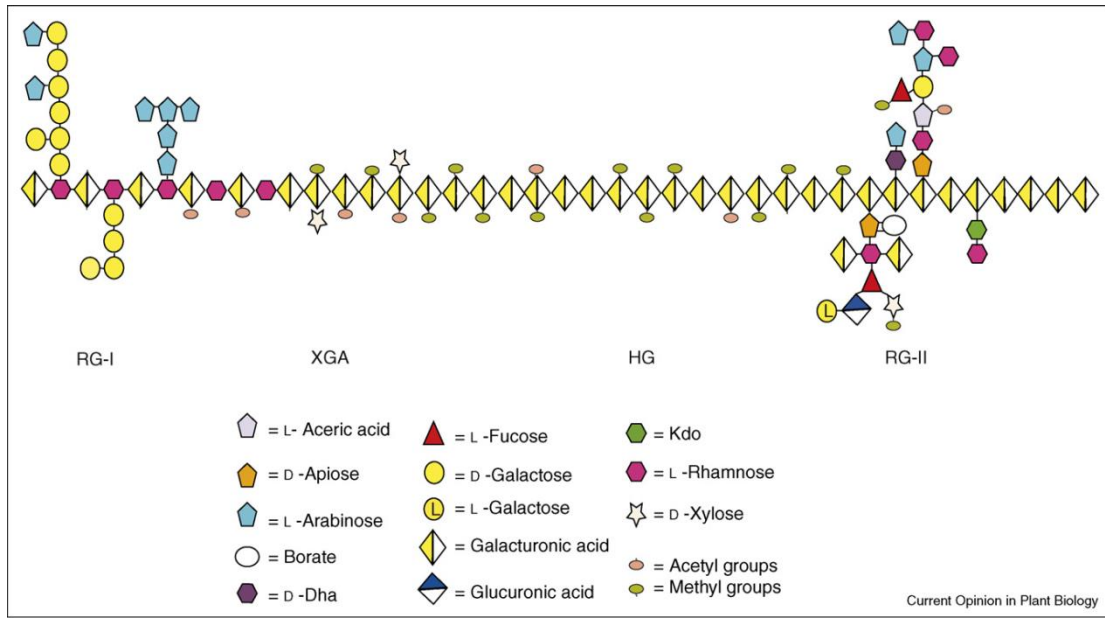
Figure 2.1.4 Schematic structure of pectin showing the four pectic polysaccharides homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) linked to each other (Mohnen, 2008).

Part of the carboxylic groups in the galacturonic chain are present in methyl ester form. The degree of methylation (DM) divides pectin into the high-methoxyl (HM) form, in which more than 50% of the carboxyl groups are methylated, and the low-methoxyl (LM), in which less than 50% are methylated. The degree of methylation is crucial for the gel formation of pectin. Low methoxyl (LM) pectins are often used in low-sugar products due to their gel-forming properties without or with a small amount of sugar and in the presence of Ca^{2+}. Esterification of carboxyl groups can also influence pectin solubility in water, which decreases with the increase of esterification degree (Abid *et al.,* 2017). Some

outstanding therapeutic effects of pectins have recently been reported, such as their anti-obesity characters through the entrapment of food components and/or catalytic enzymes (Samout *et al.,* 2016), anti-apoptotic activity in kidney toxicity induced by octylphenol (Koriem *et al.,* 2014), reductant ability in cholesterol accumulation by enhancing bile acids synthesis in the liver (Zhu *et al.,* 2017), and improvement of *Bifidobacterium* growth in the human colon resulting in higher digestion and lower inflammation (Olano-Martin *et al.,* 2002). Furthermore, a polysaccharide from pomegranate was reported as exerting an anti-tumor and hepatoprotective effect (Varghese *et al.,* 2017; Zhai *et al.,* 2018).

Pectin from natural sources	
Beneficial effects	**References**
Improvement in Bifidobacterium growth	Olano-Martin et al., 2002
Anti-apoptotic in kidney toxicity	Koriem et al., 2014
Anti-obesity	Samout et al., 2016
Reductant ability in cholesterol accumulation	Zhu et al., 2017
Pectin and polysaccharides from pomegranate	
Beneficial effects	**References**
Antiglycation and tyrosinase inhibition	Rout et al., 2007
Prebiotic	Marotti et al., 2012
Antioxidant effects	Joseph et al., 2013
Prebiotic	Di Gioia et al., 2014
Prebiotic	Khatib et al., 2017
Anti tumor	Varghese et al., 2017
Immunomodulatory	Gavlighi et al., 2018
Antiinflamamtory	Gavlighi et al., 2018
Hepatoprotection	Zhai et al., 2018
Reduced chemotherapy-induced immunosuppresion	Wu et al., 2019

Table 2.1.3 Beneficial properties of pectin from natural sources and from pomegranate.

Pomegranate pectins are gaining scientific interest thanks to their antioxidant effects (Joseph *et al.,* 2013), antiglycation and tyrosinase inhibition properties (Rout *et al.,* 2007), immunomodulatory and anti-inflammatory effects (Gavlighi *et al.,* 2018). It has recently been demonstrated that pomegranate polysaccharides can be used to lessen chemotherapy-induced immunosuppression and as immunostimulants in food and pharmaceutical industries (Wu *et al.,* 2019). Furthermore, pectin, as part of soluble fermentable dietary fiber, can exhibit prebiotic activity by stimulating the growth of beneficial bacteria in the colon and contributing to the healthy status of the gut (Di Gioia *et al.,* 2014; Marotti *et al.,* 2012). In a recent work, developed by [b]Khatib *et al*., (2017) pomegranate polysaccharides were tested to verify the ability of *Bifidobacteria* and *Lactobacilli* to use them as carbon source compared to their growth on glucose. The study revealed that both the bacterial lineages grew well on pomegranate polysaccharides with results comparable to their growing on glucose (Table 2.1.3). In light of these interesting results, pomegranate could be considered an inexpensive potential source of pectins and a valid alternative to commercial pectin which are usually extracted from apple and citrus (Grassino *et al.,* 2018). Only few studies have investigated pomegranate pectin structure and composition in depth, highlighting differences among cultivars or with respect to other vegetable sources (Shakhmatov *et al.,* 2019). Looking to the future, a better characterization of pomegranate peel in terms of its polysaccharides structures, could be useful to add value to a by product that has already become a resource.

2.2 Results

2.2.1 Purple Queen® fruits of *Punica granatum* L.: a relation between reclaimed sediments and nutraceutical properties.

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Abstract

Purple Queen® is an early ripening pomegranate cultivar growing well in soilless cultivation and appreciated for its nutraceutical properties. Plant substrates have direct effects on plant development and, due to progressive peatland exhaustion, the request for new materials suitable for plant growth and production is high. The objective of this work was to verify the effects of a new potential substrate obtained from the remediation of marine port sediments on the nutraceutical profile of Purple Queen® fruits, using 50% and 100% of reclaimed sediment. The study was carried out determining ellagitannins and polysaccharides obtained from peel after decoction and anthocyanins in aril juice. The phenolic and polysaccharides compositions were evaluated. Compared to a peat-based commercial substrate (control), the remediated port sediment preserved ellagitannin content and improved the nutraceutical profile, increasing the anthocyanin amount (up to 40% higher) and polysaccharide content up to 340 mg/g on dry fruit.
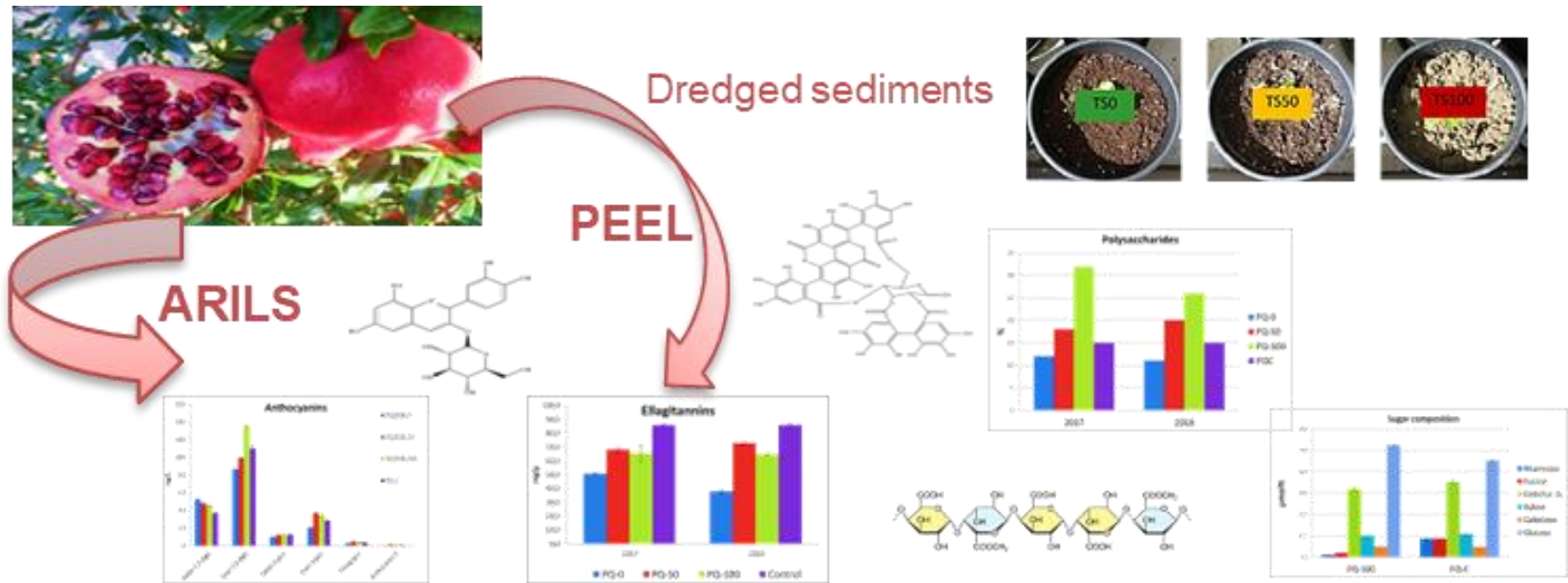
2.2.1.1 State of the art and aims of the work

Purple Queen$^®$ is a pomegranate cultivar greatly appreciated for its productivity and early ripening (second half of August in Alicante and Murcia, Spain). These peculiarities are notably functional for economic and marketing strategies since they broaden the availability of pomegranate fruits for both fresh consumption and processing. Registration has been requested for this *cultivar* in key countries outside the European Union, especially in the southern hemisphere to respond to the demand for pomegranate fruit, which has strongly increased over recent years. A characteristic of Purple Queen$^®$ is its suitability for growth also in soilless systems (i.e. in pots).

Peat is one of the main components of substrates used in agriculture. However, due to the progressive exhaustion of peatland, the demand for alternative and innovative substrates with suitable physico-chemical properties is increasing. To meet sustainability criteria in the plant nursery industry, attention is currently focused on the reutilization of waste-derived substrates. Among these, dredged remediated sediments have already been proposed as soilless growing media for the cultivation of ornamental and food crops (Tozzi *et al.,* 2019; Mattei *et al.,* 2017).

Dredging, normally performed to guarantee river and harbor navigability, involves the excavation of sediment from the bottom of water basins and its reallocation in another place (DelValls *et al.,* 2004). This process generates a huge volume of dredged spoils which must be appropriately managed following national regulations. Unpolluted sediments are generally re-used for beach nourishment and embankments while contaminated sediments, being classified as waste, are placed in landfill. Phytoremediation has proven to be a sustainable technology for reclaiming highly-polluted sediments (Bert *et al.,* 2009) and to increase their biological activities, converting sediment into a "techno-soil" able to support vegetation (Masciandaro *et al.,* 2014; Doni *et al.,* 2015). Hence, the

potential reallocation of remediated dredged materials in agriculture could provide an alternative solution to the disposal in landfills and, at the same time, reduce the intensive use of raw material as in the case of the requested peat. As reported by Mattei *et al.,* (2018), the cultivation of an ornamental crop in remediated sediments showed a significantly lower environmental impact with respect to the use of traditional peat. Recently, dredged sediments were used as growing media for soilless cultivation of Purple Queen[®]; plant productivity and the main nutritional characteristic of the juice were evaluated (Melgarejo *et al.,* 2019). The authors observed that fruit yield was partially reduced due to the high sediment bulk density which limited overall plant development. At the same time, fruits cultivated on sediment showed a significant increase of soluble solids, fructose and glucose content in the arils, indicating that the presence of sediment promoted greater dry matter accumulation in this tissue. The present study was focused on the characterization and quantification of the bioactive components of whole fruits (aryls and peel) obtained from Purple Queen[®] trees cultivated as described by Melgarejo *et al.,* (2019) for two agronomic seasons, partially or totally substituting the peat with dredged sediments. Evaluation of the nutraceutical quality of the fruit was carried out determining a pool of components, namely anthocyanins in juice as well as polysaccharides and ellagitannins in peel. To the best of our knowledge, this is the first study investigating the effect of innovative substrates on the main nutritional components of the arils and peel from pomegranate fruits obtained in soilless cultivation.

2.2.1.2 Materials and methods

Experimental design and fruit sampling

Purple Queen® plants were cultivated in a remediated dredged sediment as described by Melgarejo *et al.* (2019). Briefly, the sediments were dredged from the Livorno, Italy, port and were subjected to three years of phytoremediation as described by Masciandaro *et al.* (2014). Afterwards, the sediment underwent three months of landfarming, a bioremediation consisting in periodic aeration and irrigation of the sediment in order to homogenize the matrix. The remediated sediment was used to prepare two growing substrates: PQ-100 (PQ2017-100 and PQ2018-100 samples), the remediated sediment alone, and PQ-50 (PQ2017-50 and PQ2018-50 samples) derived from the remediated sediment mixed with a traditional peat-based commercial substrate (1:1, v/v). The same peat-based commercial substrate present in PQ-0 was used as control treatment (PQ2017-0 and PQ2018-0 samples). Moreover, a sample of Purple Queen® fruits purchased in local markets in 2018 were also analyzed (PQ-C) as further control sample. The main physico-chemical parameters of the substrates are presented in Table 2.2.1.1.

Parameters	0	50	100
pH	6,2	7.9 (±0.2)	8.21 (±0.3)
EC (µs cm^{-1})	1129 (±7)	596 (±14)	352 (±16)
NH$_3$ (mg l^{-1})	1.03 (±0.19)	0.25 (±0.03)	0.08 (±0.03)
NO$_3^-$ (mg l^{-1})	304 (±1)	190 (±18)	26 (±0.1)
TN (%)	1.3 (±0.2)	0.29 (±0.01)	0.12 (0.02)
PO$_4$ (mg l^{-1})	16.8 (±0.2)	0.4 (±0.0)	0.4 (±0.0)
Chloride (mg kg^{-1})	20.5 (±0.2)	12.4 (±2.1)	11.4 (±0.5)
K (mg kg^{-1})	65 (±5)	19 (±0.5)	9 (±0.1)
Mg (mg kg^{-1})	22.1 (±0.3)	11.4 (±0.2)	8.4 (±1.5)
Ca (mg kg^{-1})	114 (±1)	67 (±2)	31 (±4)
Cu (mg kg^{-1})	14.1 (±0.3)	43.7 (±0.9)	55.4 (±1.2)
Zn (mg kg^{-1})	13 (±1)	170 (±8)	194 (±7)
Ni (mg kg^{-1})	0.2 (±0.0)	35 (±2)	39 (±2)
Pb (mg kg^{-1})	1.4 (±0.0)	34 (±3)	38 (±6)

Table 2.2.1.1 Main chemical parameters of the commercial peat substrate (0), mixture sediments: peat v/v 1:1 (50), and remediated sediment used as pure (100). The values are the mean and standard deviation (n=3).

The fruit samples used in this experiment were harvested at full ripening at the end of two growing seasons (2017 and 2018). All plants (PQ-0, PQ-50 and PQ-100) received a complete Hoagland nutrient solution, composed of KNO$_3$, NH$_4$NO$_3$, K$_2$SO$_4$, HNO$_3$, H$_3$PO$_4$, and a complex mix of microelements (Melgarejo *et al.,* 2019). The arils and peel (mesocarp+esocarp) used for the analyses are presented in Table 2.2.1.2.

Samples	Year	Fruit total weight (g)	Aril total weight (g)	Peel total weight (g)
PQ-0	2017	302.6 (±37)	139.1 (±45.8)	163.5 (±23)
	2018	293.5 (±27)	125.2 (±42.6)	168.3 (±17)
PQ-50	2017	250.4 (±29)	108.5 (±43.3)	141.9 (±24)
	2018	264.6 (±35)	112.3 (±42.44)	152.3 (±19)
PQ-100	2017	207.3 (±31)	86.8 (±41.9)	120.5 (±23)
	2018	216.7 (±25)	88.1 (±40.7)	128.6 (±16)
PQ-C	2018	303.1 (±39)	132.8 (±43.8)	170.3 (±27)

Table 2.2.1.2 Weight of the different tissues of fruits from Purple Queen® grown on different substrates. Values are the mean of 10 fruits (n=10) and standard deviation (in brackets).

Standards and reagents

All solvents were of analytical HPLC grade from Sigma Aldrich (St. Louis, Missouri, USA). Ultrapure water was obtained by the Milli-Q-system (Millipore SA, Molsheim, France). Ellagic acid (purity ≥ 95%) and punicalagin (purity≥ 98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oenin chloride (purity ≥ 95%) was purchased from Extrasynthese (Genay, France). Dextrans at different molecular weights (2000, 1100, 410, 150, and 50 kDa) and sucrose (360 Da) used for SEC analyses were from Sigma-Aldrich, USA. The ellagitannins were quantified according to their maximum absorption at either 380 nm using a five-point calibration curve of a racemic mixture of α- and β-punicalagins (purity ≥ 99%, linearity range 2 - 5 µg, R^2=1.000) or 370 nm using a five-point calibration curve of ellagic acid (purity 95%) (linearity range 0-1.7 µg, R^2=1.000). The anthocyanins were quantified at 520 nm with a four-point calibration curve of oenin chloride (purity ≥ 95%; linearity range 0-2.6 µg, R^2=0.999).

Anthocyanins from arils

Arils from PQ2018 (0; 50; 100) and Purple Queen® commercial sample (PQ-C) were used for the preparation of juices using a domestic Hurom extractor which guarantees the preparation of juices through a rapid process at low temperatures. The arils derived from fruits collected from the different soils were divided into three aliquots and each treated separately to obtain a triplicate. Each juice was diluted (1:1 v/v) with ethanol (2% HCOOH) to better stabilize the solution before analysis. The samples were then centrifuged at 14000 rpm for 5 min. The supernatant was recovered and analyzed by HPLC-DAD-MS.

Ellagitannins and polysaccharides from peel

The peel and arils from fruits (n = 9) were manually separated, weighted and freeze-dried until constant weight was reached. The freeze-dried peel (5 g) was boiled in 200 mL of ultrapure water, for 1 h as per [b]Khatib *et al*., (2017). The supernatant was recovered after cooling and centrifugation (4500 rpm, 8 min, 4 °C) and taken to a final volume of 200 mL with ultrapure water. Ten mL were used for the analysis of ellagitannins. The remaining amount of supernatant was treated with 300 mL of ethanol and kept at 0° C to induce polysaccharides precipitation. After centrifugation (4500 rpm, 8 min, 4 °C) the recovered polysaccharides were freeze-dried and weighed to calculate the yield.

HPLC-DAD analysis of phenolic extracts

The ellagitannins in peel and juice and the anthocyanins in juice were analyzed using a HP 1200L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA) after removing suspended solids by centrifugation at 14,000 rpm for 10 min. A Kinetex, 100, EC-C18 (30 x 3 mm, 2.6 µm, Agilent, USA) column was used to determine the two phenolic subclasses by a single chromatographic run; solvent A was

CH_3CN and solvent B was H_2O acidified by HCOOH (3% v/v). The following linear gradient was applied: solvent A varied from 5% to 25% in 8 min, then was kept for 10 min at A 25%, in 2 min it reached 95%, and finally was kept in this condition for 6 min. Total time of analysis was 28 min, equilibration time 10 min, and flow rate 0.4 mL/min. Injection volume: 2 μL for ellagitannins extracts (decoction) and 10 μL for anthocyanins extracts (centrifuged juices). Chromatograms were recorded at 370 nm, 380 nm and 520 nm.

MS analysis

The extracts from decoction and juices from arils were analyzed on a quadrupole ionic trap LTQ (Thermo Finnigan) coupled to an HPLC (Thermo Finnigan Surveyor, San Jose, CA, USA); the HPLC conditions were the same as reported in paragraph 2.5. The analyses were conducted with the following ESI parameters (electrospray ionization): Sheath Gas Flow Rate: 35; Aux Gas Flow Rate: 10; Sweep Gas Flow Rate: 7; Spray Voltage: 4.20 V; Capillary temperatures: 280 °C; Capillary Voltage: -23 V; Tube Lens: -53. Acquisition for mass analysis was performed in negative and positive ions in full spectrum scan in the range of m/z from 100 to 1800.

Size Exclusion Chromatography (SEC) for polysaccharides

The samples containing the total polysaccharides were analyzed by SEC to determine the apparent molecular weight of the main constituents. Briefly, after freeze drying the samples were dissolved in distilled water at a final concentration close to 0.5 mg mL^{-1}. The samples were analyzed according to as Chamizo *et al.,* (2018) using a ProStar HPLC Chromatograph (Varian USA) equipped with a refractive index detector (mod 355), using two columns, PolySep-GFC-P 6000 and PolySep-GFC-P 4000 from Phenomenex, USA,

connected in series. The columns (700 mm length and 7.8 mm internal diameter) had separation ranges of 100 kDa to 15 MDa and 0.3 to 400 kDa. HPLC-grade water was used for the isocratic elution, with a flow of 0.6 mL min^{-1}, and total time of 70 min. Blue-dextrans at various molecular weights ranging from 50kDa to 2000 kDa were used as internal standards to determine the hydrodynamic volume.

Sugars analysis

The two samples (PQ-C and PQ2018-100) were dialysed (cut-off 12–14 kDa), freeze-dried and treated according to Nunes *et al.,* (2001) for the determination of neutral sugars after acid hydrolysis (H$_2$SO$_4$ 72%) and conversion to the corresponding alditol acetates. Gas chromatography was performed using a Hewlett-Packard 5890 with a split injector (split ratio 1:60) and FID detector. A 25-m column CP-Sil-43 CB (Chrompack, Holland) with 0.15 mm i.d. and 0.20-µm film thickness was used. With the injector and detector operating at 220 °C, the following temperature program was used: 180 °C for 5 min and 200 °C for 20 min, with a rate of 0.5 °C/min; linear velocity of the carrier gas (H$_2$) was set at 50 cm/s at 200 °C. In addition, uronic acids were colorimetrically determined using m-phenylphenol as previously reported (Nunes *et al.,* 2001).

Statistics

Each experiment was performed in triplicate and the results were expressed as mean ± SD using EXCEL software (version 2013) in-house routines. One-way ANOVA and *F*-test (p < 0.05) by Microsoft Excel statistical software and Fisher's LSD (DSAASTAT software v. 1.1, Onofri, Pisa, 2007) were used to point out significant differences between quantitative data for anthocyanins in juice and ellagitannins in peel.

2.2.1.3 Results and Discussion

This work focused on Purple Queen[®], a pomegranate cultivar widespread in southern Spain and characterized by an early ripening time (between mid-August and late September) when other widely commercial varieties such as Wonderful are not available on the market. Specifically, we wanted to verify the effects induced on the nutraceutical profile of the fruits when the plants were cultivated in pots with different percentages of remediated port sediments.

With the aim of evaluating the effect of sediments on fruit quality, three main classes of molecules were investigated: anthocyanins in arils and ellagitannins and polysaccharides in peel. These latter were gravimetrically evaluated and analyzed to determine the sugar composition and apparent molecular weight by Size Exclusion Chromatography (SEC).

Morphological characteristics of Purple Queen[®] fruits

As reported in Table 1, all the fruits showed a similar mean weight for both 2017 and 2018; control fruits (PQ-0), although the plants were cultivated in pots, showed a mean weight of 298.05 g which was comparable to that of the commercial fruits (PQ-C) at 303.1 g. The presence of sediments in both the tested percentages negatively affected fruit size. Whole fruits, arils and peel total weight followed a common trend with PQ-0>PQ-50>PQ-100 in both the years. These findings clearly indicate that the physical and chemical composition of the sediments (Table 2.2.1.1), different from peat, induced plant stress resulting in a lower plant productivity as previously highlighted by Melgarejo *et al*., (2019).

Anthocyanins in juice

In order to highlight specific differences in the amount of single anthocyanins in this study, the qualitative and quantitative evaluation of anthocyanins was carried out by HPLC-DAD. The anthocyanin chromatographic profiles of fruits collected in 2017 showed similar patterns for the produced juices independently from the substrate (data not shown). In light of these preliminary findings, the fruits collected in 2018 were used also to carry out a quantitative evaluation. As expected, the anthocyanin fingerprints were similar to those found in Purple Queen® from 2017 and to profiles reported in the literature for other varieties (Figure 2.2.1.1a) (Fischer *et al.,* 2011).

a)
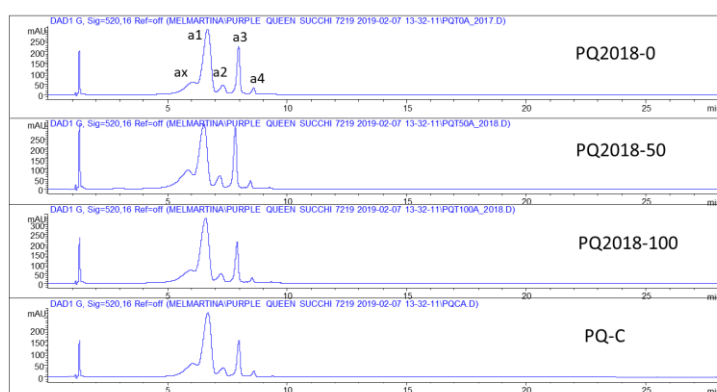
b)

Figure 2.2.1.1 HPLC-DAD profiles of Purple Queen sample, 2018 fruits.

(**a**) Anthocyanins from juice (**b**) Ellagitannins from decoction.

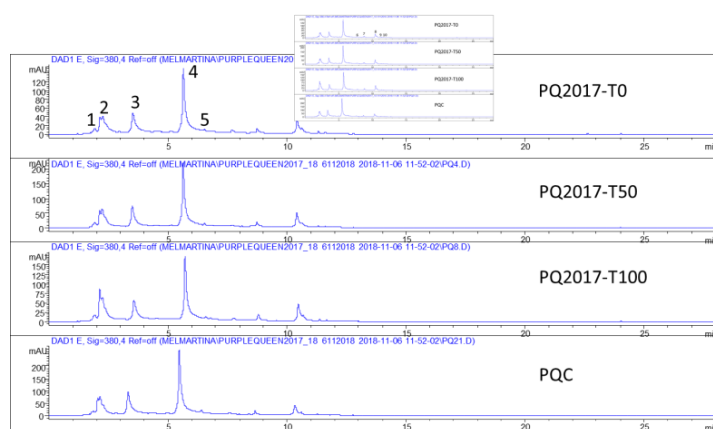The most abundant components were cyanidin-3,5-diglucoside, delphinidin-3,5-diglucoside and cyanidin-3- glucoside (Figure 2.2.1.1a).

The absence of α and β punicalagins in our samples is linked to the method applied to obtain the juices from arils only, unlike most commercial pomegranate juices which are usually made by pressing half of the whole fruit, meaning a co-extraction of some ellagitannins from peel (Fischer *et al.,* 2011). The identified anthocyanins are summarized in Table 2.2.1.3.

Analytes	rt	[MH]⁻	Identified compounds
3	3.9	1083	alpha-punicalagin
4	5.9	1083	beta-punicalagin
7	10.4	301	ellagic acid
Anthocyanins	rt	**[M]⁺**	**Identified compounds**
AX	6.1	627	delphinidin-3,5- diglucoside
A1	6.8	611	cyanidin-3,5-diglucoside
A2	7.5	465	delphinidin-3-glucoside
A3	8.1	449	cyanidin-3-glucoside
A4	8.8	433	pelargonidin-3- glucoside

Table 2.2.1.3 Main identified compounds in pomegranate samples.

The use of the sediments particularly influenced the concentration of cyanidin-3,5-diglucoside which showed the highest values (140 mg/L) in the PQ2018-100 sample; the control (PQ2018-0) showed the lowest value (90 mg/L). Moreover, Figure 2.2.1.2a shows that also cyanidin-3-glucoside was significantly increased in 2018 fruits cultivated on sediment-based media (100% and 50%) with respect to the control (PQ2018-0) and commercial fruit (PQ-C). It should be pointed out that an environmental factor, such as the growing media, positively affected the content of cyanidin, an important molecule with

beneficial qualities such as neuroprotective, antioxidant and antidiabetic properties (Cásedas *et al.,* 2019). An opposite trend was observed for delphinidin-3,5-diglucoside with resulted statistically higher in control fruit compared to the other treatments, although with slight differences (Figure 2.2.1.2a). The total anthocyanins ranged from 171 to 233 mg/L in PQ2018-0 and PQ2018-100, respectively (Figure 2.2.1.2b).

a)
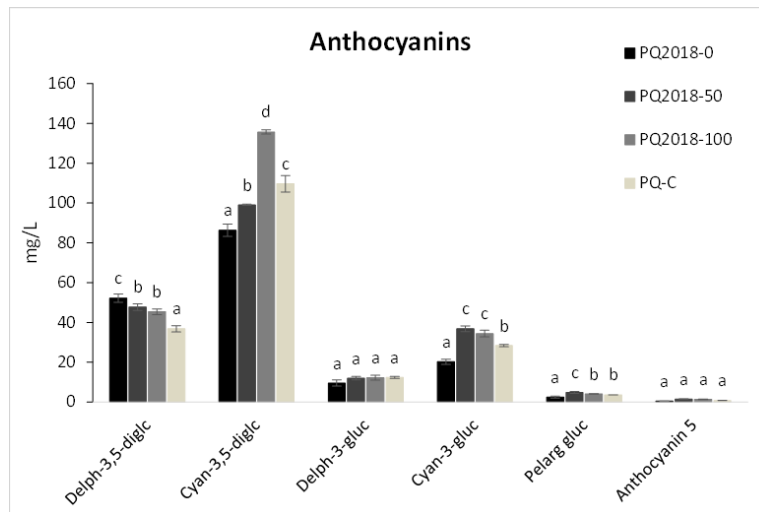
b)

Figure 2.2.1.2 Anthocyanins in juices obtained from fruits collected in 2018: **a)** distribution of the main compounds, and **b)** total anthocyanins content. The data are expressed as mg/L as a mean of triplicates. Error bars represent the standard deviation (n=3).
Different letters mean statistically significant differences ($p < 0.05$).
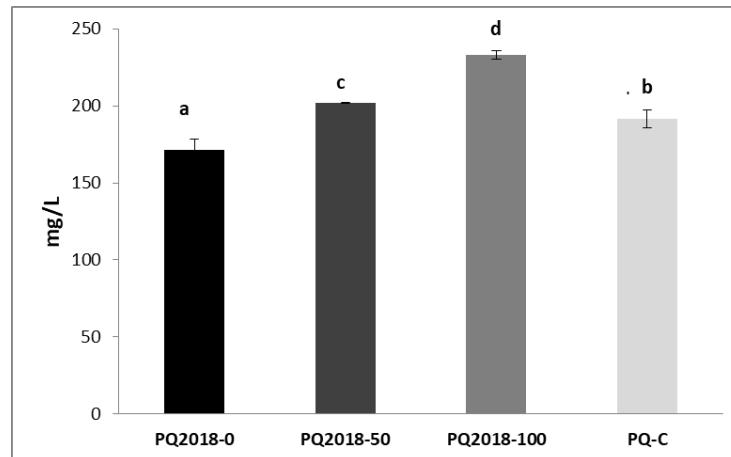
142

It is noteworthy that both PQ-100 and PQ-50 from 2018 samples were richer in anthocyanins than the control and commercial fruit (PQ2018-0 and PQ-C respectively). Based on these results, plants PQ2018-100 and PQ2018-50 synthetized a higher anthocyanins content in response to a greater stress, presumably caused by the sediment. Previous data regarding anthocyanins content in PQ grown under the same reclaimed sediment did not evidence significant variations, probably due to different extraction and analytical methods (Melgarejo *et al.,* 2019).

Findings from fruits of fifteen Iranian varieties of pomegranate indicated 252.2 mg/L as the highest anthocyanin content determined by HPLC-DAD (Alighourchi *et al.,* 2008). Comparing these results with the amount measured in Purple Queen[®], it turns out that this variety is a good source of anthocyanins, with values comparable to those of the richest Iranian varieties even when grown under only remediated dredged sediments (PQ2018-100).

Anthocyanins are well-known to have a strong positive impact on human health (Fischer *et al.,*2011) and, although there are no fixed values for anthocyanins intake, in 2013 the Chinese Nutrition Society, suggested providing a daily intake of at least 50 mg. Taking into account that red pomegranate juice is the main or only part of the fruit usually consumed fresh, the importance of finding new substrates for cultivation suitable to maintaining or even better to improving the phenolic expression, in particular of the anthocyanins, is certainly of great interest.

Ellagitannins from peel

The peel is known to be as the richest tissue of pomegranate fruit in terms of ellagitannins and polysaccharides, two classes of bioactive compounds that are well soluble and chemically stable in hot water ([a]Khatib *et al.,* 2017). Due to these properties, in our work

peel extraction was carried out by applying a decoction because this procedure resulted suitable to efficiently recover both ellagitannins and polysaccharides with high yields. The possibility of using only water as extractive solvent can be strategic to facilitate the valorization of this by-product obtained in large quantities during the production of juice (Joseph *et al.,* 2013). Applying this process, it is also possible to propose the use of the dry decoction, almost representing the 70% of the dry weight, as a new functional ingredient to enrich different foods with ellagitannins and pomegranate polysaccharides.

The components detected in Purple Queen® decoction were the same as those previously found in other varieties (Fischer *et al.,* 2011) (Table 2.2.1.3), with α+β punicalin (**2**), α punicalagin (**3**), β punicalagin (**4**), and ellagic acid (**8**) resulting as the principal ellagitannins (Table 2.2.1.4). All the chromatographic profiles related to the ellagitannins found in peel grown in 2017 and 2018 in the different substrates resulted very similar and almost completely superimposable (Figure 2.2.1.1b). It can be said that the presence of remediated dredged sediments did not induce changes in the biosynthetic pathways of these phenolic compounds. The ellagitannins content in the fruits harvested in the two seasons showed a similar trend: the presence of sediment mixed with peat (i.e. PQ-50) determined the maximum increase in both years, with a more pronounced effect on the fruits of 2018. The highest concentration of ellagitannins was found in the commercial sample (PQ-C). The amount of α and β punicalagins in Purple Queen®, cited as major ellagitannins responsible for the majority of the biological properties of pomegranate, ranged from 54% to 60% of total phenols for all the fruits grown in pots, while the percentage increased in the commercial sample (PQ-C) with approximately 70% of the total content (Table 2.2.1.4).

	Ellagitannins						
	PQ-0		PQ-50		PQ-100		PQ-C
Compounds	2017	2018	2017	2018	2017	2018	2018
ellagitannin der. (1)	1.8	0.8	2.1	2.6	2.6	2.2	1.9
α+β punicalin (2)	12.5	7.1	18.6	22.5	19.1	22.5	19.3
α punicalagin (3)	8.0	7.2	11.6	11.7	8.8	9.0	17.5
β punicalagin (4)	21.9	14.7	27.9	27.5	24.5	23.9	39.2
ellagitannin der.(5)	1.2	0.7	0.7	1.3	0.6	0.5	1.9
ellagic acid hexoside(6)	1.5	0.9	0.9	1.5	0.7	0.8	2.0
ellagic acid pentoside (7)	1.1	2.3	1.4	1.7	1.4	1.4	1.3
ellagic acid(8)	1.8	3.9	4.7	3.3	3.8	3.9	2.2
ellagic acid der.(9)	0.1	0.7	0.1	0.2	0.1	0.1	0.2
ellagic acid der.(10)	0.1	0.1	0.1	0.2	0.1	0.1	0.3

Table 2.2.1.4 Ellagitannins in the decoction of peel of Purple Queen[®] (PQ) samples collected in 2017 and 2018 grown in pots in different conditions (PQ-0; PQ-50; PQ-100); PQ-C was a commercial sample from 2018. Data (mg/g of dry weight) are means of the triplicates; the values of relative standard deviation (RSD) were below <5% for all the components.

The values reported in the literature for total ellagitannins in pomegranate peel range from 67 mg/g to 262 mg/g DW depending on the different varieties, geographical and environmental factors (Fischer *et al.,* 2011; [a]Khatib *et al.,* 2017; Singh *et al.,* 2018). Findings from a previous work on Wonderful and Laffan varieties showed that the total ellagitannins extracted with a hydroalcoholic medium (ethanol 70 %) and hot water gave very similar amounts ([a]Khatib *et al*, 2017).

In our samples, the mean values for total ellagitannins extracted by decoction ranged from 38 to 85 mg/g dry peel (Figure 2.2.1.3) confirming that Purple Queen[®] is a variety with a medium content of ellagitannins. To the best of our knowledge, this is the first study to evaluate ellagitannins in peel of the Purple Queen[®] variety and compare the content obtained from pomegranate plants grown in pots on different substrates grounds containing peat and remediated dredged sediments.
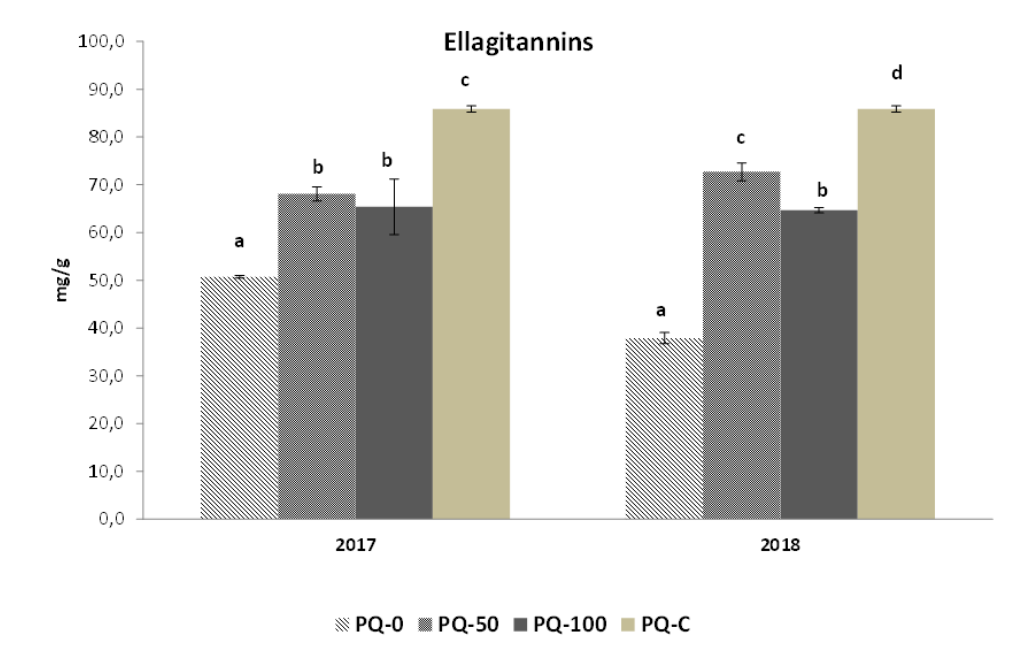
Figure 2.2.1.3 Total ellagitannins in decoction of peel: samples from 2017 and 2018 grown with different media (sediments % of 0; 50; 100) compared to Purple Queen® commercial sample (PQ-C). Data (mg/g dry weight) are means of a triplicate. Different letters indicate statistically significant differences (*p*<0.05).

Polysaccharides from peel

Pomegranate peel has been recognized to be a good source of polysaccharides, representing approximately 10-12% of the fruit dry weight and being mainly present as pectin (Gavlighi *et al.,* 2018; [b]Khatib *et al.,* 2017; Joseph *et al.,* 2013; Singh *et al.,* 2018; Shakhmatov *et al.,* 2019). Several biological properties concerning the polysaccharides of pomegranate have been recently highlighted in the literature: immunomodulatory and scavenging properties, an ability to reduce the growth of tumors in mice in combination with doxorubicin, and *in vitro* prebiotic activity contributing to maintain the health of human microbiota (Joseph *et al.,* 2013; Tortora *et al.,* 2018).

In light of these studies, although polysaccharides of pomegranate can be considered a part of the bioactive molecules of the fruit, scarce data are available on their structure and no information is available on Purple Queen® till now. In this study, initially, the yields in

total polysaccharides were gravimetrically evaluated after precipitation induced by ethanol from decoction. As shown in Figure 2.2.1.4, similar values were obtained for the 2017 and 2018 samples: the increased content of polysaccharides resulted proportional to the percentage of remediated sediment in the substrate. The amount of polysaccharides found in fruits of plants cultivated only in remediated sediment (PQ-100) showed a strong increment in both years with respect to the values measured for the commercial sample (PQ-C). The total percentage of polysaccharides expressed in the dry weight of peel ranged from approximately 12% in PQ2017-0 to a maximum of 32% in PQ2017-100. These results are clearly greater with respect to other pomegranate varieties ([b]Khatib *et al.,* 2017). In general, the increase of polysaccharides production in plants has been associated to abiotic stresses, like water deficit, however no data are available regarding the effect of reclaimed sediment. A greater accumulation of total soluble solids has been observed also in other fruits (strawberries and pomegranates) from plants grown on the same reclaimed sediment (Tozzi *et al.,* 2019; Melgarejo *et al.,* 2019), associated to the stress induced by the sediment with unsuitable physical characteristics, such as high bulk density and low porosity. Therefore, the increase of polysaccharides may contribute to the enhanced accumulation of dry matter within the fruit peel.
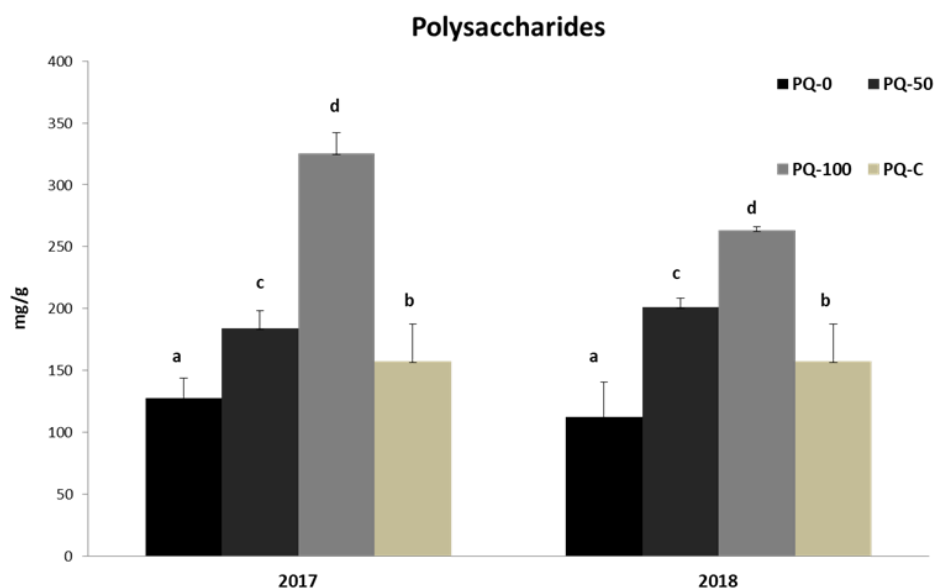
Figure 2.2.1.4 Total polysaccharides in Purple Queen® fruit samples collected in 2017 and 2018 from the three treatments compared with a commercial sample (PQ-C). Data (mg/g DM) are the mean of a triplicate. Different letters indicate statistically significant differences ($p<0.05$).

To evaluate possible changes in the apparent molecular weight (hydrodynamic volume) of the main polysaccharides, the samples were analyzed by SEC, using a pool of dextrans to determine the hydrodynamic volume of the main polysaccharides recovered after decoction from the peel of Purple Queen® samples. Figure 2.2.1.5, in reference to the two samples of 2017 and 2018 without the presence of sediment (PQ-0), shows very similar profiles with about 50% constituted by oligosaccharides (white column, <0.36 kDa) and approximately the other 50% by polysaccharides at high molecular weight (black column) with values > 2000 kDa. On the other hand, the samples grown on 100% remediated dredged sediment show different profiles, particularly for the fruits of 2017 compared to those of 2018. These latter samples have a similar polysaccharides distribution for all pot-grown plants with an almost superimposable profile compared to the commercial sample (PQ-C). In other words, the major changes in terms of molecular weight distribution were observed for the

younger plants of pomegranate (samples from 2017), while the older plants were less susceptible to the effect of the sediment.
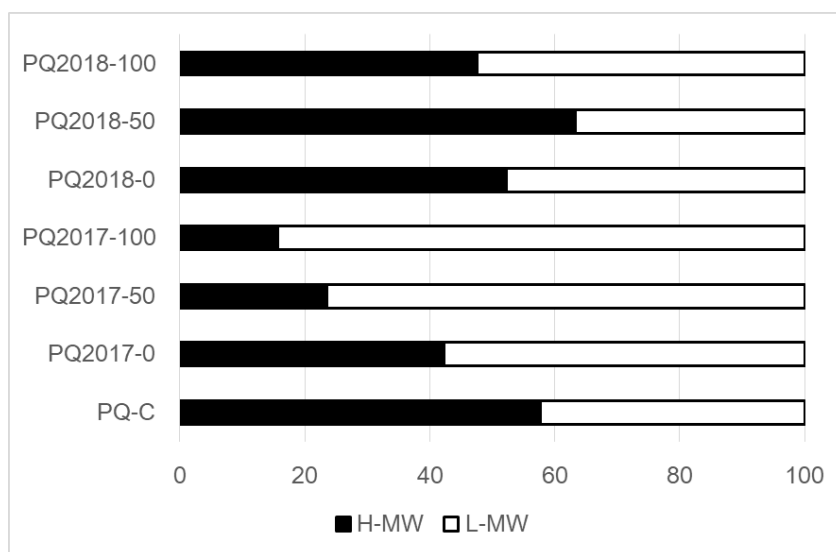
Figure 2.2.1.5 Distribution of the apparent molecular weight determined by SEC for the whole polysaccharide fractions recovered after decoction by ethanol addition; data are expressed as peak area % on total areas. *H-MW: High molecular weight; L-MW: Low molecular weight*

To further investigate the sugar composition, the total polysaccharide fractions of PQ-C and PQ2018-100 were dialysed and subjected to acidic hydrolysis. We wanted to verify if the similarity in their profiles after SEC could also be confirmed in terms of sugar composition. Our findings highlighted the presence of glucose as major neutral sugar: 45% and 52% in PQ-C and PQ2018-100, respectively (Figure 2.2.1.6). This result was predictable because of the presence of cellulose, which is reported as close to 20% of the total dietary fiber in pomegranate (Hasnaoui *et al.,* 2014). Galacturonic acid was 35% and 31% in PQ-C and PQ2018-100, respectively: galacturonic acid reported as the main polysaccharides in pomegranate (Abid *et al.,* 2017; Shakhmatov, *et al.,* 2019; Zhai *et al.,* 2018) is linked to pectin structure.
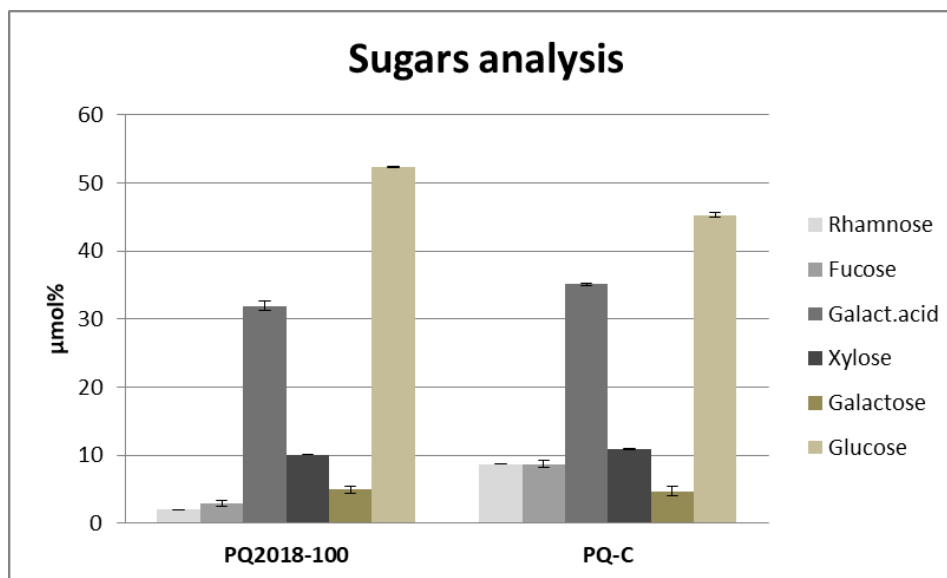
Figure 2.2.1.6 Sugars composition of Purple Queen® commercial (PQ-C) and Purple Queen® 2018 with 100% of sediment (PQ2018-100).

The results are expressed in µmol% as a mean of a duplicate.

In our case, the percentage of galacturonic acid was lower than previous values obtained for other varieties in the literature ([b]Khatib *et al.,* 2017) and it could be related to the fact that the analysis was carried out on the total crude polysaccharides and not on a purified extract containing only pectin. Furthermore, according to previous data on the absence or a very low content of starch in pomegranate fruit (Gupta *et al.,* 2015), the presence of glucose cannot be ascribable to the presence of starch. Other sugars, such as rhamnose, fucose, xylose, arabinose and galactose, are present in smaller quantities, less than 10% in both the samples.

In light of these findings, we concluded that the two fruit samples presented a similar sugar pattern, confirming that the use of dredged sediments did not influence the polysaccharides composition. So far, there are no data in the literature about the factors affecting the content of polysaccharides in pomegranate peel nor in other organs of the plants. Our results can be a starting point for planning future experiments to better clarify the mechanisms behind this effect.

2.2.1.4 Conclusions

In this work, the nutraceutical profile of pomegranate fruits from trees cultivated on reclaimed dredged sediments was studied for the first time by assessing the phenolic composition of arils and peel and the polysaccharides in peel. We attempted to evaluate if this innovative soilless substrate is suitable for growing Purple Queen® plants in pots. The presence of the sediment had a detrimental effect on fruit size and weight due to its unsuitable physical-chemical characteristics, as also observed in a previous study. However, this negative effect has been reduced by limiting the percentage of sediments added to the soil of older plants already adapted to growth on this new mixture of peat and sediment. Conversely, fruits from plants cultivated only on sediment or in a mixture with peat showed significant increases of bioactive compounds both in arils and in peel in the two consecutive seasons studied. The juice and peel showed higher concentrations of anthocyanins and ellagitannins, respectively. A similar trend was also found for the polysaccharide fraction, which was notably increased proportionally to the percentage of the remediated sediment.

Further studies are needed to better elucidate the physiological mechanisms behind the synthesis of these important bioactive compounds in pomegranate fruits in relation to the use of sediment in cultivation.

2.2.2 A comparative study on the whole fruit of fifteen varieties of *Punica granatum* L.: a focus on anthocyanins, ellagitannins and polysaccharides.

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Abstract

The pomegranate plant is the subject of a renewed commercial and scientific interest, therefore a deeper knowledge of the chemical composition of the fruits of new and little studied varieties is increasingly required. The purpose of this work was to characterize 15 varieties harvested in the same nursery by studying the composition of arils and peel. The juices were prepared only from the arils, whereas the mesocarp and exocarp were used to recover ellagitannins and polysaccharides by decoction. Juices and decoctions were submitted to HPLC-DAD, spectrophotometric and colorimetric CIEL*a*b* analyses, as well as to antioxidant and enzymatic tests. Furthermore, through successive additions of ethanol to the decoction different polysaccharide fractions were collected from each variety. Juices and decoctions were used to determine antioxidant, antiradical and metal chelating properties and the inhibitory activity against tyrosinase and alfa-amylase enzymes. Despite the morphological differences, all these varieties presented the same main phenols: four ellagitannins, including alpha and beta punicalagin, and five anthocyanins. According to HPLC-DAD and spectrophotometric data, the total amounts of anthocyanins and ellagitannins were widely variable among the varieties.

Concerning the anthocyanins in juices, a discrete correlation was obtained between h_{ab} and the ratio delphinidin/cyaniding (both expressed as sum of all the glycosides). All the

decoctions were poor in anthocyanins but rich in ellagitannins, with the greatest quantities in the Black, Provenza Francia and Acco varieties. The richest anthocyanin juice came from the Wonderful variety. The polysaccharides amounts ranged from 3% to 12% and the greatest contents were found in Acco, Ariana, Black and Grossa di Faenza varieties. After the analysis by size exclusion chromatography of four of the fifteen samples, which were selected due to their very different morphological characters, only a main polysaccharide of ca. 2 million Dalton was detected in all samples. Each decoction regardless of the variety showed better antioxidant and chelating properties than juices. Independently from the variety, juices and decoctions showed similar inhibitory capacity against the enzyme α-amylase.

2.2.2.1 State of the art and aims of the work

Pomegranate (*Punica granatum* L*.*) is a very widespread plant thanks to its tolerance to almost all soil types and great adaptability to arid regions. Nowadays, within the main producers are Iran and India, but also North and South America and Europe, particularly with the Mediterranean regions (Hussein *et al.,* 2018; Holland *et al.,* 2009). There are around 500 pomegranate cultivars all over the world with differences both from a morphological and a nutraceutical point of view. Among the peculiarities characterizing each variety or cultivar, useful to define the quality of the fruit, are certainly included the pleasant taste of juice and its anthocyanin content. Concerning the bioactive compounds as polysaccharides and ellagitannins, their increase in pomegranate peel can be considered an added value. Among the methods selected to characterize foods is included the reflectance colorimetry. The possibility to analyze food matrices as such (jellies or homogenates, turbid juices, solid form) without any treatment, represents an undoubtable advantage to understand what could alter the labile pigment component. Color expressed by a foodstuff is undoubtedly associated to its genuineness, other than the specific chemical profile which, in turn, depends by ripening stage, and cultivar. Color measurement plays a crucial role in functional foods, such as pomegranate, in which bioactive molecules are intensely colored. The possibility to evaluate this organoleptic character, without any treatment and with a very simple, fast and cheap method can give us a lot if information to match with those deriving from the quali-quantitative analysis carried out by chromatographic methods. The external part of pomegranate fruit is generally discarded during the juice production, so the knowledge of the chemical constituents extractable from exocarp and mesocarp is desirable to valorize also this by-product. According to the increased interest in search for beneficial phytochemicals present in fruit peels and utilize them in food, pharmaceutical and cosmetic industry (Singh *et al.,* 2018). Aim of this work was the

characterization of fifteen different pomegranate varieties grown in the same hatchery to shed light on the composition of the fruits. To avoid environmental interferences, all pomegranate samples were grown in the same year and the fruits collected at completed maturation. Anthocyanins and ellagitannins from arils and polysaccharides and ellagitannins from peel were evaluated. Part of the polysaccharides were characterized by Size Exclusion Chromatography and sugars analysis. In this work we also applied the reflectance colorimetry to investigate on the differences among decoctions and juices obtained by the fruit varieties.

Furthermore, the antioxidant properties were evaluated for aril juices and peel decoctions by a pool of *in vitro t*ests. Finally, juices and decoctions were used to study their biological properties by measuring the inhibition of the two enzymes, alpha-amylase and tyrosinase.

2.2.2.2 Material and Methods

The pomegranate fruits were purchased from the same hatchery (Latina, Italy). All the samples with their abbreviations, are listed in Table 2.2.2.1. The whole fruits were divided into aryls and peel (mesocarp + exocarp). The fresh tissues were stored at -22°C until the extractive procedures and the juice preparation had not been performed.

Variety	Code	Arils (%)	Mesocarp + exocarp (%)
Acco	AC	55.1	44.9
Ariana	AR	55.0	45.0
Austin	AU	58.4	41.6
Black	BL	52.5	47.5
Desertnyi	DE	50.2	49.8
Grossa di Faenza	GF	45.3	54.7
Mollar de Elche	ME	64.5	35.5
Medovyi Vahsha	MV	49.2	50.8
Parfianka	PA	54.0	46.0
Provenza Francia	PF	54.1	48.6
Sirenevyi	SI	51.0	49.0
Soft seed Maule ® 116/17	SM	47.2	52.8
Shirin Zigar	SZ	32.0	68.0
Vkusnyi	VK	55.2	44.8
Wonderful	WO	55.3	44.7

Table 2.2.2.1 Composition of the fresh fruits of the analyzed varieties of *Punica granatum* L.; each variety is identified by a code (second column in the Table), the same used in all the others Figures and Tables.

Reagents

All Ultrapure water was from Milli-Q-system (Millipore SA, Molsheim, France), solvents were from Sigma Aldrich (St. Louis, Missouri, USA), all of analytical HPLC grade. Ellagic acid (purity ≥ 95%) and punicalagin (purity≥ 98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and oenin chloride (purity ≥ 95%) was from Extrasynthese (Genay, France). Dextrans at different molecular weights (MWs: 2000, 1100, 410, 150, 50 kDa) and sucrose (360 Da) used for SEC were from Sigma-Aldrich, USA.

Juice production.

The arils of each sample were divided in three portions and each treated separately by the Hurom extractor (HU-700) through a rapid process at low temperatures to obtain triplicates of juices. Each sample was diluted (1:1 v/v) with ethanol (2% HCOOH) than centrifuged at 14000 rpm for 5 minutes and the supernatant recovered was analyzed by HPLC-DAD-MS.

Decoction from peel (mesocarp+exocarp)

The fresh mesocarp and exocarp were used to prepare a decoction according to [b]Khatib *et al.,* 2017: briefly 5 g of pomegranate peel in 200 mL were boiled for 1 h. The sample was cooled and centrifuged (4500 rpm, for 8 min, at 4°C) and the supernatant was taken to a volume of 200 mL with distilled water. 10 mL were withdrawn and used for the analysis of ellagitannins by HPLC-DAD, for the colorimetric measurements, the spectrophotometric determinations and the enzymatic tests.

Polysaccharides precipitation was performed by adding different aliquots of ethanol (100 mL each). The precipitation was completed after three aliquots of the alcohol. After centrifugation (4500 rpm, 8 min, 4 °C) the recovered fractions containing polysaccharides were collected, freeze-dried and weighted for calculating the yield.

HPLC-DAD analyses of juices and decoctions

Ellagitannins and anthocyanins were analyzed using a HP 1200L liquid chromatographic system (Agilent Technologies, Palo Alto, CA, USA). The same column, Kinetex, 100, EC-C18 (30 x 3 mm, 2.6 μm, Agilent, USA) and the same elution methods were applied to determine all the phenolic compounds; solvent A was CH_3CN and solvent B was H_2O acidified by HCOOH (3% v/v). A linear gradient was applied as follows: A varied from 5% to 25% in 8 min, then 10 min remained at 25%; in 2 min, A reached 95%, and was kept

in this condition for 6 min with total time of analysis 28 min, equilibration time 10 min, and flow rate 0.4 mL/min. The injection volumes were 2 µL for ellagitannins extracts (decoction) and 10 µL for anthocyanins extracts (centrifuged juices).

The ellagitannins were quantified according to their maximum of absorption using five points calibration curves. The racemic mixture of α- and β-punicalagins at 380 nm (purity ≥ 99%), linearity range 2-5 µg (R2=1.0); ellagic acid (purity 95%) at 370 nm, linearity range 0-1.7 µg, (R2=1.0). The anthocyanins were quantified at 520 nm with the calibration curve of oenin chloride (purity ≥ 95%), linearity range 0-2.6 µg (R^2= 0.999).

Size Exclusion Chromatography (SEC) for polysaccharides

Polysaccharides of four selected samples, Acco, Black, Wonderful and Mollar de Elche, were analysed by SEC to determine the apparent molecular weight of the main constituents. Briefly, the samples after freeze drying were dissolved in distilled water at a final concentration close to 0.5 mg mL^{-1}. The samples were analysed according to Chamizo *et al.,* (2018) by a ProStar HPLC Chromatograph (Varian USA) equipped with a refractive index detector (mod 355), using two columns, PolySep-GFC-P 6000 and PolySep-GFC-P 4000 from Phenomenex, USA, connected in series. The columns (700 mm length and 7.8 mm internal diameter) had separation ranges of 100 kDa to 15 MDa and 0.3 to 400 kDa. HPLC-grade water was used as eluent with a flow of 0.6 mL min^{-1}, and total time of 70 min. As internal standars to determine the hydrodynamic volume were used blue-dextrans at various molecular weights (50-2000 kDa).

Sugars analysis

The Black and Wonderful fractions 2 were dialysed (cut-off 12–14 kDa), freeze-dried and treated according to Nunes *et al.,* (2001) for the determination of neutral sugars, after acid hydrolysis and conversion to the corresponding alditol acetates. Gas chromatography was performed using a Hewlett-Packard 5890 with a split injector (split ratio 1:60) and a FID detector. A 25-m column CP-Sil-43 CB (Chrompack, Holland) with 0.15 mm i.d. and 0.20-μm film thickness was used. With the injector and detector operating at 220° C, the following temperature program was used: 180 °C for 5 min and 200 °C for 20 min, with a rate of 0.5 °C/min; linear velocity of the carrier gas (H_2) was set at 50 cm/s at 200° C. Furthermore, uronic acids were colorimetrically determined using m-phenylphenol as previously reported (Nunes *et al.,* 2001).

Colorimetric CIELAB analysis of juices and decoctions

CIEL*a*b* parameters (L^*, a^*, b^*, C^*_{ab} and h_{ab}) were determined on pomegranate extracts and decoctions using a colorimeter X-Rite SP-62 (X-Rite Europe GmbH, Regensdorf, Switzerland), equipped with a D65 illuminant and an observer angle of 10°. Colour description is based on three parameters as defined by the "Commission Internationale del l'Eclairage" (CIE): L^* that defines the lightness and varies between 0 (absolute black) and 100 (absolute white), a^* that measures the greenness (-a^*) or the redness (+a^*) and b* that measures the blueness (-b^*) and the yellowness (+b*). C^*_{ab} (chroma, saturation) expresses a measure of colour intensity and h_{ab} (hue, colour angle) is the attribute of appearance by which a colour is identified according to its resemblance to red, yellow, green, or blue, or a combination of two of these attributes in sequence. Cylindrical coordinates C^*_{ab} and h_{ab} are calculated from the parameters a^* and b^* using the equations $C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$ and $h_{ab} = \tan^{-1}(b^*/a^*)$ (Patsilinakos *et al.,* 2018).

The results are expressed as the mean value and RSD%. Each value registered by the instrument is the median of ten measurements performed randomly on the surface of the cell. Samples of pomegranate extracts and decoctions obtained as previously described, were used as such to re-fill the quartz cell for fluid measurements. Each experiment was performed four times and the results are expressed as the mean value ± standard deviation (SD).

Total phenolic content (TPC)

The TPC was determined using the Folin–Ciocâlteu method according to the modified method by Mocan *et al.,* (2016). A SPECTROstar Nano Multi—Detection Microplate Reader with 96-well plates (BMG Labtech, Ortenberg, Germany) was used. Briefly, a mixture solution consisting of 20 µL of extract, 100 µL of Folin-Ciocâlteu reagent and 80 µL of sodium carbonate (Na_2CO_3, 7.5% *w/v*) was homogenized and incubated at room temperature in the dark for 30 min. Afterwards, the absorbance of the samples was measured at 760 nm. Gallic acid was used as a reference standard, and the TPC was expressed as gallic acid equivalents (GAE) in mg/g dry weight (dw) of plant material.

Total flavonoid content (TFC)

The total flavonoid content (TFC) was calculated and expressed as quercetin equivalents using a method previously described by Mocan *et al.,* (2014). Briefly, a 100 µL aliquot of 2% $AlCl_3$ aqueous solution was mixed with 100 µL of juice or decoction samples. After an incubation time of 15 min, the absorbance of the sample was measured at 420 nm. Quercetin was used as a reference standard, and the TFC was expressed as quercetin equivalents (QE) in mg/g dry weight (dw) of plant material.

DPPH radical scavenging assay

The scavenge capacity of the free radical DPPH, monitored according to the method described by Martins *et al.,* (2015) with some modifications, was performed by using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). The reaction mixture in each of the 96-wells consisted of 30 µL of sample solution (appropriately diluted) and a 0.004% methanolic solution of DPPH. The mixture was incubated for 30 min in the dark, and the DPPH radical reduction was determined by measuring the absorption difference at 515 nm. Trolox was used as a standard reference and the results were expressed as Trolox equivalents per g of dry weight herbal extract (mmol TE/g dw herbal extract).

Trolox equivalent antioxidant capacity (TEAC) assay

The radical scavenging activity shown of juices and decoctions, against the stable synthetic ABTS radical cation, was measured using the method previously described by Mocan *et al.,* (2016). A Trolox calibration curve was plotted as a function of the percentage of ABTS radical scavenging activity. The final results were expressed as milligrams of Trolox equivalents (TE) per gram of dry herbal extract (mg TE/g dw extract).

FRAP assay

In FRAP assay, the reduction of Fe^{3+}-TPTZ to blue-colored Fe^{2+}-TPTZ complex was monitored by the method described by Damiano *et al.,* (2017) with slight modifications. The FRAP reagent was prepared by mixing ten volumes of acetate buffer (300 mM, pH 3.6), one volume of TPTZ solution (10 mM TPTZ in 40 mM HCl) and one volume of $FeCl_3$ solution (20 mM $FeCl_3 \cdot 6\ H_2O$ in 40 mM HCl). Reaction mixture (25 µL sample and

175 µL FRAP reagent) was incubated for 30 min in the dark at room temperature and the absorbance of each solution was measured at 593 nm using a SPECTROstar Nano Multi-Detection Microplate Reader with 96-well plates (BMG Labtech, Ortenberg, Germany). A Trolox calibration curve (0.01-0.10 mg/mL) was plotted as a function of blue-colored Fe^{2+}-TPTZ complex formation, and the results were expressed as milligrams of Trolox equivalents (TE) per milligram of extract (mg TE/mg extract).

α-Amylase inhibitory activity

Samples were added to 150 µL of phosphate buffer (0.2 M, pH 6.8) containing 17 mM NaCl. The reaction was started with adding 10 µL of porcine pancreatic α-amylase (https://www.sciencedirect.com/topics/medicine-and-dentistry/alpha-amylase-pancreas-isoenzyme) solution (25 unit/mL) and after incubation at 37 °C for 30 min stopped by addition of 20 µL NaOH (2 N) and 20 µL color reagent comprising 44 µM of 3,5-dinitrosalisylic acid, 106 µM of potassium sodium tartrate tetrahydrate and 40 µM of NaOH. The reaction mixture was incubated at 100 °C for 20 min and finally the absorbance was measured at 540 nm.

Tyrosinase inhibitory activity

Tyrosinase inhibitory activity of ecah sample was determined by method previously described by Masuda *et al.,* (2005), using a SPECTROstar Nano Multi-Detection Microplate Reader with 96-well plates (BMG Labtech, Ortenberg, Germany). Samples were dissolved in water containing 5% DMSO; for each sample four wells, designated as A, B, C, D, contained reaction mixtures (200 µL) as follows: (A) 140 µL of 66 mM phosphate buffer solution (pH=6.6) (PBS), 40 µL of mushroom tyrosinase in PBS (23 U/mL) (Tyr), (B) 160 µL PBS, (C) 80 µL PBS, 40 µL Tyr, 40 µL juice or decotion sample

and 80 μL PBS and (D) 120 μL PBS and 40 μL sample. The plate was then incubated for

10 min at room temperature; after incubation, 40 μL of 2.5 mM L-DOPA in PBS solution

were added in each well and the mixtures were incubated again for 20 min at room

temperature. The absorbance of each well was measured at 475 nm and the inhibition

percentage of the tyrosinase activity was calculated by the following equation, using as

positive control a kojic acid solution (0.10 mg/mL):

$$\%I = \frac{(A - B) - (C - D)}{(A - B)} \; x \; 100$$

The results were also expressed as mg kojic acid equivalents per gram of dry weight of

extract (mg KAE/g extract) using a calibration curve between 0.01-0.10 mg kojic acid/mL

of solution.

2.2.2.3 Results and Discussion

Juices and Decoction

One of the aims of the work was to use the same chromatographic method to determine

both the main ellagitannins and anthocyanins in juices and decoctions, in order to provide a

simple and easily applicable elution method for the quality control of the samples.

All the pomegranate samples presented superimposable HPLC-DAD profiles both for the

decoctions and for the juices with only negligible differences mainly regarding the minor

compounds. As example in Figure 2.2.2.1 the profiles obtained for Black variety are

shown. The main identified molecules were nine compounds including ellagitannins, such

as α and β punicalagins and ellagic acid, and five different anthocyanins, according to the

previous literature on pomegranate (Fisher *et al*., 2011) (Table 2.2.2.2).
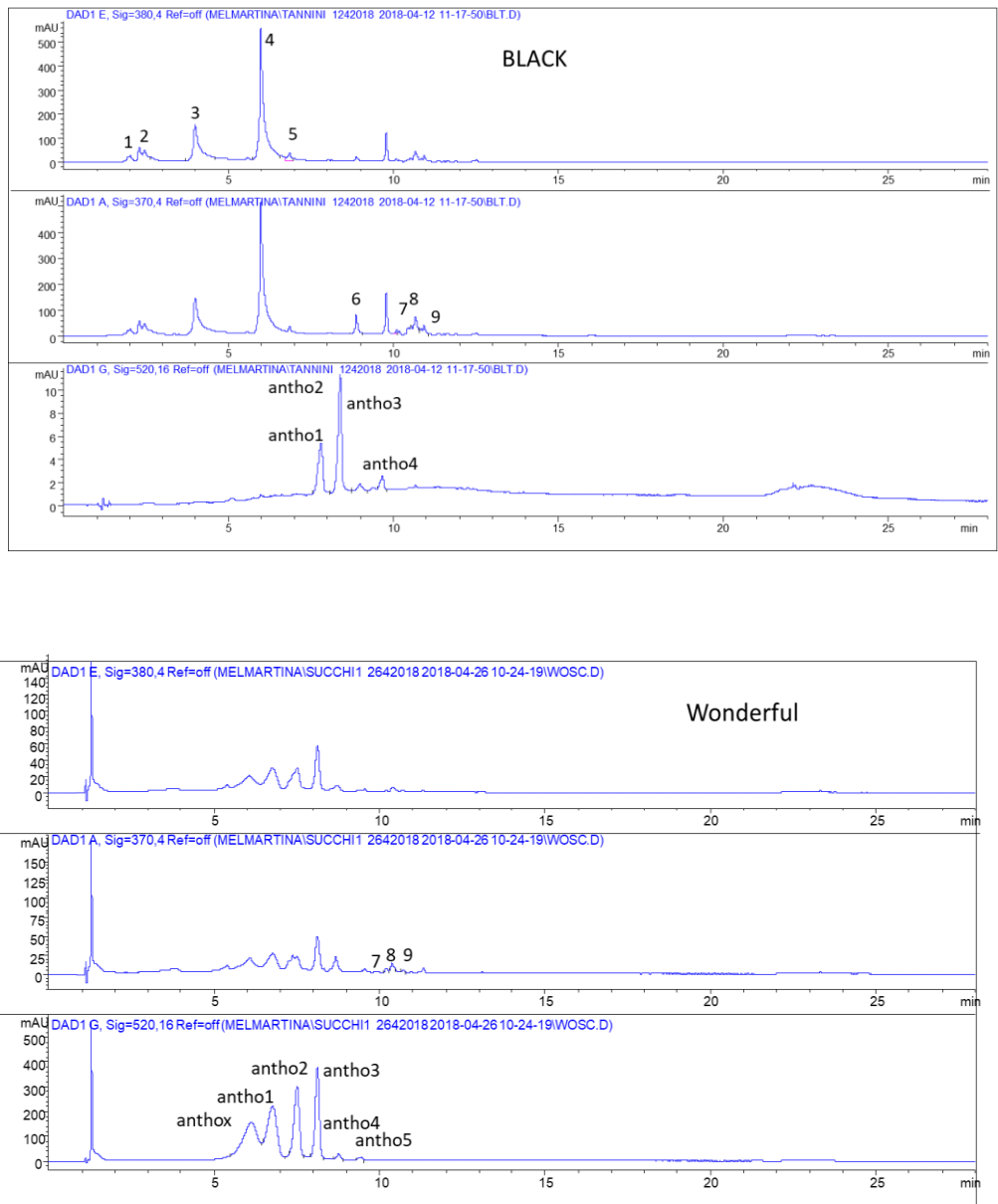
Figure 2.2.2.1 HPLC-DAD profiles at 380, 370 and 520 nm of the decoction from Black

and juice from Wonderful varieties

Ellagitannins	rt	[MH]⁻	Identified compounds
3	3.9	1083	α-punicalagin
4	5.9	1083	β-punicalagin
7	10.4	301	ellagic acid

Anthocyanins	rt	[M]⁺	Identified compounds
Anthox	6.1	627	delphinidin-3,5-*O*-diglucoside
Antho1	6.8	611	cyanidin-3,5-*O*-diglucoside
Antho2	7.5	465	delphinidin-3-*O*-glucoside
Antho3	8.1	449	cyanidin-3-*O*-glucoside
Antho4	8.8	433	pelargonidin-3-*O*-glucoside

Table 2.2.2.2 Main identified molecules in pomegranate samples

On the contrary, large differences were observed between the phenolic content of the different decoctions, as well as the amount of phenols detected in the juices of the fifteen varieties. The decoctions had greater amount of ellagitannins and a lower concentration of anthocyanins, with only few exceptions represented by those cultivars characterized by a darker skin color as Black, Austin and Soft Seed Maule® 116/17 (Figure 2.2.2.2).
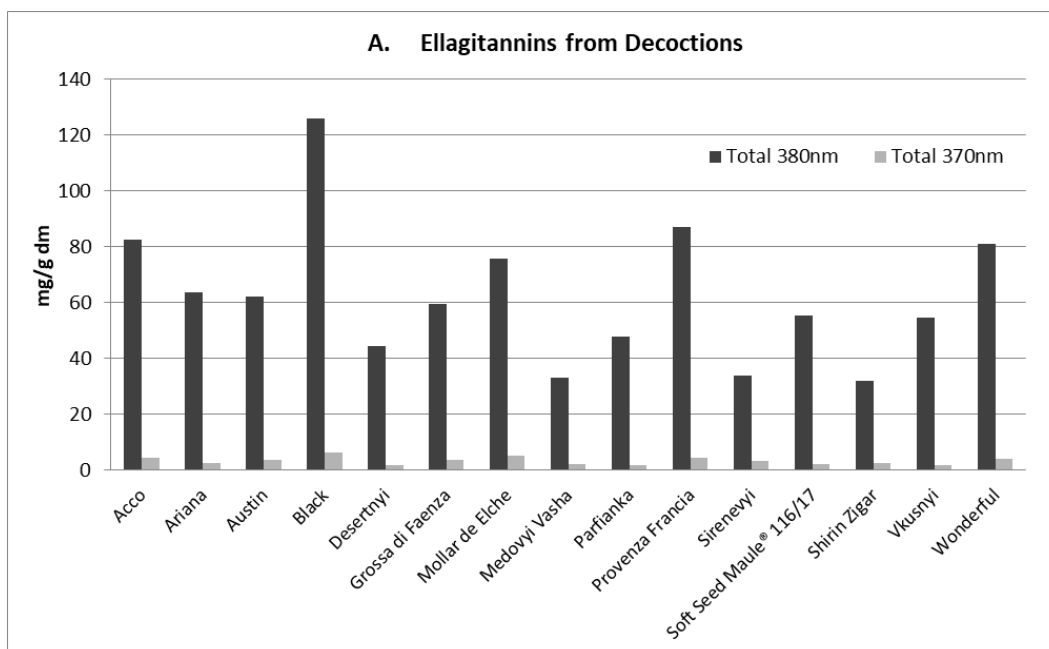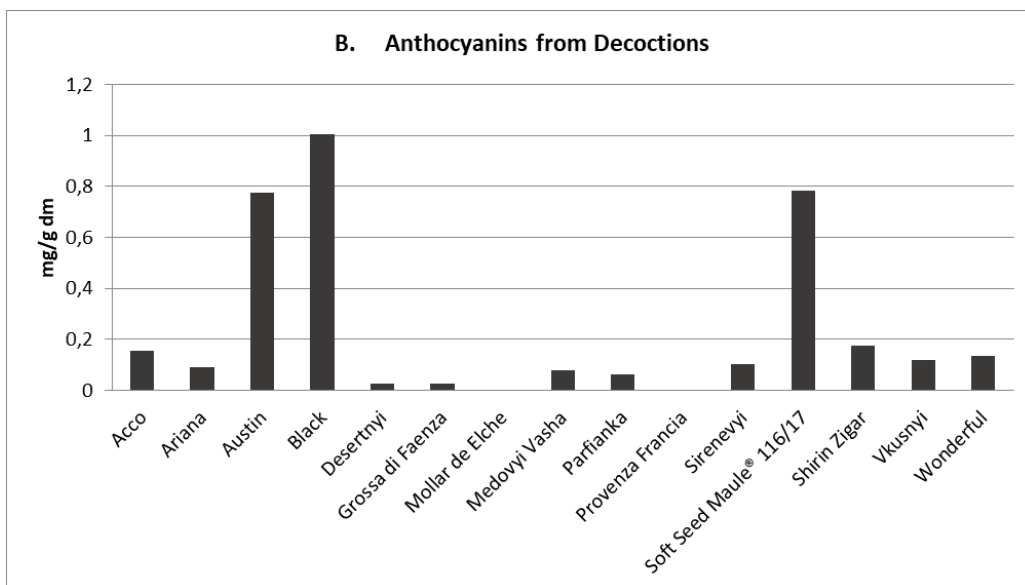
Figure 2.2.2.2 Ellagitannins (**A**) and Anthocyanins (**B**) from decoctions.

The mean RSD value evaluated on the triplicates of three varieties was < 5%

On the other side, juices were poor in ellagitannins and rich in anthocyanins, with the highest content in 116/117 sample and in Wonderful, both with values close to 600 mg/L. This result is not unexpected because the production of the juices was not obtained by pressing the whole fruit but using only the arils that are not recognized as a rich source of ellagitannins. Black was the unique variety with white arils, therefore characterized by the absence of anthocyanins (Figure 2.2.2.3) but with an appreciable quantity of ellagitannins, about ten times higher than the average value measured for the other varieties. Finally, we must take into account the fact that the ellagitannins in the juices can determine an unpleasant taste when the concentration is too high, due to the interactions with the salivary proteins typical also of other tannins.
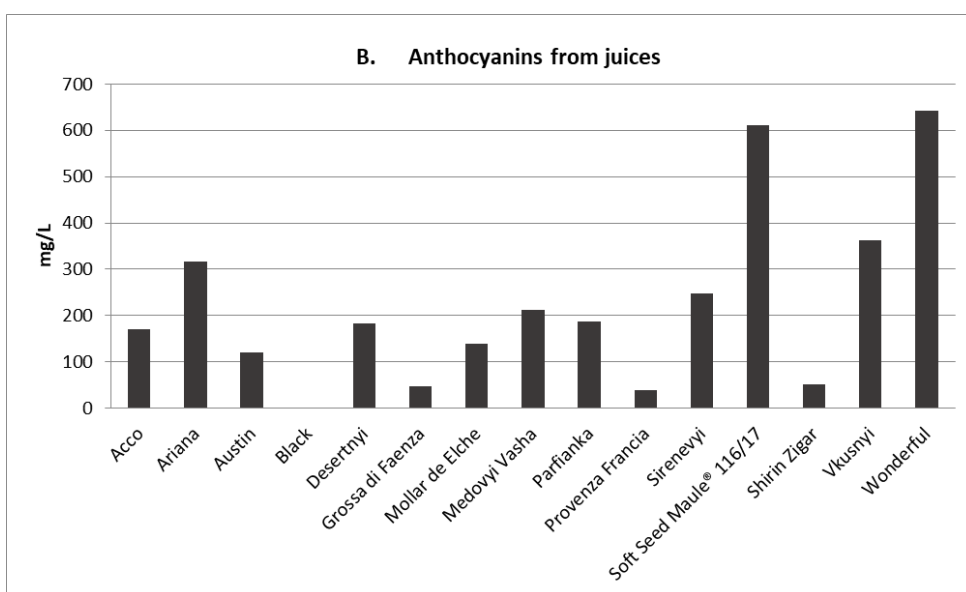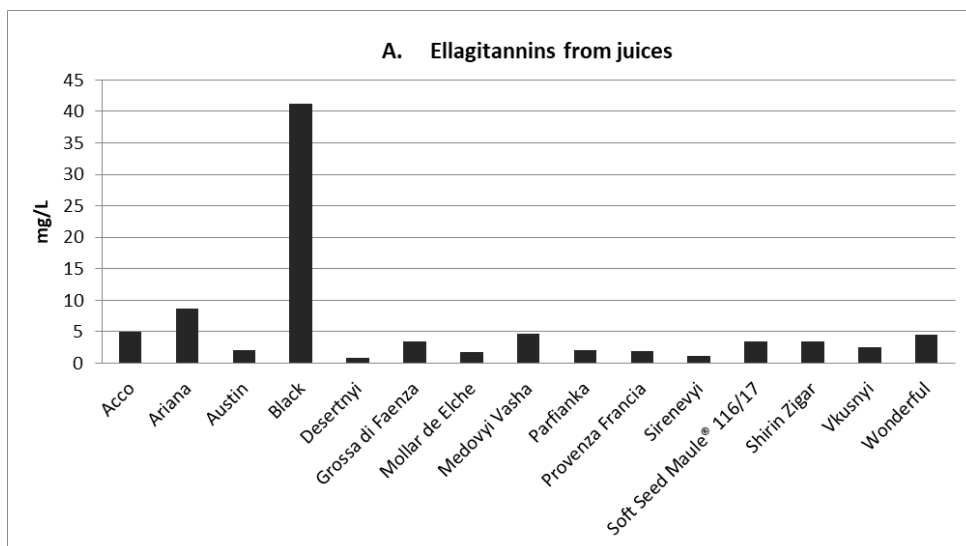
Figure 2.2.2.3 Ellagitannins (**A**) and Anthocyanins (**B**) from juices

Concerning the polysaccharides, in Table 2.2.2.3 are reported for each fractions the % yields on dry peel.

Table 2.2.2.3 Yields in polysaccharides determined after precipitation by ethanol: % of polysaccharides in each sub-fraction (D-P1, D-P2 and D-P3) and

total polysaccharides %.

Fraction	Sample														
	AC	AR	AU	BL	DE	GF	ME	MV	PA	PF	SI	SM	SZ	VK	WO
D-P1 (%)	0.43	0.28	0.60	2.61	0.54	1.80	0.40	1.21	2.02	1.23	0.31	1.09	0.28	0.97	2.00
D-P2 (%)	8.80	8.81	4.60	8.91	3.84	6.18	3.35	2.04	2.38	1.50	6.96	5.73	1.37	5.25	6.26
D-P3 (%)	1.98	1.47	1.65	0.83	2.40	2.34	4.62	4.75	3.48	1.04	1.11	1.54	1.60	1.06	0.60
Total	**11.21**	**10.56**	**6.85**	**12.35**	**6.78**	**10.32**	**8.37**	**8.00**	**7.88**	**3.77**	**8.38**	**8.36**	**3.25**	**7.28**	**8.86**

As reported in Table 2.2.2.3, fractions 2 contained the greatest amount of polysaccharides in almost all varieties, while the sum of the three fractions ranged from 3% to 12% on the dry weight of the skin. A certain variability was found between the samples, with amount in Wonderful comparable to the previous literature ([b]Khatib *et al.,* 2017). Only four samples (Acco, Black, Wonderful and Mollar de Elche) within the fifteen varieties were selected to further investigate on the apparent molecular size of polysaccharides. The choice was determined because the fruits were characterized by the greatest morphological differences, but also because these varieties produce most of the fresh fruits for the market.

Size Exclusion Chromatography and sugar analysis

Preliminary results from SEC indicated an apparent molecular weight of approx. 2 million Dalton for the principal polysaccharides in Acco, Black, Wonderful and Mollar de Elche (Figure 2.2.2.4).
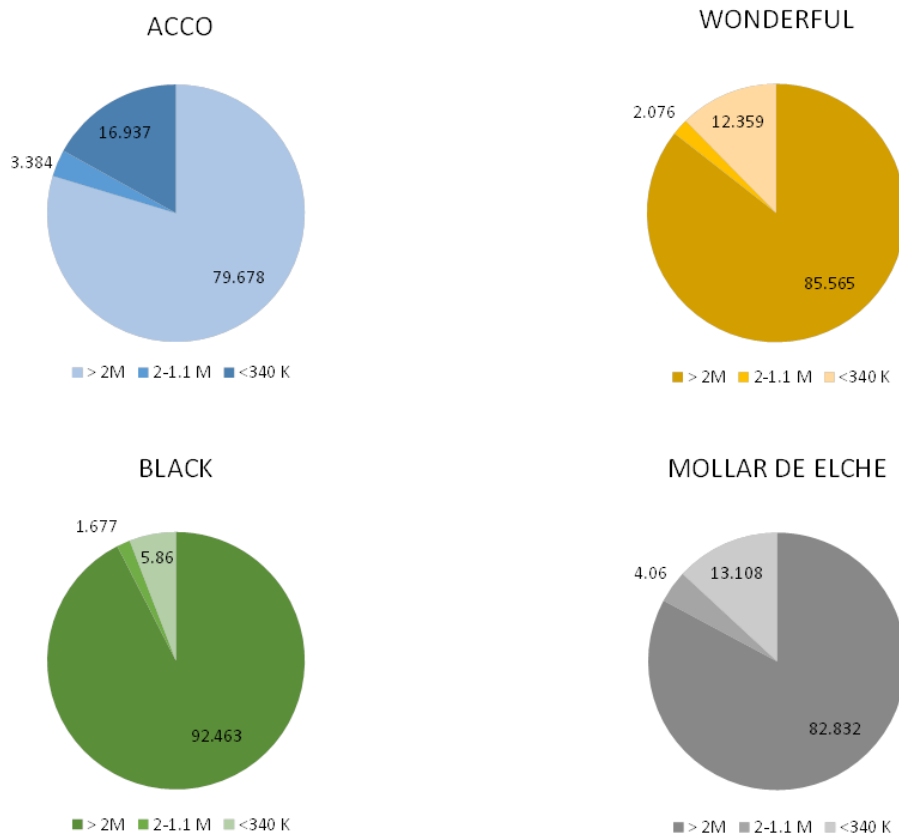
Figure 2.2.2.4 Hydrodynamic volume determined by SEC of total polysaccharides (T) in four varieties: Acco, Wonderful, Black and Mollar de Elche

The presence of high molecular weight polysaccharides as predominant molecules was also confirmed by SEC analysis also for each fraction: low percentages of oligosaccharides which presumably remained trapped in the polymer structure during precipitation after the addition of ethanol were pointed out (Figure 2.2.2.5).
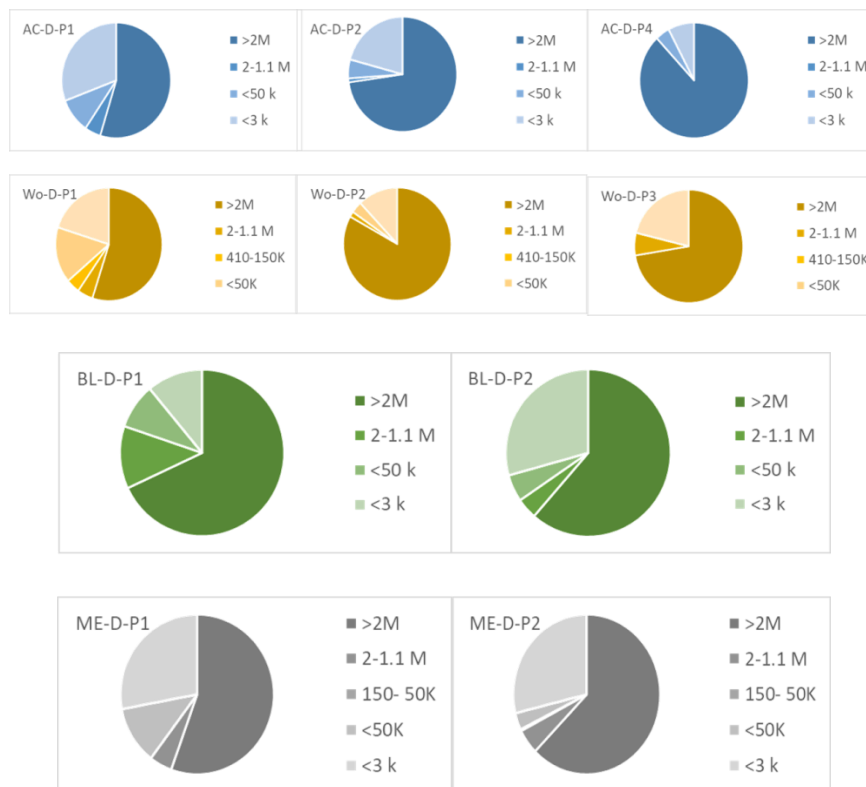
Figure 2.2.2.5 Size Exclusion Chromatography on Acco, Sicily Wonderful, Wonderful, Black, Mollar de Elche fractions D-P1,D-P2, D-P3.

Furthermore, the sugars composition was preliminarily determined only for the main fraction of the Wonderful and Black varieties. Consequently, Fraction 2 of both the varieties was preliminary dialyzed, and then hydrolyzed to determine the neutral sugars and galacturonic acid content. Despite the great morphological differences between the fruits of these two varieties, the results highlighted a similar sugars composition with galacturonic acidic and glucose as main sugars. Galacturonic acid, when reached more than 50% of the total sugar amount can confirm the presence of pectin. Furthermore, the absence of rhamnose allowed to exclude the presence of pectin belonging to the group of rhamnogalacturonan I and II (Figure 2.2.2.6).
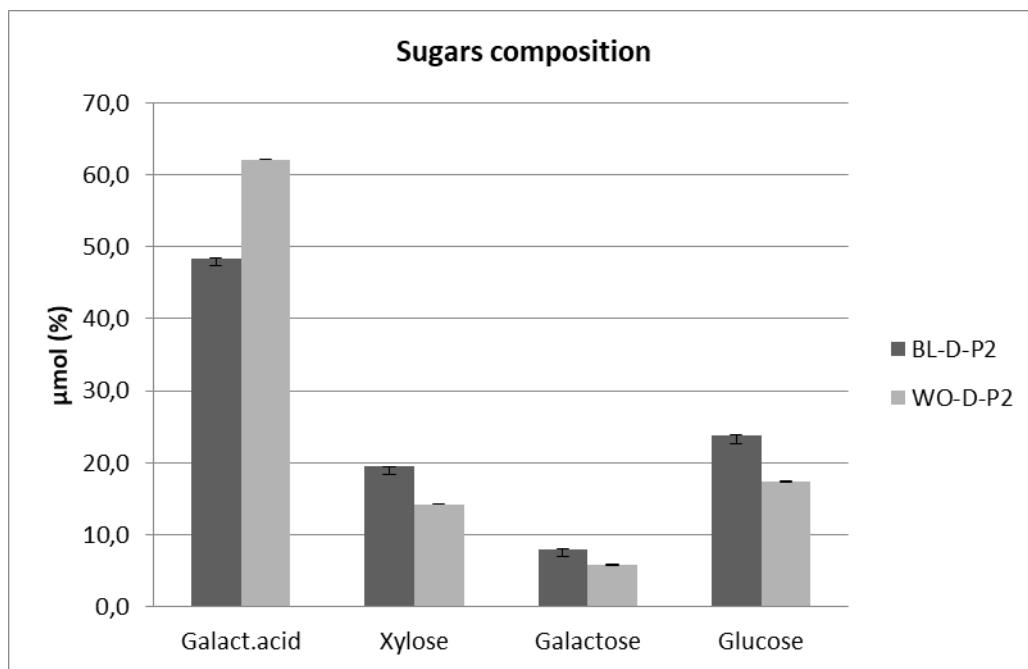
Figure 2.2.2.6 Sugars composition of the main polysaccharide fraction of Black and Wonderful varieties. Data are expressed in μmol% as a mean of a triplicate

This result concerning the sugar composition of the polysaccharide in Fraction 2, although preliminary, suggests that varieties so different in terms of shape and morphological aspect of the fruit, are characterized by the presence of similar pectin structures. Further studies must clarify the chemical structure of pectin in pomegranate fruits and evaluate their properties and suitability for specific technological purposes in the formulation of foods or for cosmetic applications.

Colorimetric CIEL*a*b* analysis

As well known, different pigments deeply characterize pomegranate fruits components. In fact, anthocyanins are contained in arils conferring them a red brilliant color, as well as yellow ellagitannins are represented both in the fruit pulp and peels contributing or determining their color. Historically, natural colorants were extracted from agricultural residues and pomegranate husks, in particular, represented a matrix largely used as natural dying for its good properties of fastness and color strength and depth (Kumar *et al.,* 2011). More recently an increasing and renewed interest was addressed to the pigment content of fruit and vegetables, for its implication in healthy process (Rymbai *et al.,* 2011).

So color, in addition to being the first character that consumers evaluate when they choose any product, represents a real fingerprint of a sum of chemical properties exerted by a foodstuff.

In the present work the pomegranate juices and decoctions, obtained and analyzed as described above, were submitted to colorimetric CIEL*a*b* analysis to evaluate how the different varieties could influence the final expressed color and if some conclusion could be drawn about the correlation among colorimetric and HPLC data. The CIEL*a*b* system is based on three parameters: L* (luminance), a* (greenness for negative values and redness for positive values) and b* (blueness for negative value and yellowness for positive values). The combination between a* and b* values will give the same color saturation (C^*_{ab}) and tonality (h_{ab}) whereas the perceived color will depend also by the expressed luminance.

Results obtained for pomegranate decoction (Table 2.2.2.4) showed L* values between 40.18 and 47.64 in a quite narrow range, whereas a* falls between 0.7 and 10.62 and b* between 2.54 and 23.48, showing significant differences among samples, which color ranges between yellow and brown.

Decoctions	L^*	a^*	b^*	C^*_{ab}	h_{ab}
D-AC	44.67 ± 0.10	1.57 ± 0.03	9.92 ± 0.09	10.04 ± 0.09	81.02 ± 0.10
D-AR	43.96 ± 0.63	2.11 ± 0.38	8.47 ± 0.87	8.73 ± 0.93	76.08 ± 1.03
D-AU	40.29 ± 0.10	7.60 ± 0.21	14.34 ± 0.36	16.23 ± 0.42	62.08 ± 0.10
D-BL	40.18 ± 1.46	2.98 ± 0.64	2.54 ± 0.24	3.92 ± 0.65	40.87 ± 3.56
D-DE	46.37 ± 0.52	1.30 ± 0.38	8.81 ± 1.53	8.90 ± 1.57	81.73 ± 1.13
D-GF	46.92 ± 0.20	0.70 ± 0.12	8.70 ± 0.63	8.73 ± 0.63	85.42 ± 0.45
D-ME	43.51 ± 0.17	6.41 ± 0.19	23.48 ± 0.50	24.34 ± 0.53	74.72 ± 0.13
D-MV	45.71 ± 0.21	1.22 ± 0.18	9.85 ± 0.91	9.93 ± 0.93	82.96 ± 0.39
D-PA	43.94 ± 0.51	4.41 ± 0.22	19.35 ± 0.73	19.84 ± 0.76	77.15 ± 0.16
D-PF	47.64 ± 0.66	1.61 ± 0.21	22.21 ± 1.14	22.27 ± 1.15	85.87 ± 0.32
D-SI	45.11 ± 0.49	5.04 ± 0.34	23.22 ± 1.02	23.76 ± 1.07	77.76 ± 0.30
D-SM	43.55 ± 0.19	10.92 ± 0.07	18.17 ± 0.09	21.20 ± 0.10	58.49 ± 0.45
D-SZ	42.31 ± 0.22	3.62 ± 0.15	14.17 ± 0.45	14.62 ± 0.47	75.66 ± 0.22
D-VK	46.85 ± 1.15	4.69 ± 0.21	21.53 ± 0.53	22.04 ± 0.55	77.72 ± 0.28
D-WO	44.73 ± 0.01	5.81 ± 0.08	19.94 ± 0.18	20.78 ± 0.19	73.74 ± 0.09
Juices	L^*	a^*	b^*	C^*_{ab}	h_{ab}
J-AC	40.91 ± 0.83	2.37 ± 0.18	3.28 ± 0.14	4.05 ± 0.22	54.19 ± 0.98
J-AR	35.02 ± 0.95	4.90 ± 0.23	1.49 ± 0.08	5.12 ± 0.25	16.92 ± 0.21
J-AU	38.28 ± 1.65	4.69 ± 0.69	3.55 ± 0.24	5.89 ± 0.69	37.32 ± 2.17
J-BL	45.96 ± 0.19	4.43 ± 0.10	5.00 ± 0.08	6.68 ± 0.13	48.47 ± 0.28
J-DE	37.02 ± 1.86	11.19 ± 0.96	1.65 ± 0.07	11.32 ± 0.96	8.44 ± 0.55
J-GF	49.31 ± 0.10	6.20 ± 0.10	5.51 ± 0.09	8.30 ± 0.14	41.60 ± 0.17
J-ME	35.02 ± 0.11	4.84 ± 0.13	4.77 ± 0.12	6.76 ± 0.14	44.35 ± 0.34
J-MV	39.47 ± 0.69	4.94 ± 0.34	1.37 ± 0.05	5.12 ± 0.34	15.56 ± 0.69
J-PA	36.53 ± 1.22	8.12 ± 0.77	1.77 ± 0.04	8.31 ± 0.76	12.36 ± 0.85
J-PF	44.65 ± 0.13	10.19 ± 0.14	5.79 ± 0.10	11.72 ± 0.17	29.63 ± 0.14
J-SI	47.34 ± 0.98	4.33 ± 0.54	2.04 ± 0.11	4.79 ± 0.49	25.46 ± 2.84
J-SM	29.74 ± 0.29	13.76 ± 0.35	2.57 ± 0.21	14.00 ± 0.38	10.56 ± 0.59
J-SZ	38.40 ± 1.28	2.55 ± 0.37	4.23 ± 0.34	4.94 ± 0.48	59.98 ± 1.73
J-VK	30.86 ± 0.13	8.02 ± 0.08	1.28 ± 0.08	8.13 ± 0.08	9.04 ± 0.48
J-WO	42.05 ± 1.63	6.98 ± 0.91	1.65 ± 0.09	7.18 ± 0.91	13.38 ± 0.84

Table 2.2.2.4 Colorimetric data from CIEL*a*b* measurement of the pomegranate juices and decoctions

Actually, it is not possible to match the obtained colorimetric data with those deriving from the yellow ellagitannins quantification, both for the presence of a residue red component, which should be considered. A partial simplification could come from the sample filtration

before performing the colorimetric analysis, here not adopted. Anyway, this step is not considered sufficient to explain the complexity of the obtained data.

On the contrary, with regards to data monitored for juices (Table 2.2.2.4) L* values fall between 29.74 and 47.34, denoting a very wide range of lightness, maybe not only ascribable to contribute of anthocyanins and ellagitannins, but also accounting for the sample turbidity. Anyway, the smallest L* value corresponds to the highest a* found. This second parameter, which ranges between 2.37 and 13.76, shows very high differences in the redness of the analyzed juices. Finally, the b* parameters falls between 1.28 and 5,79. So, when the ratio a*/b* is higher than 4 (DE, VK, SN, PA, WO), samples are perceived as basically red, when it is about 1-2 the perceived color is a red-orange and for ratio a*/b*<1 it appears orange-yellow. The attempt to match these results to the quantitative data obtained for anthocyanins and ellagitannins, returns a discrete correlation between h_{ab} and the ratio delphinidin/cyanidin expressed as sum of all the quantified glycosides (Figure 2.2.2.7).
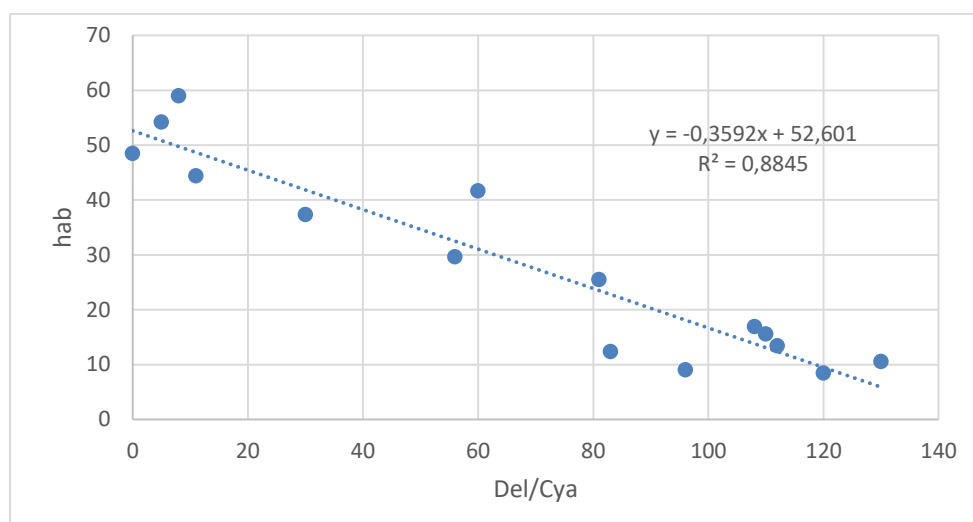

Figure 2.2.2.7 Total anthocyanins content in the fresh juices; the data are expressed as mean of three independent measurements

To our best knowledge, only some papers were published about the use of pomegranate husks as natural mordant for dying (Rather *et al.,* 2016; Mahmud-Ali *et al.,* (2012) and some other papers deal with the CIEL*a*b* analysis of pomegranate fruit, external peels and arils (Todaro *et al.,* 2016) whereas no literature is available regarding the CIEL*a*b* analysis of pomegranate decoction. If our previous results (Cesa *et al.,* 2017; Patsilinakos *et al.,* 2018) indicated that a correlation could be found between CIEL*a*b* parameters and bioactive compounds content of blueberry and goji berries, in which one only color component largely predominates, further studies are needed to better clarify if this fast, chip and simple method could be used to predict the quali-quantitative content of bioactive molecules in juices and decoctions obtained by pomegranate. In these juices, in fact, two different components contribute in a quite different manner to the color development, whereas in decoctions, browning and precipitation processes could take place during the sample storage.

Spectrophotometric determinations on juices and decoction

Collectively, decoction of pomegranate samples led to a high recovery of phenolic and flavonoid compounds compared to pomegranate juices. Values for TPC (Total Phenolic Content) and TFC (Total Flavonoid Content) reported as mg of gallic acid equivalents (mgGAE) or rutin equivalents (mgRE) per gram of extract ranged from 106.34 ± 0.42 to 263.05 ± 3.64 mgGAE/g after decoction of these varieties, whereas those of TFC varied from 18.63 ± 0.20 to 93.97 ± 0.39 mgRE/g. D-MV was the pomegranate decoction with both the lowest results registered. Conversely, the pomegranate decoctions of the other varieties characterized by high values of TPC also displayed comparable values of TFC.

Pomegranate juices showed TPC and TFC data about 20- and 300-fold inferior than decoctions. This trend was confirmed by their low antioxidant and chelating properties as

assessed by six in vitro spectrophotometric assays. On the contrary, each decoction, disregarding the variety, possessed better antioxidant and chelating abilities with respect to juices being D-MV the less interesting and D-SI the most potent. The latter was also characterized by high TPC and TFC values. Moreover, it can be extrapolated that pomegranate juices had limited anti-oxidative characteristics directly related to their low amount of phenols. Also in this case, J-MV was one of the poorest varieties and J-SI was one of the best-in-class extracts. This general trend could account for the relatively inferior stability of this processed food (Table 2.2.2.5).

Decoctions	Total phenolic content (mgGAE/g)	Total flavonoid content (mgRE/g)	DPPH (mmolTE/g)	ABTS (mmolTE/g)	CUPRAC (mmolTE/g)	FRAP (mmolTE/g)	Metal chelating (mgEDTAE/g)	Phosphomolybdenum (mmolTE)
D-AC	259.14±0.47	93.97±0.39	3.91±0.01	4.79±0.10	7.63±0.30	6.93±0.10	31.22±1.93	5.79±0.13
D-AR	213.22±1.45	61.15±0.07	3.68±0.04	3.45±0.01	5.24±0.04	4.60±0.02	13.07±2.81	4.31±0.26
D-AU	242.79±1.81	66.51±0.72	3.88±0.01	4.26±0.23	6.03±0.013	5.38±0.06	21.08±0.90	5.30±0.13
D-BL	254.91±1.19	80.53±1.52	3.89±0.01	4.78±0.14	7.33±0.09	6.16±0.09	16.61±2.64	6.53±0.18
D-DE	231.19±1.40	52.11±0.30	3.82±0.01	4.31±0.12	5.90±0.10	4.73±0.04	16.24±1.95	5.35±0.17
D-GF	251.84±0.30	77.32±0.61	3.89±0.01	4.73±0.19	7.13±0.05	5.97±0.08	17.58±4.37	6.28±0.25
D-ME	258.26±4.03	74.22±0.50	3.88±0.02	4.84±0.10	7.83±0.04	6.17±0.08	34.69±0.96	7.32±0.19
D-MV	106.34±0.42	18.63±0.20	1.46±0.01	1.41±0.06	2.08±0.02	3.19±0.01	20.71±1.82	4.20±0.18
D-PA	129.27±0.41	39.87±0.71	1.43±0.02	1.37±0.11	2.57±0.03	2.58±0.03	2.98±0.16	3.65±0.15
D-PF	226.43±1.37	52.67±0.16	3.74±0.02	3.49±0.30	5.72±0.01	4.44±0.13	15.88±2.65	5.48±0.11
D-SI	263.05±3.64	83.63±0.21	3.89±0.01	4.61±0.16	8.09±0.10	6.75±0.08	28.24±1.19	7.78±0.16
D-SM	148.14±2.14	31.69±0.06	1.83±0.06	1.43±0.18	3.01±0.05	2.49±0.01	10.27±0.81	3.60±0.11
D-SZ	233.75±1.66	52.61±0.71	3.83±0.02	4.20±0.11	6.20±0.07	4.64±0.04	16.50±2.80	5.08±0.11
D-VK	147.92±0.51	36.05±0.42	1.91±0.01	2.05±0.07	3.06±0.02	2.32±0.01	12.54±3.20	3.54±0.12
D-WO	248.93±2.98	61.42±0.45	3.91±0.01	4.02±0.31	7.30±0.21	5.71±0.08	14.16±0.43	6.44±0.06

Juices	Total phenolic content (mgGAE/g)	Total flavonoid content (mgRE/g)	DPPH (mmolTE/g)	ABTS (mmolTE/g)	CUPRAC (mmolTE/g)	FRAP (mmolTE/g)	Metal chelating (mgEDTAE/g)	Phosphomolybdenum (mmolTE)
J-AC	11.56±0.01	0.38±0.04	0.05±0.01	0.09±0.01	0.20±0.01	0.13±0.01	4.04±0.89	1.15±0.07
J-AR	16.30±0.17	0.52±0.08	0.12±0.01	0.14±0.01	0.26±0.01	0.24±0.01	na	1.06±0.03
J-AU	18.01±0.07	0.36±0.04	0.11±0.01	0.17±0.01	0.28±0.01	0.23±0.01	3.14±0.08	0.88±0.13
J-BL	14.77±0.019	0.63±0.02	0.15±0.01	0.14±0.01	0.27±0.01	0.34±0.01	na	0.28±0.03
J-DE	17.83±0.01	0.61±0.02	0.13±0.01	0.15±0.01	0.29±0.01	0.28±0.01	4.89±0.34	0.71±0.08
J-GF	18.44±0.16	0.32±0.05	0.07±0.01	0.11±0.01	0.26±0.01	0.21±0.01	2.13±0.45	0.88±0.05
J-ME	8.20±0.06	0.22±0.01	0.02±0.01	0.05±0.01	0.16±0.01	0.10±0.01	1.71±0.30	1.55±0.16
J-MV	12.73±0.08	0.32±0.07	0.07±0.01	0.10±0.01	0.22±0.01	0.16±0.01	2.48±0.02	1.46±0.02
J-PA	14.88±0.05	0.33±0.03	0.10±0.01	0.13±0.01	0.26±0.01	0.22±0.01	4.40±0.46	1.04±0.09
J-PF	14.06±0.52	0.44±0.04	0.12±0.01	0.12±0.01	0.24±0.01	0.24±0.01	4.27±1.25	0.59±0.04
J-SI	13.88±0.08	0.34±0.07	0.09±0.01	0.12±0.01	0.24±0.01	0.15±0.01	5.69±0.63	1.34±0.04
J-SM	12.66±0.13	0.33±0.08	0.10±0.01	0.11±0.01	0.23±0.01	0.17±0.01	na	0.83±0.03
J-SZ	16.52±0.08	0.44±0.03	0.12±0.01	0.16±0.01	0.28±0.01	0.19±0.01	19.67±1.34	0.48±0.02
J-VK	23.54±0.22	0.28±0.09	0.22±0.01	0.20±0.01	0.37±0.01	0.28±0.01	3.15±0.05	0.86±0.05
J-WO	18.31±0.23	0.35±0.02	0.20±0.01	0.18±0.01	0.31±0.01	0.27±0.01	4.17±0.71	1.05±0.07

Table 2.2.2.5 Total Phenolic content (TPC), Total Flavonoid content (TFC), DPPH, ABTS, CUPRAC, FRAP, Metal chelating and Phosphomolibdenum of all juices and decoction,

*Values are reported mean ± SD of three parallel experiments. GAE: Gallic acid equivalent; RE: Rutin equivalent; TE: Trolox equivalent; EDTAE: EDTA equivalent; na: not active

Enzymatic assays

Analyzing the data regarding tyrosinase inhibition (expressed in terms of mg of kojic acid equivalents for gram of extract) it is possible to confirm the better potency of decoctions over juices; it should be noted that the D-MV sample, previously described as a low source of phenols and flavonoids, proved to be the most powerful among the juices. Pomegranate decoctions displayed tyrosinase inhibition spanning from 24.44 ± 1.45 to 118.50 ± 0.81 mgKAE/g, whereas pomegranate juices hardly reached 16.56 ± 0.85 mgKAE/g, being half of the considered varieties not active against this enzyme. These results highlighted the presence of other bioactive components beyond ellagitannins to exert such an inhibitory activity of decoction (Table 2.2.2.6).

The inhibitory data against α-amylase, a key enzyme for the availability of diet glucose, were almost comparable among the pomegranate varieties for both decoctions and juices. Only slight differences have been highlighted, suggesting that pomegranate fruits of different varieties can be promising sources of functional ingredients characterized by a healthy panel of chemical and biological characteristics.

Decoctions	α-Amylase inhibition (mgACAE/g)	Tyrosinase inhibition (mgKAE/g)
D-AC	59.17±0.79	51.12±1.59
D-AR	74.49±0.63	40.99±1.46
D-AU	77.41±5.94	54.03±0.32
D-BL	63.73±3.44	76.24±1.25
D-DE	67.85±1.66	62.86±0.45
D-GF	75.10±1.22	53.54±1.54
D-ME	77.15±0.52	79.76±0.81
D-MV	57.95±0.49	118.50±0.81
D-PS	77.25±0.66	28.71±1.92
D-PF	56.70±0.90	48.70±1.06
D-SI	75.98±3.40	86.06±0.39
D-SN	54.97±0.78	28.83±1.52
D-SZ	76.88±0.40	57.53±2.51
D-VK	52.95±0.41	24.44±1.45
D-WO	56.64±0.14	70.80±2.31
Juices	α-Amylase inhibition (mgACAE/g)	Tyrosinase inhibition (mgKAE/g)
J-AC	45.17±0.62	na
J-AR	45.20±0.86	na
J-AU	47.14±0.78	na
J-BL	133.64±14.06	na
J-DE	51.23±0.65	na
J-GF	49.50±1.54	na
J-ME	43.47±0.39	na
J-MV	47.24±1.21	na
J-PA	48.39±1.12	4.86±0.56
J-PF	53.06±1.15	7.63±1.37
J-SI	44.85±0.14	8.41±1.13
J-SN	51.33±1.04	6.85±1.16
J-SZ	54.39±1.41	7.35±1.46
J-VK	77.09±0.96	16.56±0.85
J-WO	51.07±0.55	5.87±0.70

Table 2.2.2.6 Amylase and Tyrosinase inhibition of both juices and decoction

[*]Values are reported mean ± SD of three parallel experiments. ACAE: Acarbose equivalent; KAE: Kojic acid equivalent; na: not active

2.2.2.4 Conclusions

This work compared for the first time fifteen varieties of pomegranate obtained from the same hatchery, having fruits with very different morphological characters, with some of them never studied so far. The work compared the chemical composition in terms of ellagitannins and anthocyanins detected in juices and peel and the content of polysaccharides in peel. The chromatographic profiles, both of ellagitannins and anthocyanins, resulted qualitatively very similar and, in some cases, almost superimposable. At the same time, according to the noticeable morphological differences among the selected varieties, a wide variability in terms of total content of ellagitannins, anthocyanins and polysaccharides was highlighted. The reflectance colorimetry resulted suitable to analyze the juices as such, showing a good correlation with the ratio sum delphinidin glycosides/sum cyanidin glycosides evaluated by HPLC-DAD. Concerning the polysaccharides, it was proposed and applied a fractionation method to collect three main polysaccharide fractions from each sample. The preliminary analysis by size exclusion chromatography applied to four varieties having very different morphological characters, pointed out in all the sample a large predominance of polysaccharides at high apparent molecular weight close to two million Dalton. Some of the varieties with very different morphological characteristics showed polysaccharides with similar hydrodynamic volume (apparent molecular weight). Overall, the quantitative results indicated a wide variability in terms of total phenols and polysaccharides amount, which was also confirmed by the colorimetric analysis. The total polyphenolic content was low in pomegranate juices and consistently higher in the decoctions. Applying different *in vitro* methods, it was possible to acquire a more complete description of the antioxidant potency of juices and decoctions. Regarding the enzymatic tests, the anti-alfa-amylase activity was quite

comparable among all the samples, while decoctions were generally much more active as anti-

tyrosinase agents.

2.2.3 An optimized fractionation process to recover and investigate on the polysaccharides from pomegranate fruits by-products of the Wonderful and Purple Queen® varieties

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Unpublished results

Abstract

Polysaccharides recovered from by-products of two widely cultivated pomegranate varieties, namely Wonderful and Purple Queen®, were studied in this research. Polysaccharides were extracted from fruits' peel by decoction with hot water and fractionated by precipitation with ethanol in order to provide different fractions of polysaccharides in a green and easy way, thus obtaining three fractions for each sample. Polysaccharides' yield was higher for the Purple Queen® sample (up to 648.9 mg/5g), with fraction 2 as the most abundant one for all samples. The different fractions were characterized by size exclusion chromatography (SEC), light scattering (LS), ^{1}H-NMR, determination of the simple sugars and methylation analysis. The SEC analysis, carried out on the dialyzed fractions, showed the presence of structures slightly higher than commercial pectin, while LS analysis allowed differentiating between the molecular sizes of the different fractions. ^{1}H-NMR allowed excluding the presence of significant amounts of rhamnogalacturonans, proteins and phenolic compounds; the same analysis, carried out after dialysis, highlighted a high level of the methoxyl groups typically linked to glucuronic acid in pectin with a high degree of methylation, and lower levels of *O*-acetyl groups, with fractions from Apulian Wonderful sample showing different profiles. Simple sugar analysis showed that galacturonic acid was approx. 50% in all Fractions 3,

confirming the presence of pectin; the high xylose content also pointed out the presence of xylogalacturonan in addition to homogalacturonan, while methylation analysis allowed pointing out the presence of Arabinoxylan, Xyloglucan and Arabinogalactan, and the presence of higher content of cellulose in the Purple Queen sample than in the Wonderful samples

2.2.3.1 State of the art and aims of the work

The interest toward polysaccharides from pomegranate (*Punica granatum* L.) fruit has strongly increased in the last years: they can be included among the bioactive components of the fruit along with phenolic compounds (Joseph *et al.,* 2013; Varghese *et al.,* 2017; Wu *et al.,* 2019). Amounts of polysaccharides range from 10% to 12% on the weight of dried mesocarp ([b]Khatib *et al.,* 2017), while, according to other authors, the amounts of total fiber in 12 Tunisian varieties range from 40% to 60% on the weight of dry peel (Hasnaoui *et al.,* 2014). So far, the chemical features of the polysaccharides in the peel have not been fully explored, but most of the authors reported pectin as the main constituent (Güzel *et al.,*2019; Abid *et al.,* 2016; [b]Khatib *et al.,* 2017; Abid *et al.,* 2017; Shakhmatov *et al.,* 2019; Pereira *et al.,* 2016). Pectins consist of a complex mixture of macromolecules with different structures and chemical-physical properties, such as molecular weight, gelling ability and water solubility, which are strongly associated to the applied extraction method, as summarized in a recent review (Dranca *et al.,* 2018). The authors of this review also reported the application of green extraction using hot water to efficiently recover the greatest part of pectin from orange sour and durian rinds. Even in the case of pomegranate, hot water was recently proposed to extract pectin from the peel (Shakhmatov *et al.,* 2019). At the same time, it was demonstrated that the boiling treatment did not affect the polysaccharide profile, particularly the hydrodynamic volume associated to the apparent molecular weight determined by size exclusion chromatography ([b]Khatib *et al*., 2017). It has recently been emphasized that pectin has a multiplicity of structures (Chan *et al.,* 2017; Dranca *et al.,* 2018). More in general, knowledge on the composition and structure of pomegranate's polysaccharides is so far limited, particularly when related to a specific variety. Even a recent study, focusing on the

determination of the chemical structure of pectin extracted from pomegranate, was applied to a sample obtained from commercial fruits of unknown variety (Shakhmatov *et al.,* 2019).

Nowadays the pomegranate peel is recognized as one of the most valuable by-product of food industry, but it needs to be further investigated regarding the polysaccharide content. The collection of useful information on the structural characteristics of polysaccharides such as pectin requires the application of different analytical methods, many of which require long procedures. (Dranca *et al.,* 2018). To improve the knowledge on natural polysaccharides, the possibility to identify and apply more rapid analytical procedures is strongly required.

So far, the Wonderful variety constitutes the greatest production in North and South America, as well as in Israel, or in Mediterranean countries as Italy and Greece. It is characterized by a high productivity associated with an adequate average fruit size and a pleasant taste of the juice with a sweet/sour note that is widely appreciated (Hussein *et al.,* 2018). Recently, the new Purple Queen® variety, originated in the South of Spain, has been proposed on the market; it is appreciated for its productivity and early ripening time (half of August) and currently requested for registration outside the European Union (Melgarejo *et al.,* 2019). These peculiarities of the Purple Queen® variety are functional for the market since they broaden the availability of the fruits both for fresh consumption and juice production contributing to supply the fruit demand, increased over these last years.

The aim of this research was to study the polysaccharides of two widely cultivated pomegranate varieties, Wonderful and Purple Queen®. Decoction was applied as a green method to extract these compounds from the peel, then they were fractionated by precipitation with different amounts of ethanol. This latter step aimed at proposing a simple method able to provide fractions with different polysaccharides composition. The chemical investigation on the collected fractions was carried out by means of size exclusion chromatography (SEC),

light scattering (LS), ^{1}H-NMR and determination of the simple sugars. As far as we know, this approach was applied for the first time to polysaccharides extracted from the peel of pomegranate of Wonderful and Purple Queen® varieties.

2.2.3.2 Materials and methods

Fruits

Three batches of fruits (approx. 4 kg each) of pomegranate of the varieties Wonderful and Purple Queen® were purchased from different producers in the same year (2018). Wonderful variety was purchased from different geographical areas of Italy: Apulia (code WoA) and Sicily (code WoS, Marsala (TP), Italy); Purple Queen® variety was purchased from Murcia, Spain (code PQS).

Standards and reagents

All solvents of analytical HPLC grade were from Sigma Aldrich (St. Louis, Missouri, USA). Ultrapure water was obtained by the Milli-Q-system (Millipore SA. Molsheim. France). Dextrans at different molecular weights (2000, 1100, 410, 150 and 50 KDa) and sucrose (342 Da) used for the SEC analysis were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The commercial pectin at different degree of esterification (55-70% and >85%) were purchased from Merck. The dialysis kit (Spectra/Pro) 12-14 KD was purchased from Spectrum Laboratories, Inc. (Breda, The Netherlands).

Extraction and fractionation of polysaccharides

The peel (mesocarp + exocarp) of the fruits was separated from arils and used to prepare a decoction. Briefly, the freeze-dried pomegranate peel was boiled in water (5g in 200 mL) for 1h. The supernatant was recovered after cooling in ice bath and centrifugation (5000 rpm, 15 min, 4°C), then distilled water was added to reach a total volume of 200 mL of decoction ([b]Khatib *et al.,* 2017). Hot water was chosen as extractive medium with the aim of recovering the most soluble fraction of fiber, and reducing the co-presence of insoluble fiber, as cellulose and lignin.

Preliminarily, the decoction from the WoA sample was used for defining the fractionation method. The sample was dissolved in water, then increasing amounts of ethanol were added, keeping the solutions for 3 h at 0°C. The precipitated fractions obtained from each step were recovered after centrifugation at 5,000 rpm, at 5°C for 15 min.

The fractionation procedure defined in the preliminary trial was then applied to the three decoctions recovered from the peel of WoA, WoS and PQS samples. Different fractions were obtained after 3 consecutive additions of ethanol (100 mL each) in ice bath. The addition of the fourth volume of ethanol (100 mL) did not induced any further precipitation, thus a total of three polysaccharide fractions were collected from each sample. The fractions were freeze-dried, weighted, then dialyzed (cut off 10K-14KD) and freeze-dried again. All the obtained fractions are listed in Table 2.2.3.1.

^{1}H-NMR analyses

The proton spectra were recorded working on the dialyzed fractions dissolved in D_2O at a concentration ranging from 5 to 6 mg/mL; maleic acid was added as internal standard (0.13

mg/mL) for quantitative evaluations applying the protocol previously used for other matrices (Khatib *et al.,* 2016) .

Size Exclusion Chromatography (SEC)

All the freeze-dried samples were dissolved in distilled water at a final concentration of 0.5 mg/ mL. The obtained solutions were analyzed by a ProStar HPLC Chromatograph (Varian USA) equipped with a refractive index detector (mod 355); the two columns PolySep-GFC-P 6000 and 4000 (Phenomenex, USA) connected in series were used according to a previous work (Chamizo *et al.,* 2018). Two columns, PolySep-GFC-P 6000 and PolySep-GFC-P 4000 (700 mm length and 7.8 mm internal diameter) from Phenomenex, USA, were connected in series to obtain separation ranges of 100 kDa - 15 MDa and 0.3- 400 kDa. HPLC-grade water was the eluent and the flow was 0.6 mL min-1; total analysis time, 70 min. Blue-dextrans at various molecular weights (50-2000 KDa) were used as internal standards to determine the hydrodynamic volume.

Size and polydispersity index by Light Scattering (LS)

The particle sizes of the different fractions (F1-F3) of each variety were measured by DLS, Zetasizer Nano series ZS90 (Malvern Instruments, Malvern, UK), with a JDS Uniphase 22 mW He-Ne laser that worked at l=632.8 mm with an optical fiber-based detector and a digital LV/LSE-5003 correlator; the temperature controller (Julabo water bath) set at 25.8 °C (kept constant by a Haake temperature controller). Time correlation functions were analyzed to obtain the hydrodynamic diameter of the polysaccharides and the size distribution (polydispersity index, PdI, or polydispersity, PD) by using the ALV-60X0 software package (Malvern, version 7.2). Autocorrelation functions were analyzed by the Cumulants method by

fitting a single exponential to the correlation function to obtain the size distribution; polydispersity values were calculated for each peak as peak width/mean diameter. Scattering was measured in a borosilicate cell of optical quality, 4 mL volume at 908 angle by diluting the samples in 10 nm NaCl solution. For all the samples, a mean value of three measurements at the stationary level was taken.

Sugar and Linkage analysis

The third fraction of each pomegranate variety was dialysed (cut-off 12–14 kDa), freeze-dried and used for the determination of neutral sugars and linkage analysis according to Nunes *et al.,* (2001). Neutral sugars were determined after acid hydrolysis (H_2SO_4 72% w/w) and conversion to the corresponding alditol acetates. Glycosidic linkage analysis was performed by methylation of the polysaccharides in order to obtain partially methylated alditol acetates that were subsequently analysed by gas chromatography-mass spectrometry (GC–MS). GC analysis was performed using a Hewlett-Packard 5890 with a split injector (split ratio 1:60) and a FID detector. A 25-m column CP-Sil-43 CB (Chrompack, Holland) with 0.15 mm i.d. and 0.20-μm film thickness was used, with the injector and detector operating at 220° C. The following temperature program was used: 180°C for 5 min and 200°C for 20 min, with a rate of 0.5°C/min; linear velocity of the carrier gas (H_2) was set at 50 cm/s at 200°C. Furthermore, uronic acids were colorimetrically determined using *m*-phenylphenol as previously reported (Nunes *et al.,* 2001).

2.2.3.4 Results and Discussion

Preliminary fractionation

Polysaccharide profiles were studied selecting commercial fruits of well-known origin and representative of the major productive areas in Italy and Spain. In particular, Apulia and Sicily regions, as representative geographical areas in Italy for the production of fresh fruits from Wonderful variety, and Alicante and Murcia in the South of Spain to collect fruits of Purple Queen® variety, were selected. Purple Queen® variety is a less known cultivar, increasingly appreciated for its productivity, early ripening time and pleasant tasty (Melgarejo *et al.,* 2019).

A preliminary fractionation process was applied to fruits of the WoA sample to verify if the gradual addition of small amounts of ethanol was useful to recover the main polysaccharides in a partially purified form. This approach allowed us to recover several fractions. Nevertheless, only two of them were obtained in appreciable amounts: P-F5 and P-F7. Both these fractions, analyzed after dialysis by SEC, showed only one peak with the same retention time, indicating the presence of compounds in a range from 1.1 to 2 MDa, (Figure 2.2.3.1). The percentage of the mean peak on the total molecular range (2000 KDa-50KDa), was 89.2% and 91.1% for F5 and F7, respectively, highlighting a high degree of purity of these two fractions.
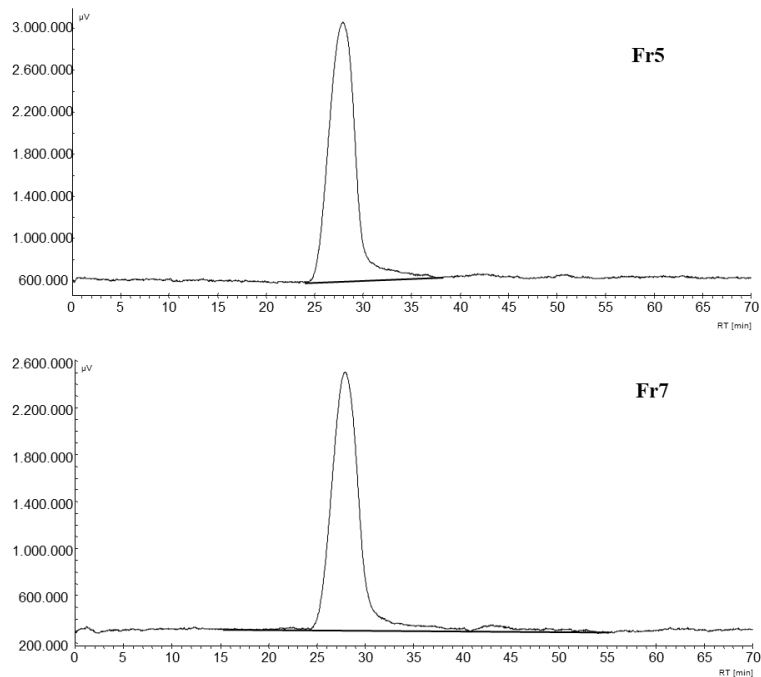
Figure 2.2.3.1 SEC profiles of the main fractions from the preliminary fractionation process.
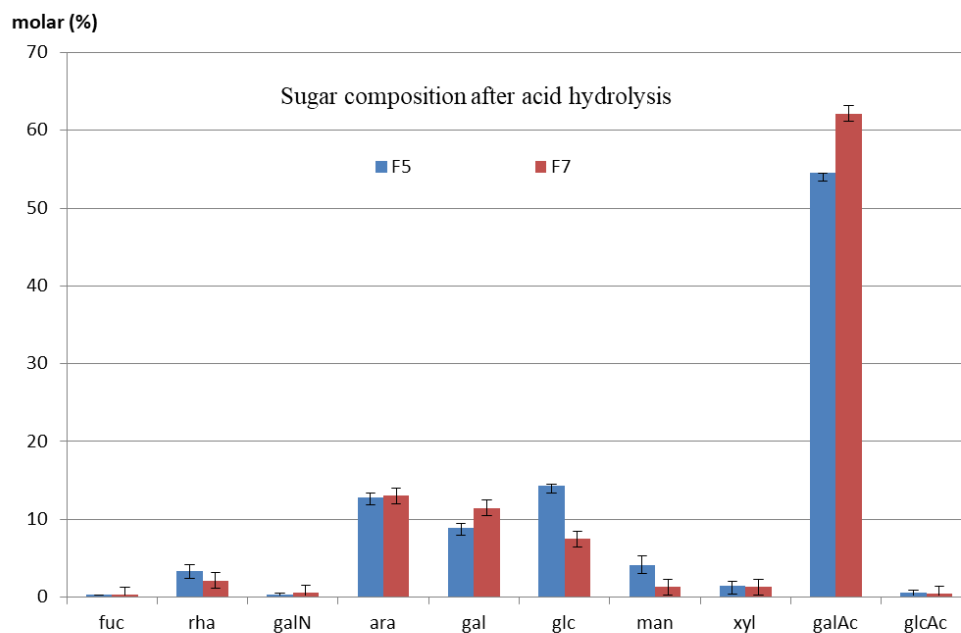

Figure 2.2.3.2 Sugars composition of P-F 5and P-F7 fractions.

Data are expressed in molar % as a mean of triplicate.

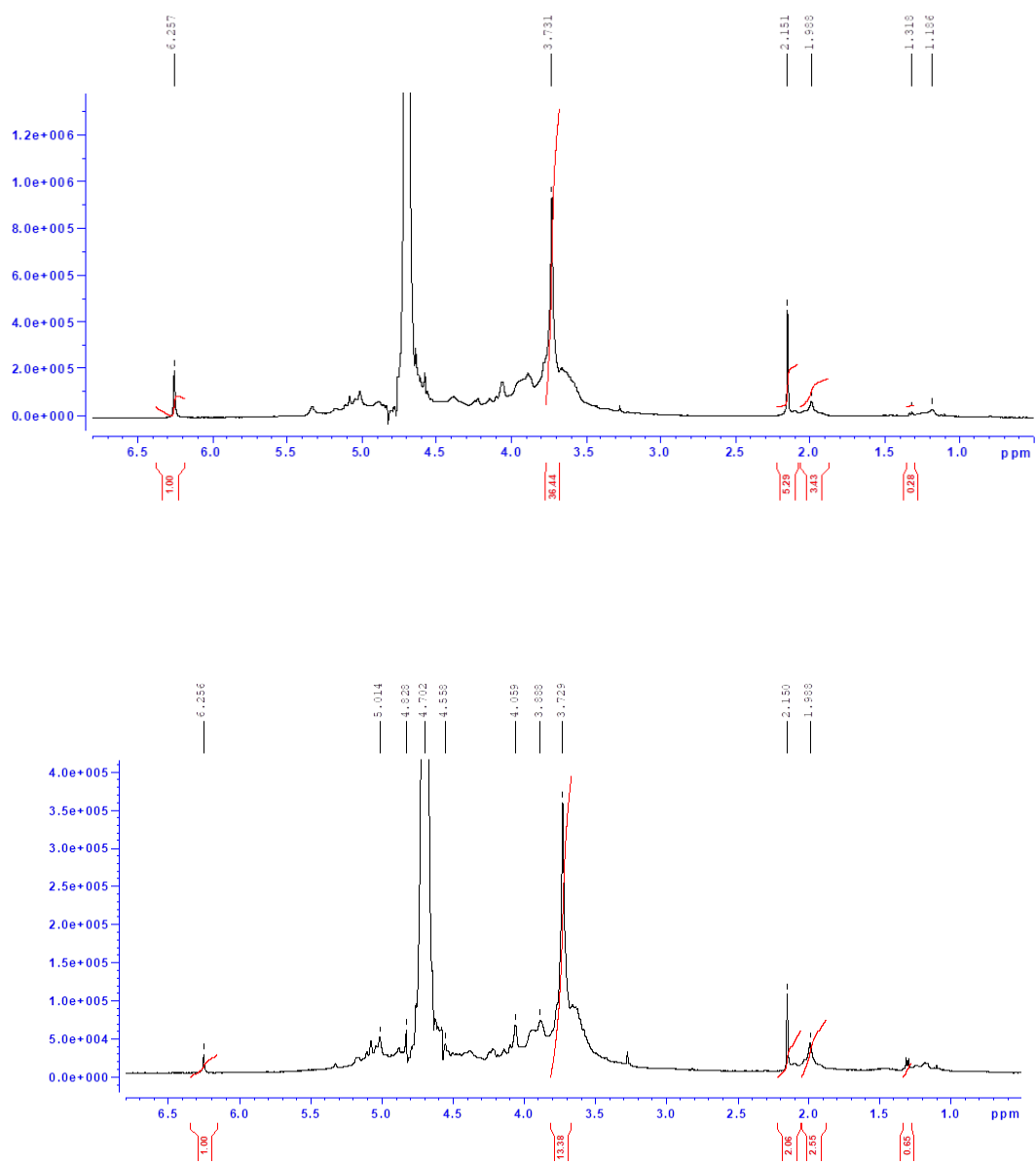Furthermore, both F5 and F7 samples, when analyzed by ^{1}H-NMR showed very similar spectra (Figure 2.2.3.3).

Figure 2.2.3.3 ^{1}H-NMR spectra of the dialyzed P-F5 (**top**) and P-F7 (**down**) fractions.

This finding is in agreement with results obtained from analysis of sugars composition, which showed only slight differences (Figure 2.2.3.2), and a very low intensity of signal at 1.2 ppm in the ^{1}HNMR spectra, indicating the rhamnose as a minor sugar in both the samples. The major sugars as glucose, galactose and arabinose showed similar concentrations, with galacturonic acid reaching percentages higher than 50% of total sugars, confirming both these fractions as rich in pectin, in agreement with previous works on pomegranate fruit ([b]Khatib *et al.,* 2017; Abid *et al.,* 2017; Pereira *et al.,* 2016; Shakhmatov *et al.,* 2019).

Concerning the presence in pomegranate peel of polysaccharides other than pectin, the literature reports the absence, or the presence in very low concentrations of starch (USDA 2011; Gupta *et al.,* 2015), allowing considering as negligible the contribution of starch on the recovered polysaccharide fractions. Moreover, a study on the pomegranate peel of twelve Tunisian varieties reported a mean content of cellulose close to 20% on dry peel and a ratio between soluble and insoluble fiber of approx. 1.0, confirming that the soluble fiber was in a comparable amount with respect to the insoluble one (Hasnaoui *et al.,* 2014). Finally, according to most of the works dealing with polysaccharides of pomegranate, pectin as the main constituent of the soluble fraction of the fiber can be recovered applying different water media, as water acidified by nitric acid (Abid *et al*., 2017), hot water and enzymatic methods (Gavlighi *et al.,* 2018) or simply hot water (Shakhmatov *et al*. ,2019; [b]Khatib *et al*., 2017).

Fractionation process applied to all the samples

After the preliminary analysis, a final simplified fractionation was applied and only three fractions were collected from each decoction by adding increasing volumes of ethanol. In particular, 0.5:1 v:v to obtain the fractions 1 (F1), 1:1 v:v to recover the fraction 2 (F2) and 1:1.5 v:v to collect the fractions 3 (F3). The collected fractions are listed in Table 2.2.3.1 Our

results expressed on dry peel pointed out that the sum of the total water-soluble polysaccharides extracted from the decoction process ranged from 5.8% to 6.5%, with the mean yields for each fraction before dialysis, and their sum after dialysis reported in Table 2.2.3.1. F2 was always the most abundant fraction, and after dialysis similar amounts were found for the Purple Queen and Wonderful Apulia samples, not so different from those reported by Shakhmatov *et al.,* (2019), which worked on fruits of unknown variety using hot water as extractive medium. On the contrary, Wonderful Sicily showed a lower content of total polysaccharides after dialysis with a percentage of 2.8%.

Samples	Acronyms	Yields before dialysis *(mg/5 g dw)*	Yields* after dialysis *(mg/5 g dw)*	SEC (KDalton)	LS size in nm *(major peak)*	LS size in nm *(minor peak)*
Purple Queen Fraction 1	PQS-F1	112.3±24.2		2000	-	-
Purple Queen Fraction 2	PQS-F2	402.3±67.8	260	2000	2099	334
Purple Queen Fraction 3	PQS-F3	134.3±18.5		2000	1290	-
Wonderful Apulia Fraction 1	WoA-F1	111.7±31.8		2000	1438	398
Wonderful Apulia Fraction 2	WoA-F2	226.3±26.8	293	2000	953	-
Wonderful Apulia Fraction 3	WoA-F3	77.0±10.6		2000	1205	-
Wonderful Sicily Fraction 1	WoS-F1	13.0±4.6		2000	995	-
Wonderful Sicily Fraction 2	WoS-F2	230.5±35.9	140	2000	1013	-
Wonderful Sicily Fraction 3	WoS-F3	50.0±14.1		2000	2439	407

Table 2.2.3.1 List of the collected polysaccharide fractions and applied analyses after dialysis (cut off 10KDalton); *the yields are expressed in mg/5 g as a mean value of three independent precipitations.

The chromatographic profiles obtained by SEC for the dialysed fractions showed in all cases only one peak, which according to retention times (24-25 min), suggested the presence of structures with similar hydrodynamic volumes. In Figure 2.2.3.4 the profiles obtained for the F3 fractions are shown as examples.
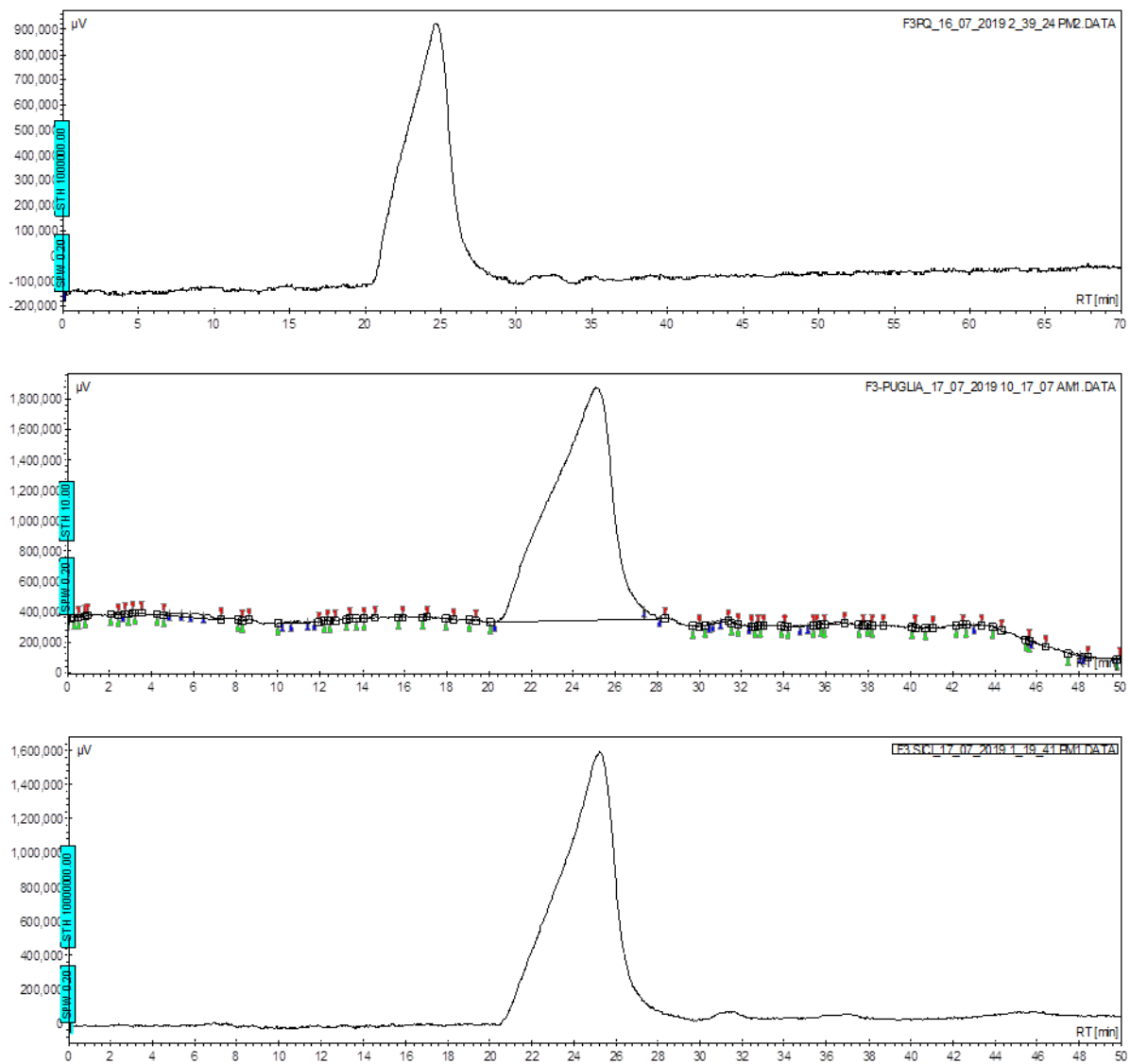

Figure 2.2.3.4 Size Exclusion Chromatography profiles of some fractions from PQS, WoA and WoS (from the top to the bottom).

Noteworthy, both the commercial pectin at different degree of esterification (DE), 55-75% and 85%, showed only one peak in SEC, with retention times of 26 min and 27.2 min respectively. These retention times, higher than those of pomegranate fractions, indicate lower hydrodynamic volumes with respect to those observed for the polysaccharides isolated from pomegranate.
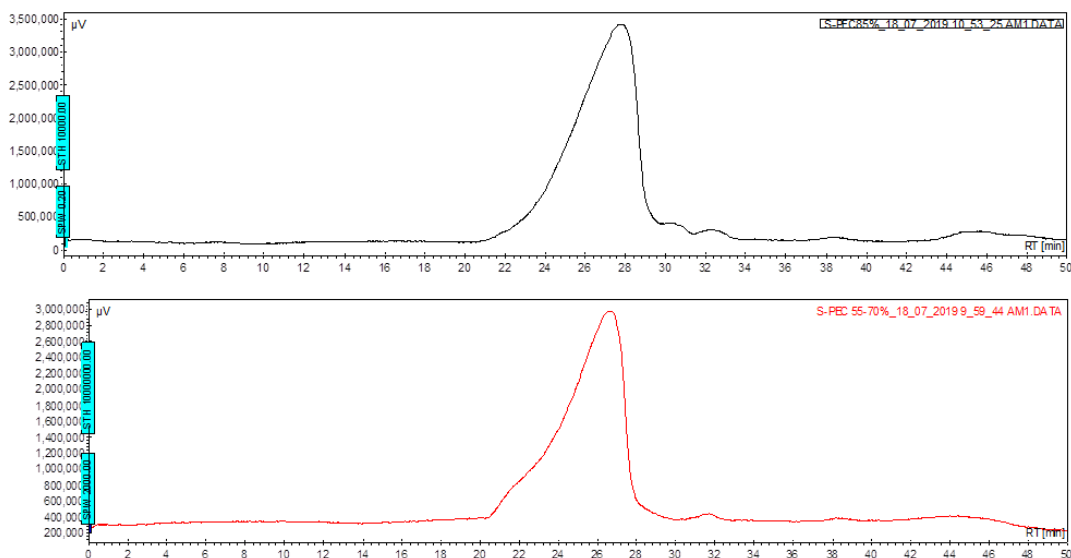

Figure 2.2.3.5 Size Exclusion Chromatography Profiles of two commercial pectin used as reference standards with known degree of esterification (**up**) DE> 85% ; (**down**) 55% <DE <70%

Despite the ability to discriminate between the commercial pectin, the applied SEC method was not able to highlight the differences among the fractions isolated from these pomegranate fruits. Consequently, a further investigation on the molecular size and shape of these molecules was carried out by light scattering (LS) technique, which proved to be suitable for highlighting the differences between the structures of the isolated fractions (Table 2.2.3.1 and Figure 2.2.3.6).

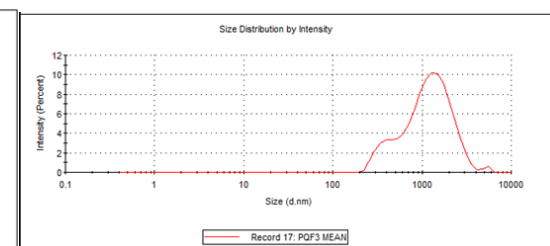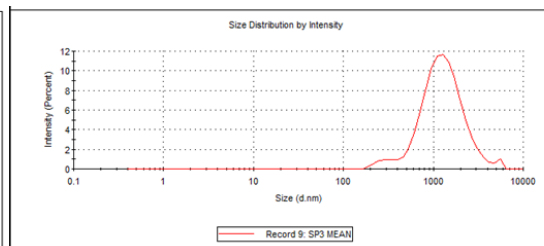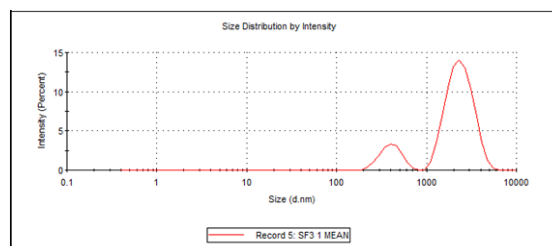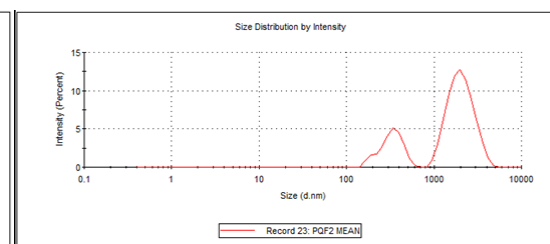**WoS**   **WoA**   **PQS**
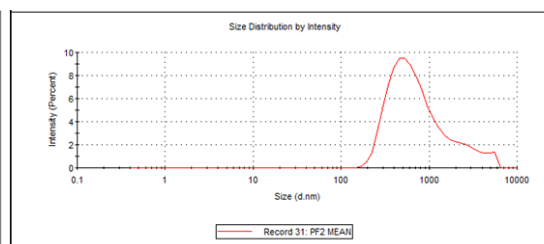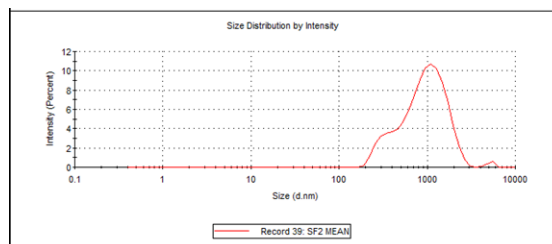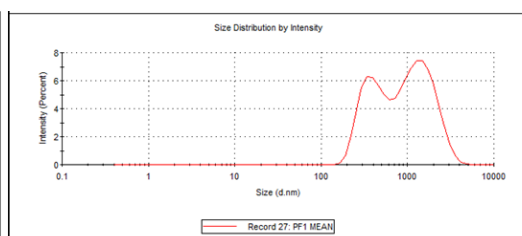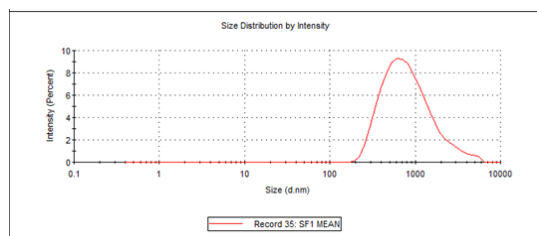
Figure 2.2.3.6 Light scattering results concerning the fractions isolated from Wonderful Sicily (WoS), Wonderful Apulia (WoA), and Purple Queen from Spain (PQS).

Concerning PQS-F2, the LS profile presented one main peak with a size of 2099 nm (76 %) and a minor peak with a lower size of 334 nm (24 %), while PQS-F3 presented a lower size with a value of 1290 nm. According to Figure 2.2.3.6 concerning WoA, similar mean sizes from 953 nm to 1438 nm were obtained for the three fractions.Again, for two WoS fractions (F1 and F2) it was possible to find a similar size close to 1000 nm, while F3 fraction presented a major component (86 %) with a higher mean size, 2439 nm, and a minor component at 407 nm (16 %).

Concerning the two reference polysaccharides, after the LS analysis the pectin with the highest DE (85%) showed an average size of 339 nm, the lowest value among all the pomegranate fractions and also with respect to the pectin at 55-75% of DE; this latter compound resulted with a molecular size more similar to those of pomegranate fractions, with a mean size of 942 nm (Figure 2.2.3.7).
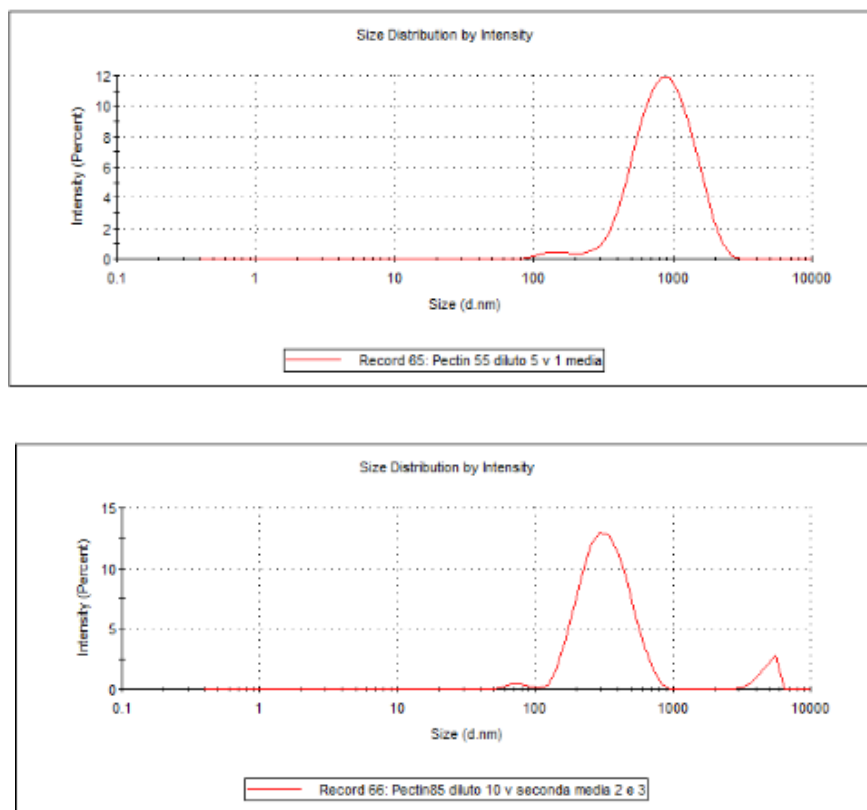
Figure 2.2.3.7 LS analyses of the commercial pectin.

DE: 55-75% (**up**) 85% (**down**)

The findings from LS analyses confirmed the ability of this technique to furnish the mean size of these polysaccharides dissolved in water solution in a very rapid way, and it resulted more effective that SEC in highlighting some structural differences among the isolated polysaccharides from pomegranate.

^{1}H-NMR analysis

All fractions were analyzed by ^{1}H-NMR after dialysis to evaluate differences or similarities through some diagnostic signals and the results were compared with those from the proton spectra of the commercial pectin. The presence of diagnostic signals in the proton spectra of

pectin can help to recognize some peculiarities in the chemical structure of these compounds. The amount of rhamnose can be easily evaluated by the intensity of the signal at approx. 1.2 ppm, corresponding to the methyl group on carbon C6, helping to confirm or to exclude the presence of rhamnogalacturonan structures, typical of several pectin. Analogously, information on the amount of the acetyl groups in the molecule can be extrapolated by measuring the intensity of the signal close to 2 ppm, corresponding to the singlet of the methyl group. Finally, an high percentage of methoxyl groups in the structure of the pectin can be pointed out by the presence of an intense singlet close to 3.69 ppm in an crowded area of the spectrum, containing most of the proton signals of oligosaccharides and polysaccharides. The absence (or presence with low intensities) of signals at values over 5.5 ppm can help to exclude the presence of aromatic amino acids belonging to protein structures co-precipitated in appreciable amount along with polysaccharides.

Concerning our fractions, rhamnose resulted almost completely absent or present in traces amount only in PQ-F2, WoA-F3 and WoS-F2, confirming the absence of significant amounts of rhamnogalacturonan. The proton spectra of the fractions did not show any signal over 5.5 ppm, indicating the absence of significant amounts of proteins or aromatic compounds such as ellagitannins, trapped during the precipitation process. For almost all the fractions, an intense signal was observed at 3.69 ppm, indicating the presence of a large number of methoxyl groups usually present on galacturonic acid residues and suggesting a high degree of methylation (DM).

Nevertheless, the resolution obtained for the many signals in the range 3.4-4.2 ppm did not permit to accurately measure the intensity of the signal of methoxyl group at 3.6-3.7 ppm.

The fractions from WoS, showed a similar distribution between methoxyl and acetyl groups: this information was extrapolated by measuring the ratio between the integral of the signals at

3.69 ppm and 1.95 ppm respectively, obtaining values from 6.4 to 7.0. Among the polysaccharides from WoA, even if both obtained from the variety Wonderful, only F3 fraction showed a proton spectrum similar to those of the fractions from WoS. Concerning F2 fraction, it was characterized by a very intense signal of the methoxyl moiety and a negligible signal related to the acylated group, as confirmed by the high ratio between the corresponding integrals (14.7). Finally, the F1 fraction presented a very different proton spectrum: the signal at 3.69 ppm almost disappeared and two groups of signals with similar intensity appeared at 3.82 and 3.62 ppm, suggesting the presence of methoxyl groups not linked to the glucuronic acid moiety. Furthermore, the presence of an intense signal at 5.29 ppm indicated the presence of H1 anomeric protons with alpha-linkage (Cui, 2005). Concerning F1 and F2 fractions from PQS, both the samples showed similar proton spectra with the same pattern of signals observed for the F1 fraction of WoA. The proton spectrum of Fraction 3 from PQS resulted very similar to F3 fractions from WoA and WoS, with only slight differences in the range of 3.5-4.3 ppm.

To better investigate the chemical structure of these last samples, all the F3 fractions were further analyzed to determine the composition in simple sugars and the main sugar linkages.

Sugars composition

To better verify if the applied fractionation procedure allowed separating different polysaccharide structures, a couple of samples with the higher difference in terms of water solubility, was chosen to determine the sugars composition after hydrolysis. To this aim, the F1 and F3 fractions from the WoS samples were chosen. The results pointed out similar percentages of galacturonic acid, ranging from 53% to 58%, again confirming the presence of pectin in the two fractions. It is noticeable the higher amount of glucose in F1 than in F3,

passing from 13% to 3%. Together with a lower content of glucose, F3 showed a higher percentage of xylose with respect to F1 (Figure 2.2.3.6). Considering that the pomegranate peel used as raw material also contains cellulose that could be easily precipitated by the first addition of ethanol, the percentage of glucose higher in F1 fraction than in F3 fraction was expected. The lower amount of glucose found in F3 fraction (below 5% of total µmols), confirms the hypothesis that cellulose, because of its low solubility in water, is almost completely excluded as component of F3 fraction.
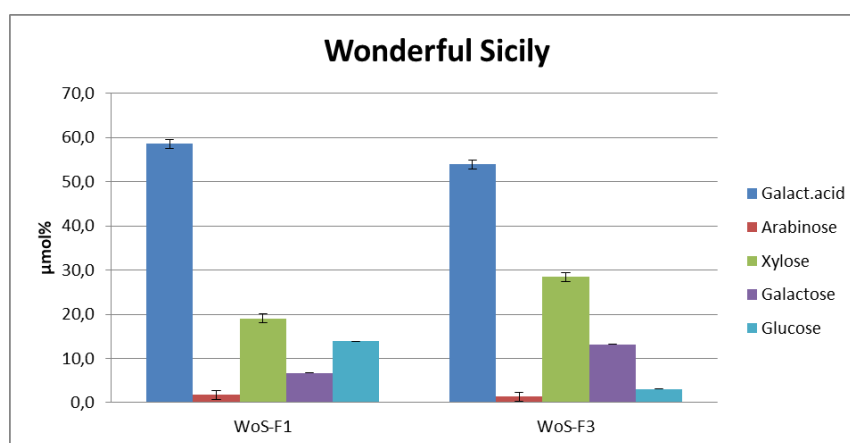
Figure 2.2.3.8 Sugars composition in F1 and F3 samples from Wonderful Sicily (WoS). Data are expressed in µmol% as a mean of duplicate.

In light of these results, in order to exclude the presence of cellulose in the final fractions, the sugar composition was also determined for WoA-F3 and PQS-F3.

In the two Wonderful varieties, galacturonic acid reached approx. 50%, galactose ranged from 13% to 15 % and xylose from 27% to 28%, while glucose was present in a very low amount. The percentage of xylose higher than the other neutral sugars is in agreement with other literature data concerning different pomegranate cultivars (Hasnaoui *et al.,* 2014). Considering the high percentage of xylose, the presence of homogalacturonan and xylogalacturonan

(included within the typical pectin structures) as main constituents of the F3 fractions from Wonderful variety can be confirmed. Furthermore, the absence of significant amounts of rhamnogalacturonans of type I or II it was confirmed, also according to ^{1}H-NMR data. WoA-F3 and WoS-F3 showed similar sugars composition and ^{1}H-NMR spectra (Figure 2.3.2.8), and both were obtained with negligible amount of cellulose. In contrast, PQS-F3, although characterized by galacturonic acid as the main sugar (49%) thus confirming the presence of pectin, still showed 23% glucose together with a xylose content of 18% (Table 2.2.3.2).

Samples	Galact.acid µmol%	Arabinose µmol%	Xylose µmol%	Galactose µmol%	Glucose µmol%
PQS-F3	49.1	1.4	17.8	7.8	23.7
WoA-F3	50.2	1.8	27.3	15.4	5.3
WoS-F3	53.9	1.4	28.5	13.1	3.1

Table 2.2.3.2 Sugars composition of PQS, WoA and WoS Fractions 3.
Data are expressed in µmol% as a mean of duplicate. RSD<5%

In light of these results it can be concluded that the proposed fractionation process applied to Purple Queen®, was not suitable to avoid cellulose interference in F3 fraction. We can hypothesize that such different behaviour is due to a higher amount of cellulose in the peel of Purple Queen® than in the peel of Wonderful.

Linkages	WoA-F3	Wo-SF3	PQS-F3
tAra f	14.4	14.1	12.7
1,2 Ara f	0.3	0.3	1.2
1,3 Ara f	2.2	2.4	2.4
1,2,5 Araf	2.7	2.5	2.3
Total Ara	**19.6**	**19.3**	**18.6**
t Fuc	0.7	0.5	0.9
1,2,4 Fuc	0.9	1.3	1.5
Total Fuc	**1.6**	**1.8**	**2.4**
t Xyl	10.3	12.8	10.5
1,4 Xyl	17.4	8.0	6.6
1,2 Xyl	4.2	1.6	0.7
1,3,4 Xyl	8.9	8.5	3.6
1,2,3,4 Xyl	0.5	5.7	5.1
Total Xyl	**41.3**	**36.6**	**26.5**
t Glc	2.3	1.1	3.3
1,4 Glc	9.6	5.2	21.0
1,2,4 Glc	0.5	0.4	0.3
1,4,6 Glc	2.5	4.1	4.1
Total Glc	**14.9**	**10.8**	**28.7**
t Gal	8.4	11.9	6.7
1,6 Gal	11.9	10.2	10.7
1,3,6 Gal	0.3	5.0	4.2
1,3,4,6 Gal	0.8	2.6	2.6
Total Gal	**21.4**	**29.7**	**24.2**
Total	**100.0**	**100.0**	**100.0**

Table 2.2.3.3 Glycosidic linkages of Apulia, Sicilia Wonderful and Purple Queen, Fractions 3. Data are expressed as a mean of duplicate in µmol%. RSD<5%

Furthermore, methylation analysis was performed on Fraction 3 of the three samples to define

the glycosidic linkages in the main polysaccharide chains. The results summarized in Table

2.2.3.3 allow highlighting the presence of 3 polysaccharides of hemicellulose nature in each

fraction. In particular, the two fractions from Wonderful, as expected, present more similarities with respect to Purple Queen variety.

Comparing the total sugars composition obtained after sugars and methylation analyses, several differences had been pointed out, mainly related to the inability of methylation analysis to identify galacturonic acid. In particular, a significantly higher percentage of arabinose was highlighted after methylation analysis with respect to the sugar analysis. Indeed, arabinose is the sugar that much more seem to undergo degradation after strong acidic condition used in neutral sugars analysis.

The higher percentage of (1→4) Xyl, (1→3,4) Xyl and (1→3) Araf can be associated to the presence of arabinoxylan, already reported in pomegranate peel (Hasnaoui *et al.,* 2014). It is possible to observe that, even if there are the same linkages, the two Wonderful samples have some differences in the percentage of branches: the higher amount of (1→3,4) Xyl with respect to (1→4) Xyl in WoS-F3 can be linked to a more ramified structure, with respect to the Apulian sample (WoA). The (1→4) Glc, (1→4,6) Glc, (1→2) Xyl and tXyl linkages can be attributed to the xyloglucan structure. Within the two Wonderful samples, the Sicilian one presented higher branched structure represented by the higher percentage of (1→4,6) Glc with respect to (1→4) Glc linkages. Furthermore, the higher percentage of (1→4) Glc in the Purple Queen sample indicates the presence of higher content of cellulose in this variety with respect to the Wonderful ones. Moreover, the high percentage values of (1→6) Gal and t Gal can be linked to the arabinogalactan structure detected in both the varieties. Such a finding is in agreement to the recent work of Shakhmatov *et al*., (2019), in which similar structures were found in pomegranate fruits of unknown variety.

2.2.3.4 Conclusions

Polysaccharides recovered from by-products of two widely cultivated pomegranate varieties, namely Wonderful (from Apulia and Sicily) and Purple Queen® (from South of Spain), were studied in this research. Polysaccharides were extracted from fruits' peel using hot water, thus obtaining a decoction in a green and easy way. Polysaccharides were then precipitated from the decoction and fractionated applying a precipitation process optimized adding the minimum amount of ethanol. Three different polysaccharides fractions were obtained for each sample, with the highest yields obtained for the medium fraction in all samples.

Size Exclusion Chromatography allowed pointing out the presence of structures slightly higher than commercial pectin in the dialyzed fraction, while Light Scattering has been proposed for differentiating between the molecular size of the polysaccharide structures in the different fractions. ^{1}H-NMR analysis of the dialyzed fractions allowed excluding the presence of significant amounts of rhamnogalacturonans, proteins and residual ellagitannins. Furthermore, it allowed pointing out a high level of methoxyl groups, typically linked to glucuronic acid in pectin structures with a high degree of methylation, and lower levels of *O*-acetyl groups. All the fractions showed the presence of signals attributable to pectin structures. The high xylose content in last fractions pointed out the presence of xylogalacturonan in addition to homogalacturonan, and a very low glucose content in the Wonderful samples. Finally, methylation confirmed the presence of arabinoxylan and xyloglucan with different percentages of branches; arabinogalactan was only detected in Purple Queen sample with also a residual content of cellulose. Fractions without cellulose as interference were obtained only for Wonderful variety, while the same result was not obtained for Purple Queen presumably due to the high content of cellulose in the peel of this variety.

The proposed fractionation method can be applied to differentiate the main polysaccharides in pomegranate peel and can help to plan the use of the isolated fractions for biological tests, which requires high quantities of samples as those for the *in vitro* evaluation of the prebiotic properties.

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Oral presentation

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Poster presentation

Balli, D., Tozzi, F., Khatib, M., Adessi, A., Melgarejo, P., Masciandaro, G., Giordani, E., Innocenti, M., Mulinacci, N. (2019). *Ellagitannins, anthocyanins and polysaccharides in fruits of Purple Queen® pomegranate cultivar grown in substrates with dredged sediments*. XX Euro Food Chem, 17th-19th June 2019, Porto, Portugal.

Balli, D., Khatib, M., Cecchi, L., Innocenti, M., Mulinacci, N. (2018). *Polysaccharides, ellagitannins and anthocyanins from pomegranate: a study of the whole fruits of 16 varieties*. CHIMALI 2018 (XII Italian Food Chemistry Congress), September 24th-27th, 2018, Camerino, Italy. Book of Abstract, (PO.12). ISBN: 978-88-676809-7-5.

Balli D. (2018). *Millet from Burkina Faso: optimization of the extractive procedures for the recovery of phenolic compounds*. 9 PhD-Day, 31st May 2018, Sesto Fiorentino.

Bellumori, M., Innocenti, M., Balli, D., Chasquibol Silva, N., Pieraccini, G., Mulinacci N. (2017). *Determination of phenolic compounds from camu-camu fruit (Myrciaria dubia) from Peru by HPLC-DAD-ESI-MS/MS*. 5MS Food day 11th-13th October, Bologna

Balli, D., Bellumori, M., Paoli, P., Pieraccini, G., Mulinacci, N., Innocenti, M. (2017). *Lisosan® G, a food supplement from wheat: the chemical profile and the antidiabetic properties*. 5MS Food day 11th-13th October 2017, Bologna

Balli, D., Bargiacchi, E., Miele, S., Innocenti, M., Mulinacci, N. (2017). *Dry grape pomace as bioactive ingredient of new food formulations: the case of pasta and rice bar*. The 8th International Conference on Polyphenols and Health, 9th October 2017, Quebec, Canada

Balli, D. (2017). *Lisosan® G, a food supplement from wheat: the chemical profile and the antidiabetic properties.* PhD day, 24th May 2017, Sesto Fiorentino

Balli, D. (2016). *Effect of fermentation on the bioactive metabolites of cereals from Tuscan and Sub-Saharan Africa tradition.* Congresso Nazionale di Chimica degli Alimenti, 4th-7th October 2016, Cagliari.

Congress participation

La chimica degli alimenti e i giovani ricercatori: II Edizione. Palazzo Greppi sala Napoleonica Milano, 23rd-24th September 2019.

XX Euro Food Chem. June 17th-19th 2019, Porto, Portugal.

Green extraction of natural products. GENP2018, University of Bari, 12th-13th November 2018.

CHIMALI 2018 (XII Italian Food Chemistry Congress). 24th-27th September 2018, Camerino, Italy.

XXVIII Congresso Nazionale di Scienze Merceologiche. 21st-23rd February 2018, Firenze.

5th MS-Food Day. 11th-13th October 2017, Bologna

La chimica degli alimenti e i giovani ricercatori: nuovi approcci in tema di qualità, sicurezza e aspetti funzionali di ingredienti alimentari. Palazzo Greppi sala Napoleonica, September 25th-26th 2017, Milano.

Abroad stages

Winner of doctoral students to participate in the NAVA programme implemented at Lodz University of Technology in the academic year 2018/2019. 7th-14th September 2019, Lodz, Poland.

Intership in the University of Aveiro, Portugal, Chemisty Department. 25th March-25th July 2019.

Tutor of master degree thesis

1. *Alla riscoperta del miglio e delle sue molteplici proprietà: uno studio sui biofenoli di 14 varietà*. **Relatore** Nadia Mulinacci **Correlatore** Maria Bellumori, Diletta Balli **Candidato** Francesca Corrieri. Anno accademico 2018/2019. Tesi magistrale in Scienze dell'Alimentazione

2. *Composizione fenolica di un gruppo di varietà di grani antichi coltivati in condizioni agronomiche diverse*. **Relatore** Nadia Mulinacci **Correlatore** Marzia Innocenti, Diletta Balli **Candidato** Mirna Palazzo. Anno accademico 2018/2019. Tesi magistrale in Farmacia

3. *Ricerca del glutine come contaminante in alimenti a marchio "gluten free"*. **Relatore** Nadia Mulinacci **Correlatore** Diletta Balli **Candidato** Giulia Neirotti. Anno accademico 2017/2018. Tesi magistrale in Scienze dell'Alimentazione

4. *Polisaccaridi, Ellagitannini e Antociani da melograna: uno studio sui frutti interi di 16 varietà.* **Relatore** Nadia Mulinacci **Correlatore** Diletta Balli **Candidato** Martina Carone. Anno accademico 2017/2018. Tesi magistrale in Scienze dell'Alimentazione

5. *Miglio grano farro: ottimizzazione delle procedure estrattive della frazione fenolica.* **Relatore** Nadia Mulinacci **Correlatore** Diletta Balli **Candidato** Elisa Mani. Anno accademico 2017/2018. Tesi magistrale in Farmacia

6. *Componente fenolica nei prodotti fermentati a base di cereali e legumi: miso di miglio e ceci.* **Relatore** Marzia Innocenti **Correlatore** Diletta Balli **Candidato** Alessandra Grifoni. Anno accademico 2017/2018. Tesi magistrale in Scienze dell'Alimentazione.

7. *Componente fenolica nei prodotti fermentati a base di legumi e cereali: miso di riso e soia.* **Relatore** Nadia Mulinacci **Correlatore** Diletta Balli **Candidato** Alessandro Verderame. Anno accademico 2017/2018. Tesi magistrale in Scienze dell'Alimentazione.

8. *Uno studio di caratterizzazione chimica sul Lisosan$^{®}$ G: un integratore alimentare a base di frumento fermentato.* **Relatore** Nadia Mulinacci **Correlatore** Marzia Innocenti, Diletta Balli **Candidato** Loredana Daniele. Anno accademico 2015/2016 Tesi magistrale in Scienze dell'Alimentazione.

Schools

Formare le competenze trasversali: soft skills per l'imprenditività 2019. October 2019.

Corso pratico-teorico Accademia della Tisana. 14th-17th June 2017, Anghiari (AR).

3 MS NatMed School. 26th-27th October 2017, laboratori Aboca, Pistrino (AR).

Seminari di Spettrometria di Massa (Direttore Centro Spettrometria di Massa Dott. Giuseppe Pieraccini), *NMR* (Professor Fabrizio Melani).