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Development of functional foods and ingredients using by-products from plant foods

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General abstract

The aim of this PhD project was the valorization of by-products from some mediterranean plant foods through their re-utilization in new functional foods and ingredients having the ability to modulate the oxidative processes and nutrient metabolism in the gastrointestinal tract (GiT).

Obesity is an urgent social problem and functional foods able to modulate oxidative stress and energy homeostasis are promising tools to control inflammatory status and body weight.

Several evidence shows that polyphenols (PPs) and dietary fiber (DF) may control energy intake and appetite in humans through several mechanisms. PPs may quench free radicals forming in GiT, may counteract subclinical oxidative stress and high-fat diet induced inflammation, and may also influence the activity of key digestive enzymes thus modulating nutrients bioavailability and the related neuro-hormonal signals. In this way, PPs are hypothesized able to modulate appetite cues in the short term, and to control body weight in the long term. However, food processes as well as the physicochemical interconnections of PPs with DF and other food components are crucial factors influencing their bioaccessibility and their functionality along the GiT.

Plant foods by-products from agricultural and food industry are a natural and cheap source of PPs and DF. In this thesis the possible reutilization of pomegranate peels (PPe), artichoke leaves (ALe) and stems (AS), and olive leaves (OLe) was investigated by developing three studies.

In a first study the bioaccessibility of PPs from PPe and a PPe-enriched cookie (PPeC), their potential total antioxidant capacity (TAC) along the GiT, as well as the ability of PPeC to modulate glucose and lipid metabolism was assessed. To this purpose PPeC were developed and *in vitro* sequential enzyme digestion coupled to LC/MS/MS and biochemical analyses of the soluble and insoluble fractions collected at each digestion steps were carried out. Data showed the chemical transformations induced by baking process on PPe PPs, the potential release of antioxidants along the GiT and their ability to inhibit α-glucosidase, α-amylase and lipase activities in the duodenal tract.

In a second study the development of breads enriched with AS at 3%, 6% and 9% was performed and the intestinal fate of PPs and cynaropicrin was studied *in vitro* using a simulated human digestion model coupled to High Resolution Mass Spectrometry (HRMS) analysis of digestive extracts. The potential metabolic fate of PPs and cynaropicrin from AS-

enriched breads and the ability of the extracts from the duodenal step to inhibit α-glucosidase activity was assessed.

A third study was conducted in collaboration with Prof. Bruce Hamaker and Prof. Osvaldo Campanella at Purdue University, (Indiana, USA) and consisted in the development of new antioxidant DF by including PPs-rich extracts from PPe, OLe and ALe in starch-alginate based microspheres (MS). The physicochemical properties of the new ingredients were compared to those of 6 commercially available DF concentrates as well as to whole wheat bran and oat bran in order to position the new ingredients on the actual market of DF concentrates and dietary source of DF.

All in all data obtained in this thesis demonstrated that plant foods by-products are promising source of PPs and DF to develop foods and ingredients that may control oxidation and nutrient metabolism along the GiT.

Chapter 1

Introduction

1.1 An overview on an urgent social problem: The spread of overweight and obesity

In 2014, the World Health Organization (WHO) estimated that more than 1.9 billion of adults were overweight. Of these over 600 million were obese. The tremendous increase of overweight and obesity worldwide is associated to an increase within the population of non communicable chronic diseases (NCCDs), mainly including cardiovascular diseases, type 2 diabete, hypertension and some types of cancer.[1] Consequently, the rising prevalence of overweight and obesity is associated to social and financial burdens, such as higher health-care costs for diseases treatment as well as increased loss of work.[2]

The primary cause of overweight and obesity is the imbalance between energy intake and energy expenditure. Several factors may co-work to innescate this condition such as early life factors, individual predisposition (genetic and epigenetic characters), social factors, lifestyle as well as food nutritional properties.[3,4]

To manage this global social challenge, from many years international and national authorities started campaigns for the promotion of physical activity and of guidelines for a healthy diet. A healthy diet should include 400 g/day of fruits and vegetables, legumes, nuts and wholegrains, less than 10% of daily energy intake from free sugars, less than 30% of daily energy intake from fats, reducing saturated fats to less than 10% of total energy intake and trans fats to less than 1% of total energy intake, and replacing both with unsaturated fats, and less than 5 g/day of salt.[5]

In this context the food industry may play a crucial role. It should increase the efforts to market food products with a composition in line with the international nutritional guidelines, and should promote the research and the development of new functional foods that, beyond their nutritional role, are able to prevent metabolic syndrome by modulating physiological processes underlying the control of appetite and body weight gain.[6-8]

It is well know that on the basis of their specific chemical composition (types and amount of macronutrients, micronutrients and non-nutrients bioactive compounds) as well as their physical and sensory properties, foods can affect cognitive, hedonic, neuroendocrine and homeostatic factors underlying the regulation of appetite and of energy intake. For example, solid foods are more satiating than liquid foods and among macronutrients proteins are recognised as more satiating than carbohydrates and fats.[9,10] Additionally, some non-nutritional bioactive food components, mainly including dietary fiber (DF) and polyphenols (PPs), may act as modulators of physiological signals involved in the regulation of energy intake.[11,12]

1.2 Non-nutritional bioactive compounds for the weight management

PPs and DF are two classes of food components well known for their ability to interact with the human intestinal microbial population and for their important role in the prevention of some common NCCDs.[13-15] Although these compounds are both present in plant foods, such as fruits vegetables and cereals, they are often considered separately, regardless their potential synergistic beneficial effects along the gastrointestinal tract (GiT).[16]

Several evidences indicate that beyond their role in the prevention of metabolic diseases, PPs and DF may modulate appetite through multiple and fine mechanisms.[17-21]

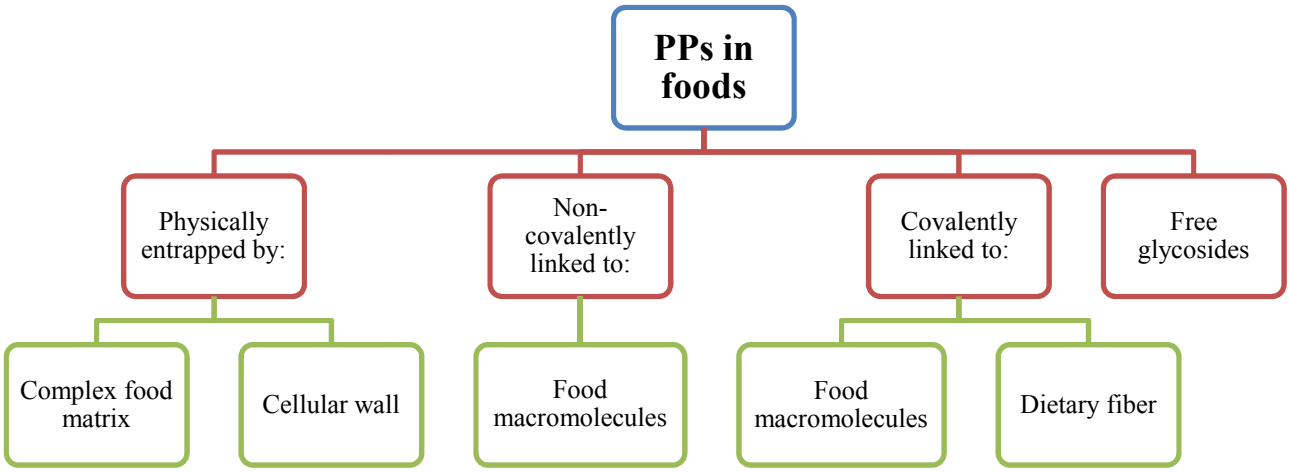

Figure 1.1. Physicochemical interactions of PPs with other components in food matrices.

As graphically shown in **Figure 1.1.**, PPs in foods occur in free form (mainly as glycosides) or covalently bound to cell wall structural components.[22] Additionally, PPs can be physically entrapped or linked to food macronutrients (i.e. starch, proteins and lipids) mainly through non-covalent interactions.[23,24]

The chemical structure of PPs, as well as their disposition in the food matrix, highly influence their bioaccessibility in the GiT, i.e. the amount of these compounds that is available to be absorbed. So the action of the digestive and bacterial enzymes, by breaking up the food matrix and delivering PPs in the GiT, is fundamental for PPs to act both systematically (after absorbtion) as well as locally in the GiT.[25,26] This latter is the first apparatus to be exposed to dietary PPs after their release from the food matrix.

The area of food science studying how a food component influences and is influenced in its bioactivity by the food matrix, is called nutrydinamics. The term nutridynamics originates from pharmacodynamics, that is the branch of pharmacology related to the effects of drugs and the

mechanisms of their action in the human body. However, contrary to a drug that contains a single component in a pure form and that exerts a strong bioactive effect on a single target, foods consist of a variety of components that can have multiple small interactions within many human targets upon consumption as well as show a wide range of interactions among each other.[27]

Figure 1.2. summarizes the potential pathways whereby dietary PPs can affect the energy intake. In the intestinal lumen, PPs may act as antioxidant and anti-inflammatory molecules. They can quench the free radicals continuously forming in the GiT thus counteracting subclinical oxidative stress and intestinal high-fat diet induced-inflammation, which are correlated to obesity exacerbation and insulin resistance.[28-33] Furthermore, in the GiT PPs may influence the activity of digestive enzymes such as pancreatic α-amylase, α-glucosidase and pancreatic lipase, thus modulating nutrients bioavailability and the neuro-hormonal signals underpinning appetite mechanisms in the short term, and the body weight in the long term.[34-38]

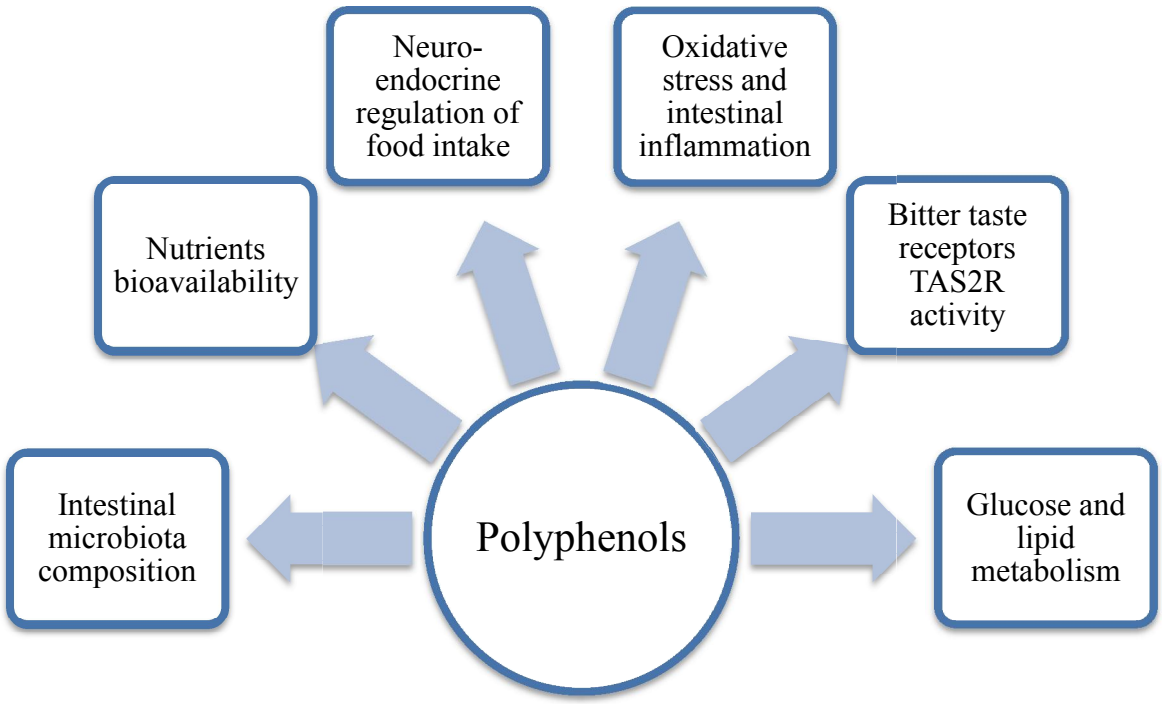

Figure 1.2. Effects of polyphenols on physiological processes potentially leading to a control of weight gain.

Starch is the most abundant digestible polysaccharide in foods and its digestion slowly starts in the mouth and is completed in the intestinal lumen where it is hydrolyzed by pancreatic α-amylase. The resulting oligosaccharides and disaccharides are hydrolysed into absorbable glucose by α-glucosidases located in the brush-border surface membrane of the intestinal cells. The inhibition of these enzymes leads to the modulation of glucose release and absorption in the

small intestine thus influencing post-prandial blood glucose and the related hormonal response, with effects on fats oxidation and satiety.[39,40]

Similarly, pancreatic lipase is the enzyme responsible of hydrolysis of dietary fats, mainly triglycerides (90–95%). The hydrolysis of fats starts in the mouth, then continues in the stomach by gastric lipase, and in the duodenum through the synergistic actions of gastric and pancreatic lipases, leading to the formation of monoglycerides and free fatty acids. These compounds are absorbed by the enterocytes to synthesize new triglyceride molecules, which are transported to the different organs via lipoproteins, especially chylomicrons. The inhibition of pancreatic lipase lows digestion of triglycerides, resulting in a lower absorption of fatty acids and a reduced energy intake.[34]

Some classes of PPs and other plant-derived bioactive compounds, mainly including secoiridoids, iridoids and other terpenoids, can bind specific bitter receptors located along the GiT, which can elicit the secretion of hunger and satiety hormones regulating gastrointestinal motility and response to food as well as appetite.[41-47] Recent evidence shows that PPs reaching the low GiT, i.e. the polymerized PPs or those covalently bound to DF, may counteract intestinal and systemic inflammation correlated to metabolic syndrome and obesity by influencing the abundance of microbial species towards a healthy profile.[28,48-54] So, DF can be considered as a functional carrier of bioactive PPs along the GiT.[16]

DF were defined as "*carbohydrate polymers with ten or more monomeric units which are not hydrolysed by the human enzymes in the small intestine and belong to the following categories*:" 1) Edible carbohydrate polymers naturally occurring in the food as consumed; 2) Carbohydrate polymers, which have been obtained from food raw material by physiological, enzymatic or chemical means and which have been shown to exert physiological benefits as demonstrated by generally accepted scientific evidence to competent authorities; 3) Synthetic carbohydrate polymers with a demonstrated beneficial physiological effect on health.[55,56]
On the basis of the water solubility, DF can be divided into soluble DF and insoluble DF, mainly including cellulose, hemicelluloses, and lignin. According to the water-solubility and other physicochemical properties, DF may influence the physiological processes underlying food intake and weight gain through different mechanisms (see **Figure 1.3.)**.[17,57-59]

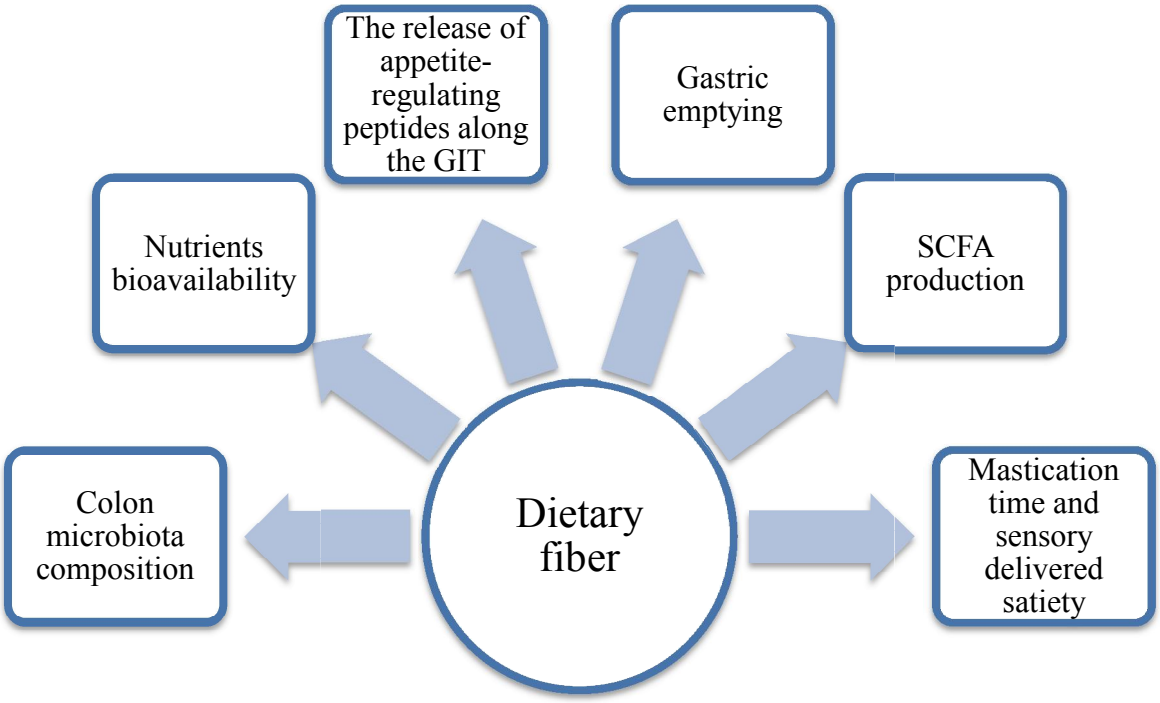

Figure 1.3. Effects of dietary fiber on physiological processes potentially leading to a control of weight gain.

Beyond water-solubility, other DF properties include viscosity, water-holding capacity (WHC), oil holding capacity (OHC) and fermentability. Such properties may not only affect the short-term satiating capacity of DF as well as appetite and energy intake in the long-term, but could also impact on technological properties of foods.[60-62] **Table 1.1.** summarises the technological effects and the potential physiological implications based on some DF physico-chemical properties. Generally, insoluble DF presents a high WHC and OHC, low viscosity and low fermentability. Conversely, soluble DF shows higher viscosity and higher fermentability compared to insoluble DF.

From a physiological point of view an high intake of DF is associated to increased mastication time, prolonged intestinal transit, reduced bioacessibility and absorption of ingested carbohydrates and lipids, as well as changes of composition and activity of colonic microflora.[17,63-65]

Specifically, short-chain fatty acids (SCFA) are a product of microbial metabolism on fermentable DF in the colon. High production of SCFA may have a role in appetite regulation via activation of SCFA-activated G-coupled protein receptors free fatty acid (FFA) receptor 2 and FFA receptor 3, which are co-localised in many human tissues, mainly including L-cells in the colon and adipocytes.

The activation of FFA receptor 3 in adypocites, and FFA receptor 2 and FFA receptor 3 in the colonic mucosa, stimulates the production of two anorexigenic gut-hormones, leptin and peptide

YY, respectively. Moreover, the activation of these SCFA-activated G-coupled protein receptors was associated with an increased secretion of glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).[66,67]

Table 1.1. Physicochemical properties of DF affecting technological properties of food and physiological processes. Adapted by Elleuch et al., (2011).[62]

Physicochemical properties of DF	**Technological properties in foods**	**Potential physiological implications**
High water holding capacity	Avoids syneresis and affects viscosity and texture of foods	Increases the fecal bulk volume and the mastication time; Laxative effects
High oil holding capacity	Stabilizes emulsions in high-fat foods	Effects on fat digestion and absorption in the GiT
High water-solubility/viscosity	Influences the food texture potentially forming gels and acting as emulsifier	Affects bioacessibility, bioavailability and metabolic fate of carbohydrates and lipids
Antioxidant activity	Improves the oxidative stability of foods and prolongs the shelf life	Influences the colonic microbiota composition

1.3 Vegetable by-products as sustainable source of non-nutritional bioactive compounds

Food wastes are defined as "*Wholesome edible material intended for human consumption, arising at any point in the food supply chain, that is instead discarded, lost, degraded or consumed by pests as well as the edible material that is intentionally fed to animals or that is a by-product of food processing diverted away from the human food*".[68]

In line with the European legislation, the waste products resulting from a production process, may be considered as by-products, only according to certain conditions: 1) The use of the products is certain; 2) The products can be used directly without any further processing other than normal industrial practice; 3) The product is produced as an integral part of a production process; 4) Its use is lawful and will not lead to overall adverse environmental or human health impacts.[69] The industrial processing of fruits and vegetables for the production of oils, juices, jams or canned foodstuffs has as a consequence the production of large amounts of food processing by-products, mainly including peels, leaves, stems, pomace, processing waters and seeds. The large disposal of this materials results in high costs for the food industry and can have a negative environnmental impact.[70,71]

The estimated percentage of food wastes and by-products ranges from 30% to 50% for fruit and vegetable juices production, from 5% to 30% for fruit and vegetable processing and preservation and from 40% to 70% for vegetable oils production.[72]

It is worth to notice that fruits from the temperate areas, as the mediterranean region, are usually characterized by a moderate amounts of waste material whereas considerably higher ratios of by-products arise from tropical and subtropical fruits processing.[73]

Globe artichoke (*Cynara cardunculus* L. var. *scolymus)* and olive (*Olea europea* L.) are two vegetable products distributed worldwide and mainly cultivated in the Mediterranean region. Italy is the first world producer of globe artichoke (451461 tons/year) followed by Egypt (266196 tons/year) and Spain (234091 tons/year).[74] The immature inflorescence, also termed capitula or head, represents the edible part of globe artichoke and is consumed worldwide as a fresh, frozen, or canned foodstuff.[75] Conversely, artichoke by-products from industrial processing, mainly including stems and external leaves account for about 80-85 % of the total fresh weight of the plant.[76] Concerning the world production of olives, Spain is the first world producer (4560400 tons/year) followed by Italy (1963676 tons/year) and Grece (1780560 tons/year).[74] Olives are mainly cultivated for the extraction of extra virgin olive oil, that

represents the major final product of industrial olives processing. Olive leaves mainly result from the pruning of olive trees as well as from the harvesting and cleaning of olives prior to oil extraction. Specifically, the production of olive leaves from pruning has been estimated to be 25 kg per olive tree.[77] However, an additional amount of leaves equal to 10% of the total weight of the harvested olives, is collected at the oil mill step.[78]

In the last decade, due to the recognized health benefits associated to the juice consumption, the cultivation of pomegranate trees (*Punica Granatum* L.), beyond in the Mediterranean countries where they has been cultivated extensively from long time (Tunisia, Turkey, Egypt, Spain, and Morocco), it increased in Italy, mainly including in the Southeastern regions.[79-82] The pomegranate fruits are majorly processed by the food industry to obtain the juice from the arils, while PPe, representing about 50% of the fresh fruit weight, are discarded and are commercially available as a dietary supplement.[83]

Considering the huge amounts of these processing products as well as the estimated percentage of by-products resulting by the industrial processing, their disposal represents a serious problem for the respective industries.

In recent decades, the growing demand by consumers of functional foods enriched with bioactive compounds, as well as the increased interest of food industry for the development of more environmentally friendly food processes, led to the search of new sustainable solutions for the exploitation of by-products resulting from plant foods processing.[84-86] In this frame, plant foods by-products are rich low-cost sources of PPs and DF and can be used for the recovery of proteins and colorants. However, the utilization of vegetable by-products in food applications could present some criticisms related to their safety of use. The individuation of potentially hazardous constituents inside the by-products could allow the selection of safe doses in the final food products.[70, 73,87] Microbial issues of the by-product can be avoided submitting the material to washing and drying steps by oven or lyophilization. The dried products can be milled and submitted to a solvent extraction for the recovery of bioactive compounds, or directly used in their whole form as functional flours for the formulation of new DF-rich foods.[71, 88, 89] Drying of the ingredients can be also combined with other processes on the final product (such as for example thermal treatments) aiming to reduce the overall microbial load.

In **Figure 1.4.** the estimated total amount of DF in some plant foods by-products was reported. It is worthy to notice that, excluding bran from rice and oat and kiwi pomace, the total amount of DF in plant by-products, is always more than 40%, calculated on the basis of dry weight.

Despite there is a large number of studies about the inclusion of bioactive compounds from plant foods by-products in functional foods, there is a lack of knowledge concerning the interactions of these bioactive compounds with the other components of the food matrix as well as their potential health benefits along the GiT. [70, 90-99]

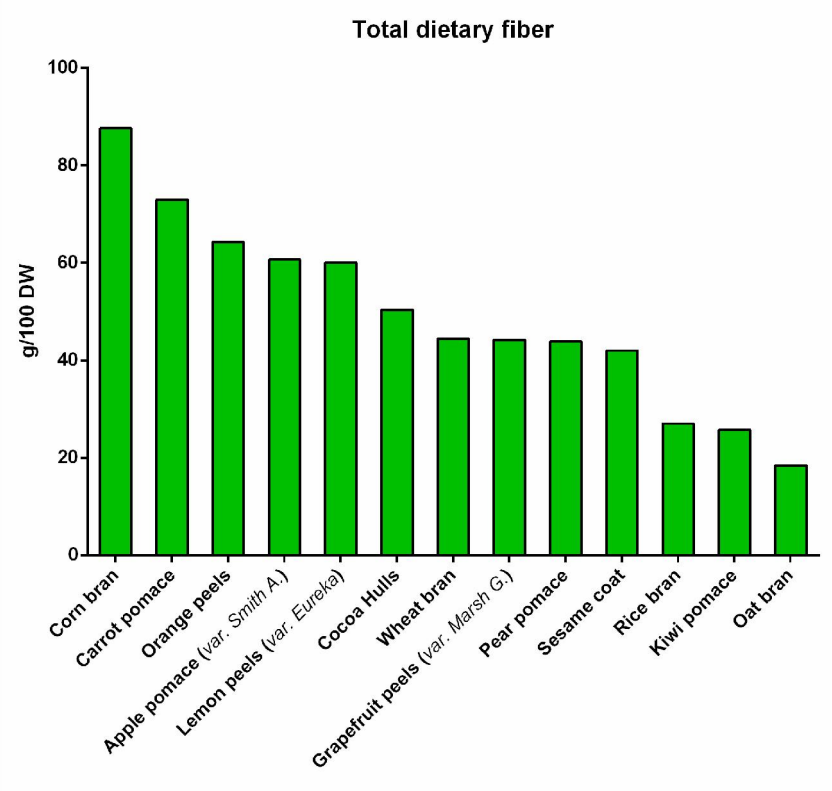

Figure 1.4. Amount of total dietary fiber in some plant foods by-products. Data were from Elleuch *et al.*, 2011 [62] and Sharma *et al.*, 2016 [88]

1.4 Functional foods and health claims

The term functional foods was first coined in Japan in 1984 in order to define "*Foods fortified with special constituents that possess advantageous physiological effects*".[100]
In 1999, a more complex definition of functional foods was introduced by the European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE). In its definition the Commission has better clarified the concept of "functional" applied to food science. Specifically, according to FuFoSE "*a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule just as normal food form*".[101] More recently, the Functional Food Center/Functional Food Institute, located in Dallas (Texas, USA) defined functional foods as "*Natural or processed foods that contains known or unknown biologically-active compounds; which, in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease*". [102]

In Japan, functional foods are considered a special food category and are defined "Foods for Specific Health Uses (FOSHU). The Japanese Ministry of Health and Welfare is the legislative institution appointed to release a FOSHU designation after an evaluation process that typically requires one year. In the United States the Food and Drug Administration (FDA) recognizes dietary supplements and medical foods and is the legislative institution that, based on the strength of the scientific evidence, takes the final decision concerning the proposals of health claim for food products with beneficial effects on health.[102]
In the European (EU) context, functional foods are not considered a specific category of foods, therefore the general EU legislation on foods can be applicable to this products depending on their specific nature. However, from 2006 the utilization of nutrition and health claims appearing in food labels is under specific EU regulations (EC regulation 1924/2006, EC regulation 353/2008, EC regulation 1169/2009, EC regulation 432/2012, EC regulation 1047/2012).[103-107]
The EC regulation 1924/2006 defined the difference between "Nutrition claim" and "Health claim". Specifically, a "*nutrition claim means any claim which states, suggests or implies that a food has particular beneficial nutritional properties due to:*
(a) the energy (calorific value) it

- *provides;*

- *provides at a reduced or increased rate; or*
- *does not provide; and/or*

(b) the nutrients or other substances it

- *contains;*
- *contains in reduced or increased proportions; or*
- *does not contain.*

On the other hand a "*health claim means any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health*".

The European Commission, taking into account the independent assessments and advices supplied by the European Food Safety Authority (EFSA), authorizes the utilization of nutrition and health claims on food labelling. According to the EU functional food legislation, a list of authorised and not-authorised nutrition and health claims made on foods has been published in a register consultable online by all the EU state members.[108]

The United States, Japan and Europe are considered the world's largest markets of functional foods, accounting for the 90% of the total world sales. Within the Europe the major markets are the United Kingdom, Germany, France and Italy.[109]
Considering that the total costs for the development of a conventional new food product range between 1 and 2 million of US dollars, it can be estimated that the costs for the development and marketing of a functional food are higher by far. In fact, the development and marketing of functional foods is a multi-disciplinary and multi-steps process that requires research efforts finalized to the identification of the functional compounds and their inclusion on a final product that is sensory acceptable and effective in human body.[110] Thus, researches on food processing and transformation as well as on validation of the efficacy through clinical trials are necessary to gain approval for health-enhancing marketing claims. The scale-up of the technologies, the marketing of the new products as well as the legislative aspects regulating the nutritional informations required on the label and the types of allowed nutritional and health-claims have to be taken into account for the estimation of total costs in the development stage and before the launch on the market of the final product.[111]

According to Betoret et al., (2011) the technological strategies used in food processing for the development of new functional foods can be divided in three principal groups:[112]

- Technologies traditionally used in food processing, mainly including formulation, blending as well as cultivation and animal breeding techniques finalized to obtain improved food products;
- Technologies designed to prevent the deterioration of physiologically active compounds, mainly including microencapsulation, development and utilization of edible films and coatings or the vacuum impregnation, the latter considered a useful way to introduce desirable solutes into the porous structure of foods, thus modifying their original composition;
- Others more recent technologies that contribute to customize designed functional foods, mainly including nutrigenomics.

1.5 Abbreviations

DF: Dietary fiber;

EFSA: European Food Safety Authority

FDA: Food and Drug Administration

FFA: Free fatty acid;

FOSHU: Foods for Specific Health Uses

GIP: Glucose-dependent insulinotropic peptide.

GiT: Gastrointestinal tract;

GLP-1: Glucagon-like peptide 1;

NCCDs: Non communicable chronic diseases;

OHC: Oil holding capacity;

PPs: Polyphenols;

SCFA: Short-chain fatty acids;

WHC: Water-holding capacity;

WHO: World Health Organization;

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Chapter 2

In vitro bioaccessibility and functional properties of polyphenols from pomegranate peels and pomegranate peels-enriched cookies

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Abstract

Obesity is an urgent social problem and new functional foods providing polyphenols and dietary fibers (DF) may be promising tools to modulate oxidative stress, inflammation and energy homeostasis. Pomegranate peels (PPe) are an agro-industrial by-product containing polyphenols such as ellagitannins (ETs), gallic acid (GA), ellagic acid (EA) and its derivatives (EAs), as well as DF. In this study, PPe enriched cookies (PPeC) were developed and the bioaccessibility of their polyphenols as well as the ability to exert antioxidant activity along the gastro-intestinal tract (GiT) and to modulate digestive enzymes was evaluated *in vitro*. Data showed that the potential bioaccessibility of ETs was 40% lower from PPeC than PPe whereas EAs and GA bioaccessibility increased by 93% and 52% for PPeC compared to PPe. The concentration of the polyphenols at each digestion step was associated with the total antioxidant capacity of the potentially bioaccessible material. Moreover the polyphenols released in the simulated duodenal phase upon PPeC digestion exhibited inhibitory activity towards α-glucosidase, α-amylase and lipase, being for α-glucosidase > α-amylase > lipase. In conclusion data demonstrated that the inclusion of PPe at 7.5% in a bakery product potentially leaded to a high bioaccessibility of ETs degradation products (mainly EA and EAs) in the duodenum, with a consequent antioxidant protection along the GiT and modulation of glucose metabolism. Further human studies are warranted to evaluate whether these effects also occur *in vivo*.

2.1 Introduction

Obesity consists in excessive body fat accumulation resulting from an imbalance between individual energy intake and expenditure. In 2014, more than 1.9 billion of adults were overweightand over 600 million of these were obese.[1] The tremendous increase of obesity worldwide is associated to an increase among population of non communicable chronic diseases (NCCD), such as cardiovascular diseases, type 2 diabetes, and some types of cancer.[2,3] Subclinical oxidative stress and inflammation are key factors in the pathogenesis of obesity-related diseases.[4] Healthy lifestyle, including energy balanced and variegate dietary regimens, together with a sufficient level of physical activity, are known to be the best strategies to fight overweight and associated diseases.[1] However, educational campaign and promotion of physical activity failed among population. Thus, the consumption of foods providing bioactive compounds, such as polyphenols and dietary fibers (DF), may represent an effective strategy to prevent obesity and the risk of associated NCCD.[5-8]

Jointly with DF, polyphenols are very promising ingredients for the formulation of functional foods aiming to target gastro-intestinal tract (GiT) and metabolic pathways underpinning appetite and body weight control.[8-13] In fact, beside their ability to quench free radicals forming in the GiT, thus acting as antioxidants, polyphenols may influence the activity of digestive enzymes, such as α- amylase, α-glucosidase and pancreatic lipase, thus modulating nutrients bioavailability and the hormonal response triggered by foods.[4,14-20]

The chemical structure of polyphenols, as well as their disposition in the food matrix can highly influence their availability to react against free radicals and to inhibit digestive enzymes in the GiT. Therefore, the study of bioaccessibility of polyphenols along the GiT, also using *in vitro* human digestion models, is very useful to foresee their functional effects in human body.

Fruits, vegetables and whole grains are the most important dietary sources of polyphenols.[21,22] Nevertheless, by-products of plant food processing may represent a natural and cheap promising source of polyphenols for nutraceutical purpose.[23] They can be extracted to recover purified biophenols or, more advantageously, they may be used as whole powder and added as functional ingredients in food formulation, providing also a considerable amount of DF.[24,25]

In this frame, pomegranate peels (PPe) represent a very promising agro-industrial by-product, due to their high content of bioactive compounds such as ellagitannins (ETs), mainly including punicalagin, gallic acid (GA), ellagic acid (EA) and its derivatives (EAs), as well as DF (33-62% of PPe on dry weight).[26-28] The pomegranate fruits are majorly processed by

food industry to obtain the juice from the arils, while PPe, representing about 50% of fresh fruit weight, are discarded and are commercially available as a dietary supplement.[29] PPe have higher content of total polyphenols and higher antioxidant capacity than the pulp fraction.[30] Several evidence indicate that ETs and EA from pomegranate may be protective against many chronic diseases, such as some types of cancers, type 2 diabetes, atherosclerosis and cardiovascular diseases.[31,32] Recent *in vitro* studies pointed out that the benefits of ETs and EA from pomegranate was due to their anti-inflammatory, antioxidant and digestive enzyme inhibitory action along the GiT.[15,31,33-40]

Only few studies proposed PPe powder as functional ingredient to increase the antioxidant capacity, the nutritional value and the shelf-life of some foods, such as sunflower oil, wheat bread, cookies and meat products.[41-46] A very recent *in vitro* study showed that a PPe flour could release ETs and EA during an *in vitro* sequential enzyme digestion, might modify antioxidant status and lead to the production of short-chain fatty acids inside the GiT.[47] However, a lack of knowledge still exists in the literature regarding the potential functionality of PPe when used as functional ingredient in baked food products.

This study aimed at assessing the bioaccessibility of polyphenols from a PPe powder and PPe-enriched cookies (PPeC), their potential antioxidant capacity along the GiT as well as the ability of PPeC to modulate glucose and lipid metabolism. To this purpose, PPeC were developed and *in vitro* sequential enzyme digestions coupled to LC/MS/MS and biochemical analyses of the fractions collected at each digestion steps were used to determine the chemical transformations induced by baking process on PPe polyphenols and to assess the step-by-step release of antioxidant activity as well as the overall potential antioxidant capacity of PPeC.

2.2 Material and methods

2.2.1 Chemicals

Water and methanol used for the analysis were of HPLC grade (Merck, Germany). Ethanol and formic acid were purchased from VWR international (Fontenay-sous-Bois, France). Cellulose powder was obtained from Fluka (Buchs, Switzerland). Total dietary fiber assay kit was purchased from Megazyme International (Wicklow, Ireland). Calcium chloride, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl, 95% (DPPH), sodium bicarbonate, hydrochloric acid, acetic acid, celite, 5,5′-dithiobis(2-nitrobenzoic acid), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 2,3-dimercapto-1-propanol tributyrate, starch Azure, 4-morpholinoethanesulfonic acid (MES), para-nitrophenyl-α-d glucopyranoside, sodium phosphate dibasic heptahydrate were purchased from Sigma-Aldrich (St. Louis, MO). Standards of punicalagin, EA and GA were purchased from Sigma-Aldrich (St. Louis, MO). Lipase from *Aspergillus niger* (187 U/g), α-amylase from porcine pancreas (type VI-B ≥ 10 U/mg), α-glucosidase from *Saccharomyces cerevisiae* (Type I ≥ 10 U/mg), bacterial protease from *Streptomyces griseus* (Pronase E, Type XIV ≥ 3.5 U/mg), cell wall degrading enzyme complex from *Aspergillus* sp. (Viscozyme L), pancreatin from porcine pancreas (4 X USP) and pepsin from porcine gastric mucosa (≥ 250 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). The ingredients for cookies preparation were purchased from a local market

2.2.2 PPe powder dietary fiber

A commercially available PPe powder was used in this study. It was purchased from Detrade UG (Bremen, Germany). The content of total dietary fiber (TDF), including soluble dietary fiber (SDF) and insoluble dietary fiber (IDF), was determined using the protocol described by Prosky *et al*., (1987).[48]

2.2.3 PPeC preparation

PPeC and control cookies (CTC), were prepared according to a recipe described in AACC (American Association of Cereal Chemists), method 10-54 (AACC, 2000) with some modifications.[49] Particularly, dough was prepared mixing 40 g of wheat flour, 5 g of PPe, 17.5 g of sugar, 10 g of butter, 0.8 g of NaHCO3, 0.2 g of NaCl and 18 g of water. In CTC, PPe powder was substituted with the same amount of inert cellulose powder. To achieve

maximum homogeneity between the two samples, each dough was rolled between two bars with a height of 3 mm and was shaped in a disk of 30 mm diameter. CTC and PPeC were baked simultaneously at 170°C for 12 min in a forced-air circulation oven. The final concentration of PPe powder in PPeC was 7.5%.

2.2.4 In-vitro sequential enzyme digestion of PPe powder and PPeC

The sequential enzyme digestions of PPe, PPeC and CTC were performed following the method described by Papillo *et al*. (2014), slightly modified.[50] The scheme of the adopted experimental protocol is reported in **Figure 2.1.**.

Briefly, four sequential digestive phases, namely simulated salivary phase (SSP), gastric phase (SGP), duodenal phase (SDP) and colon phase (SCP), including Viscozyme L and Pronase E steps, were carried out. PPe (2.5 g), grinded PPeC (2.5 g), cellulose (2.5 g, as control for PPe) and grinded CTC (2.5 g) were submitted to the enzymatic digestion. At each step, the supernatants were collected for the HPLC analysis and for the measure of the total antioxidant capacity (TAC), whereas the pellets were analyzed for the evaluation of insoluble TAC. Before the analysis, the supernatants were further centrifuged (4000 rpm for 30 min at 4°C), ultra-centrifuged (14800 rpm for 15 min at 4°C) and different aliquots (one milliliter each) of digestive extracts were carefully dried under nitrogen flow, in order to preserve the samples.

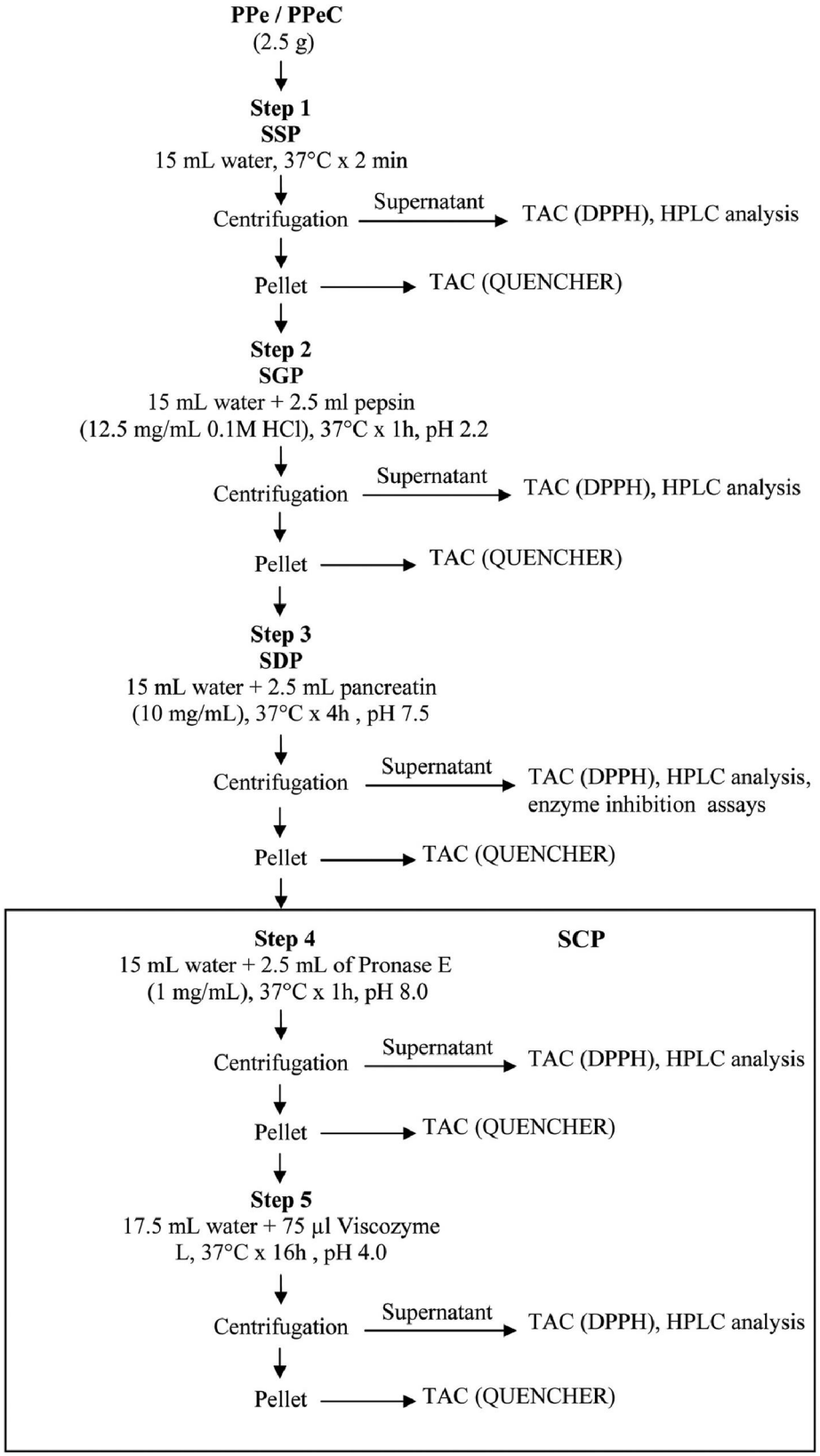

Fig. 2.1. Flow chart of the experimental design. Enzyme inhibition assays were performed only for PPeC.

2.2.5 HPLC analysis

For the HPLC analysis of soluble fractions, the aliquots of digestive extracts were re-suspended in 1 mL of a solution water/methanol (50:50, v/v) at 0.5 % of formic acid and properly diluted. The chromatographic separation of ETs, EA, EAs and GA from the supernatants at each digestion step was obtained according the methods described by Seeram *et al*. (2004), with slight modifications.[51,52] The HPLC system consisted in two binary pumps (LC-10AD, Shimadzu, Kyoto, Japan), equipped with a photo-diode array detector (SPD-M10A, Shimadzu, Kyoto, Japan) and a C-18 reversed phase column Gemini 5µ 250 mm x 4.6 (Phenomenex, Torrance, CA) was used. The flow rate was 0.8 mL/min and the mobile phases were water at 2% of formic acid (phase A) and methanol (phase B); the following gradient was applied: [min]/[%B] - (0/0), (20/10), (30/30), (35/40), (40/70), (45/98), (48/98), (50/0), (52/0). The injection volume was 20 µl. ETs, EA, EAs and GA were identified by LC-MS/MS with an API 3000 Triple Quadrupole mass spectrometer (Applied Biosystem Sciex). Mass spectrometry conditions were optimized, according to Fischer *et al.*, (2011).[26] ETs, including punicalin (anomers α and β), punicalagin (anomers α and β), pedunculagin (anomers α and β) and a compound previously identified by Fischer *et al*., (2011) as a castalagin derivative, were detected and quantified at 378 nm. For EA, EAs and GA, the wavelengths used were 366 nm and 280 nm, respectively. Calibration curves of punicalagin, EA and GA were obtained. Punicalagin, EA and GA were identified and quantified with the corresponding standards. Punicalin anomers, pedunculagin anomers, castalagin derivative and total ETs were quantified as punicalagin equivalents. EAs, including ellagic acid hexoside, pentoside and deoxyhexoside, were quantified as EA equivalents.

2.2.6 Total antioxidant capacity of soluble and insoluble fractions

The total antioxidant capacity (TAC) of soluble and insoluble fractions obtained by each digestive step, was measured with a spectrophotometer (PG Instruments, UK) set at 517 nm, using respectively the DPPH method and DPPH QUENCHER method, as previously described by Papillo *et al.*, (2014).[50] Before the analysis, the dried aliquots of the soluble fractions were re-suspended in 1 mL of water and the insoluble pellets obtained after each digestion step were freeze dried. The results were expressed as mmol of Trolox equivalents (TE)/kg of dry matter (DM).

2.2.7 α-glucosidase inhibition assay

The assay was carried out as reported by Chandran *et al.* (2015), with slight modifications.[53] The aliquots of extract from SDP, previously dried under nitrogen flow, were re-suspended in distilled water and appropriately diluted. Subsequently, 125 µL of this solution were added to 870 µL of 0.1 M phosphate buffer (pH 6.8). Then, 125 µL of a 0.1 M phosphate buffer solution (pH 6.8) containing the enzyme α-glucosidase from *Saccharomyces cerevisiae* (1 U/mL) were added. The reaction was started adding to the mixture, 125 µL of a 0.1 M phosphate buffer solution containing para-nitrophenyl-α-d-glucopyranoside (3 mM) as substrate for the enzyme. The samples were incubated for 20 min at 37°C, in a shaking thermostatic bath. The amount of released p-nitrophenol was determined spectrophotometrically, measuring the absorbance of the solution at 405 nm. Percent inhibition was calculated as follows:

$$\frac{[(\text{Abs } 100\% - \text{Abs } 0\%) - (\text{Abs sample} - \text{Abs blank})]}{(\text{Abs } 100\% - \text{Abs } 0\%)} \times 100$$

where:

Abs 100% was the absorbance of 100 % enzyme activity (in the reaction mixture only the enzyme and the substrate); Abs 0% was the absorbance of 0% enzyme activity (in the reaction mixture only the substrate without enzyme); Abs sample was the absorbance of the reaction mixture, containing substrate, enzyme and the tested extract; Abs blank was the absorbance of the reaction mixture without enzyme and containing only the substrate and tested extract.

2.2.8 α-amylase inhibition assay

The capacity of SDP to inhibit the activity of α-amylase was investigated as reported by Dey *et al.,* (2014), with slight modifications.[54]

The dried aliquots of SDP extracts were reconstituted in 1 mL of distilled water and properly diluted. The starch Azure was used as substrate. Particularly, 4 mg of starch Azure were suspended in 0.4 mL of a 0.1 M Tris-HCl buffer (pH 6.9), containing calcium chloride (0.01 M) and the solution was boiled for 5 minutes and pre-incubated 5 minutes at 37°C. Then, 0.4 mL of extract diluted in 0.1 M Tris–HCl buffer (pH 6.9) were added to the substrate. Finally, the reaction was started adding 0.2 mL of a 0.1 M Tris-HCl buffer solution (pH 6.9), containing α-amylase from porcine pancreas (2 U/mL). The reaction mixture was incubated

10 minutes at 37 °C, in a shaking thermostatic bath. The reaction was stopped by adding 1 mL of 50% (v/v) acetic acid solution. Afterwards, the samples were ultra-centrifuged at 14800 rpm for 10 min. at 4°C. The absorbance of supernatant was measured at 595 nm with a spectrophotometer (PG Instruments, UK). Percent inhibition was calculated, as follows:

$$\frac{[(\text{Abs } 100\% - \text{Abs } 0\%) - (\text{Abs sample} - \text{Abs blank})]}{(\text{Abs } 100\% - \text{Abs } 0\%)} \times 100$$

where:

Abs 100% was the absorbance of 100 % enzyme activity (in the reaction mixture only the enzyme and the substrate); Abs 0% was the absorbance of 0% enzyme activity (in the reaction mixture only substrate without enzyme); Abs sample was the absorbance of the reaction mixture, containing substrate, enzyme and the tested extract; Abs blank was the absorbance of the reaction mixture without the enzyme and containing only the substrate and tested extract.

2.2.9 Lipase inhibition assay

Lipase inhibition assay was carried out with a method adapted by Mennella *et. al.,* (2014).[55] Preliminarily, one milliliter of SDP extract, previously dried under nitrogen flow, was resuspended in one milliliter of distilled water and appropriately diluted. One milliliter of 0.1 M Tris–HCl buffer solution (pH 8.3), containing 0.3 mM 5-5′-dithio-bis (2-nitrobenzoic acid) (DTNB), was mixed with 50 µl of 0.1 M Tris–HCl buffer solution (pH 8.3) containing the enzyme (0.05 U/mL) and 40 µl of the solution of intestinal extract. Then, 80 µL of 20 mM 2,3-dimercapto-1-propanol tributyrate (dissolved in ethanol) were added in order to start the reaction. The samples were incubated in a shaking thermostatic bath for 30 min at 37°C. The lipase released 2,3-dimercapto-1-propanol that reacted with DTNB to release TNB anions (yellow colour). Tubes were centrifuged at 14800 rpm for 10 min at 4°C and the absorbance was measured at 412 nm with a spectrophotometer (PG Instruments, UK). Percent inhibition was calculated as follows:

$$\frac{[(\text{Abs } 100\% - \text{Abs } 0\%) - (\text{Abs sample} - \text{Abs blank})]}{(\text{Abs } 100\% - \text{Abs } 0\%)} \times 100$$

where:

Abs 100% was the absorbance of 100 % enzyme activity (in the reaction mixture only the enzyme and the substrate); Abs 0% was the absorbance of 0% enzyme activity (in the reaction mixture only substrate without enzyme); Abs sample was the absorbance of the reaction

mixture containing substrate, enzyme and the tested extract; Abs blank was the absorbance of the reaction mixture without enzyme and containing only the substrate and tested extract.

2.2.10 Statistical analysis

According to Papillo *et al.,* (2014),[50] the TAC of all the soluble fractions plus the TAC of the last insoluble residue were used to calculate the overall potential TAC (OP-TAC), of which the FREE-TAC was related to the SSP, while the BOUND-TAC was due to the soluble fractions collected in all the steps plus the last residue. In addition, the total amount of ETs, EAs and GA released at all digestion steps were used to calculate the overall bioaccessible ETs (OB-ETs), EAs (OB-EAs) and GA (OB-GA), respectively. The amount of each class of compounds released in the SSP were the FREE-ETs, FREE-EAs and FREE-GA, while the amount released after SSP (from stomach to colon) were the BOUND-ETs, BOUND-EAs and BOUND-GA. The sequential enzymatic digestion was performed in triplicate and all the samples were always analysed in triplicate. The data were analysed by ANOVA, and the mean values were compared by Tukey test ($\alpha = 0.05$), using XLStat statistical software (Addinsoft, New York, NY). The Pearson correlation coefficient was used for the bivariate correlation analysis between variables ($\alpha = 0.05$).

2.3 Results

2.3.1 PPe dietary fiber

TDF content of PPe powder was 48 g/100g of PPe, whose 72.9% was IDF and 27.1% was SDF.

2.3.2 *Bioaccessibility of polyphenols from PPe and from PPeC*

Punicalin anomers, punicalagin anomers, pedunculagin anomers, castalagin der., GA , EA and three EAs were identified and quantified in the extracts of each step of simulated *in vitro* digestion of PPe and PPeC whereas they were absent in cellulose and CTC. The results were summarized in **Table 2.1.**

The total amount of bioaccessible ETs, EAs and GA from PPe were 7666.3 mg/100g of PPe powder, of which the 42.8% (3278.7 mg/100g of PPe powder) were immediately released in the SSP, the 52.0% were potentially bioaccessible in the upper GiT and the 5.2% were potentially bioaccessible only after in the SCP.

The total amount of bioaccessible ETs, EAs and GA from PPeC was 491.1 mg/100g of PPeC, of which 223.2 mg (45.4%) were from the salivary phase, thus being potentially bioaccessible early in the proximal GiT, while the remaining part 267.9 mg (54.6%) were released in the next steps. In particular, 216.8 mg/100g of PPeC (44.1% of the total polyphenols released) were potentially bioaccessible in the upper GiT (stomach and duodenum) after the action of pepsin and pancreatin, while 51.1 mg (10.4% of the total polyphenols released) were potentially bioaccessible 233 in the lower GiT (colon) after Pronase E and Viscozyme L action. The extract from SSP was mainly constituted by both punicalagin and EA, whereas the subsequent extracts were mainly constituted by EA.

In **Table 2.2.**, the values of overall bioaccessible (OB)-polyphenols (i.e. OB-ETs, OB-EAs, OB-GA), as well as the percentage of free-polyphenols (i.e. FREE-ETs, FREE-EA, FREE-GA) and bound polyphenols (i.e. BOUND-ETs, BOUND-EAs and BOUND-GA) for both PPeC and for PPe were reported. Data showed that despite huge differences of repartition between free and bound fractions of specific polyphenols out of the overall bioaccessible amount from PPe and PPeC, similar percentages for total polyphenols were found.

Table 2.1. Concentrations (mg/100g) of polyphenols released upon enzymatic digestion of PPe and PPeC. For PPeC the percentage variation (Var.) of each concentration *vs* the expected concentration was also reported. Expected concentrations were calculated on the basis of data obtained by PPe *in vitro* digestion, taking into account the enrichment of PPe in PPeC (7.5% by weight). Values were expressed as mean ± SD. For each compound, values in the same row followed by different lowercase letter (for PPe digestion) or different uppercase letter (for PPeC digestion) are significantly different ($p < 0.05$) according to Tukey's test.

	Simulated salivary phase			**Simulated gastric phase**			**Simulated duodena lphase**			**Simulated colon phase**			**Total**		
Compounds	**PPe**	**PPeC**	Var.	**PPe**	**PPeC**	Var.	**PPe**	**PPeC**	Var.	**PPe**	**PPeC**	Var.	**PPe**	**PPeC**	Var.
	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)	mg/100g	mg/100g	(%)
Punicalin	855.9±193.0 ^{a}	33.1±1.4 ^{A}	-48	924.1±60.9 ^{a}	11.8±2.5 ^{B}	-83	240.1±17.9 ^{b}	4.3±0.1 ^{C}	-76	62.3±2.9 ^{b}	< LOD	n.a.	2082.4±166.3	49.2± 1.2	-68
Punicalagin	1491.6±122.4 ^{a}	57.0±8.0 ^{A}	-48	1039.8±62.1 ^{b}	22.9±1.4 ^{B}	-70	426.6±54.9 ^{c}	14.9±3.2 ^{B}	-53	74.1±16.7 ^{d}	< LOD	n.a.	3032.1±218.2	94.8±9.8	-58
Pedunculagin	62.1±14.4 ^{a}	2.4±0.4 ^{A}	-48	23.9±3.1 ^{b}	1.1±0.5 ^{B}	-39	15.3±3.1 ^{b,c}	< LOD	n.a.	0.7±0.1 ^{c}	< LOD	n.a.	102.0±18.4	3.5±0.3	-53
Castalaginder.	73.3±6.3 ^{c}	43.4±6.5 ^{B}	704	15.9±2.3 ^{d}	1.5±0.4 ^{D}	25	569.2±18.4 ^{a}	61.3±2.7 ^{A}	46	181.7±7.1 ^{b}	16.3±0.8 ^{C}	22	840.1±19.8	122.4± 8.7	97
ETs	2482.9±70.9 ^{a}	135.9±16.0 ^{A}	-26	2003.7±114.1 ^{b}	37.2±4.0 ^{C}	-75	1251.2±78.5 ^{c}	80.5±5.9 ^{B}	-13	318.8±20.7 ^{d}	16.3±0.8 ^{D}	-31	6056.6±156.9	270.1±17.2	-40
Ellagic acid HE	88.2±4.6 ^{a}	3.8±0.7 ^{A}	-42	41.4±0.9 ^{b}	2.1±0.2 ^{B}	-32	20.2±0.2 ^{c}	1.9±0.1 ^{B}	27	5.3±0.3 ^{d}	0.6±0.0 ^{C}	50	155.1± 5.2	8.4±0.4	-27
Ellagic acid PE	23.3±0.4 ^{a}	1.6±0.3 ^{A}	-6	< LOD	0.3±0.1 ^{C}	n.a.	8.0±0.1 ^{b}	1.0±0.1 ^{B}	67	2.8±0.1 ^{c}	0.3±0.0 ^{C}	50	34.1±0.3	3.2±0.4	28
Ellagic acid DHE	29.6±2.8 ^{a}	1.7±0.1 ^{A}	-23	12.3±3.6 ^{b}	0.7±0.1 ^{C}	-22	9.9±1.8 ^{b}	1.0±0.1 ^{B}	43	2.6±0.1 ^{c}	0.2±0.0 ^{D}	0	54.4±8.2	3.6± 0.1	-10
Ellagic acid	537.5±12.3 ^{a}	55.9±5.3 ^{B}	40	341.4±71.3 ^{b}	14.6±0.6 ^{D}	-42	136.8±1.1 ^{c}	69.1±0.3 ^{A}	584	59.6±5.2 ^{d}	33.5±0.7 ^{C}	661	1075.2±86.4	173.1±4.0	117
EAs	678.6±13.7 ^{a}	63.0±6.4 ^{B}	25	395.1±68.5 ^{b}	17.7±0.8 ^{D}	-39	174.9±3.0 ^{c}	73.1±0.3 ^{A}	467	70.3±4.9 ^{d}	34.6±0.8 ^{C}	565	1318.8±83.0	188.4±4.6	93
Gallic acid	117.3±14.2 ^{a}	24.3±1.9 ^{A}	179	103.2±1.7 ^{a}	7.5±1.2 ^{B}	-1	57.5±1.2 ^{b}	0.8±0.2 ^{C}	-81	12.9±0.1 ^{c}	< LOD	n.a.	291.0±16.5	32.6±0.9	52
ETs + EAs + GA $^{\#}$	3278.7±90.0 ^{a}	223.2±24.3 ^{A}	-8	2502.0±77.7 ^{b}	62.5±6.1 ^{C}	-66	1483.6±80.0 ^{c}	154.3±6.0 ^{B}	41	402.0±25.5 ^{d}	51.1±1.5 ^{C}	72	7666.3±122.9	491.1±22.7	-13

$^{\#}$ mg of punicalagin equiv.+ mg of EA equiv. + mg of GA equiv. HE: Hexoside; PE: Pentoside; DHE: Deoxyhexoside; < LOD: Lower than the limit of detection; n.a.: not available.

Table 2.2. Overall bioaccessible-ETs (OB-ETs), Overall bioaccessible-EAs (OB-EAs) and Overall bioaccessible-GA (OB-GA), for PPe and PPeC,. FREE-ETs, FREE-EAs and FREE-GA corresponded to the amount released in the SSP while the amount released later on the digestion were the BOUND-ETs, BOUND-EAs and BOUND-GA.

	Compounds	**Overall bioaccessible (mg/100g)**	**Free (mg/100g)**	**Free (%)**	**Bound (mg/100g)**	**Bound (%)**
PPe	ETs	6056.6 ± 156.9	2482.9 ± 70.9	41	3573.5 ± 169.4	59
	EAs	1318.8 ± 83.0	678.6 ± 13.7	51	640.3 ± 70.4	49
	GA	291.0 ± 16.5	117.3 ± 14.2	40	173.7 ± 2.7	60
	Total	7666.3 ± 122.9	3278.7 ± 90.0	43	4387.6 ± 116.7	57
PPeC	ETs	270.1 ± 17.2	135.9 ± 16.0	50	134.2 ± 1.6	50
	EAs	188.4 ± 4.6	63.0 ± 6.4	33	125.4 ± 1.8	67
	GA	32.6 ± 0.9	24.3 ± 1.9	75	8.3 ± 1.0	25
	Total	491.1 ± 22.7	223.2 ± 24.3	45	267.9 ± 2.4	55

2.3.3 Total antioxidant capacity

Figure 2.2. reported the TAC of soluble and insoluble fractions after each step of the *in vitro* digestion of both PPe that PPeC.

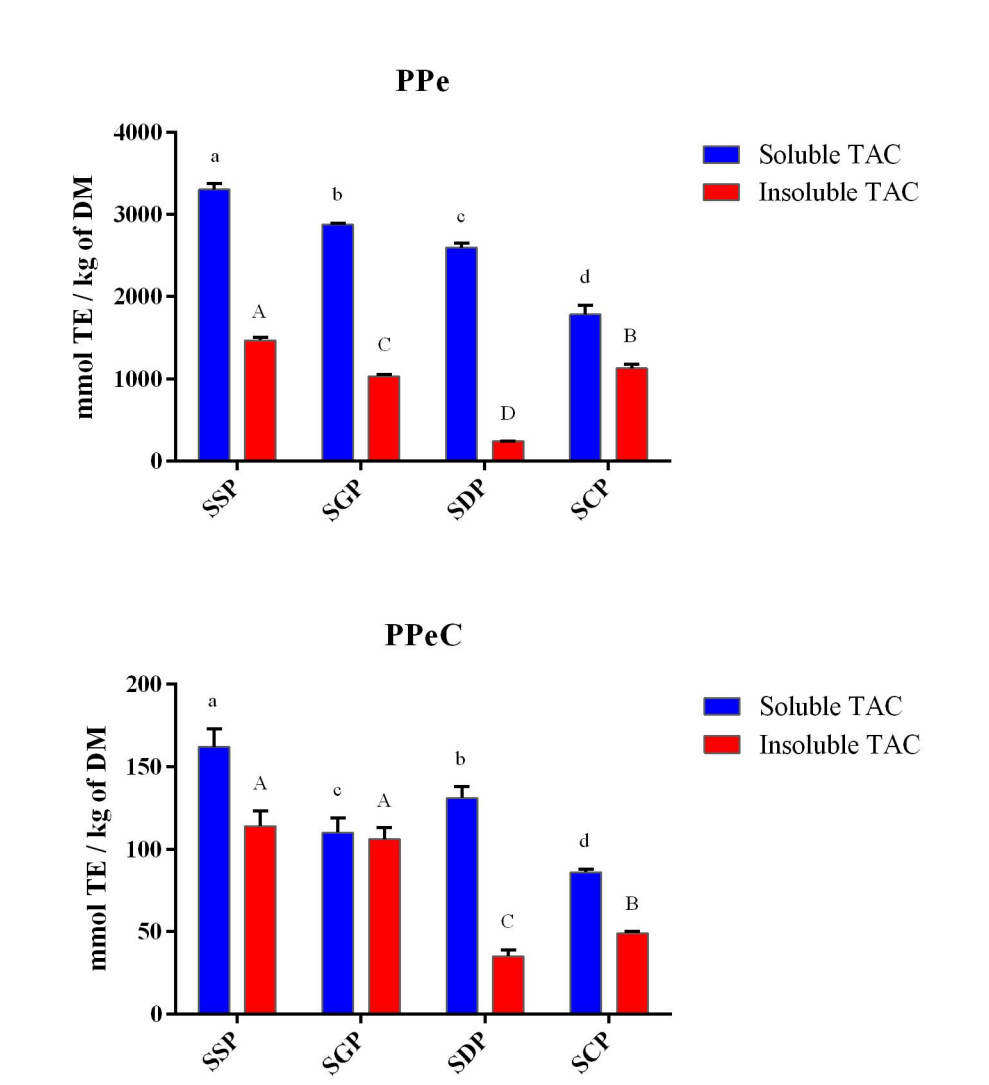

Figure 2.2. TAC of soluble and insoluble fractions from simulated gastrointestinal digestion of PPe and PPeC. Mean values were expressed as mmol of TE/kg of DM. Both for PPe and for PPeC digestion steps, different lower case letter (for soluble TAC) and upper case letter (for insoluble TAC) indicate significantly different values ($p < 0.05$) according to Tukey's test.

Data obtained by PPe digestion showed that the TAC of fractions solubilized gradually decreased along the GiT. The TAC of insoluble fraction (pellet) after SCP (colon) was higher than that obtained after SGP, thus indicating that a great amount of TAC was released after the disruption of the DF matrix in PPe.

Similarly, during PPeC digestion, the TAC of the fraction solubilized in the SSP was always higher than the others but a higher TAC of the SDP than SGP extract was observed. The insoluble TAC found for PPeC followed the same trend along the digestive steps as for PPe, except the TAC of SCP pellet that was only a bit higher than that relative to SDP.

The FREE-TAC and BOUND-TAC were evaluated both for PPe and for PPeC. For PPe, the values of FREE-TAC and BOUND-TAC were 3307 mmol TE/kg of DM (29% of OP-TAC) and 7933 mmol TE/kg of DM (71% of OP-TAC), respectively. BOUND-TAC calculated for the extracts after the SGP, SDP and SCP were 26%, 23% and 16% of the total OP-TAC, respectively.

Likewise, for PPeC, FREE-TAC and BOUND-TAC were 162 mmol TE/257 kg of DM (31% of OP-TAC) and 360 mmol TE/kg of DM (69% of OP-TAC), respectively. The values of BOUND-TAC calculated for the extracts after the SGP, SDP and SCP were 21%, 25% and 17% of the total OP-TAC, respectively.

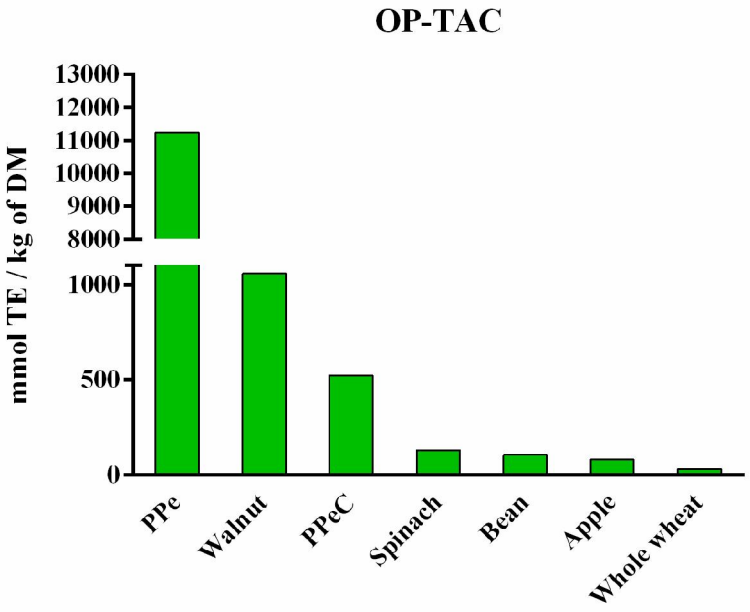

Figure 2.3. Comparison among OP-TAC values of PPe and PPeC and OP-TAC of 5 common plant foods as reported by Papillo *et al*., (2014).[50] Values are expressed as mmol of TE per kg of DM.

The OP-TAC calculated for PPe and PPeC were 11240 mmol TE/kg of DM and 522 mmol TE/kg of DM, respectively. In **Figure 2.3.**, the values of OP-TAC calculated for PPE and PPeC were compared with the values of OP-TAC, calculated for five common plant foods.

2.3.4 Inhibition of enzyme activity

SDP extracts from PPeC digestion were tested in order to assay their capacity to inhibit *in vitro* α- amylase, α-glucosidase, and lipase. The results were reported in **Figure 2.4.** Data demonstrated that SDP strongly inhibited α-glucosidase activity, while a weak inhibitory capacity against α-amylase and lipase was found. This effect was due to the whole mixture of polyphenols (ETs, EA, EAs and GA) present in SDP extract. It was calculated that 0.007 mg of polyphenols in SDP extract inhibited by 84% the activity of 1 U of α-glucosidase, whereas about 5 (0.036 mg) and 80 (0.578 mg) greater amounts of the same mixture of polyphenols were necessary to inhibit by 72% and by 13% 1 U of α-amylase and 1 U of lipase, respectively.

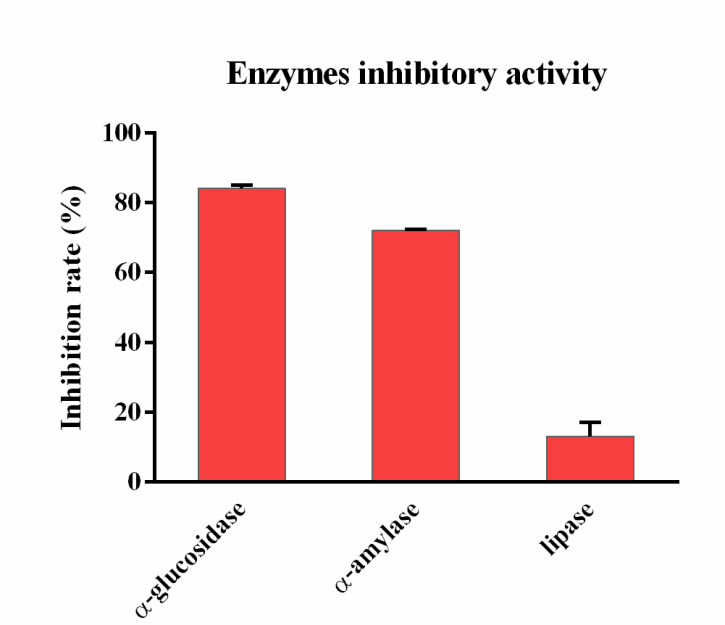

Figure 2.4. Enzyme inhibition rate of extracts from SDP of PPeC. The dilution factors of the tested extracts, compared with the initial concentration, were 30, 6 and 6, for α-glucosidase, α-amylase and lipase, respectively. Data were corrected for the inhibition elicited by CTC.

2.4 Discussion

For the first time in this study the potential TAC and bioaccessibility of polyphenols from a PPe powder and a PPe-enriched baked food, as well as their ability to inhibit the activity of α-amylase, α-glucosidase and lipase, were evaluated upon an *in vitro* simulated gastrointestinal digestion. PPeC developed in this study contained 7.5% of PPe, providing 491.1 mg/100g of PPeC of overall bioaccessible polyphenols (mainly ETs and EA), and 3.6 % of DF according to Hasnaoui *et al.* and

Viuda-Martos *et al.*.[28,56] This formulation guaranteed sensory acceptability of the product according to Ismail *et al.*[41] and Srivastava *et al.*[57] and allowed the nutritional claim of *"source of dietary fiber"* according to European Commission Regulation 1924/2006 and European Food Safety Authority (EFSA).[58,59]

Previous *in vivo* study demonstrated that under physiological conditions ETs are partially hydrolyzed and release EA. EA is then gradually metabolized in the intestine to produce bioactive urolithin D, urolithin C and finally urolithin A and urolithin B in the distal parts of the intestine.[31] Interestingly, our data showed that the potential bioaccessibility of ETs was 40% lower while that of EAs and GA was respectively 93% and 52% higher from PPeC than PPe. In particular, comparing the release of polyphenols obtained from PPeC upon the *in vitro* digestion with the expected values extrapolated from the data obtained for PPe, and taking into account the enrichment of PPe in PPeC (7.5% by weight), data showed (**Table 2.1.**) a 13.4% reduced overall release of polyphenols from PPeC compared to the expected concentration. This total difference was due to a halved release of ETs, and a doubled and a 1.5 fold increased release of EAs and GA from PPeC compared to the expected values, respectively. It is likely that during the process to produce cookies, ETs (mainly punicalin and punicalagin) and EA present in PPe, partially hydrolyzed by forming EA and GA, respectively, in PPeC.[60] Moreover, it was hypothesized that some of ETs, such as castalagin der., could become more bioaccessible from PPe under cookies processing because a double amount (122.4 mg/100g of PPeC vs 62.2 mg/100g of PPeC) was retrieved in the solubilized fractions after *in vitro* digestion compared to the expected amount. Interestingly, looking at the step by step release of castalagin der. upon the simulated gastrointestinal digestion, data showed that the 63.1% of the increased release occurred in the SSP whereas the 31.9% and the 4.8% were released in the SDP and in the SCP, respectively. On the other hand, the newly formed GA in PPeC was completely released in the SSP (140%) while the newly formed EAs were released by 66% and by 32% in the SDP and SCP respectively and only the 14% was present in the SSP.
These data demonstrated that the complex food matrix of PPeC, mainly including starch and the gluten network together with PPe, strongly influenced the release of EAs, mainly EA, so that the enzyme action of pepsin and pancreatin was necessary to allow their delivery from the food matrix.
This happened because polyphenols can interact with food macronutrients through covalent and non-covalent interactions, the resultant affinity being also influenced by the water solubility of polyphenols: higher is the water-solubility lower is the affinity with non-polar food matrices.[61-64] EA is much less soluble in water than ETs and GA, thus potentially forming stronger hydrophobic

interactions with the water insoluble gluten matrix of PPeC, and being mostly released after the disruption of that matrix compared to its more water-soluble glycosylated derivatives (mainly detected in the SSP).[29,31] In other words, data showed that the use of PPe as ingredient in the cookies determined a different bioaccessibility of the constituent polyphenols compared to the PPe powder, thus influencing the related potential antioxidant protection. In fact, a significant correlation among the total amount of ETs, EAs and GA, released at each digestive step, and the values of TAC measured for the corresponding soluble extracts, both for PPe ($r = 0.983$; $p = 0.017$) and for PPeC ($r = 0.969$; $p = 0.031$) was observed. Moreover, the effect of digestive enzymes on the two types of matrix (PPe and PPeC) caused a different behavior of the un-solubilized materials at each digestive step. In particular, results showed that, insoluble fractions of PPe and PPeC after the SCP, i.e. after the action of bacterial proteases and cellulolytic enzyme mixture, exhibited a higher antioxidant activity than the insoluble material obtained by the SDP.

The potential antioxidant protection in the gut demonstrated *in vitro* in this study might be at the basis of the reduced colon inflammation and positive modulation of the gut microbiota induced by PPe in obese mice fed with a high-fat diet.[65]

In PPe, like in cereals, fruits and vegetables, polyphenols are found free or covalently bound to cell wall structural components, such as arabinoxylans, cellulose and lignin, or to proteins.[66] So, once ingested the action of digestive enzymes allows the polyphenols to be delivered by the food matrix and act against reactive oxygen species (ROS), along the GiT.[12,13,50] On the other hand the destruction of the matrix operated by the enzymes allows the bound polyphenols to dispose on th surface of the un-solubilized material and to act as a sponge of free radicals along the GiT before arriving to the colon and be delivered by the gut microbiota enzymes.[13]

Mounting evidence indicate that the interactions among the gut microbiota, DF and polyphenols may have implications on the prevention of obesity risk. The potential antioxidant activity along the GiT, demonstrated in this study by PPe and PPeC, is perfectly in line with the conclusion driven by Al-Muammar and Khan, that the prevention of obesity risk, associated with consumption of pomegranate products, is correlated to their antioxidant compounds released along the GiT.[38] Data of the present study showed that values of FREE-TAC were 29% and 31% of OP-TAC, for PPe and PPeC, respectively. These results were similar to those obtained for apple (32%) and spinach (29%) in a previous study from our research group, and a bit lower than those relative to walnuts (42%).[50] However, PPe exhibited an OP-TAC that was about ten times higher than the OP-TAC calculated for the walnuts (see **Figure 2.3.**). This was in line with the huge abundance of the whole mix of antioxidants (ETs, EA, EAs and GA) in pomegranate by-products (85 g/kg of d.w.) compared to

walnuts (1.8 to 3.0 g for kg of fresh weight), particularly to the higher relative abundance of hydrolysable tannins in pomegranate by-products (about 99.5%) than in walnuts kernels (about 76.3%).[26,67] In fact, compared to other polyphenols, hydrolysable tannins are recognized highly antioxidants and very active inhibitors of free radicals. These effects were directly correlated to their molecular weight and to the number of reactive galloyl groups in the molecules.[68] Punicalagin is recognized as one of strongest antioxidant compound and major responsible of the high antioxidant capacity of pomegranate juice being 16 folds more antioxidant than EA and 7 folds more antioxidant than GA.[26,27,69]

Together with the antioxidant potential, our data also indicated that PPeC could influence glucose metabolism and lipid digestion by modulating activity of α-glucosidase, α-amylase and lipase.

Starch is the most common complex carbohydrate in foods, and its digestion in the GiT is mainly mediated by α-amylase and α-glucosidase. The rate of glucose release in the GiT modulate energy homeostasis and fine physiological mechanisms underlying hunger and satiety.[70]

The findings of the present study indicated that the soluble extract from SDP strongly inhibited α-glucosidase and, at a lesser extent, α-amylase. The results on α-glucosidase inhibition were consistent with those previously obtained by Li *et al*.[39] with PPe phenolics and those obtained by Cam *et al.*[71] with a functional ice cream containing PPe phenolics-enriched microcapsules.

The use of α-glucosidase from *Saccharomyces cerevisiae* in the present study could be considered as a limitation because it is known that the extent of activity of α-glucosidase inhibitors may vary according to the origin of α-glucosidase, i.e. if they are α-glucosidase from yeast or from mammalians.[72-75] However, in previous studies polyphenols from PPe extracts mainly including punicalagin, punicalin and ellagic acid strongly inhibited *in vitro* rat intestinal α-glucosidase and recombinant human maltase–glucoamylase but exhibited weak or no inhibitory effects against porcine pancreatic α-amylase.[34,36,76] So, it might be speculated that the results found in the present study using a pure yeast α-glucosidase might be found also using a mammalian enzyme, but future studies are warranted to demonstrate this hypothesis.

On the other hand, lipase is the major enzyme responsible for the gastrointestinal digestion of dietary triglycerides into monoglycerides and free fatty acids. The inhibition of pancreatic lipase along the GiT could result in a reduced lipid absorption and in a consequent reduced energy intake.The weak inhibitory effect of SDP extract on lipase activity was in line with results previously obtained *in vitro* and with the *in vivo* evidence that consumption of a pomegranate leaf extract could inhibit the development of obesity and hyperlipidemia in high-fat diet induced obese mice also through inhibition of the pancreatic lipase activity.[15,40]

2.5 Conclusion

In conclusion, the findings of the present study support the use of PPe at a dose of 7.5% by weight in bakery food products, as a functional ingredient to counteract oxidative stress along the GiT. The potential antioxidant protection may involve all the organs of the GiT due to the release of ETs, EA, EAs and GA along all the digestive system. Moreover it was demonstrated that the baking process increased the amount of EA in the PPeC due to chemical degradation of ETs, thus allowing its increased release in the duodenal and colon phase compared to the raw ingredient. In fact, food matrix strongly influenced both the release of polyphenols in the different steps as well as the antioxidant potential of the un-solubilized material. Together with a high antioxidant potential, the mix of polyphenols solubilized in the SDP also acted as strong inhibitors of α-glucosidase thus possibly influencing in a positive manner the glucose metabolism *in vivo*. I*n vivo* studies are warranted to evaluate the sensory properties of the PPeC developed in this study and its effects on oxidative stress and on glucose and lipid metabolism.

2.6 Abbreviations

CTC: Control cookies

DF: Dietary fiber

EA: Ellagic acid

EAs: Ellagic acid derivatives

ETs: Ellagitannins

GA: Gallic acid

GiT: Gastrointestinal tract

PPe: Pomegranate peels

PPeC: Pomegranate peels-enriched cookies

SCP: Simulated colon phase

SDP: Simulated duodenal phase

SGP: Simulated gastric phase

SSP: Simulated salivary phase

TAC: Total antioxidant capacity

TE: Trolox equivalents

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Chapter 3

Potential functionality and bioaccessibility of polyphenols and cynaropicrin from breads enriched with artichoke (*Cynara cardunculus* L. var. *scolymus*) stems

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Abstract

Globe artichoke (*Cynara cardunculus* L. var. *scolymus)* by-products from industrial processing mainly include stems and external leaves. They may be natural and sustainable source of polyphenols (PPs) and dietary fiber for the formulation of functional foods.

In this study an artichoke stem powder (ASP) was used at three concentrations (3%, 6% and 9%), in substitution of wheat flour, for the formulation of new breads. The potential bioaccessibility of PPs and cynaropicrin from the ASP-enriched breads was evaluated *in vitro* by using a digestion model coupled to High Resolution Mass Spectrometry (HRMS) analysis. The overall total antioxidant capacity (TAC) of the bioaccessible and unsolubilized fractions obtained at the duodenum and colon steps were tested together with the ability of ASP-enriched breads to modulate α-glucosidase activity.

Data showed that most of PPs and cynaropicrin (about 82% and 74% of total bioaccessible amount) were mainly released at the duodenum step of digestion but caffeic acid that was mainly released in the colon (about 88%). PPs released in the duodenal step showed an antioxidant activity and an inhibitory capacity towards α-glucosidase correlated with the amount of ASP added in the bread.

In conclusion it was demonstrated that ASP-enriched breads allowed the release of PPs, cynaropicrin and antioxidant activity along the gastro-intestinal tract and modulated carbohydrate metabolism *in vitro*.

3.1 Introduction

Globe artichoke (*Cynara cardunculus* L. var. *scolymus*) is a vegetable distributed worldwide and mainly cultivated in the Mediterranean regions. Italy is the first world producer (451461 tons/year) followed by Egypt (266196 tons/year) and Spain (234091 tons/year).[1] The immature inflorescence (capitula or head) represents the edible part of the plant and is consumed fresh, frozen, or canned.[2] Stems and external leaves represent about 80-85 % of the total fresh weight of the plant and are the main artichoke by-products from industrial processing.[3]

Both the edible parts and the by-products are well known sources of dietary fiber (DF) and polyphenols (PPs), mainly including caffeoylquinic acids (CQA), di-caffeoylquinic acids (DCQA) and flavones.[3-6] Chlorogenic acid (5-*O*-caffeoylquinic acid), 1,5-di-*O*-caffeoylquinic acid and 3,5-di-*O*-caffeoylquinic acid are the most abundant phenolic acids in artichoke tissues, whereas luteolin and apigenin glycosides and rutinosides derivatives are the most abundant flavones.[3,4,7,8] These molecules exert *in vitro* antioxidant, antimicrobial and anticarcinogenic activities, they can modulate the endothelial-type nitric-oxide synthase gene expression and act as natural inhibitors of key enzymes in metabolic syndrome development.[9-17] Moreover, several animal and human studies highlighted the health benefits associated to the intake of artichoke bioactive compounds, mainly including prebiotic effects on colonic microbiota as well as hepatoprotective, antioxidative, hypolipidemic, hypoglycemic, anti-inflammatory, anti-dyspeptic, cholesterol lowering, anti-obesity and bile-expelling effects.[18-33] These effects were mostly associated to the ability of artichoke caffeoylquinic acids and flavonoids to modulate cellular antioxidant defense systems and the activity of some digestive enzymes.[34]

Sesquiterpene lactones, including guaianolides, represent another class of compounds present in artichoke (mainly in the leaves) and having several biological properties such as anti-hyperlipidemic and anti-photoaging activities.[35,36] Cynaropicrin is the most abundant, accounting for the 80% of bitter taste of artichoke.[37,38] This compound and the grosheimin can activate *in vitro* the human bitter taste receptor hTAS2R46 at very low concentrations.[39] This property may contribute to the metabolic benefits mostly associated to the consumption of artichoke DF and PPs. Indeed, mounting evidence shows that bitter compounds modulate neurohormonal response behind gastrointestinal motility, glucose homoeostasis and appetite control through the activation of bitter taste receptors located along the gastrointestinal tract (GiT).[40-44]

The presence of a multitude of bioactive compounds in artichoke by-products make them a good source of functional ingredients to add in food as already proposed by some researchers.[5,45-49] Despite the undisputable social value to valorize agricultural by-products, their use as functional ingredients in new food matrices need a bio-efficacy validation. In fact, both food processing and structure may influence the chemical composition and the bioavailability of bioactive compounds from the enriched-foods.[50,51] The bioaccessibility, the bioavailability and the potential antioxidant effects along the GiT of PPs from artichoke heads, were previously studied both *in vivo* and *in vitro*.[21,52-54] Scientific literature is still lacking on the bioaccessibility and potential bio-efficacy of bioactive compounds from foods enriched with artichoke by-products.

In this study the stems were selected among artichoke by-products and used at 3%, 6% and 9% in the formulation of new types of bread. The potential bioaccessibility of PPs and cynaropicrin from the breads as well as the overall total antioxidant capacity (TAC) and potential ability to modulate α-glucosidase activity in the GiT were assessed.

3.2 Material and methods

3.2.1 Chemicals

Water, methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Ethanol and formic acid were purchased from VWR international (Fontenay-sous-Bois, France). Cellulose powder was obtained from Fluka (Buchs, Switzerland). Total dietary fiber assay kit was purchased from Megazyme International (Wicklow, Ireland). Calcium chloride, potassium chloride, sodium chloride, potassium phosphate monobasic, magnesium chloride hexahydrate, ammonium carbonate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl, 95% (DPPH), sodium bicarbonate, sodium hydroxide, hydrochloric acid, celite, 5,5′-Dithiobis(2-nitrobenzoic acid), *tris*(hydroxymethyl)-aminomethane hydrochloride, 4-morpholinoethanesulfonic acid (MES), para-nitrophenyl-α-d-glucopyranoside, sodium phosphate dibasic heptahydrate (≥ 99,99% trace metals basis) were purchased from Sigma-Aldrich (St. Louis, MO). α-amylase from porcine pancreas (type VI-B ≥ 10 U/mg), α-glucosidase from *Saccharomyces cerevisiae* (Type I ≥ 10 U/mg), protease from *Streptomyces griseus*, (Pronase E, Type XIV ≥ 3.5 U/mg), cell wall degrading enzyme complex from *Aspergillus* sp. (Viscozyme L), pancreatin from porcine pancreas (4 X USP) and pepsin from porcine gastric mucosa (≥ 250 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). Analytical standards of cynarin, chlorogenic acid, and luteolin 7-O-glucoside were purchased from Sigma-Aldrich (St. Louis, MO). The analytical standard of cynaropicrin was purchased from Extrasynthese (Lyon, France). The calibration solutions for HRMS analysis were obtained from Thermo Fisher Scientific (Bremen, Germany). The ingredients for the preparation of white-breads and enriched breads were purchased from a local market.

3.2.2 Plant material and extraction of bioactive compounds

Fresh globe artichokes were from the varietal type "Tondo di Paestum". External leaves and floral stems were separated from the artichoke heads (including outer bracts, inner bracts and the receptacle). The three fractions were washed and freeze-dried and afterwards were minced with a Grindomix knife mill (10000 rpm for 30 sec.) until to obtain three different dry powders. The extraction of PPs and cynaropicrin from artichoke powders was carried out with a method adapted by Palermo *et al.*, (2013) and Menin *et al.*, (2012).[55,56] Specifically, 10 mL of a solution methanol/water (75:25, v/v) acidified with 0.1% of formic acid were added to 500 mg of dry

sample. Ultrasound assisted extraction was performed for 20 minutes. Then, the samples were centrifuged 10 min at 4000 rpm at 4°C. Two milliliters of supernatant were collected and further ultra-centrifuged 10 min at 14800 rpm at 4°C before the analysis. The identification and quantification of PPs and cynaropicrin was carried out using an U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Santonin was used as internal standard for the evaluation cynaropicrin extraction recovery.

3.2.3 Total phenols and antioxidant activity of artichoke fractions

One milliliter of the extracts obtained as reported above was dried under nitrogen flow and subsequently was re-suspended in 1 mL of methanol and appropriately diluted before the analysis. The amount of total phenols was measured using the Folin–Ciocalteu assay as described by Ferracane *et al*., (2010).[57] Specifically, 125 µL of sample properly diluted were mixed with 125 µL of Folin–Ciocalteau phenol reagent and allowed to react for 6 min. After 6 min, 1.25 mL of saturated Na_2CO_3 solution (7.5%) was added. The samples were incubated for 90 minutes at room temperature, before that the absorbance of the reaction mixture was measured with a spectrophotometer (PG Instruments, UK) set at 760 nm. The results were expressed mg of gallic acid equivalents (GAE)/g of dry weight (DW). The antioxidant capacity (AC) of the extracts was measured spectrophotometrically by using the DPPH assay according to Papillo *et al.,* (*2014*).[58] The results were expressed as µmol Trolox equivalents (TE)/ g of DW.

3.2.4 Insoluble, soluble and total dietary fiber of artichoke by-products

The determination of soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and total dietary fiber (TDF) in artichoke dry powders, was carried out according to the protocol described by Prosky *et al*., (1987).[59] The results were reported as mg/g of DW. For each sample the IDF/SDF ratio was calculated.

3.2.5 Food preparation

Three types of artichoke stem powder (ASP) enriched bread were formulated by using a traditional bread recipe and replacing wheat flour with 3%, 6% and 9% of ASP on total ingredients. The 3 recipes were reported in **Table 3.1.**. The ingredients were mixed for 12 min

and portions (150 g) of dough were left to rise at room temperature for one hour before baking (200°C for 20 min and 185°C for 15 min) in an air ventilated oven.

Table 3.1. Recipes of ASP-enriched breads and control bread.

Bread	Water (g)	Wheat flour (g)	Dry yeast (g)	Salt (g)	Artichoke stem powder (g)
Control	50	100	1.2	1	/
3% ASP	50	95.45	1.2	1	4.55
6% ASP	50	90.09	1.2	1	9.10
9% ASP	50	86.3	1.2	1	13.7

3.2.6 In-vitro simulated gastrointestinal digestion

The gastrointestinal digestion of the ASP-enriched breads was performed by using the method described by Minekus *et al.,* (2014) and briefly modified.[60] For the oral phase 2.5 g of freeze-dried sample were minced and mixed for 2 min at 37°C in a thermostatic shaking bath with 1.75 mL of simulated salivary fluid (SSF), 0.25 mL of α-amylase from porcine pancreas (1500 U/mL), 12.5 µl of 0.3 M $CaCl_2$ and 488 µl of water. For the simulated gastric phase, oral bolus was mixed with 3.75 mL of SGF, 2.5 µl of 0.3 M $CaCl_2$ and 0.8 mL of a solution of porcine pepsin (25000 U/mL) made up in simulated gastric fluid (SGF). The pH was adjusted to 3.0 with 1 M HCl, the volume was filled up to 10 mL with distilled water and the samples were incubated for 2 hours at 37°C in a thermostatic shaking bath. For the simulated duodenal phase (SDP) the gastric chyme was mixed with 5.5 mL of simulated intestinal fluid (SIF), 2.5 mL of pancreatin solution (800 U/mL), 1.25 mL of a bile salts solution (10 mM in the final volume), 20 µl of 0.3 M $CaCl_2$ and 650 µl of distilled water. The pH was adjusted to 7.0 with 1 M NaOH and the samples were incubated for 2 hours at 37°C in a thermostatic shaking bath. At the end of SDP step, the samples were centrifuged at 4000 rpm for 10 minutes, at 4°C. The soluble fractions were separated from the insoluble pellets, and collected for the analysis. The insoluble pellets were submitted to a further digestion as previously reported by Colantuono *et al.,* (2016), in order to simulate the release of bioactive compounds in the post duodenal phase.[61] Specifically, the residual pellets were added with 15 mL of distilled water and 2.5 mL of Pronase E solution (1 mg/mL) and incubated at 37 °C for 1 h (pH 8.0). Subsequently, the samples were centrifuged at 4000 rpm for 3 min and the soluble extracts and the pellets were separated. The pellets were further added with 17.5 mL of distilled water and 75 µl of Viscozyme L, to obtain

the complete disruption of the food matrix, including the DF fraction. The samples were incubated at 37 °C for 16 h (pH 4.0) and subsequently the pellets and the soluble fractions were separated by centrifugation (4000 rpm for 3 min), before the analysis. For the identification and quantification of PPs in the soluble fractions, 500 µL of each soluble extracts, respectively from SDP and simulated colon phase (SCP), were dried under nitrogen flow and re-suspended in 1 mL of a solution of methanol/water (75:25, v/v) acidified with 0.1% of formic acid, and properly diluted before the HRMS analysis. For the identification and quantification of cynaropicrin in the extracts from digestion, 200 µL of each soluble extracts from SDP and SCP, were extracted with 1 mL of ethyl acetate. The samples were mixed for 4 min with a vortex and after 10 min the upper fraction was recovered. The procedure was repeated twice for each sample. The three supernatants were combined and dried under nitrogen flow. Finally, the samples were re-suspended in 200 µL of a solution of methanol/water (75:25, v/v) acidified with 0.1% of formic acid, before the HRMS analysis. Rosmarinic acid and santonin were used as internal standards for assessing the recovery of PPs and cynaropicrin from the digestive extracts, respectively.

3.2.7 High resolution mass spectrometry (HRMS) analysis

The method was adapted from Troise *et al.*, (2014) with slight modifications.[62] LC-MS data were acquired on an Accela U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (8 °C) and a column oven. Chromatographic separation was carried out on a Gemini C18-110Å 5 µm column (150 mm × 2.0 mm) (Phenomenex, Torrance, CA) thermostated at 25 °C. Both for PPs and guaianolides analysis, 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) were used. Gradient elution was linearly programmed as follows 10% B (1 min), 10–90% B (17 min), constant to 90% B (2 min), 90–10% B (2 min). The flow rate was set to 200 µL/min and the injection volume was 10 µL. The U-HPLC was directly interfaced to an Exactive Orbitrap MS equipped with a heated electrospray interface. Mass spectrometer operated in the full spectral acquisition mode, where the positive and negative ionization mode was used in the same run in the mass range of *m/z* 150–800. The resolving power was set to 50,000 full width at half-maximum (FWHM, *m/z* 200) resulting in a scan time of 1 s., automatic gain control (AGC), balance (target value of 1 106), and scan speed, 4 Hz.; maximum injection time was 100 ms. The interface parameters were as follows: the spray voltage was + 3.2 kV and − 3.0 kV in positive and

negative ion mode, respectively; the tube lens was at 100 V (− 100 V in negative ion), the capillary voltage was 30 V (− 50 V in negative ion), the capillary temperature was 275 °C, and a sheath and auxiliary gas flow of 30 and 15 arbitrary units were used. Metabolite identification was performed by using exact mass values value up to the fifth decimal digit with mass tolerance ± 5 ppm and in **Table 3.2.** molecular formula, retention time, theoretical mass, experimental mass and error have been reported. Data were evaluated by Xcalibur 3.0 software (Thermo Fisher Scientific, Bremen, Germany).

Table 3.2. High-Resolution Mass Spectrometry Identification of Phenolic Acids,flavonoids and cynaropicrin achieved by Orbitrap MS.

Compound	Molecular formula	Theoretical		Experimental	Mass accuracy (ppm)	Retention time (min)
		$[M-H]^-$ *m/z*	$[M+H]^+$ *m/z*			
Caffeoylquinic acid isomers	$C_{16}H_{18}O_9$	353.08781		353.08838	1.61	6.28
Caffeic acid	$C_9H_8O_4$	179.03498		179.03487	-0.61	7.22
Dicaffeoylquinic acid isomers	$C_{25}H_{24}O_{12}$	515.11950		515.11938	-0.23	7.3-9.01
Luteolin 7-O-rutinoside	$C_{27}H_{30}O_{15}$	593.15119		593.15240	2.04	7.83
Luteolin 7-O-glucoside	$C_{21}H_{20}O_{11}$	447.09328		447.09399	1.59	8.23
Luteolin	$C_{15}H_{10}O_6$	285.04046		285.04065	0.67	11.08
Cynaropycrin	$C_{19}H_{22}O_6$		347.14891	347.14883	-0.23	10.53

3.2.8 Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of the soluble and insoluble fractions from the SDP and SCP were measured by the DPPH and DPPH QUENCHER methods, respectively.[58] Before the analysis the soluble extracts were properly diluted with water and the insoluble pellets were freeze-dried. The results were expressed as μmol of TE/g of dry weight (DW). Overall TAC was calculated as the sum of the antioxidant capacity of soluble extracts and insoluble pellets from the SDP and SCP.

3.2.9 α-glucosidase inhibition assay

The *α-glucosidase* inhibition assay was carried out as reported by Colantuono *et al.* (2016), with slight modification.[61] Specifically, 250 µL of sample from SDP were added to reaction mixture instead of 125 µL. Percentage variation of inhibition compared to the control was calculated as previously reported.[61]

3.2.10 Statistical analysis

The data were analyzed by ANOVA, and the means were compared by Tukey test ($\alpha = 0.05$), using XLStat statistical software (Addinsoft, New York, NY). All the experiments were performed in triplicate. The Pearson product-moment correlation coefficient was used for the bivariate correlation analysis among the variables ($\alpha = 0.05$).

3.3 Results

3.3.1 Characterization of the artichoke by-products

In **Table 3.3.** the content of dietary fiber, total PPs and cynaropicrin as well as the antioxidant capacity of the artichoke by-products (leaves and stem) in comparison to the head, was reported. ASP showed the highest amount of total polyphenols and the highest AA compared to the other artichoke fractions. The amount of total phenols in ASP was 1.6 and 3.9 folds higher that the amount of total phenols in ALP and AHP, respectively. According to the amount of total phenols, the AC was 1.6 and 4.7 folds higher for ASP compared to ALP and AHP, respectively. Cynaropicrin abundance was in ALP > ASP > AHP whereas the TDF was in AHP > ALP > ASP. In all the fractions the IDF was higher than the SDF but ASP showed the lowest IDF/SDF ratio.

Table 3.3. Chemical characterization and antioxidant capacity (AC) of the artichoke head and by-products (leaves and stem)**.**Values are expressed as mean ± SD. Values in the same column followed by different lower case letters are significantly different ($p < 0.05$) according to Tukey's test.

Artichoke parts	IDF (mg/g DW)	SDF (mg/g DW)	TDF (mg/g DW)	IDF/SDF	AC (µmol TE/g of DW)	Total PPs (mg GAE/g DW)	Total cynaropicrin (mg/g DW)
Head	429.2 ± 12.5 [a]	182.0 ± 1.8 [a]	611.2 ± 12.1 [a]	2.4	31.6 ± 1.7 [c]	8.8 ± 0.6 [c]	0.014 ± 0.002 [c]
Leaves	363.6 ± 35.5 [b]	105.2 ± 13.6 [c]	468.8 ± 49.1 [b]	3.5	91.1 ± 5.6 [b]	21.6 ± 2.1 [b]	20.8 ± 0.5 [a]
Stem	251.1 ± 22.4 [c]	141.9 ± 9.0 [b]	393.0 ± 20.0 [b]	1.8	147.7 ± 12.6 [a]	34.7 ± 1.1 [a]	2.7 ± 0.2 [b]

3.3.2 Bioaccessibility of PPs and cynaropicrin from ASP-enriched breads

Table 3.4. summarized the data about PPs bioaccessibility. Data showed that ~97% of bioaccessible PPs from all ASP-enriched breads were phenolic acids. DCQA isomers were the most abundant compounds released in the SDP followed by CQA isomers and luteolin 7-rutinoside, the relative abundance being averagely ~61% for DCQA isomers, ~36% for CQA isomers, and ~2% for luteolin-7-rutinoside. About the 18% of totally bioaccessible PPs were released in the SCP except caffeic acid that was released at the 88%. Data about the bioaccessibility of cynaropicrin were reported in **Figure 3.1.**. It shows that the bioaccessible cynaropicrin both in the duodenum and in the colon phase linearly increased with the ASP added in the bread. Seventy four percent of totally bioaccessible cynaropicrin was released in the SDP .

Table 3.4. Concentrations (µg per g) of polyphenols released upon *in vitro* digestion of ASP-enriched breads. Values are expressed as mean ± SD. For each compound, different lower case letters indicate significantly different values ($p < 0.05$) according to Tukey's test.

Compound	3% SDP (µg/g DW)	3% SCP (µg/g DW)	Total 3% (µg/g DW)	6% SDP (µg/g DW)	6% SCP (µg/g DW)	Total 6% (µg/g DW)	9% SDP (µg/g DW)	9% SCP (µg/g DW)	Total 9% (µg/g DW)
CQA isomers	244.4 ± 3.2^{c}	31.4 ± 2.3^{f}	275.8 ± 5.1	496.4 ± 11.5^{b}	95.0 ± 8.1^{e}	591.5 ± 12.1	674.5 ± 22.9^{a}	138.9 ± 15.6^{d}	813.4 ± 25.0
DCQA isomers	379.1 ± 14.3^{c}	44.7 ± 2.2^{f}	423.8 ± 16.2	876.2 ± 16.9^{b}	152.2 ± 8.0^{e}	1028.4 ± 18.1	1218.2 ± 39.0^{a}	257.0 ± 23.3^{d}	1475.2 ± 48.4
Caffeic acid*	5.1 ± 0.4^{d}	40.4 ± 1.3^{c}	45.5 ± 0.2	11.0 ± 0.3^{d}	65.5 ± 9.6^{b}	76.4 ± 9.5	14.1 ± 1.4^{d}	111.5 ± 8.3^{a}	125.6 ± 8.3
Phenolic acids	628.6 ± 17.7^{c}	116.5 ± 2.6^{f}	745.1 ± 19.9	1383.6 ± 23.8^{b}	312.7 ± 25.1^{e}	1696.3 ± 31.7	1906.8 ± 17.6^{a}	507.4 ± 36.6^{d}	2414.2 ± 42.3
Luteolin 7-Rut.**	12.0 ± 0.2^{c}	2.0 ± 0.1^{f}	14.0 ± 0.2	23.5 ± 0.5^{b}	3.6 ± 0.1^{e}	27.1 ± 0.4	32.6 ± 0.8^{a}	5.0 ± 0.8^{d}	37.7 ± 0.9
Luteolin 7-Glu.	1.4 ± 0.0^{c}	0.8 ± 0.0^{d}	2.2 ± 0.0	2.5 ± 0.2^{b}	1.6 ± 0.1^{c}	4.0 ± 0.3	4.0 ± 0.3^{a}	2.4 ± 0.2^{b}	6.4 ± 0.4
Luteolin	7.3 ± 0.3^{c}	2.2 ± 0.1^{f}	9.4 ± 0.3	13.4 ± 0.5^{b}	3.9 ± 0.2^{c}	17.3 ± 0.7	15.2 ± 1.3^{a}	5.7 ± 0.8^{d}	20.9 ± 1.6
Flavonoids	20.7 ± 0.5^{c}	4.9 ± 0.1^{f}	25.6 ± 0.5	39.4 ± 0.7^{b}	9.1 ± 0.4^{c}	48.5 ± 1.0	51.8 ± 2.2^{a}	13.1 ± 1.8^{d}	64.9 ± 2.8
Total PPs	649.3 ± 18.1^{c}	121.3 ± 2.6^{f}	770.6 ± 20.2	1423.0 ± 24.2^{b}	321.8 ± 25.5^{c}	1744.8 ± 32.5	1958.6 ± 16.2^{a}	520.5 ± 38.1^{d}	2479.1 ± 43.9

*Expressed as µg of chlorogenic acid equivalents;**Expressed as µg of luteolin-7-Glu equivalents;

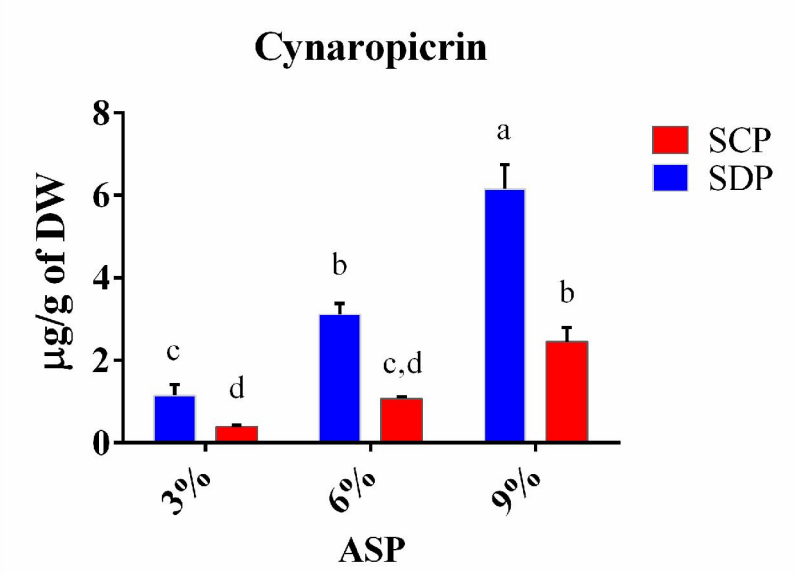

Figure 3.1. Cynaropicrin concentrations (µg/g of dry bread) in the simulated duodenal phase (SDP) and simulated colon phase (SCP). Values are expressed as mean ± SD. Different lower case letters indicate values significantly different ($p < 0.05$) according to Tukey's test.

3.3.3 Overall Total Antioxidant Capacity (TAC) upon digestion

The overall TAC of the ASP-enriched breads and the contribution of the soluble and of the unsoluble fraction at the duodenum and colon steps were graphically shown in **Figure 3.2.**. Data showed a similar repartition of the antioxidant activity among the evaluated steps for all the breads. As expected a significant correlation between the antioxidant capacity of the soluble extracts from the SDP and SCP and the concentrations of total PPs released under the same digestive steps ($r = 0.992$; $p < 0.0001$) was found.

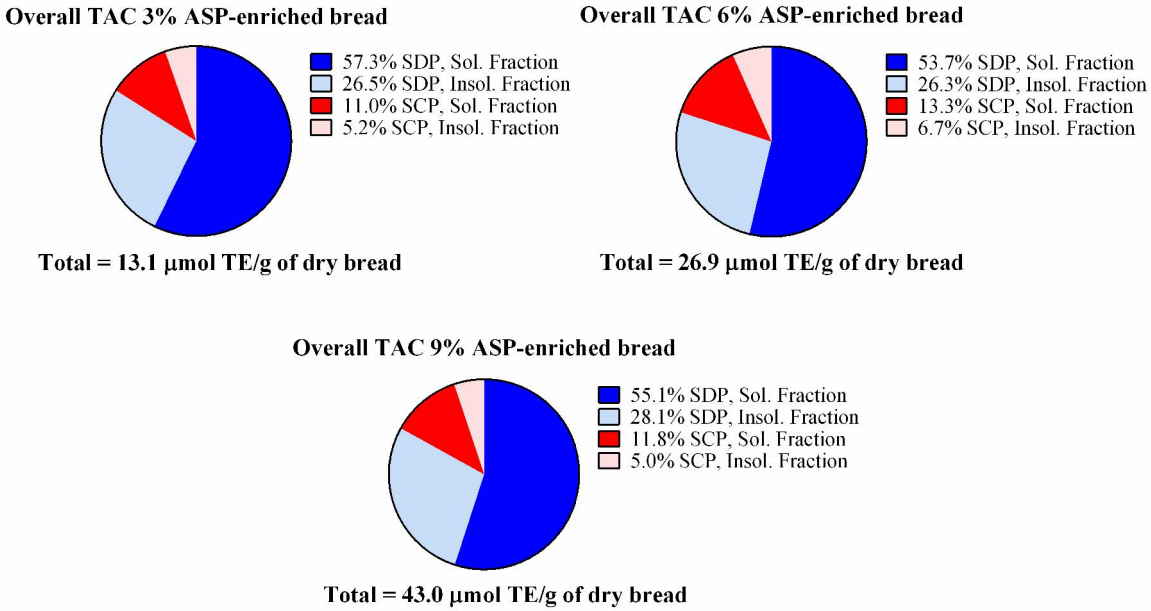

Figure 3.2. Overall TAC released in the intestinal steps upon *in vitro* digestion of ASP-enriched breads. Mean values are expressed as µmol of TE/g of dry bread. Data were corrected for the antioxidant activity of control bread.

3.3.4 Inhibition of α-glucosidase

The results were reported in **Figure 3.3.**. Data showed that 0.121 mg, 0.269 mg and 0.384 mg of total PPs released in the SDP extracts, from 3%, 6% and 9% ASP-enriched breads, were able to inhibit by 6.7%, 10.6% and 16.9% 1 U of α-glucosidase, respectively. A significant correlation between the amount of total PPs released in the SDP extracts and the α-glucosidase inhibition rate ($r = 0.952$; $p = 0.003$) was found.

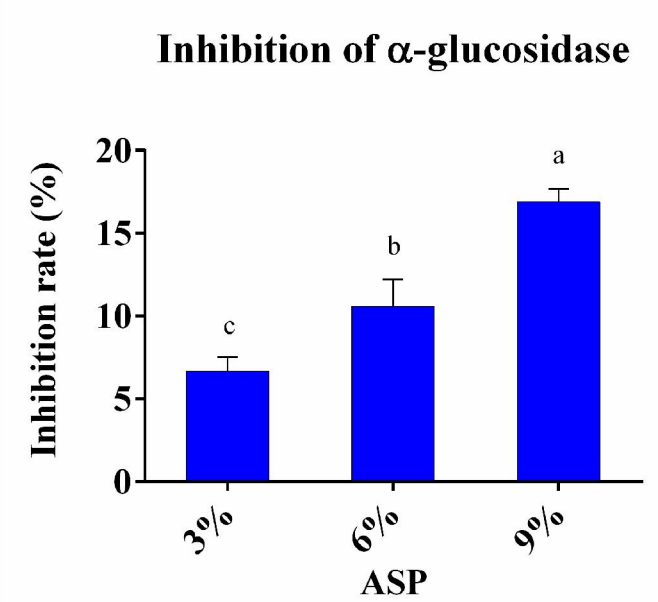

Figure 3.3. Enzyme inhibition rate of extracts from SDP. Data were corrected for the inhibition elicited by control bread. Different lower case letters indicate values significantly different ($p < 0.05$) according to Tukey's test.

3.4 Discussion

In this study the artichoke stem was used as food ingredient in bread formulation and the bioaccessibility of PPs and cynaropicrin, the overall TAC and the ability of breads to inhibit α-glucosidase in the intestinal tract were tested *in vitro*.

The artichoke stem was selected as possible functional ingredient between the artichoke by-products for its DF and PPs content. Data showed that ASP had a higher amount of total PPs, mainly including phenolic acids, and TAC as well as a more balanced IDF/SDF ratio than the artichoke leaves. These findings confirmed previous literature.[4,6,7,45] Moreover, the composition of the DF was particularly interesting because an IDF/SDF ratio ranging from 1 to 2.3 is considered the best to get the health benefits associated to DF consumption.[63] On the contrary the artichoke leaves possessed the highest amount of the bitter sesquiterpene cynaropicrin, as previously reported by Eljounaidi *et al.*, (2015).[64]

Bioaccessibility data and the antioxidant capacity of the extracts solubilized along the intestinal tract *in vitro*, showed that the amount of PPs and the antioxidant activity released from the ASP-enriched breads correlated with the amount of ASP used in the bread formulation.

Despite the highest amount of PPs from the breads were released in the duodenal step, a part of PPs ranging between 16% and 21% of totally bioaccessible compounds, was released in the colon step of digestion following the enzyme action of Pronase E and Viscozyme L (including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase) on the undigested material from the duodenum step. Interestingly, the compound mostly released in SCP was the caffeic acid accounting for the 25% of total PPs bioaccessible in the SCP. This finding could be explained by the breakdown of the linkages between caffeic acid and the IDF of ASP following the action of the cell wall degrading enzymes on the pellets from the SDP, similarly to what was previously reported for unripe apples, winemaking by-products, highland barley and quinoa seeds.[65-68] On the other hand caffeic acid might result also from the hydrolysis of chlorogenic acid due, in our system, to the side esterase activity of Pronase E,[69-71] simulating the activity of microbial esterases *in vivo*.[72-75] From a physiological point of view the potential delivery of caffeic acid in the colon is worth of notice because caffeic acid has a stronger antioxidant capacity and is more easily absorbed from the colon than chlorogenic acid.[76-78]

The potential antioxidant activity along the GiT found in this study was in accordance to previous *in vitro* and *in vivo* evidence showing that artichoke indigestible fraction may affect in a positive way the intestinal health by modulating bacterial enzymatic activities and antioxidant status of rat cecum.[18,53] Interestingly the increase of soluble TAC occurred also in the colon step and went in parallel with a ~5 fold-reduction of the antioxidant capacity of the insoluble pellets from SDP. This finding was in accordance to Mrabet *et al.*, (2017) and suggested that a further destroying of the food matrix as obtained by the enzyme activity of Viscozyme L may act in vivo through microbiota action and modification.[79]

The bitter sesquiterpene cynaropicrin was also found to be released in the duodenum and colon steps in amount that was proportional to the ASP added in the bread. However, considering the amount of this compound in the ASP, it was calculated that the total amount retrieved upon digestion was meanly about 2% of the amount present in the breads. It was hypothesized that cynaropicrin was mainly degraded by the simulated physiological conditions, or it was not effectively extracted by the aqueous digestive fluids as already reported by Thormann *et al.,* (2014) for the sesquiterpene lactone nobilin.[80] Appropriate studies are needed to clarify the mechanisms behind this result in order to potentiate the bioaccessibility of this compound. In fact, cynaropicrin presence in the GiT may have a functional role because it possesses a wide range of pharmacological properties that may work from the intestine (such as anti-hyperlipidemic, anti-trypanosomal, anti-malarial, antifeedant, antispasmodic, anti-photoaging, and anti-tumor action), also through activation of bitter sensory receptors.[81]

Finally, this study also demonstrated that the PPs-rich extracts resulting from the digestion of ASP-enriched breads exerted a inhibitory activity towards α-glucosidase that was proportional to the amount of ASP in the breads. It was calculated that the ratio between the amount of total PPs in the tested extracts and 1 unit (U) of enzyme was equal to 0.121 mg/1 U, 0.269 mg/1 U and 0.384 mg/1 U, for the α-glucosidase inhibition rate found for the 3%, 6% and 9% ASP-enriched bread, respectively. On the other hand, in a previous paper, it was calculated that 0.007 mg of PPs in the extract resulting from the SDP of a 7.5% pomegranate peels-enriched cookies *in vitro* digestion inhibited the activity of 1 U of α-glucosidase by 84%, thus indicating that pomegrante PPs, mainly including ellagitannins and ellagic acid, are stronger natural inhibitor of this enzyme.[61] These findings were in accordance with the evidence that the specific chemical structure of PPs influence the α-glucosidase inhibition. Generally, a high numbers of galloyl and

hydroxyl groups in the molecular structure increase the enzyme inhibitory capacity of PPs while hydrogenation and glycosylation work in a opposite way.[15,82-84] Despite the use of α-glucosidase from *Saccharomyces cerevisiae* could be considered as a limitation of this study, because it is known that the extent of activity of α-glucosidase inhibitors may vary according to the origin of α-glucosidase,[85] previous evidence demonstrated *in vivo* that artichoke products have effective hypoglygemic effect.[29,31,33,86,87] That effect was correlated to the phenolic fraction, mainly including CQA, DCQA and flavonoids well known inhibitors of mammalian α-glucosidases.[15,88, 89] Intestinal α-glucosidase is located in the brush-border surface membrane of the intestinal cells and it is a key enzyme for the conversion of oligosaccharides and disaccharides, into absorbable monosaccharides. The inhibition of this enzyme leads to the modulation of glucose absorption in the small intestine, and therefore the postprandial blood glucose level and the related neuro-hormonal response. Controlling the blood glucose level is considered an effective way to prevent diabetes and obesity exacerbation.[90,91]

3.5 Conclusion

In conclusion, in this study an artichoke stem powder was used as ingredient for the formulation of new breads. Data showed that the potential release of PPs and cynaropicrin as well as the antioxidant activity and α-glucosidase activity in the small intestine were proportional to the amount of ASP added to the bread recipe. Interestingly, a part of these bioactive compounds are potentially released in the colon thus suggesting an effect of the new foods that may be mediated by the intestinal microbiota.

Further studies are needed to assess the biological effects of ASP-enriched breads in vivo as well as the specific role of the microbiota.

3.6 Abbreviations

AC: Antioxidant capacity;

AHP: Artichoke head powder

ALP: Artichoke leaves powder;

ASP: Artichoke stem powder;

CQA: Caffeoylquinic acids;

DCQA: di-caffeoylquinic acids;

DF: Dietary fiber

GAE: Gallic acid equivalents;

GiT: Gastrintestinal tract;

IDF: Insoluble dietary fiber;

PPs: Polyphenols

SCP: Simulated colon phase;

SDF: Soluble dietary fiber;

SDP: Simulated duodenal phase;

SGF: Simulated gastric fluid;

SIF: Simulated intestinal fluid;

SSF: Simulated salivary fluid;

TAC: Total antioxidant capacity;

TDF: Total dietary fiber;

TE: Trolox equivalents;

3.7 References

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Chapter 4

Development and functional characterization of new antioxidant dietary fibers from pomegranate, olive and artichoke by-products

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Abstract

Polyphenols (PPs) and dietary fibers (DF) are food components with well known health benefits. A novel ingredient based on starch-alginate microspheres (MS), working *in vivo* as DF, was recently developed.

The main objectives of this work were to develop new antioxidant DF and to assess their potential functionality.

Three types of antioxidant MS were obtained by inclusion of pomegranate peels (PPe), olive leaves (OLe) and artichoke leaves (ALe) extracts at 20% (w/w) in the formulation of the basic MS. The physico-chemical properties of the new MS were compared with those of 6 commercially available DF concentrates and with wheat and oat bran. In order to evaluate the potential efficacy to release PPs along the gastrointestinal tract (GiT), PPe-microspheres (PPe-MS) were submitted to an *in vitro* simulated gastrointestinal digestion.

Data showed that the three types of MS exerted higher free antioxidant capacity (free-AC) than commercial DF rich products, and the bound antioxidant capacity (bound-AC) of PPe-MS was even comparable to that of wheat bran and 4.4 folds higher than that of oat-bran. The release of ellagitannins (ETs) along the GiT decreased from the salivary up to the small intestine phase whereas gallic acid, ellagic acid (EA) and its derivatives had an opposite trend. However, a residual amount of PPs was found in the pellet obtained from the small intestine step of the digestion. This finding suggested that *in vivo* a part of ETs and EA may reach the colon, and may be transformed by the local microbiota in biologically active urolithins.

In conclusion, in this study three types of antioxidant DF constituted by PPs extracted from three common plant by-products, and included in starch-alginate based MS, were developed. Further studies are warrented to asses the impact of the MS in food formulation and to evaluate *in vivo* the antioxidant efficacy and the impact on health.

Keywords: antioxidant dietary fiber, functional ingredients, plant by-products, polyphenols, starch-based microspheres

4.1. Introduction

Polyphenols (PPs) and dietary fibers (DF) are two classes of food components well known for their beneficial effects on human health.

PPs are secondary metabolites of plants and represent the most abundant antioxidant compounds in foods.[1,2] Whole grains, fruits and vegetables are the major dietary sources of PPs.[3] Several *in vitro* and *in vivo* studies support the role of PPs in the prevention of non communicable chronic diseases and obesity.[4-9] Even though certain systemic health effects of PPs are correlated to the extent of their absorption along the gastrointestinal tract (GiT) and post-absorptive metabolism,[2,10] the gastrointestinal mucosa is the primary site for the beneficial effects associated to these phytochemicals, since it is directly exposed to them after their release from the food matrix.[11,12] In the intestinal lumen, PPs exert antioxidant and anti-inflammatory activity, thus counteracting the local subclinical oxidative stress and inflammation. Furthermore, PPs may affect the activity of key digestive enzymes, influencing the bioavailability and the metabolism of carbohydrates and fats.[13-19] For these reasons, several technological strategies were developed, aiming to obtain new functional foods and ingredients for the targeted delivery of PPs along the GiT.[20,21] In fruits, vegetables and whole grains, PPs occur in free form (mainly as glycosides) or covalently bound to cell wall structural components.[22] Additionally, PPs may be found physically entrapped or linked to food macronutrients (i.e. starch, proteins and lipids) mainly through non-covalent interactions.[23,24] Free and non-covalently bound PPs may be delivered in the upper GiT by direct solubilization in the intestinal fluids or after the action of digestive enzymes on food matrix. Conversely, PPs covalently bound to DF pass unmodified through the upper intestine and reach the colon. In this site, bound PPs are delivered by the action of microbial enzymes, thus creating a reducing environment and being available for absorption in their original chemical forms or as microbial metabolites.[25-28] In the large intestine, PPs may also positively change the composition of the microbiota and, in turn, the host physiology.[29-31]

So from a physiological perspective plant foods having PPs bound to DF can be considered as natural carriers of PPs along the GiT. That the reduced risk of several non communicable-chronic diseases found by epidemiological studies associated to a regular consumption of fruits and vegetables and whole grains are due to their content of both DF and micronutrients (including PPs and minerals) is actually well known.[32] As a consequence for general

population a minimum daily intake of 25 g/day of DF was recommended by the European Food Safety Authority (EFSA), Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO)[33] and the development and marketing of functional foods enriched with PPs and DF is always a hot topic for food scientists and industry.[34-37] In this perspective the recent inclusion, by the CODEX Alimentarius Commission, among the definitions of DF of "*synthetic carbohydrate polymers showing health benefits*" along with the classical definitions of "*edible carbohydrate polymers naturally occurring in foods*" and "*carbohydrate polymers, obtained from raw foods by physiological, enzymatic or chemical means and showing health benefits*" opened new opportunities in the field of functional ingredient development.[33,38]

In this frame, Rose *et al.* (2009) developed a new source of DF, named starch-entrapped microspheres (MS), constituted by electrostatically cross-linked alginate matrix forming a web-like structure that is filled with native corn starch. The entrapped corn starch may partially escape the digestion in the small intestine thereby potentially acting like resistant starch in the colon. Compared to other DF, including corn bran arabinoxylans and long-chain β-glucans, the starch-entrapped MS showed a beneficial fermentation profile, inhibiting the growth of potentially harmful bacteria and increasing short-chain fatty acids (SCFAs) production during an *in vitro* faecal fermentation.[39-41] This innovative source of DF was recently patented by Hamaker and coworkers.[42] Furthermore, corn native starch was previously reported as an effective filling agent of calcium alginate matrix, able to enhance the entrapment capacity of probiotic bacteria, PPs and other bioactive chemical compounds.[43-50] Some plant by-products from agricultural and food industry are rich and cheap sources of PPs and DF. These materials can be dehydrated and submitted to solvent extraction for the recovery of PPs-rich extracts, or directly used in their whole form as functional ingredients for the formulation of new foods.[51-56]

In this framework, the main objective of this study was to develop new antioxidant DF-ingredients by using agricultural by-products as natural sources of PPs. For this purpose,three PPs-rich extracts were obtained from pomegranate peels (PPe), olive leaves (OLe) and artichoke leaves (ALe), and were used as a functional filling for the development of new starch-alginate based MS. The physico-chemical properties of the new ingredients were compared to those of 6 commercially available DF concentrates, as well as to wheat bran and

oat bran. PPe-microspheres (PPe-MS) were submitted to an *in vitro* simulated gastrointestinal digestion in order to evaluate their potential efficacy to deliver PPs and to exert antioxidant activity along the GiT.

4.2. Materials and Methods

4.2.1. Chemicals

HPLC-grade methanol, acetonitrile and water were purchased from Merck (Darmstadt, Germany). Ethanol, formic acid, ethyl acetate and acetone were obtained from VWR international (Fontenay-sous-Bois, France). Total DF assay kit was purchased from Megazyme International (Wicklow, Ireland). Calcium chloride, celite, hydrochloridric acid (HCl), 4-morpholineethanesulfonic acid (MES), 2,2-diphenyl-1-picrylhydrazyl, 95% (DPPH), *tris*(hydroxymethyl)aminomethane *hydrochloride (Tris-HCl),* sodium alginate, sodium hydroxide and *6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid* were purchased from Sigma-Aldrich (St. Louis, MO). Standards of punicalagin, ellagic acid (EA), gallic acid (GA), oleuropein, chlorogenic acid, caffeic acid, cynarin, ferulic acid, p-cumaric acid, rutin, apigenin, hydroxytyrosol, luteolin and protocatechuic acid were purchased from Sigma-Aldrich (St. Louis, MO). The standard of cynaropicrin was obtained from Extrasynthese (Lyon, France). The standard of luteolin-4-O-glucoside was obtained from Indofine Chemical Company (Somerville, NJ). Corn starch was obtained from Tate & Lyle (Decatur, IL). PPe powder was obtained from Detrade UG (Bremen, Germany). Organic wheat bran and oat bran (Fior di loto, Orbassano, Italy) were purchased in a local market. A barley β-glucans concentrate (Glucagel) was obtained from DKSH (Miribel Cedex, France). Potato DF, pea cortical DF, citrus DF, carrot DF, bamboo DF were purchased from ITALI (Reggio Emilia, Italy). ALe and OLe dry powder were purchased in a local herbalist's shop.

4.2.2. Preparation of plant by-products extracts

PPe extract: One liter of a solution water/ethanol, (50:50, v/v) with 0.5% formic acid was added to 250 g of PPe powder. The mixture was sonicated for 30 min and subsequently filtered through a filter paper (VWR international, qualitative filter paper 600). The filtered extract (590 mL) was recovered and concentrated under vacuum in a rotary evaporator (T <

30°C); the residual acqueous fraction was finally freeze-dried. Prior to the preparation of PPe-MS, the dry extract was solubilized in 590 mL of distilled water.

OLe extract: One liter of a solution ethanol/water (70:30, v/v) was added to 250 g of OLe powder. The mixture was sonicated for 30 min and subsequently filtered through a filter paper (VWR international, qualitative filter paper 600). The filtered extract (490 mL) was recovered and concentrated under vacuum in a rotary evaporator (T < 30°C); the residual acqueous fraction was finally freeze-dried. Prior to the preparation process of OLe-microspheres (OLe-MS), the dry extract has been solubilized in 490 mL of distilled water.

ALe extract: One liter of a solution ethanol/water (75:25, v/v) with 0.1% formic acid was added to 250 g of ALe powder. The mixture was sonicated for 30 min and subsequently filtered through a filter paper (VWR international, qualitative filter paper 600). The filtered extract (1149 mL) was recovered and concentrated under vacuum in a rotary evaporator (T < 30°C); the residual acqueous fraction was finally freeze-dried. Prior to the preparation process of ALe-microspheres (ALe-MS), the dry extract was dissolved in 670 mL of distilled water.

4.2.3. Preparation of functional starch-based MS

Starch-alginate based MS were prepared following a protocol previously described by Rose *et al.*, (2009).[39] Briefly, three different suspensions were obtained by mixing at room temperature distilled water, native corn starch, sodium alginate and the acqueous extracts from PPe, ALe and OLe, respectively. The suspensions were prepared including the extracts at 20% (w/w) in the formulation of the basic MS constituted by distilled water/native corn starch/sodium alginate (69:9:2, w/w/w).

With continuous stirring, each suspension was pumped by a peristaltic pump through a 22 gauge needle (EFD, Nordon Company) into a solution of calcium chloride (4% w/v). Hydrogel MS were kept in the calcium chloride solution for 1 h, before harvesting and washing with distilled water. Subsequently, the MS were dried in an oven at 38 ± 2 °C for 24 h. Control-MS were prepared mixing distilled water, native corn starch and sodium alginate (89:9:2, w/w/w). Before the analysis, the dry MS were powdered with a Grindomix knife mill (10000 rpm for 30 s).

4.2.4. Determination of insoluble, soluble and total dietary fiber

The amount of soluble dietary fiber (SDF), insoluble dietary fiber (IDF) and total dietary fiber (TDF) was determined according to the gravimetric enzymatic method as described by Prosky *et al*., (1987).[57] For each sample the IDF/SDF ratio was calculated.

4.2.5. Water holding capacity, water solubility and oil holding capacity

The water holding capacity (WHC) was determined using the method described by Fuentes-Alventosa *et al.,* (2009) slightly modified.[58] Briefly, 200 mg of sample were placed in a 50 mL centrifuge tube and mixed with 12 mL of distilled water. After 24 h of stirring at room temperature, the suspension was centrifuged at 4000 rpm for 45 min. Then, the supernatant was carefully removed, and the wet pellet was weighed. WHC was calculated as mL of water per gram of sample. For the determination of the water-solubility, the wet pellet resulting from the WHC analysis, was freeze-dried and weighed. The water-solubility was calculated considering the weight difference between the weights of the residual pellet and of the initial sample. The results were expressed as a percentage of solubilized sample.

The oil holding capacity (OHC) was determined using the method described by Fuentes-Alventosa *et al.,* (2009) slighty modified.[58] 200 mg of each sample were suspended in 12 mL of sunflower seeds oil. After 24 h of stirring at room temperature, the suspension was centrifuged at 4000 rpm for 45 min. The supernatant was carefully removed, and the oil-imbibed pelletwas weighed. OHC was expressed as mL of sunflower seeds oil per gram of sample. The analyses were performed in triplicate.

4.2.6. Antioxidant characterization of DF and MS products

4.2.6.1. Extraction of free and bound antioxidant compounds

The hydroalcoholic extraction and the alkaline hydrolysis of samples was performed as reported by Papillo *et al*., (2014) with slight modifications.[59] For all the samples, free antioxidant compounds were extracted with 4 mL of a solution methanol/water (70:30, v/v) added to 100 mg of sample. Then the mixture was sonicated for 30min using an ultrasound bath. Then, the samples were centrifuged at 4000 rpm for 10 min at 4°C, and the supernatants were recovered for the analysis. The resulting pellet was hydrolyzed by adding 2 mL of 4 M NaOH in agitation for 2 h. After the alkaline hydrolysis the pH of each sample was adjusted

to 2.0 with 6 M HCl. Subsequently, the samples were centrifuged at 4000 rpm for 10 min and the supernatants were recovered and extracted three times with 15 mL (5 mL for 3 times) of ethyl-acetate. The combined extracts were dried under nitrogen flow and re-suspended in 1 mL of methanol/water (70:30, v/v) prior to perform the analysis.

4.2.6.2. Free and bound antioxidant capacity of MS and DF products

For all the samples, the antioxidant capacity of free polyphenols (free-AC) and the antioxidant capacity of bound polyphenols (bound-AC) were evaluated by using the DPPH method according to Papillo *et al.* (2014).[59]

Briefly, a DPPH solution was prepared by diluting 0.01 mmol of DPPH in 10 mL of pure methanol and adjusting the absorbance of the DPPH radical solution to 0.9 ± 0.02 at 517 nm. Two hundred microliters of sample were added to 1 mL of DPPH solution, and after 10 min, the absorbance was measured with a spectrophotometer (PG Instruments, UK) set at 517 nm. Free-AC and Bound-AC values for PPe-MS, OLe-MS and ALe-MS were obtained by deduction of those obtained for the control MS. Data were reported as μmol Trolox equivalent (TE)/100g of product. The overall total antioxidant capacity (TAC) of the products was calculated as the sum of free-AC and bound-AC.

4.2.7. High Resolution Mass Spectrometry (HRMS) analysis of free and bound PPs in MS

The hydroalcoholic extracts and the the extracts from alkaline hydrolysis were analyzed for the determination of free and bound PPs in PPe-MS, ALe-MS and OLe-MS. LC-MS data were acquired on an Accela U-HPLC system coupled to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (10 °C) and a column oven. Chromatographic separation was carried out on a Gemini C18-110A 5 μm column, 150 mm x 2.0 mm (Phenomenex, Torrance, CA) heated to 30°C. The mobile phase consisted of 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B), the flow rate was set to 200 μL/min and the injection volume was 10 μL.

For the analysis of PPs from PPe-MS, the extracts were dried under nitrogen flow and resuspended in a solution methanol/water (50:50, v/v). The gradient elution was as follows: 0 % B (1 min), 0–10 % B (1-2.5) min, 10–30 % B (2.5-7.5 min), 30-40 % B (7.5-9) min, 40-70

% B (9-10) min, 70-98 % B (10-11.5) min, constant at 98% B (11.5-13) min, 98-0 % B (13-16) min. For PPs and cynaropycrin from ALe-MS analysis, the gradient elution was as follows: 10 % B (1 min), 10-90 %B (1-18) min, constant at 90 % B (18-20) min, 90-10% B (20-22) min. For OLe-MS PPs analysis, the gradient elution was as follows: 10% B (1 min), 10-90% B (1-8) min, constant to 90% B (8-10) min, 90-10% B (10-12 min).

The U-HPLC was directly interfaced to an Exactive Orbitrap MS equipped with a heated electrospray interface (HESI). Acquisition was performed in both positive and negative ionization modes, in the mass range of *m/z* 100–1300. The resolving power was set to 50,000 full width at half-maximum (FWHM, m/z 200) resulting in a scan time of 1 s. The automatic gain control was used in balanced mode (1×10^6 ions); maximum injection time was 100 ms. The interface parameters were as follows: spray voltage was at 3.0 kV (positive mode and negative mode) for artichoke and olive and 3500 kV for pomegranate analysis, capillary voltage 50V, capillary temperature at 275 °C, sheath gas at 35 arbitrary units, and auxiliary gas at 15 arbitrary units. The instrument was externally calibrated by infusion with two different calibration solutions: for positive ion calibration mode, the solution consisted in caffeine, Met–Arg–Phe– Ala (MRFA), Ultramark 1621, and acetic acid in a mixture of acetonitrile/methanol/water (2:1:1, v/v/v); instead the negative ion mode calibration solution consisted in sodium dodecyl sulfate, sodium taurocholate, and Ultramark 1621 in acetonitrile/methanol/water solution (2:1:1, v/v/v) containing 1% acetic acid.

Chromatographic data acquisition and peak integration were performed using Xcalibur software (Thermo Fisher Scientific, San Jose, USA). Calibrations curves of punicalagin, EA, GA, oleuropein, chlorogenic acid, caffeic acid, cynarin, ferulic acid, p-coumaric acid, rutin, apigenin, hydroxytyrosol, luteolin, protocatechuic acid, luteolin-4-O-glucoside and cynaropicrin were constructed in the linearity range 100-5000 ng/mL. Metabolite identification was performed by using exact mass values value up to the fifth decimal digit with mass tolerance ± 5 ppm and in **Table 4.1.** molecular formula, retention time, theoretical mass, experimental mass and error have been reported.

Table 4.1. High-Resolution Mass Spectrometry Identification of bioactive compounds achieved by Orbitrap MS.

Compound	Molecular formula	Theoretical		Experimental	Mass accuracy (ppm)	Retention time (min)
		$[M-H]^-$ *m/z*	$[M+H]^+$ *m/z*			
PPe-MS						
Punicalin	$C_{34}H_{22}O_{22}$	781.05300		781.05389	1.14	6.3-6.7
Punicalagin	$C_{48}H_{28}O_{30}$	1083.05926		1083.05994	0.63	7.6-7.9
Causarinin	$C_{41}H_{28}O_{26}$	935.07960		935.08118	1.69	8.2
Galloyl-HHDP-hexoside	$C_{27}H_{22}O_{18}$	633.07334		633.07410	1.20	8.6
Granatin B	$C_{41}H_{28}O_{27}$	951.07452		951.07556	1.09	9.2
Ellagic acid hexoside	$C_{20}H_{16}O_{13}$	463.05181		463.05225	0.95	8.7
Ellagic acid pentoside	$C_{19}H_{14}O_{12}$	433.04125		433.04135	0.23	9.6
Ellagic acid deoxyhexoside	$C_{20}H_{16}O_{12}$	447.05690		447.05701	0.25	9.7
Ellagic acid	$C_{14}H_6O_8$	300.99899		300.99915	0.53	10.4
Gallic acid	$C_7H_6O_5$	169.01425		169.01378	-2.78	6.4
OLe-MS						
Protocatechuic acid	$C_7H_6O_4$	153.01933		153.01865	-4.44	4.7
Verbascoside	$C_{29}H_{36}O_{15}$	623.19814		623.19818	0.06	6.1
p-coumaric acid	$C_9H_8O_3$	163.04007		163.03964	-2.64	6.6
Ferulic acid	$C_{10}H_{10}O_4$	193.05063		193.05042	-1.09	6.74
Luteolin rutinoside	$C_{27}H_{30}O_{15}$	593.15119		593.15149	0.51	6.05
Rutin	$C_{27}H_{30}O_{16}$	609.14611		609.14575	-0.59	6.07
Apigenin rutinoside	$C_{27}H_{30}O_{14}$	577.15628		577.15643	0.26	6.31
Luteolin glucoside	$C_{21}H_{20}O_{11}$	447.09328		447.09222	-2.37	6.57
Apigenin glucoside	$C_{21}H_{20}O_{10}$	431.09837		431.09863	0.60	6.55
Hydroxytyrosol	$C_8H_{10}O_3$	153.05572		153.05499	-4.77	4.12
3,4-DHPEA-AC	$C_{10}H_{12}O_4$		197.08084	197.08118	1.73	5.29
Elenolic acid	$C_{11}H_{14}O_6$		243.08631	243.08647	0.66	5.81
p-HPEA-EA	$C_{19}H_{22}O_7$		363.14383	363.14392	0.25	6.54
Oleuropein	$C_{25}H_{32}O_{13}$		541.19157	541.19159	0.04	6.68
Ligstroside	$C_{25}H_{32}O_{12}$		525.19665	525.19647	-0.34	7.13
methyl- DHPEA-EA	$C_{20}H_{24}O_8$		393.15439	393.15427	-0.31	7.17
ALe-MS						
Caffeoylquinic acid isomers	$C_{16}H_{18}O_9$	353.08781		353.08835	1.53	6.28
Caffeic acid	$C_9H_8O_4$	179.03498		179.0345	-2.68	7.22
Dicaffeoylquinic acid isomers	$C_{25}H_{24}O_{12}$	515.11950		515.11816	-2.60	7.3-9.01
Luteolin 7-O- rutinoside	$C_{27}H_{30}O_{15}$	593.15119		593.15094	-0.42	7.83
Luteolin 7-O-glucoside	$C_{21}H_{20}O_{11}$	447.09328		447.09207	-2.71	8.23
Luteolin 7-O-glucuronide	$C_{21}H_{18}O_{12}$	461.07255		461.07205	-1.08	8.53
Apigenin 7-O-glucoside	$C_{21}H_{20}O_{10}$	431.09837		431.09766	-1.65	8.93
Luteolin 7-O-malonyl-gluco.	$C_{24}H_{22}O_{14}$	533.09368		533.09497	2.42	9.06
Apigenin 7-O-glucuronide	$C_{21}H_{18}O_{11}$	445.07882		445.07748	-3.01	9.29
Luteolin	$C_{15}H_{10}O_6$	285.04046		285.04105	2.07	11.08
Apigenin	$C_{15}H_{10}O_5$	269.04555		269.04596	1.52	12.26
Cynaropycrin	$C_{19}H_{22}O_6$		347.14891	347.14886	-0.14	10.53

4.2.8. In vitro gastrointestinal digestion of PPe-MS

Before the *in vitro* gastrointestinal digestion, 2.5 g of PPe-MS and control-MS were cooked at 100°C for 20 min in 25 mL of distilled water as reported by Venkatachalam *et al.*.[60] 5 g of cooked PPe-MS and control-MS were submitted to the *in vitro* gastrointestinal digestion as reported by Minekus *et al.,* (2014) with slight modifications.[61] Specifically, α-amylase from porcine pancreas and the simulated intestinal fluid (SIF) weres used in the oral phase and in the intestinal phase in substitution of salivary α-amylase and bile salts, respectively. At each digestive step an aliquot of sample was withdrawn and analyzed by HRMS as described above for PPs from PPe-MS. The pellet obtained after the simulated intestinal phase (SIP) was hydrolyzed with 15 mL of 4 M NaOH in agitation for 2 h. After adjusting to pH 2.0 with 6 M HCl, the sample was centrifuged at 4000 rpm for 10 min and the supernatant was recovered and extracted for 4 times with 20mL of ethyl-acetate. The combined extracts were dried under vacuum and re-suspended in 2 mL of a solution methanol/water (50:50, v/v) immediately prior to the HRMS analysis.

4.2.9. Statistical analysis

Data were expressed as mean ± standard deviation. All the analysis were performed in triplicate.The data were analyzed by ANOVA, and the mean values were compared by Tukey test ($\alpha = 0.05$), using XLStat statistical software (Addinsoft, New York, NY). The Pearson correlation coefficient was used for the bivariate correlation analysis between variables ($\alpha = 0.05$). Exploratory Principal Component Analysis (PCA) of the normalized variables was performed using XLStat statistical software (Addinsoft, New York, NY).

4.3. Results

4.3.1. Physico-chemical characteristics of MS and DF products

Table 4.2. shows the main physico-chemical characteristics of the newly developed MS and 8 commercial products. The mean amount of TDF in powdered MS was about 13.1 % and was lower than that (71.7%) found in commercial DF products ranging from 19.1 % of oat bran up to 97.0 % of bamboo DF (mainly IDF). Barley β-glucans concentrate possessed the highest amount of SDF followed by citrus DF (17.1 g/100g of sample) and carrot DF (15.9 g/100g of sample) whose SDF accounted for 19.0% and 19.3% of TDF, respectively. Significant differences between wheat bran and oat bran for relative content of TDF and IDF, were found. Potato DF showed the highest values of WHC and OHC, whereas the powdered MS possessed the lowest values. Citrus DF and carrot DF also showed high WHC values, the one of carrot being higher than that of citrus DF. Bamboo DF possessed a low WHC, but a quite high OHC, after potato DF and barley β-glucans. As expected, barley β-glucans concentrate possessed the highest water solubility, followed by wheat bran and powdered PPe-MS, whereas bamboo DF was at the bottom of the ranking list. Positive correlations between the amount of TDF and the WHC ($r = 0.749$; $p = 0.005$) and OHC ($r = 0.648$; $p = 0.023$), and between the amount of SDF and the water solubility ($r = 0.908$; $p < 0.0001$) were found.

PPe-MS showed the highest TAC (5798.7 ± 365.6 μmol TE/100g) followed by OLe-MS (993.4 ± 25.9 μmol TE/100 g) and wheat bran (499.1 ± 5.6 μmol TE/100 g). Conversely, bamboo DF (12.8 ± 3.3 μmol TE/100 g) citrus DF (35.4 ± 4.0 μmol TE/100 g) and carrot DF (69.4 ± 7.5 μmol TE/100 g) possessed the lowest TAC values

Table 4.2. Physico-chemical characteristics of the powdered MS and commercial DF concentrates. Values are expressed as mean ± SD. Values followed by the same letter within the same column are not significantly different ($p > 0.05$) according to Tukey's Test.

Products	IDF (g/100 g)	SDF (g/100 g)	TDF (g/100 g)	IDF/SDF	WHC (mL/g)	OHC (mL/g)	Solubility (%)	TAC (μmol TE/100 g)
Oat bran	15.8 ± 1.3^{f}	$3.3 \pm 0.4^{d,e}$	19.1 ± 0.9^{h}	4.8	2.2 ± 0.4^{f}	$1.7 \pm 0.2^{c,d}$	$18.6 \pm 0.3^{c,d}$	$155.6 \pm 7.0^{d,e}$
Wheat bran	43.8 ± 0.8^{e}	$3.0 \pm 0.1^{d,e}$	46.8 ± 0.9^{g}	14.8	5.1 ± 0.3^{d}	3.1 ± 0.2^{b}	28.5 ± 0.6^{b}	499.1 ± 5.6^{c}
Potato DF	65.6 ± 2.4^{d}	8.6 ± 1.7^{c}	74.2 ± 0.7^{f}	7.6	11.5 ± 0.7^{a}	6.3 ± 0.3^{a}	$15.2 \pm 2.3^{d,e}$	$174.2 \pm 11.8^{d,e}$
Citrus DF	72.8 ± 1.7^{c}	17.1 ± 2.0^{b}	89.9 ± 0.3^{b}	4.3	8.3 ± 0.2^{b}	2.1 ± 0.2^{c}	$15.2 \pm 0.8^{d,e}$	35.4 ± 4.0^{e}
Bamboo DF	95.4 ± 0.1^{a}	$1.6 \pm 0.2^{d,e}$	97.0 ± 0.1^{a}	58.7	3.5 ± 0.3^{e}	3.3 ± 0.1^{b}	6.0 ± 1.7^{f}	12.8 ± 3.3^{e}
Pea cortical DF	82.1 ± 0.6^{b}	$4.8 \pm 0.2^{c,d}$	87.1 ± 0.3^{c}	17.3	5.2 ± 0.1^{d}	1.5 ± 0.1^{d}	11.4 ± 0.3^{e}	98.2 ± 6.6^{e}
Carrot DF	66.5 ± 1.6^{d}	15.9 ± 1.9^{b}	82.4 ± 0.3^{d}	4.2	8.4 ± 0.3^{b}	3.2 ± 0.2^{b}	15.7 ± 1.2^{d}	69.4 ± 7.5^{e}
Barley β-glucans	1.6 ± 0.8^{g}	75.5 ± 0.8^{a}	77.1 ± 1.6^{e}	0.02	7.0 ± 0.7^{c}	3.5 ± 0.1^{b}	77.4 ± 2.9^{a}	80.3 ± 3.5^{e}
PPe-MS	12.4 ± 0.4^{f}	$1.7 \pm 0.5^{d,e}$	14.1 ± 0.1^{i}	7.4	2.2 ± 0.1^{f}	0.7 ± 0.1^{e}	21.3 ± 0.9^{c}	5798.7 ± 365.6^{a}
ALe-MS	11.9 ± 0.1^{f}	0.6 ± 0.1^{e}	12.5 ± 0.2^{i}	21.5	2.4 ± 0.2^{f}	0.7 ± 0.1^{e}	$18.6 \pm 0.7^{c,d}$	$329.1 \pm 11.1^{c,d}$
OLe-MS	12.1 ± 0.1^{f}	0.9 ± 0.1^{e}	13.0 ± 0.2^{i}	13.7	2.2 ± 0.1^{f}	0.7 ± 0.1^{e}	16.1 ± 1.8^{d}	993.4 ± 25.9^{b}
Control-MS	12.2 ± 0.5^{f}	0.5 ± 0.1^{e}	12.7 ± 0.6^{i}	24.0	2.2 ± 0.1^{f}	0.7 ± 0.1^{e}	16.2 ± 0.6^{d}	n.a.

4.3.2 Free and bound PPs in MS

Tables 4.3., **4.4.** and **4.5.** report the main PPs present in powdered PPe-MS, Ole-MS and ALe-MS, respectively. The most abundant PPs were ellagitannins (ETs), mainly including punicalin and punicalagin, EA and its derivatives, in PPe-MS, secoiridoids and phenolic acids in OLe-MS and flavonoids and phenolic acids in ALe-MS. The total amount of PPs was higher in OLe-MS, followed by PPe-MS and ALe-MS. PPs were not detected in control-MS. In all the MS, PPs were mainly found in the free form.

Table 4.3. PPs in PPe-MS. The values were expressed as mean ± SD. ETs were expressed as μg equivalents of punicalagin. Ellagic acid derivatives were expressed as μg equivalents of EA. Total PPs were expressed as the sum of μg equivalent of punicalagin + μg equivalent of EA + μg of GA.

Compound	Free (μg/g DM)	Bound (μg/g DM)	Total (μg/g DM)
Punicalin	5708.0 ± 335.0	3.8 ± 1.4	5711.8 ± 334.2
Punicalagin	1396.8 ± 72.2	n.d.	1397.3 ± 72.2
Causarinin	131.6 ± 6.9	n.d.	131.6 ± 6.9
Galloyl-HHDP-hexoside	1249.8 ± 117.7	23.6 ± 19.2	1273.5 ± 98.5
Granatin B	651.8 ± 65.8	n.d.	651.8 ± 65.8
ETs	**9138.1 ± 482.1**	**27.9 ± 20.5**	**9166.0 ± 470.8**
Ellagic acid hexoside	142.7 ± 13.5	n.d.	142.7 ± 13.5
Ellagic acid pentoside	14.2 ± 3.1	n.d.	14.2 ± 3.1
Ellagic acid deoxyhexoside	65.6 ± 7.1	n.d.	65.6 ± 7.1
Ellagic acid	740.4 ± 19.0	13.8 ± 2.1	754.2 ± 17.0
EAs	**963.0 ± 38.5**	**13.8 ± 2.1**	**976.8 ± 37.1**
Gallic acid	619.5 ± 83.5	12.4 ± 7.7	631.9 ± 90.5
Total PPs	**10720.6 ± 426.1**	**54.1 ± 29.0**	**10774.7 ± 414.0**

Table 4.4. PPs in OLe-MS. The values were expressed as mean ± SD. Apigenin and luteolin glycosides were expressed as µg equivalents of luteolin-4-O-glucoside. Secoiridoids were expressed as µg equivalents of oleuropein. Total PPs were expressed as the sum of µg equivalent of phenolic acids + µg equivalent of flavonoids + µg equivalent of secoiridoids + µg equivalent of Hydroxytyrosol.

Compound	Free (µg/g DM)	Bound (µg/g DM)	Total (µg/g DM)
Protocatechuic acid	3.2 ± 0.1	0.1 ± 0.0	3.3 ± 0.1
Verbascoside	80.1 ± 1.7	n.d.	80.1 ± 1.7
p-coumaric acid	n.d.	6.6 ± 1.7	6.6 ± 1.7
Ferulic acid	7.0 ± 0.7	13.4 ± 5.8	20.4 ± 6.4
Phenolic acids	**90.3 ± 1.8**	**20.1 ± 7.5**	**110.4 ± 9.0**
Luteolin rutinoside	317.1 ± 13.8	4.8 ± 0.4	321.9 ± 14.0
Rutin	65.1 ± 1.9	n.d.	65.1 ± 1.9
Apigenin rutinoside	242.1 ± 43.0	5.0 ± 1.4	247.1 ± 41.9
Luteolin glucoside	3443.7 ± 136.7	90.2 ± 13.7	3533.9 ± 123.2
Apigenin glucoside	82.6 ± 0.8	9.6 ± 2.3	92.2 ± 1.7
Flavonoids	**4150.5 ± 188.0**	**109.6 ± 17.0**	**4260.1 ± 171.0**
3,4-DHPEA-AC	3372.6 ± 398.6	45.2 ± 2.5	3417.8 ± 399.2
Elenolic acid	6870.6 ± 277.0	n.d.	6870.6 ± 277.0
p-HPEA-EA	1988.4 ± 107.7	0.5 ± 0.3	1988.9 ± 108.0
Oleuropein	38132.3 ± 871.2	n.d.	38132.3 ± 871.2
Ligstroside	194.0 ± 39.6	n.d.	194.0 ± 39.6
Methyl-DHPEA-EA	793.4 ± 28.2	2.4 ± 0.8	795.1 ± 28.4
Secoiridoids	**51351.4 ± 1097.2**	**47.3 ± 3.4**	**51398.7 ± 1100.0**
Hydroxytyrosol	48.3 ± 1.8	n.d.	48.3 ± 1.8
Total	**55640.5 ± 1284.2**	**177.1 ± 8.9**	**55817.5 ± 1275.4**

Table 4.5. PPs in ALe-MS. The values were expressed as mean ± SD. Luteolin and apigenin glycosides were expressed as μg equivalents of luteolin and apigenin, respectively. Caffeoylquinic acid isomers were expressed as μg equivalents of chlorogenic acid. Dicaffeoylquinic acid isomers were expressed as μg equivalents of cynarin. Total PPs were expressed as the sum of μg equivalent of phenolic acids + μg equivalent of flavonoids.

Compound	Free (μg/g DM)	Bound (μg/g DM)	Total (μg/g DM)
Caffeoylquinic acid isomers	232.2 ± 51.6	0.14 ± 0.07	232.2 ± 51.6
Caffeic acid	0.5 ± 0.1	14.1 ± 0.3	14.6 ± 0.4
Dicaffeoylquinic acid isomers	280.6 ± 61.1	1.6 ± 0.6	282.2 ± 60.4
Phenolic acids	**513.3 ± 112.1**	**15.8 ± 0.4**	**529.1 ± 111.8**
Luteolin 7-O- rutinoside	0.8 ± 0.2	0.2 ± 0.1	1.0 ± 0.2
Luteolin 7-O-glucoside	136.6 ± 18.5	49.1 ± 8.3	185.7 ± 10.2
Luteolin 7-O-glucuronide	6.0 ± 0.9	2.4 ± 0.7	8.4 ± 0.2
Apigenin 7-O-glucoside	1.6 ± 0.3	3.0 ± 0.3	4.6 ± 0.2
Luteolin 7-O-malonyl-glucoside	89.9 ± 11.6	n.d.	89.9 ± 11.6
Apigenin 7-O-glucuronide	n.d.	0.5 ± 0.1	0.5 ± 0.1
Luteolin	5.6 ± 0.8	0.8 ± 0.1	6.4 ± 0.9
Apigenin	0.3 ± 0.2	0.1 ± 0.01	0.4 ± 0.2
Flavonoids	**240.7 ± 32.5**	**56.2 ± 9.0**	**296.8 ± 23.5**
Cynaropycrin	251.1 ± 25.7	n.d.	251.1 ± 25.7
Total PPs	**754.0 ± 143.3**	**72.0 ± 9.3**	**826.0 ± 134.1**

4.3.3. Free and bound antioxidant capacity of MS and DF products

Figure 4.1. shows the free-AC and bound-AC of all the products.

Among the commercial DF products, potato DF showed the highest value of free-AC (148.5 ± 9.8 μmol TE/100g), followed by pea cortical DF (80.4 ± 7.7 μmol TE/100g) and barley β-glucans concentrate (59.8 ± 2.6 μmol TE/100g). Conversely, bamboo DF possessed the lowest values of free-AC and bound-AC. Concerning cereal brans, as expected wheat bran free-AC (218.2 ± 7.6 μmol TE/100g) was more than double of that of oat bran (100.3 ± 8.3

µmol TE/100g) and the bound-AC of wheat bran was more than 5 folds higher of oat bran bound-AC (280.9 ± 5.1 vs 55.2 ± 1.6 µmol TE/100g, respectively). Interestingly, powdered PPe-MS showed a bound-AC (242.3 ± 28.9 µmol TE/100g) comparable to wheat bran and 4.4 folds higher than oat bran bound-AC (55.2 ± 1.6 µmol TE/100g). Citrus DF, wheat bran and carrot DF were the products with the highest percentage of bound-AC accounting for 62%, 56% and 44% of the respective TAC (sum of free-AC and buond-AC).

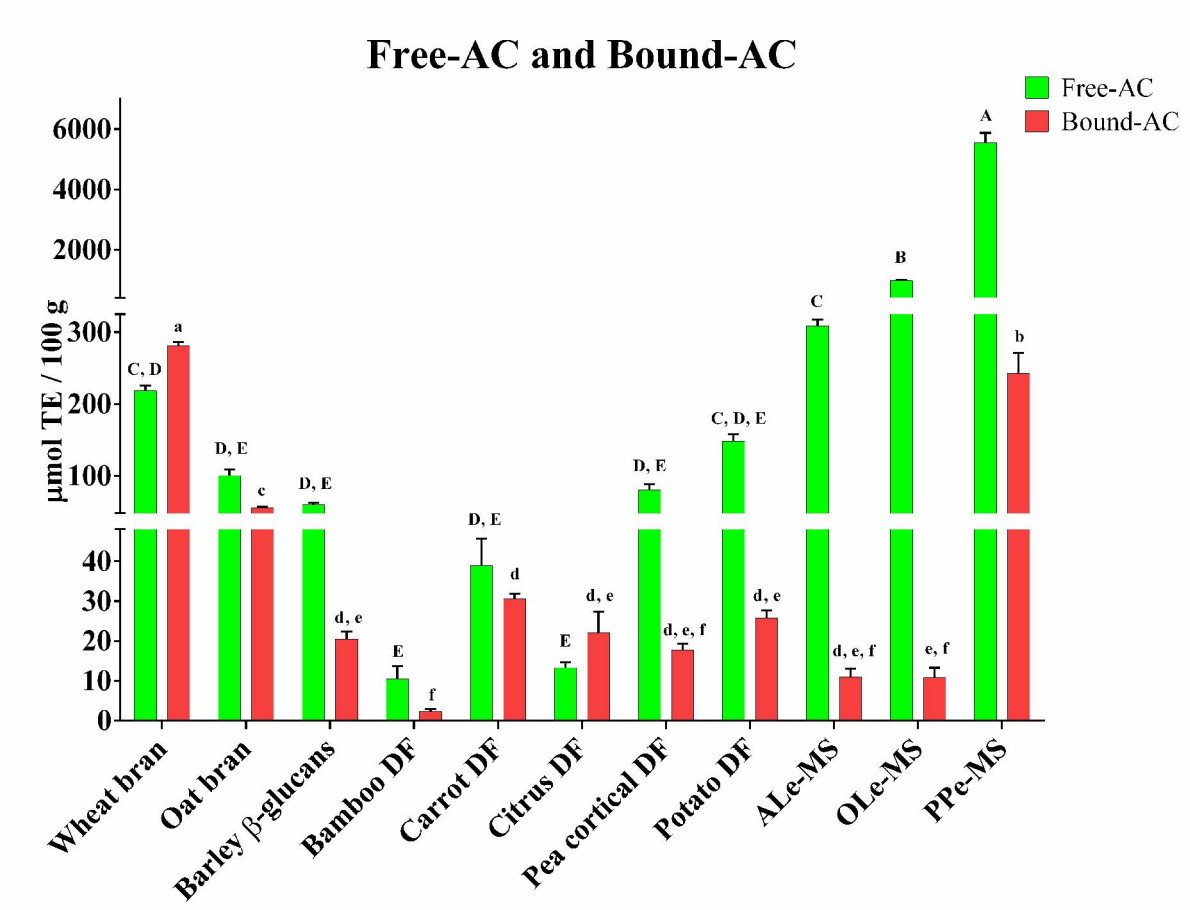

Figure 4.1. Free-AC and Bound-AC calculated for the products. Resultes were expressed as µmol of Trolox Equivalents (TE)/100g of product. Different lower case letter (for bound-AC values) and different upper case letter (for free-AC values) indicated statistically significant differences ($p < 0.05$) among the samples, according to Tukey's Test.

4.3.4 Potential impact of newly developed MS

Figure 4.2. reports the result of an explorative principal component analysis (PCA) including all the data obtained. The first two principal components (axis PC1 and PC2) explained approximately 65% of the total data variance including variance linked to each variable, the one common among variables, and error variance. The left box in the **figure 4.2.** shows the relationship between the analysed products and the variables (loadings plot) while the right

box report the PCA scores plot. This analysis revealed a defined clustering behavior except for one sample in the first quarter that was considered an outlier (β-glucans concentrate). Data showed that the MS were clustered together with wheat bran and oat bran in the left part of the scores plot graph. Specifically, PPe-MS, wheat bran and oat bran were located in the same quarter, where the value of PC1 was negative and the value of PC2 was positive. Conversely, excluding barley β-glucans, the other products were grouped in the right part of the scores plot graph. Citrus DF, pea cortical DF and bamboo DF were placed in the second quarter where PC1 was positive and PC2 was negative. Carrot DF and potato DF were located in the first quarter (positive values for PC1 and PC2). Barley β-glucans concentrate was considered an outlier due to its high percentage of SDF and to the high water solubility, which contributed for the 29.3% and 34.0% of the PC2, respectively.

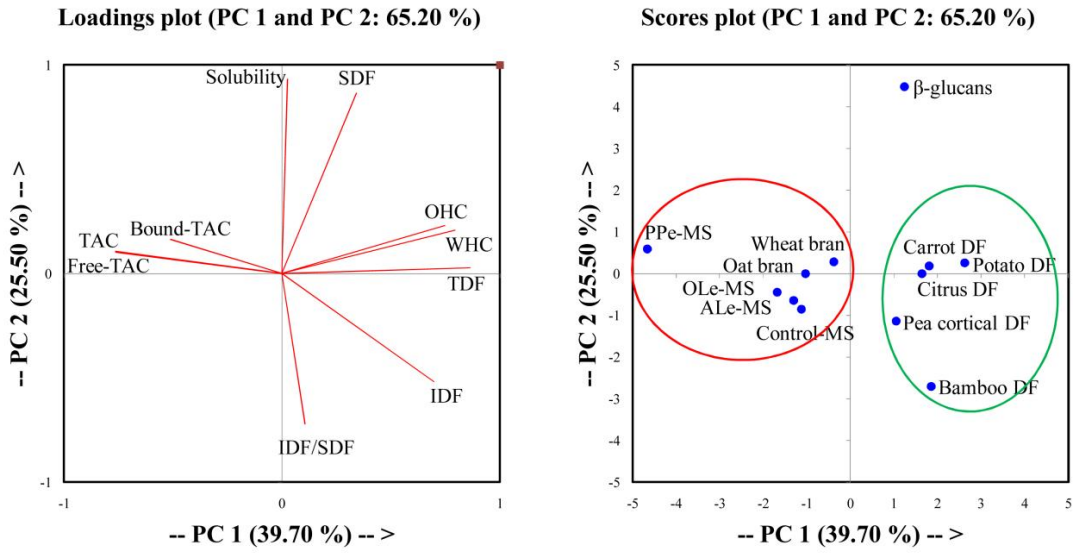

Figure 4.2. Exploratory Principal Component Analysis (PCA).

4.3.5. In vitro gastrointestinal digestion of PPe-MS

Figure 4.3. shows the concentration of PPe-MS PPs released at each step of the gastrointestinal *in vitro* digestion. Punicalagin, punicalin and other ETs from PPe-MS were mainly released in the simulated oral phase (SOP). Only a small residual fraction of punicalin (40.9 ± 7.5 µg of punicalagin equivalent/g of dry MS) was detected in the simulated intestinal phase (SIP). Conversely, EA and EA-derivatives were mainly released in the simulated gastric phase (SGP) and in the SIP. GA was not detected in the SOP extract but was mainly released in the SGP (141.5 ± 4.2 µg /g of dry MS) and in the SIP (39.5 ± 1.7 µg/g of dry MS).

The pellets resulting from the SIP were hydrolyzed in order to analyze the amount of residual PPs entrapped in the matrix after the gastrointestinal *in vitro* digestion. Interestingly, data reported in **Table 4.6.** showed that small residual fractions of EA (11.2 ± 0.02 μg/g of dry MS), punicalin (4.1 ± 0.1 μg of punicalagin equivalent/g of dry MS) and punicalagin (1.2 ± 0.1 μg /g of dry MS), were released after the alkaline hydrolysis of the residual pellets thus demonstrating that a residual fraction of PPs from PPe-MS may be potentially released in the colon. PPs were not found in the digestive extract of control-MS.

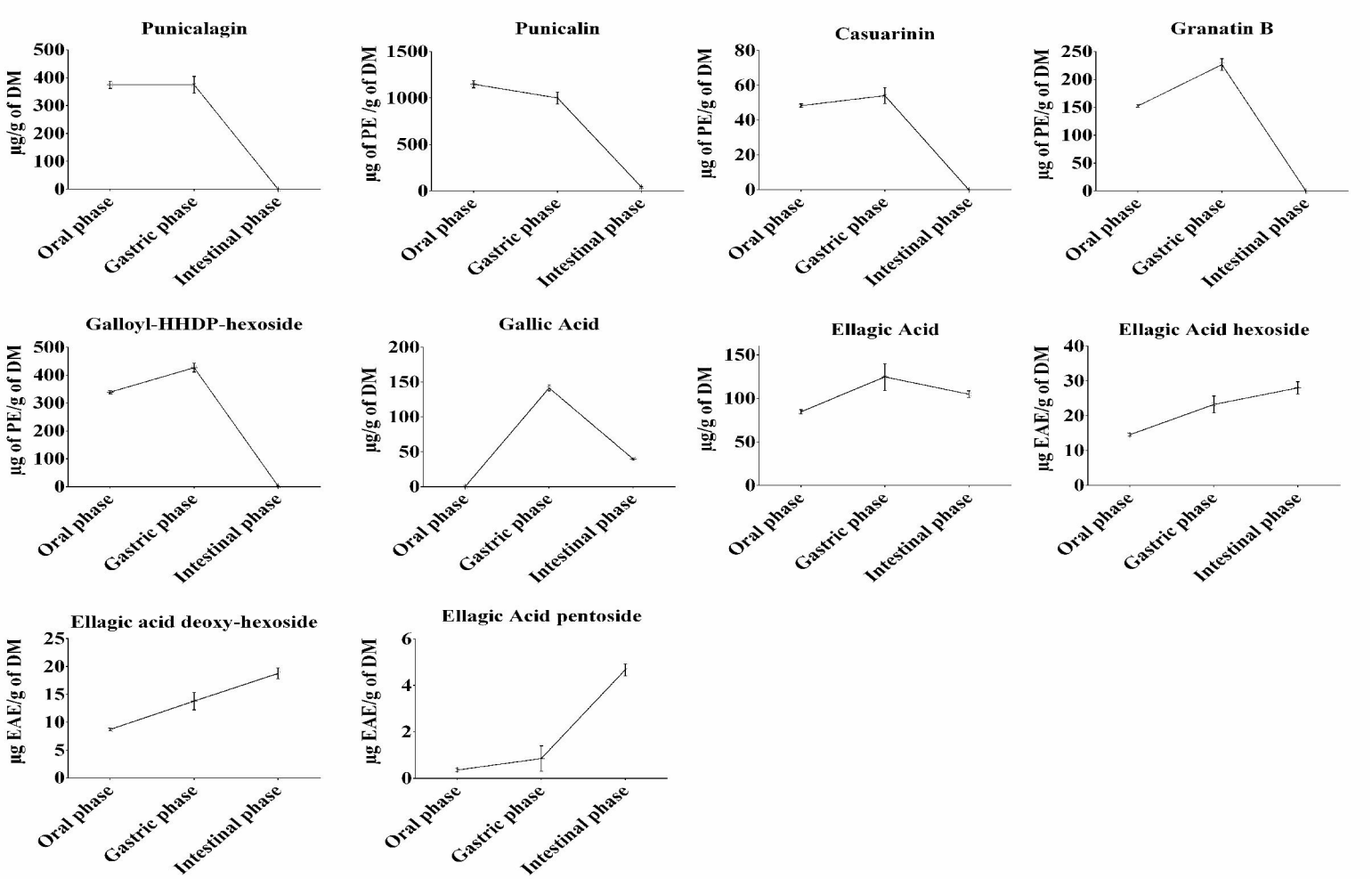

Figure 4.3. PPs released during in vitro gastrointestinal digestion of PPe-MS. Punicalin, Causarinin, Granatin B and Galloyl-HHDP-hexoside were reported as µg of punicalagin equivalent. EA derivatives were reported as µg of EA equivalents.

Table 4.6. PPs released after hydrolysis on the pellets resulting by the simulated intestinal phase of MS in vitro digestion.

Compound	Buond PPs released after *in vitro* digestion (µg/g dry MS)
Punicalin	4.1 ± 0.1
Punicalagin	1.2 ± 0.1
Causarinin	n.d.
Galloyl-HHDP-hexoside	0.8 ± 0.4
Granatin B	n.d.
ETs	**6.0 ± 0.7**
Ellagic acid hexoside	n.d.
Ellagic acid pentoside	n.d.
Ellagic acid deoxyhexoside	n.d.
Ellagic acid	11.2 ± 0.02
EAs	**11.2 ± 0.02**
Gallic acid	n.d.
Total PPs	**17.2 ± 0.7**

4.4. Discussion

In this study for the first time three types of antioxidant DF-rich ingredients were designed and developed. The new ingredients were MS based on the combined inclusion of native corn starch and PPs in a calcium-alginate matrix. The basic structure of the new MS, i.e. corn starch entrapped in the spherical alginate matrix, was previously demonstrated to limit the extent of starch digestion in the small intestine thus allowing its fermentation in the colon.[39,40] Moreover, starch-based MS were able to decrease postprandial blood glucose 60 min after ingestion.[60]

Data of the present study demonstrated that the inclusion of PPs-extracts in MS formulation resulted in new starch-alginate based MS that could be considered an antioxidant DF. The three types of powdered MS showed higher free TAC than commercial DF, and the bound-TAC of PPe-MS was even comparable to that of wheat bran and 4.4 folds higher than oat-bran.

This finding is very interesting because wheat bran is one of the best natural source of bound-PPs, mainly including ferulic acid, and it is well-recognized for its health properties along the GiT.[26,28,62] Similarly, oat bran possesses PPs bound to IDF and that can be released after a chemical or enzymatic hydrolysis, as well as by microbial fermentation in the colon.[26,63-65] Many epidemiological studies have demonstrated that regular consumption of whole grains and whole grain products is associated with reduced risks of various types of non-communicable chronic diseases such as cardiovascular diseases, type 2 diabetes, and some types of cancer, mainly including colon cancer.[66] The health benefits of whole grains have been attributed in part to their phytochemicals, most of which are covalently bound to the IDF portion of the bran fraction.[67,68] Moreover, the consumption of whole grain products, mainly including wheat bran and oat bran, was associated with beneficial modifications of the colonic microbial community, including an enhanced growth of probiotic bacteria, as well as to improvements of antioxidant and inflammatory status, both in animals and in humans.[28,69-73]

Although OLe-MS possessed a higher amount of total PPs than PPe-MS, the latter showed a TAC that was 6 and 18 folds higher than the TAC of OLe-MS and Ale-MS, respectively. These findings can be explained by considering the huge variability of the antioxidant capacity associated to various PPs.[74,75] Phenolic acids, mainly including ferulic acid, are the most abundant phenolic compounds covalently associated to the IDF of wheat bran and oat bran and can be released after an alkaline hydrolysis.[65,76] Secoiridoids, mainly including

oleuropein, were the most abundant compounds detected in OLe-MS. ETs, EA and GA were the main PPs detected in PPe-MS. EA and GA are recognized as more effective antioxidant compounds and active inhibitors of free radicals compared to ferulic acid and PPs detected in OLe-MS.[77,78] On the other hand, punicalagin is one of the strongest natural antioxidant compounds, exhibiting an antioxidant capacity that is 16, 7, 16 and 13 folds higher than the antioxidant capacity measured for EA, GA, protocatechuic acid and p-coumaric acid, respectively.[79]

On the basis of their great antioxidant capacity, the potential efficacy of PPe-MS to deliver PPs along the GiT was tested by applying an *in vitro* gastrointestinal digestion. In order to simulate the utilization of PPe-MS as functional ingredient in foods, they were cooked before the analysis. As it could be expected, data showed thata fraction of total PPs was partially lost during the cooking process. However, it was also demonstrated that the release of residual ETs decreased from the SOP to the SIP whereas GA, EA and EA-derivatives had an opposite trend. This finding was in accordance with previous evidence showing that ETs can be hydrolyzed under physiological conditions, thus mainly releasing GA, EA and EA glycosidic derivatives in the SIP.[80] Moreover, accordingly with *in vivo* evidence demonstrating that only a part of EA is absorbed in the intestinal lumen while the other part reaches the colon, the alkaline hydrolisis of the final pellet resulting from the SIP showed that residual fractions of EA and ETs could potentially reach the colon. So, it could be speculated that the consumption of the PPe-MS may lead to a release of EA and ETs to the colon, consequently creating a reducing environment in the gut, as well as promoting the microbial transformation of these PPs into the more adsorbable bioactive urolithins.[80] Further studies are warrented to test these hypothesys *in vivo*.

Another important finding of this study regarded the positioning of the new MS in the scenario of the commercially available DF as regards the content of TDF, WHC, OHC, other than TAC and Free-TAC. In this respect exploratory PCA data indicated that the three MS could be engrouped with oat bran and wheat bran in the left part of the scores plot graph, whereas the others DF-concentrate, (excluding β-glucans that was considered an outsider) constituted a separate group in the right part of the scores plot graph graph (see **Figure 4.2.**). Specifically in the left part of the graph the products with higher antioxidant activity and lower amount of TDF were reported, conversely the other products were grouped in the right part of the graph. Furthermore, observing the loadings plot graph it is possible to see that, as expected OHC and WHC are parameters strictly related to the amount of TDF.

That the average content of TDF in MS, wheat bran and oat bran was lower and that, consequently also the WHC and OHC could be lower, than those of the 6 commercial DF-concentrates, was an expected finding. However, the TAC of MS, wheat bran and oat bran was higher compared to the TAC measured for the 6 commercially available DF-concentrates. This cluster behavior could be explained by considering both the individual characteristics of the productsas well as the processing conditions that commercial DF could undergo in order to optimize the technological properties. It is well known that chemical, mechanical, thermal and enzymatic processing can modify the properties of DF, mainly including WHC, OHC, solubility and TAC.[81]

IDF was the predominant fraction of TDF in MS and it was mainly constituted by calcium-alginate, an insoluble salt of alginic acid. Alginic acid is a linear indigestible anionic polysaccharide extracted from brown seaweed, constituted by β-(1,4)-mannuronic acid and α-(1,4)-guluronic acid and is depolymerized by the action of specific alginate lyases.[82,83] According to Park and Jhon, (2009), also bamboo TDF was mainly constituted by IDF (98.3%) whereas barley β-glucans concentrate was mainly constituted by SDF (97.9%).[84] That the content of TDF and IDF was higher in wheat bran compared to oat bran was also in line with the literature.[26]

On the other hand MS possessed a low water-solubility, WHC and OHC compared to the other DF.The WHC and the OHC indicate the amount of water and oil retained by a certain weight of product under specific conditions.[81] These properties, together with the water solubility, have both technological and physiological relevance. DF with a high WHC can be useful to avoid the synaeresis and modify the viscosity and texture of food formulations.[85,86] From a physiological perspective it may induce a prolonged mastication and increase faecal bulk volume, thus potentially influencing satiation and appetite.[87-89] DF possessing a high OHC is useful to stabilize food emulsions, as well as to decrease fat digestion and absorption along the GiT.[81,85,90]

As regards the water solubility, a highly water-soluble DF can affect the viscosity of foods and from the pysiological point of view, may influence nutrients absorption in the GiT.[81] Those functions are well recognized in the literature for barley β-glucans concentrate, showing the highest water-solubility among the tested products.[91, 92]

4.5. Conclusions

In this study three types of antioxidant DF, constituted by PPs extracted from three common plant by-products and included in starch-alginate based MS, were developed. The new ingredients showed a TAC that could act both in the food matrix and along the GiT, as demonstrated *in vitro* for the PPe-MS. In the actual scenario of commercially available DF, data showed that the new antioxidant MS were very similar to wheat bran and oat bran for the main physical-chemical properties other than for the antioxidant capacity. This comparison let us hypothesize that these ingredients are very promising for both technological and physiological pespective. Further studies are warranted to assess the impact of the MS in food formulation and to demonstrate *in vivo* the antioxidant efficacy and the impact on health.

4.6 Abbreviations

ALe: Artichoke leaves;

ALe-MS: ALe-Microspheres;

DF: Dietary fiber;

EA: Ellagic acid;

EAs: Ellagic acid derivatives;

ETs: Ellagitannins;

GA: Gallic acid;

GiT: Gastrointestinal tract;;

IDF: Insoluble dietary fiber;

MS: Microspheres;

NCCDs: Non communicable chronic diseases;

OHC: Oil holding capacity;

OLe: Olive leaves;

OLE-MS: OLe-Microspheres

PCA: Principal component analysis;

PPe: Pomegranate peels;

PPe-MS: PPe-Microspheres;

PPs: Polyphenols;

SCFAs: Short-chain fatty acids;

SDF: Soluble dietary fiber;

SGP: Simulated gastric phase;

SIP: Simulated intestinal phase;

SOP: Simulated oral phase;

TAC: Total antioxidant capacity;

TDF: Total dietary fiber;

TE: Trolox equivalents;

WHC: Water-holding capacity;

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Chapter 5

Summary, conclusions and future perspectives

5.1 Summary, conclusions and future perspectives

In the last decades, a growing number of functional foods promising a huge variety of health properties beyond their nutritional aspects, was developed and entered on the market.[1]

Both food processing and food composition may affect the food quality and the behavior of nutrients and non-nutrients in the gastrointestinal tract (GiT).[2-8] On the other hand, the environmental conditions (i.e. pH changes, time of transit, permeability of the gastrointestinal mucosa, presence of salts, mucine, enzymes and emulsifying molecules in the intestinal lumen) associated to the physiological processes taking place along the GiT (i.e. the gastrointestinal motility, activities of digestive enzymes and host microbiota) are also key factors influencing the bioaccessibility and bioavailability of macronutrients, micronutrients and bioactive non-nutritional compounds in foods.[8-10]

So the lacking information about the influence of food processing and matrix on the bioactive compounds together with the fate and the potential functional properties along the GiT, may be an important cause of costly late-stage failures in functional foods development process. Therefore, it has become widely appreciated that those parameters should be elucidated as early as possible.[11, 12]

Human trials are necessary to evaluate the physiological responses and the effects following the consumption of specific bioactive compounds. However *in vitro* assays are a useful tool to elucidate a specific mechanism underlying a physiological response, or to foresee the effects of food processing and food matrix on the functionality and bioaccessibility of food bioactives in the GiT.[13]

In vitro digestion models, coupled to chemical analysis and biochemical assays allow us to study the potential fate of targeted food components along the GiT and the potential mechanisms behind modulation of oxidative and inflammatory processes as well as nutrient metabolism.[14-19]

This approach was used during the implementation of this work that was described in 5 chapters and whose main conclusions are summarized as follows.

In **Chapter 1** a brief introduction on the worldwide spread of obesity and an overview on the role of bioactive compounds, mainly including polyphenols (PPs) and dietary fiber (DF), to counteract weight gain and metabolic syndrome was presented. Furthermore, the concept of plant foods by-products as rich sources of bioactive compounds was introduced.

In **Chapter 2** pomegranate peels (PPe) were proposed as functional ingredient for the development of functional cookies. The findings supported the use of PPe at a dose of 7.5% by weight in bakery food products, as a functional ingredient to counteract oxidative stress along the GiT. The potential antioxidant protection may involve all the organs of the GiT due to the release of PPs from PPe along all the digestive system. Moreover, it was demonstrated that the baking process increased the amount of ellagic acid in the PPe-enriched cookies due to chemical degradation of ellagitannins, thus allowing its increased release in the duodenal and colon phase compared to the raw PPe. Together with a high antioxidant potential, the mix of PPs solubilized in the simulated duodenal phase also acted as strong inhibitors of α-glucosidase, thus possibly influencing in a positive manner the glucose metabolism *in vivo*.

In **Chapter 3** artichoke stems from "Tondo di Paestum" were selected among artichoke by-products and used at 3%, 6% and 9% in the formulation of new types of bread. The potential bioaccessibility of PPs and of the bitter sesquiterpene lactone cynaropicrin from the breads as well as the overall total antioxidant capacity (TAC) and potential ability to modulate α-glucosidase activity in the GiT were assessed. Data showed that the potential release of PPs and cynaropicrin as well as the antioxidant activity and α-glucosidase activity in the small intestine correlated with the amount of ASP added to the bread recipe. Interestingly, a part of these bioactive compounds were potentially released in the colon step after a biochemical conversion induced by enzymes of microbial origin, thus suggesting an effect of the new foods that may be mediated by the intestinal microbiota *in vivo*. Finally, it was demonstrated that PPs from artichoke stem were less active against α-glucosidase compared to PPs from PPe.

In **Chapter 4**, three PPs-rich extracts were obtained from PPe, olive leaves (OLe) and artichoke leaves (ALe), and were used as a functional filling for the development of new starch-alginate based microspheres. The physico-chemical properties of the new ingredients were compared to those of 6 commercially available DF concentrates, as well as to wheat bran and oat bran. PPe-microspheres (PPe-MS) were submitted to an *in vitro* simulated gastrointestinal digestion in order to evaluate their potential efficacy to deliver PPs and to exert antioxidant activity along the GiT. The new ingredients exhibited antioxidant activity inside the food and along the GiT, as demonstrated *in vitro* for the PPe-MS. In the actual scenario of commercially available DF, data showed that the new antioxidant MS were very similar to wheat bran and oat bran for the main physical-chemical properties other than for the

antioxidant capacity. This comparison let us hypothesize that these ingredients were very promising for both technological and physiological perspective.
In **Chapter 5** the concluding remarks and future perspectives of the work were reported.

For a general conclusion it is important to stress that due to the main limitation of *in vitro* model systems to fully mimic the overall processes occurring *in vivo* (such as absorption, hormonal and nervous responses, feedback mechanisms, mucosal cell activity, complexity of peristaltic movements, and involvement of the local immune system), human trials are needed to confirm findings from *in vitro* studies here presented and for the final validation of the new foods/ingredients designed and tested in this work.

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