Chapter 2. Non-extractable polyphenols: A relevant group with health effects.

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Abstract

Current research on dietary polyphenols is mostly focused on the so-called extractable polyphenols (EPP), those ones relased from food matriz by several solvent combination. However, this ignores a relevant fraction of polyphenols remaining in the residues of those extractions, the non-extractable polyphenols (NEPP) or macromolecular antioxidants. They are either polymeric structures (mostly high molecular weight proanthocyanidins) or small phenolic structures closely linked to macromolecules such a proteins or dietary fibre. This partial approach to these dietary bioactive constituents may hamper the understanding of the whole relevance of dietary polyphenols. The present chapter provides an overview on the current knowledge of NEPP. In particular, the following aspecs are explored: strategies for the release and analysis of NEPP; contribution of these compounds to total polyphenol content in foods and, therefore to total polyphenol intake in different populations; evidences of their metabolic fate as well as specific features as compared to that of EPP; mechanism of biological action (antioxidant effects, modulation of colonic microbiota, biological activities of NEPP-derived metabolites, synergy with dietary fibre); evidences of their biological activity (considering both local effects in the digestive tube and systemic effects through their metabolites). Finally, the chapter provides some perspectives of the

main aspects that should be considered in order to advance in the scientific knowledge on NEPP in the near future.

Keywords: non-extractable polyphenols; colonic metabolites; gastrointestinal health; cardiometabolic health.

2.1. Introduction: the concept of non-extractable polyphenols (NEPP)

Research on food polyphenols has experienced a huge development during the last century. Thus, from the initial studies more focused on phytochemical aspects, the consideration of polyphenols as anti-nutrients, etc., research evolved towards describing the relevance of polyphenols to improve several health markers by a combination of mechanisms. These advances implied many kinds of studies (*in vitro*, preclinical, clinical or epidemiological) and with several approaches (mechanistic, bioavailability, health effects). Nevertheless, in most of these studies, an important part of polyphenols was and still is, neglected: the non-extractable polyphenols (NEPP). This partial approach to these dietary bioactive constituents partially hampers the understanding of the whole relevance of dietary polyphenols. For instance, if they are not systematically considered when evaluating food polyphenol content when a clinical trial is designed based on a certain polyphenol intake, only a fraction (extractable polyphenols, EPP) is actually considered. Thus, although whole information on the outcomes of the intervention may be achieved, that on the incomes will remain incomplete.

But what does the term NEPP mean and why they are not considered in most polyphenol studies? When food polyphenols started to be studied, different strategies for their release from food matrix were explored, until the best combinations of solvents were found for obtaining overall polyphenol profile or for extracting specific polyphenol classes. For instance, it is known that acidified acetone: water (70:30) is the most efficient combination for extracting proanthocyanidins (Hümmer and Schreier, 2008). This approach, common for studying food constituents, was based upon the consideration that, although these food extractions provided a residue, the polyphenol content in it could be considered as negligible. However, further research showed that a fraction of food polyphenols remained in those residues, and they could even constitute a major fraction of total polyphenols in certain food items (Saura-Calixto, 2018). It should be highlighted that this fraction remains, whatever the solvents are used or even

applying new extraction techniques, such as ultrasound, subcritical water extraction, etc., (Wu *et al*., 2018; Sanz-Pintos *et al*., 2017). The reason for this is how NEPP are present in foods.

Briefly, NEPP may be divided into two categories: hydrolysable polyphenols (HPP) and non-extractable proanthocyanidins (NEPA). HPP are small phenolic compounds which are associated with food macromolecules -proteins, but, especially dietary fibre- by a combination of weak and strong bonds (Pérez-Jiménez and Torres, 2011). They have been mostly studied in cereals (where they are also known as insoluble or bound phenolics), but they have been reported in other food groups (Arranz *et al*., 2010). Regarding NEPA, they are high molecular weight proanthocyanidins, i.e., a polymeric chain of flavanols which may reach several dozens of units which also limits their release from food matrix (Saura-Calixto, 1988). Both HPP and NEPA have a common characteristic, which is that they are present in foods forming macromolecules (NEPA are macromolecules *per se*, while HPP are associated with macromolecules). For this reason, during the last years, the term "macromolecular antioxidants" have also been suggested to describe NEPP; besides, this term has higher application potential in a nutritional and medical context (Saura-Calixto, 2018).

Thus, from a chemical point of view, HPP and NEPA do not constitute specific chemical entities, but they are present in the food matrix in a different way (either by molecular weight or by association with food matrix) than the extractable polyphenols (EPP), i.e., low molecular weight phenolic compounds easily released from the food matrix. This, as it will be further discussed, will give place to specific metabolic features associated with NEPP and not with EPP and, ultimately, it may have consequences in the health effects of NEPP. Therefore, the relevance of considering NEPP is not only a chemical or theoretical discussion but also it has practical implications for the health effects associated with plant food consumption. Indeed, independently of the practical problems that researchers still have for a comprehensive characterization of polyphenol profile, the fact is that, when consuming food, we are consuming both EPP and NEPP; this is something that should not be disregarded.

2.2. Contribution of NEPP to total polyphenol content and intake

A first step towards unraveling the relevance of NEPP for health and nutrition is to determine their content in common foods, as well as their quantitative contribution to total polyphenol content, i.e., their proportion as compared to that of EPP. These data may be used for different purposes, such as identifying NEPP-rich foods, designing clinical trials focused on NEPP supplementation, or evaluating NEPP intake in different populations. Indeed, some preliminary studies already made estimations of NEPP intake as compared to that of EPP intake (Arranz *et al*., 2010; Saura-Calixto *et al*., 2007; Hervert-Hernández *et al*., 2011) . Nevertheless, for performing all these determinations, a critical step is to have a routine methodology for the analysis of NEPP in foods, which is still far to be achieved (Pérez-Jiménez and Torres, 2011).

2.2.1. Strategies for the extraction and analysis of NEPP

For the isolation and analysis of NEPP, several steps are needed. The first one, common to the analysis of EPP, is generally a particle size reduction since this diminution allows to increase the contact surface between matrix and solvent (Pérez-Jiménez *et al*., 2008). Also, for fat-rich samples, such as nuts, a previous defatting step is recommended since the fat may behave as interference in further determinations (Arranz *et al*., 2008).

The second step consists in the removal of EPP and other soluble substances like soluble sugars, vitamins, organic acids, and others. This step is usually performed combining successive solid-liquid extractions, such as acid-methanol/water (50:50) for the extraction of the most polar polyphenols, e.g. phenolic acids, followed by extraction with acetone/water (70:30) or acetone/water/acetic acid (70:29.5:0.5) to extract more apolar polyphenols such as proanthocyanidins. After centrifugation, the residue is treated for the release of NEPP, which include, as described above, HPP and NEPA.

For both HPP and NEPA release, the most common procedures include the use of acids at high temperatures, with different purposes. Thus, in the case of HPP, such treatments allow the release of polyphenols attached to the plant matrix, releasing small phenolic structures that may be further determined by spectrophotometry (Folin assay) or by HPLC or HPLC-MS procedures (Pérez-Jiménez *et al*., 2008; Arranz *et al*., 2009). Thus, a standard procedure for the release of HPP from the residue of the EPP release is

the treatment with methanol/H_2SO_4 (90:10, v/v) at 85 °C for 20 hours with agitation (Hartzfeld *et al*., 2002). The derived hydrolysate, exhibiting strong acidity, must be partially neutralized before determining polyphenol content by some of the mentioned techniques. Indeed, in the case of chromatography analysis is going to be applied, an additional solid phase extraction procedure is needed, to remove the high concentration of salts released during the acid treatment (Pérez-Jiménez and Saura-Calixto, 2015).

It should be indicated that, for HPP analysis, alkaline treatments in the residues of extraction have been more commonly used than acid treatment, particularly in the case of cereals. However, although this may be more useful for the specific case of ferulic acid (a major constituent in cereal samples), for most phenolic acids attached to the plant cell wall, acid treatment is the most efficient way to release HPP (Arranz and Saura-Calixto, 2010).

As regards to NEPA release from the residue of EPP extraction, the aim of the butanol/HCl/$FeCl_3$ procedure (based on the Porter method) (Porter, 1988) is the depolymerization of the long polymeric structures, releasing the corresponding coloured anthocyanidins. Then, absorbance is measured at both 450 and 555 nm to calculate the concentration in relation to a known standard (Zurita *et al*., 2012). An alternative procedure for the release of NEPA is the nucleophilic depolymerization, for which several reagents may be used. This procedure was applied for determining NEPA content in a wide number of samples (Hellström and Mattila, 2008), but it seems to be less efficient than the butanolysis procedure (Pérez-Jiménez and Torres, 2011).

One of the problems arising from the use of strong acid treatments on the residues of EPP removal is that, in the case of HPP, either significant losses can occur due to degradation of some polyphenols -usually benzoic and hydroxycinnamic acids- or some artifacts may be generated (Pérez-Jiménez and Saura-Calixto, 2015). This makes that the structures finally measured in HPP hydrolyzates may not be exactly those originally present in foods. In the same way, the depolymerization of NEPA originates structures different to those ingested, which makes difficult the application of *in vitro* procedures to determine their biological activities. Nevertheless, such strong conditions are, up to this moment, the only valid ones for a comprehensive characterization of NEPP since

other procedures, such as enzymatic treatments, are shown to be much less effective (Pérez-Jiménez *et al*., 2009a).

Another important aspect for the characterization of NEPP, since a fraction of them is strongly associated with food matrix, is the understanding of the linkages involved in this association and the extension of them. Indeed, this is a subject still being studied. For instance, it was recently reported that the association between NEPP and the cell wall increases during pear overipening (Brahem *et al*., 2019).

Overall, the most important problem for the analysis of NEPP is that there is not yet an accepted standard methodology (Wu *et al*., 2018; Domínguez-Rodríguez *et al*., 2017; García-Villalba *et al*., 2015; Chen *et al*., 2015;de Mira *et al*., 2009; de Camargo *et al*., 2016). As shown in **Table 2.1**, many differences may be found between the procedures used in the literature, first for EPP removal, and then for NEPP release. Such standard methodology would help to increase the number of laboratories routinely measuring NEPP in their phenolic compound determinations. And this would increase the available amount of data on polyphenol content in foods, needed for instance for proper estimations of NEPP intake, as discussed below.

2.2.2. NEPP content in common foods

The available data on NEPP content in common foods, although scarcer than those for EPP, have shown the wide distribution of NEPP in the different groups of plant foods (fruits, vegetables, legumes, cereals, and nuts). When mixtures of individual foods corresponding to the mean intake in Spain of each food group were prepared, and NEPP content was determined, it was found that the richest food group in NEPP are fruits (880 mg/g dry weight) followed by legumes (568 mg/100 g), vegetables (326 mg/100 g), nuts (333 mg/100 g), and cereals (210 mg/100 g) (Arranz *et al*., 2010).

Indeed, NEPP are not only found in most common foods, but they are present at similar or even higher concentration than EPP. **Figure 2.1** shows, for a selection of common fruits and vegetables, the proportion of EPP, HPP, and NEPA. It can be seen that all samples contained NEPP as HPP and, in the case of fruits, some of them also contained NEPA (these were not detected in any of the samples of vegetables). And in a

40% of the samples NEPP constituted at least half of total polyphenol content; indeed, in samples such as banana, nectarine or pear, this fraction was above 80% of total polyphenol content (Pérez-Jiménez and Saura-Calixto, 2015). Cereals are also a food group where a very high proportion of polyphenols are present as NEPP; for instance, in wheat bran, the content of HPP has been reported to be up to ten-fold that of EPP (Arranz and Saura-Calixto, 2010). Overall, the specific contribution of NEPP to total polyphenol content in different food items may be due to the variations in polyphenol profile and subcellular location in different foodstuffs, as well as to the various food processes and experimental methodologies used (Pérez-Jiménez and Torres, 2011).

NEPP have also been reported in other less common food items, such as the tropical fruits acerola, açai or cashew apple (Maria do Socorro *et al*., 2010; Maria do Socorro *et al*., 2011). And they were found to represent about 40% of total polyphenol content in edible seaweeds (Sanz-Pintos *et al*., 2017). Interestingly, cocoa, which is widely studied for its high EPP content, also presents a relevant proportion of NEPP in the form of NEPA (Tabernero *et al*., 2006). Still, more data on NEPP content in common foods are needed. For instance, although a detailed list was published for fruits and vegetables(Pérez-Jiménez and Saura-Calixto, 2015), similar data have not been provided for common legumes or nuts. And, in the case of cereals, since this analysis was commonly performed by using alkaline conditions which, as discussed above, may not be as efficient as acid conditions, more data on NEPP content in cereals and derived products by using this procedure should be produced. Also, it should not be disregarded that beverages also contain a small proportion of NEPP, being associated with dietary fibre (Díaz-Rubio and Saura-Calixto, 2011), which also has to be considered in the characterization of these food items.

Besides, another important source of NEPP are different food wastes. Indeed, many fruit peels present a relevant content of these compounds, even higher than that of EPP, as it is the case for banana, kiwi, melon, orange, pear and watermelon peels (Pérez-Jiménez and Saura-Calixto, 2018). Recently, prickly pear peels of different tonalities (yellow-orange, red and green) were reported to present a high content of NEPP, being gallic acid 3-O-gallate, cinnamic acid, hesperidin and myricetin 3-O-rhamnoside the main individual contributors (Amaya-Cruz *et al*., 2019). Other byproducts such as grape pomace from wine processing or the by-product of peach juice production also present a

very high NEPP content (Pérez-Jiménez *et al*., 2009a) and detailed information by HPLC-MS analysis have been provided for them (Pérez-Ramírez *et al*., 2018; Rodríguez-Gonzáles *et al*., 2018). These results show that such byproducts should be further studied for their potential as new functional ingredients as well as for the economic and environmental advantage of an integral use of vegetal materials.

2.2.3. Estimation of NEPP intake in different populations

The existing data on NEPP content in foods have been used to estimate NEPP intake in some populations. The most detailed study was performed in Spain, based on the reported data from mean food consumption in this country (**Table 2.2**). It was obtained that the daily intake of total polyphenols from solid foods was 1,207 mg/day/person where 949 mg were NEPP, and 258 mg were EPP. Adding to this value the intake of phenolic compounds provided by the consumption of beverages and oils in the Spanish previously reported (Saura-Calixto *et al*., 2007), the total polyphenol intake increased to a value of about 1840 mg/day, of which 898 mg would be EPP and 949 mg NEPP. Although legumes and cerels were the food groups showing the highest NEPP content, the major contributors to NEPP intake were fruits (46%) and cereals (30%), followed by vegetables (13%) (Arranz *et al*., 2010).

Another study evaluated the intake of NEPP from fruits and vegetables in four European countries (Spain, France, Germany, and the Netherlands). It was found that this intake was higher in the Netherlands and Spain than in France and Germany (about 500 mg/day/person *vs*. 400 mg/day/person, respectively). Among the countries with the highest consumption, fruits were the main contributors in Spain, while vegetables were the main contributors in Netherlands (Pérez-Jiménez and Saura-Calixto, 2015). The intake of NEPP from fruit and vegetables was also estimated in a Mexican rural population; in this case, NEPP intake from these foods was about 640 mg/day/person, with NEPA being provided only by fruits and a similar contribution of fruit and vegetables for HPP intake. Interestingly, NEPP intake was three-fold that of EPP (Hervert-Hernández *et al*., 2011).

Up to this moment, no other study has estimated NEPPP intake in other populations, so there is a need to update data in this sense. For this purpose, a specific

database on NEPP content -similar to the existing ones for EPP content in foods (Neveu *et al*., 2010) would be very useful. Finally, data on polyphenol intake should be used in observational studies in order to evaluate potential association with health outcomes, as it has been done for EPP in many nutritional cohorts (Zamora-Ros *et al*., 2016; Karam *et al*., 2018).

2.3. Metabolic fate of NEPP. A key process for their health effects

To exert their health effects, polyphenols must be available to a certain extent in the target tissue. Therefore, their biological properties, as those of other dietary bioactive compounds, are closely linked to the different steps of their metabolic fate: release from the food matrix by the action of digestive enzymes (small intestine) or bacterial microbiota (large intestine), thus becoming bioaccessible, followed by partial absorption and further transformations. The metabolic transformations of EPP have been widely studied and their key aspects are already known: only a fraction is absorbed in the small intestine, while many of them are absorbed in the colon, after microbiota transformation; the metabolites absorbed may be transported through the portal vein to the liver where other transformations take place, giving rise to phase II metabolites; after extensive circulation in the human body, the absorbed metabolites are excreted in urine, while non-absorbed metabolites or unfermented substrates are excreted through the feces (Rodríguez-Mateos *et al*., 2014).

Regarding NEPP, their transformations through the gastrointestinal tract must be studied to understand their potential biological effects. In particular, the following aspects should be considered: their solubilization in gastrointestinal fluids, which may take place both in the small and in the large intestine; their transformation by the action of the colonic microbiota, since the colon is the key organ for their transformation; the absorption of the resulting metabolites (Pérez-Jiménez *et al*., 2013). However, it should be mentioned that each of these points is in function of several factors such as NEPP specific structure or the association of NEPP with other macromolecules, as it will be discussed in detail.

2.3.1. Current evidence of the metabolic transformation of NEPP

As already explained for other aspects regarding NEPP research, studies on their metabolic fate have been scarce as compared to those regarding EPP. Nevertheless, there is already a certain level of evidence on the fact that NEPP are at least partially transformed and further absorbed after their intake, which was recently reviewed (Ludwig *et al*., 2018). Based on this evidence, it is possible to suggest a metabolic fate for NEPP, as depicted in **Figure 2.2**. After intake, there may be partial solubilization of NEPP either in the stomach or in the small intestine, which would anyway affect a minor fraction of these compounds. This may take place by the action of intestinal esterases (Andreasen *et al*., 2001a); this would allow that some of the HPP (small phenolic compounds linked to the cell wall by covalent linkages) were released from food matrix. The other possible way of solubilization of NEPP throughout the digestive tube is still controversial, since *in vitro* and *in vivo* studies have provided contradictory resulta regarding the depolymerization of NEPA (Williamson and Clifford, 2017; Tsang *et al*., 2005; Mateos-Martín *et al.,* 2012) Therefore, further research on the transformations of polymeric proanthocyanidins (either as extractable proanthocyanidins or as NEPA) to effectively answer this question, remains to be performed. Anyway, although solubilization of NEPP in the upper part of the digestive tube may be followed by absorption of these compounds, this will still be a minor fraction of the metabolic fate of NEPP.

These processes for the release of NEPP from food matrix correspond to the metabolic pathway 1 of NEPP transformation: release from food matrix without transformations of the chemical structures. This pathway takes place both in the small and in the large intestine since esterases are also present there (Andreasen *et al*., 2001b).

At the same time, another process takes place in the colon: the metabolic pathway 2 of NEPP transformation (**Figure 2.2**), which corresponds to the transformation of NEPP structures by microbiota action. These transformations may affect both HPP and NEPA.

In the case of HPP, there is already accumulated evidence on the colonic transformation of phenolic compounds -either if they were originally free in the food matrix or they were released from it (Rodríguez-Mateos *et al*., 2014), although still new metabolites are reported (Gómez-Juaristi *et al*., 2018). Moreover, how the overall food

matrix affects the colonic transformation of HPP still deserves more research. For instance, a preclinical study with durum wheat showed that the particle size of the aleurone fraction tested greatly affected the transformation and final bioavailability of ferulic acid linked to the cell wall (Calani *et al*., 2014).

As regards to the metabolic pathway 2, it must be considered that the constituents of NEPA are indeed the same ones as those of extractable proanthocyanidins, being the only difference in the degree of polymerization. Therefore, those compounds described as the most common microbial metabolites of extractable proanthocyanidins, i.e., (epi)catechin and several phenolic acids, would be expected to be also formed from NEPA (Monagas *et al*., 2010). This was first confirmed in an *in vitro* study where the colonic fermentation of a grape product and a concentrate of proanthocyanidins from *Ceratonia siliqua* L. was performed (Saura-Calixto *et al*., 2010). It was found that the major metabolites were hydroxyphenylacetic acid and hydroxyphenylvaleric acid typically formed during colonic transformation of flavanols. The colonic transformation of NEPA was later confirmed in an *in vivo* study, where rats were fed with a diet supplemented with a grape by-product devoid of extractable proanthocyanidins (Mateos-Martín *et al*., 2012). It was found that NEPA are a precursor source of bioavailable compounds -phenolic acids and (epi)catechin-for at least 24 hours after intake. In particular, twenty metabolites from different families were identified in urine. Interestingly, some of these metabolites were conjugated, indicating that, after absorption, these compounds were further transformed by hepatic enzymes. Additionally, two phenolic metabolites derived from microbial transformation were detected in faeces: 4-hydroxyphenylpropionic acid and 3,4-dihydroxyphenylpropionic acid. As it will be discussed later, even if these compounds are finally excreted, their contact with colonic epithelium during a prolonged period may also have biological implications. Indeed, another preclinical study performed with a grape product where most proanthocyanidins were present as NEPA, detected microbial metabolites in caecal content (the fluid previous to faeces formation) 24 h after supplementation (Touriño *et al*., 2011). This means that more than one day after NEPA intake, there are still microbial-derived metabolites in contact with colonocytes.

The relevance of pathway 2 (colonic transformation of NEPP) should be still explored for the specific case of non-extractable ellagitannins. Thus, it is known that

ellagitannins are transformed by colonic microbiota, yielding specific metabolites, the urolithins (García-Villalba *et al*., 2013). At the same time, it has been reported that ellagitannins are distributed in foods between extractable and non-extractable ellagitannins (García-Villalba *et al*., 2015). Therefore, it may be hypothesized that non-extractable ellagitannins contribute to the colonic production of urolithins, although this aspect has not been specifically explored.

Finally, it should not be disregarded that colonic transformation is a two-way process, i.e., the formation of microbial metabolites implies effects on the growth or activity of specific bacterial species. This aspect, and its relation to potential biological effects of NEPP will be later discussed.

2.3.2. Specific features of the metabolic fate of NEPP

As far as we know, the metabolic transformations of EPP and NEPP give place, as shown above, to the same chemical structures. This is logical, since the structures of the starting compounds are similar, changing only the molecular size or the association with dietary fibre. However, these specific facts of NEPP are the origin of differential features of their metabolic fate, as compared to that of EPP: delayed absorption and interactions with dietary fibre (Pérez-Jiménez *et al*., 2013).

Firstly, the delayed absorption of NEPP may be expected, considering other known facts on the metabolic fate of EPP: proanthocyanidin oligomers need to reach the colon to be transformed, giving place to longer circulation times of their metabolites than monomers or dimers (Monagas *et al*., 2010), or glycosides may be less available than their corresponding aglycone since an additional hydrolysis step is needed (Steensma *et al*., 2006). Thus, since NEPP have either high molecular weight or associations with food matrix, this implies additional steps in their metabolic transformation. This was demonstrated in a preclinical study, where rats were fed with a diet supplemented either with ferulic acid or with wheat bran, where a major fraction of ferulic acid is associated with cell wall, i.e., present as HPP (Rondini *et al*., 2004). It should not be disregarded that, although phenolic acids are the major constituents of the HPP fraction, other compounds such as ellagitannins or different flavonoid classes have also been reported in several foods. Indeed, it has been observed that, in food matrixes rich in pectin (a

soluble dietary fibre), there is a delay in the release of some phenolic compounds due to their retention in the gel formed by this substance (Mosele *et al*., 2016).Overall, in the case of HPP, even if they may not be covalently linked to dietary fibre but exhibit weaker associations, aspects such as physical entrapment, increased viscosity or volume will contribute to this delayed release (Bohn, 2014).

Regarding NEPA, acute supplementation to healthy volunteers with a grape product where most polyphenols were present as NEPA produced a significant increase in plasma antioxidant capacity 8 h after the intake (Pérez-Jiménez *et al*., 2009b), a time longer than the 1-2 hours described for the peak in polyphenol concentration when supplementing with extractable proanthocyanidins (Rodríguez-Mateos *et al*., 2014). Additionally, it has been suggested that another aspect that may be involved in the delayed absorption of NEPP, besides their intrinsic structural aspects (high molecular weight or association with dietary fibre) are the potential interactions that these compounds may establish with intestinal tissues or mucin, also avoiding the access to them by gut microbiota (González-Sarrías *et al*., 2017). From a practical point of view, this delayed absorption of NEPP will give place to a sustained circulation of potentially active metabolites as a particular characteristic of NEPP metabolic fate.

The other specific feature of the metabolic fate of NEPP is derived from their close association with dietary fibre. Thus, at the same time that phenolic compounds are transformed by the microbiota, dietary fibre is simultaneously fermented, and mutual enhancements may take place between both processes. As regards to the effect of this interaction on NEPP transformation, it was observed in an *in vitro* model that the colonic transformation of NEPA (adjusting the results per g of these compounds) was higher in the presence of a combination of soluble and insoluble dietary fibre than in the presence of only insoluble dietary fibre (Saura-Calixto *et al*., 2010); since it is known that soluble dietary fibre is much more fermented than the insoluble fraction, it seems that the transformation of this dietary fibre also contributed to the transformation of NEPA. Similarly, a study in rats supplemented with resistant starch (a fermentable insoluble dietary fibre) showed higher levels of plasma phenolic metabolites in rats subjected to this supplementation than in the control group, finding significant associationd between the levels of some phenolic metabolites and those of short-chain fatty acids derived from dietary fibre fermentation (Vitaglione *et al*., 2019). Moreover,

the association of NEPP with dietary fibre would not only affect the rate of transformation of these phenolic compounds but also their transport and absorption; thus, it was recently reported that sustained exposition to short-chain fatty acids derived from dietary fibre fermentation increased the transport of ferulic acid in a monolayer model of epithelial cells, as well as the formation of hesperetin conjugates and their efflux towards the basolateral compartment (Van Rymenant *et al*., 2017). This agrees with previous results indicating that butyric acid increased the transepithelial transport of ferulic acid through upregulation of specific transporters (Ziegler *et al*., 2016). Conversely, NEPP may also enhance the fermentation of dietary fibre (Aprikian *et al*., 2003), which would be an additional biological effect of these compounds, as it will be discussed in the next section.

2.4. How NEPP may exhibit health effects

The potential health effects of NEPP have been attributed to four different mechanisms, some of these specific of them, and others shared with EPP: antioxidant effect, modulation of the colonic microbiota, biological activities of the metabolites released during NEPP catabolism and the synergistic effects with other macromolecules such as dietary fibre. They should not be considered as independent effects, but as combined processes that will take place simultaneously after the consumption of NEPP-rich foods.

2.4.1. Antioxidant effects

The most widely known property of dietary polyphenols is their antioxidant action. Although the determination of antioxidant capacity in foods or in biological samples cannot be automatically associated with health effects as it was commonly done, a certain number of studies have found significant associations between the antioxidant capacity of a diet and several health outcomes such as inflammation management (Brighenti *et al*., 2005) or their association with chronic diseases (Nascimento-Souza *et al*., 2018). Nevertheless, these studies have been mostly based on the evaluation of the antioxidant capacity derived from EPP, ignoring that corresponding to NEPP.

Indeed, the studies evaluating the antioxidant capacity of NEPP in common foods have found that these compounds have an important contribution to total food antioxidant capacity, being equivalent to that of EPP. A study found that, considering overall consumption of foods and beverages in Spain (Pérez-Jiménez *et al*., 2015), the antioxidant capacity intake from soluble antioxidants was equivalent to that from macromolecular antioxidants. Beverages (53%) and fruits (16%) were the main contributors to antioxidant capacity intake from soluble antioxidants, while cereals (43%) and fruits (23%) were the main contributors to antioxidant capacity derived from macromolecular antioxidants (**Figure 2.3**).

Additionally, some studies evaluated the antioxidant capacity of NEPP in specific foods, providing additional information such as the high antioxidant capacity values associated with NEPP in tropical fruits (Maria do Socorro *et al*., 2010; Páez-Peñuñuri *et al*., 2016) or in fruit peels (Liu *et al*., 2018; Pérez-Jiménez and Saura-Calixto, 2018). Another important aspect regarding the antioxidant capacity associated with NEPP in foods is that processing may differently affect these values, sometimes increasing them and others, decreasing them, as shown in different studies (Pérez-Jiménez, 2018). Anyway, it is remarkable that when antioxidant capacity derived from EPP and NEPP in several Italian dishes -as commonly prepared- was determined, in order to calculate the contribution of each fraction to total antioxidant capacity, there was a major contribution of NEPP to total antioxidant capacity (Durazzo *et al*., 2017).

The above data may be used as the basis for studies evaluating potential associations between the antioxidant capacity of diets derived from NEPP and several health outcomes, as done for EEP. But, obviously, for obtaining the strongest evidence of the antioxidant effects of NEPP, this must be measured by *in vivo* studies based on supplementation with different NEPP-rich products. In this case, the antioxidant capacity of intact NEPP during the upper part of the digestive tube will be similar to that measured by *in vitro* techniques. But, after colonic transformation, NEPP-derived antioxidant capacity should be estimated from that of their metabolites, lower than that of their parent compounds (Dueñas *et al*., 2011).

Studies evaluating *in vivo* antioxidant capacity after supplementation with NEPP-rich products are scarce, but they provide interesting results. First, regarding local

modifications of antioxidant capacity, a study in rats supplemented with a NEPP-rich grape pomace found a five-fold increase in cecum antioxidant capacity (Goñi and Serrano, 2005). Additional preclinical studies found that this kind of supplementation also enhanced the endogenous antioxidant system and decreased lipid oxidation in colonic mucosa (López-Oliva *et al*., 2010; López-Oliva *et al*., 2013). Interestingly, a recent study found significant increases in the antioxidant capacity of all the sections of the digestive tube after supplementing rats with high molecular weight melanoidins (Patrignani *et al*., 2019); since it has been shown that these complexes also contain a fraction of NEPP (Pérez-Jiménez *et al*., 2014), it may be hypothesized that these compounds partially contributed to the observed effect.

Besides, it may be expected that NEPP, once metabolized, give place to increases in plasma antioxidant capacity. In this sense, the supplementation to rats with NEPP from persimmon significantly increased this parameter (Matsumura *et al*., 2016). And supplementation with a grape product rich in NEPP caused a sustained increase in plasma antioxidant capacity in healthy volunteers as compared to a non-supplemented control group (Pérez-Jiménez *et al*., 2009b). Anyway, much more *in vivo* studies should be done with NEPP-derived products to deeply understand their effect on oxidative status, either locally in the digestive tube or the bloodstream after absorption.

2.4.2. Microbiota modulation

During the last decades, clear evidence has shown the importance of microbiota regarding both the development of obesity and associated chronic diseases; this affects both the need of avoiding dysbiosis and of preserving gut microbes functionality (Khan *et al*., 2016; Weiss and Hennet, 2017). Therefore, the modulation of microbiota is one of the main mechanisms by which polyphenols can produce their beneficial health effects. It is improtant to remark that there are two improtan aspect to take into account when evaluating the interactiosn between microbiota and NEPP: first, the importance of identifying bacteria responsible of the transformation and, second, the modulation of the microbiota by NEPP.

At the moment, information on the gut bacteria involved in the colonic transformation of polyphenols is still limited, although there is already a certain level of

knowledge of the modifications caused by these dietary constituents (Espín *et al*., 2017) and even some specific bacterial species involved in the transformation of ellagitannins have been reported (Selma *et al*., 2014; Selma *et al*., 2017; Gaya *et al*., 2018). Since, as previously stated, the chemical structures of EPP and NEPP reaching the colon are similar (differing in chain length or association with macromolecules), it may be assumed that they will be transformed by the same species. Thus, NEPP would contribute to the overall modifications in gut microbiota originated by all dietary polyphenols. Nevertheless, some studies have explored specific characteristic of the metabolic fate of whole-grain cereals (rich in NEPP), which deserve some commentaries.

When whole-grain cereals reach the colon, as previously explained, the first step needed is the release of HPP from the cell wall, what needs the direct action of feruloyl esterase activity with a potential combination of xylanase activity. These enzymes have been reported in bacterial species belonging to the genera *Lactobacillus* and *Roseburia* (*Firmicutes*), *Bifidobacterium* (*Actinobacteria*) and *Bacteroides* and *Prevotella* (*Bacteroidetes*) (Chassard *et al*., 2007; Couteau *et al*., 2001; Dodd *et al*., 2011; Flint *et al*., 2012). For instance, it was observed that fermentation of whole-grain barley or oat groats in the presence of three lactic acid bacteria (*L. acidophilus, L. johnsonii,* and *L. reuteri*) increased the production of free phenolic acids (Hole *et al*., 2012). Additionally, when evaluating the bacterial species involved in the metabolic transformation of HPP, this should be considered as a whole, also evaluating those directly modified by the presence of dietary fibre. In this sense, a recent preclinical study where rats were supplemented with a whole-grain flour rich in resistant starch and HPP found that, although the common genera involved in the transformation of HPP were not modified, there was an increase in members of *Ruminococcaeae/Ruminococcus,* known to be involved in the transformation of resistant starch, and this finally caused an increase in the levels of dihydroferulic acid, a colonic metabolite of ferulic acid (Vitaglione *et al*., 2019).

As regards to the other relevant aspect in the interaction between NEPP and microbiota, i.e., hown NEPP affect the gwoth of different bacterial species, a clinical trial with overweight/obese subjects supplemented with whole grains (rich in HPP) observed sustained increases in the levels of *Firmicutes* and also *Bacteroidetes*,

accompanied by a decrease in *Clostridia* (Vitaglione *et al*., 2015). And regarding the effects of NEPA on colonic microbiota, an *in vitro* study evaluated the effect of whole Grape Antioxidant Dietary Fibre (GADF) (contanining 80% of polyphenols as NEPP), the EPP fraction, and the NEPP fraction on the growth of different beneficial microbes. It was found that NEPP specifically enhanced the growth of *L. reuteri*. But the most interesting results was that it was the whole GADF and not the separated fractions (EPP and NEPP) which had the highest effect on the growth of *L. acidophilus*, suggesting a synergy between both fractions (Pozuelo *et al*., 2012). Nevertheless, another study isolated NEPP from corn and tested their role on the growth of several bacterial species, finding no significant differences on the effect on *L. helveticus* and *B. longum*, as compared to a control (Galvez-Ranilla *et al*., 2017). Besides the differences in the microbes tested, since NEPP in corn are mostly HPP while in GADF they were mostly NEPA, the particularly potential role of the different fractions of NEPP on colonic microbiota deserves further research.

Finally, it should not be disregarded that, besides stimulating the growth of certain bacterial species, polyphenols (including NEPP) may contribute to shaping the gut microbiota by other mechanisms: antimicrobial effects which may be of health relevance, inhibition of microbial enzymes, alterations of mucus viscosity or disruption of quorum sensing (Havlik and Edwards, 2018).

Much more information is still needed regarding the gut microbes involved in the transformation of NEPP as well as on the effects than NEPP have in the overall microbiota profile. Moreover, certain limitations of previous studies should also be considered. For instance, common microdilution methods originally developed for the study of antibiotics may not be as useful in the context of colon microbiota, since they were developed for compounds with better solubility and slower metabolism, they use isolated microbial strains, and there are many differences in the methodologies used; for these reasons, more clinical studies evaluating microbiota after the supplementation with NEPP-rich derived products should be performed. Finally, it is very relevant to ensure that *in vitro* models use concentrations similar to those circulating in the human body after polyphenol intake, since many studies have been based in much higher doses, limiting the relevance of the reported results (Havlik and Edwards, 2018).

2.4.3. Biological activities of microbial metabolites

The metabolites formed from NEPP by the action of colonic microbiota have been reported to be at least partially absorbed, as shown by their detection first in the colonic tissue and later in urine (Touriño *et al*., 2011; Touriño *et al*., 2009). Once absorbed, these metabolites show relevant biological effects (Giménez-Bastida *et al.,* 2012; Sala *et al*., 2015; Singh *et al*., 2019; di Gesso *et al*., 2015; Krga *et al*., 2016; Lee *et al*., 2017; González de Llano *et al*., 2019; Wang *et al*., 2018; Pourová *et al*., 2018; Houghton *et al*., 2018; Waldecker *et al*., 2008; Russell *et al*., 2008; Esteban-Fernández *et al*., 2017; Dal-Pan *et al*., 2017; Verzelloni *et al*., 2011). As explained, the chemical nature of these metabolites and, therefore, their biological activities, will be similar to those derived from EPP, but it may be expected that they will be absorbed and transferred to the bloodstream at delayed periods as compared to EPP-derived metabolites.

Several studies have evaluated the biological effects of polyphenol metabolites, considering colonic transformations as well as hepatic conjugations. These modifications may either increase or decrease the biological activity of the parent compound (Lodi *et al*., 2009). In the specific case of conjugated metabolites, it must be considered that a deconjugation process before cell entry has been reported (Menendez *et al*., 2011), so it might happen that these compounds ultimately exert their potential biological effects as de-conjugated structures (Galindo *et al*., 2012). A summary of the biological activities reported for microbial phenolic metabolites is shown in **Table 2.3**. Briefly, these studies have reported anti-inflammatory, anti-cancer, antiglycative, neuroprotective, inhibition of lipid synthesis or insulin modulating activities, by a combination of mechanisms of action; of course, these effects should be further confirmed in *in vivo* studies. Besides, these effects may be relevant in different target tissues but also locally, since some of them are beneficial for the colonic environment; for instance, urolithin A has shown to enhance gut barrier integrity (Singh *et al*., 2019). Since these studies were mostly developed in a context of evaluating mechanistic aspects, they were performed in *in vitro* models; nevertheless, it is important that these models reflect the actual concentrations of phenolic metabolites circulating in the body after absorption (Kroon *et al*., 2004; Álvarez-Cilleros *et al*., 2018). Interestingly, as

shown in **Table 2.3**, some *in vivo* studies have found significant associations between the circulating levels of some of these metabolites and certain health outcomes.

It should also be considered when evaluating the biological activities of NEPP, how their interactions with food matrix take place. For instance, it was recently shown that NEPA linked to the cell wall by non-covalent bonds showed an anti-inflammatory activity nearly equivalent to extractable proanthocyanidins, while this activity was reduced in NEPA associated with cell wall by covalent linkages (Le Bourvellec *et al*., 2019).

A final aspect to be considered is that, although the circulating phenolic metabolites are the compounds ultimately responsible for the action of either EPP or NEPP, they exert their activities in combination with the other mechanisms of action of these compounds, including modulation of dysbiosis. Thus, equol (a colonic metabolite of isoflavones) supplementation to non-equol producers did not originate the same health effects as those observed when the subjects were able to produce this metabolite; similarly, several discrepancies were found between the *in vitro* biological activities of certain ellagitannin metabolites and the health status of subjects with the highest production of those metabolites (Tomas-Barberan *et al*., 2018). Therefore, these metabolites should be considered in the overall context of the combination of mechanisms of action that finally give place to the biological effects of NEPP.

2.4.4. Synergy with dietary fibre

While the previously described mechanisms of action of NEPP are mostly shared with extractable polyphenols, there is a specific effect derived from the close association between NEPP and dietary fibre, i.e., the synergy that may take place between both food constituents.

When describing NEPP metabolic fate, it was explained that the presence of dietary fibre might stimulate the formation of microbial-derived metabolites from NEPP. Indeed, it has been suggested that an additional physiological effect of dietary fibre would be to behave as a carrier of phenolic compounds until the short intestine, allowing such transformations to take place (Saura-Calixto, 2011).

It may be expected that this interaction between dietary fibre and phenolic compounds does not only affect the generation of phenolic metabolites, already explained, but also that of short-chain fatty acids from dietary fibre. Regarding this aspect, some studies suggested that NEPP may stimulate the fermentation of dietary fibre. Thus, an improvement in the total production of SCFAs was observed after the *in vitro* incubation of chlorogenic acid, rutin, caffeic acid, quercetin, and olive oil polyphenols (Parkar *et al*., 2013; Zampa *et al*., 2006). A similar tendency was observed when rats were supplemented with pectin and freeze-dried apple or only with pectin (Aprikian *et al*., 2003). And when several flavonoids were added to maize cell walls artificially lignified, forming new linkages with the cell wall, this led to an increase in the fermentability of the cell wall (Grabber *et al*., 2012).

Moreover, a recent study found that proanthocyanidins not bound to cell wall produced, after an *in vitro* fermentation, less short-chain fatty acids, particularly butyrate, that those associated with the cell wall either by covalent or non-covalent linkages (and, at the same time, led to a higher production of phenolic microbial-derived metabolites, showing the complex interactions with the microbiota) (Le Bourvellec *et al*., 2019)¡**Error! Marcador no definido.**. In contrast, other studies found that the presence of proanthocyanidins inhibited the formation of short-chain fatty acids (Bazzocco *et al*., 2008) or that the co-occurrence of tea polyphenols and β-glucans did not stimulate the microbial transformation of soluble dietary fibre (Jalil *et al*., 2019). But, at the same time, several *in vivo* studies have found synergystic effects between polyphenols and dietary fibre regarding cardiometabolic markers (Vitaglione *et al*., 2015); this would imply that there was no inhibition of the fermentation of dietary fibre since its colonic metabolites are the main agents in the cardiometabolic effects of dietary fibre.

These divergences show that the potential interactions between NEPP and dietary fibre should be much more explored, considering the different proportions of both substrates that may be found in a real situation. In this sense, it should be indicated that many *in vitro* studies on colonic transformations studied separately either phenolic compounds or dietary fibre, i.e., providing a single source of carbon; however, this situation is quite far from the one after consuming foods, which makes that these results may not be completely translated to real situations (Edwards *et al*., 2017). Thus, the simultaneous effects that NEPP and dietary fibre have on microbiota should be

evaluated, considering not only its profile but also its activity, since polyphenols may also affect the activity of bacterial enzymes (Kosmala *et al*., 2014). Additionally, it would be interesting to determine not only the total formation of short chain fatty acids in the presence of NEPP but also the section of the short intestine where they are formed; for instance, it was hypothesized that a potential retardation of this fermentation in the proximal colon by the action of polyphenols, with a higher production in the distal colon, would avoid the action of proteolytic bacterial species present in that section of the colon and associated with harmful metabolites (Tuohy *et al*., 2012). Finally, and showing the complexity of this matter, although several beneficial effects of short-chain fatty acids have been reported, an excess of them may increase caloric intake; therefore, there is a narrow equilibrium between a deficit and an excess of these compounds (Mosele *et al*., 2015). In this sense, the concept of hierarchy in the preference of gut bacterial by one or another substrate (Hamaker and Tuncil, 2014) becomes relevant, since depending on the overall food intake the same bacterial species might show a higher preference for the transformation of dietary fibre or of polyphenols, particularly when they are associated in a single complex, as it is the case of NEPP.

2.5. Studies on the health effects of NEPP

2.5.1. Local vs. systemic effects

Based on the metabolic fate and mechanisms of action of NEPP previously reported, their potential health effects will take place mainly in two different ways (Pérez-Jiménez *et al*., 2013), summarized in **Figure 2.4**:

a) Local effects in the digestive tract. These effects may be due either to the completely intact NEPP structures (since, for instance, they exhibit antioxidant capacity) or to the metabolites produced after the action of colonic microbiota. While intact NEPP may exert an effect throughout the whole digestive tube, the derived metabolites will only be able to exert an effect in the colon. Nevertheless, most studies, in this sense have focused on colon health.

b) Systemic effects after absorption. After NEPP transformation, a fraction of the generated metabolites is absorbed and transferred into the bloodstream. These metabolites may originate several systemic effects during their circulation through the body (and eventually enter into the cell or accumulate into target tissues). The studies, in this sense have focused on cardiometabolic makers.

Current evidence on both ways of action, based on preclinical and clinical studies, will be summarized in the following headings. It should be indicated that, due to the specific characteristics of NEPP regarding their close association with dietary fibre, it is quite difficult to perform studies with NEPP isolates. Indeed, in the case of HPP, if they were isolated, they would lose their specific macromolecular characteristics, i.e., their association with dietary fibre. For this reason, most studies are performed with NEPP-rich products, but which also present an important proportion of dietary fibre.

Besides, although the scope of this chapter is circumscribed to the relevance of NEPP for human health, it should not be disregarded that some studies have also reported a promising role for NEPP in animal nutrition. Thus, from the traditional view of polyphenols as compounds that should be removed from animal studies, some experiments performed during the last years have found that supplementing chicken broilers with grape pomace (where most polyphenols are present as NEPP) have beneficial effects in intestinal microbiota and gut morphology -among other parameters- without compromising growth and performance (Viveros *et al*., 2011; Brenes *et al*., 2016) and significantly increasing the levels of circulating polyphenol-derived metabolites (Muñoz-González *et al*., 2019). Therefore, this is a complementary aspect of the biological relevance of NEPP.

2.5.2. Effects on gastrointestinal health

Once foods are consumed, they will stay in contact with the digestive tubes for hours. As explained, while a fraction of EPP may be absorbed in the small intestine, almost all NEPP will reach the colon unaltered. This means that the original structures will be available to exert, for hours after intake, radical-scavenging capacities. In this way, several *in vitro* digestion have shown that NEPP contribute to antioxidant capacity in the different stages of the digestive system (Serrano *et al*., 2007; Saura-Calixto *et al*.,

2007; Fogliano *et al*., 2011; Solari-Godiño *et al*., 2017). Besides this effect, NEPP may also exert inhibitory activities on certain digestive tube enzymes. This effect has been widely studied during last years for dietary polyphenols, but, to the best of our knowledge, only one study specifically explored it in a NEPP concentrate (Yan *et al*., 2018).

But, regarding gastrointestinal health, the main target for NEPP is the colon, where they will remain for longer periods. In this context, it is worthy of highlighting that an epidemiological study exploring the association between (extractable) proanthocyanidin intake and cancer colon risk observed that the highest decrease in cancer risk was found for polymeric structures with more than ten units (Rossi *et al*., 2010). This seems logical since they will arrive in a higher proportion to the colon and will stay longer; although this study did not include NEPA, it should not be disregarded that they are also high molecular weight proanthocyanidins reaching the colon in an intact state.

Studies on the effects of NEPP in the colon have mostly used grape-derived products, e.g., grape pomace or GADF, where NEPP constitute the major fraction of phenolic compounds reaching the colon after the intake of these products. One of the most explored aspects was their effect on colonic oxidative status; this is a relevant outcome since, for instance, it has been reported that colonic cancer patients exhibit impaired colon oxidative status as compared to healthy subjects (Chang *et al*., 2008). Thus, supplementation to rats with grape seeds significantly improved the caecal antioxidant capacity as compared with the control group, with NEPP being responsible for the 50% of the antioxidant capacity found in these samples (Goñi and Serrano, 2005). In the same way, supplementation to healthy rats with GADF significantly decreased the levels of lipid oxidation and increased the endogenous antioxidant defenses (López-Oliva *et al*., 2010; López-Oliva *et al*., 2013). Additionally, the previously explained effects of NEPP on colonic microbiota are also another way to improve gastrointestinal health.

But the reported local effects of NEPP go further, and they have been directly related to the modulation of certain diseases. Supplementation with GADF to healthy mice significantly modified several biochemical routes in the colonic mucosal in a beneficial way, as measured by gene expression analysis: it up-regulated genes involved

in the metabolism of xenobiotics compounds, regulation of plasma glucose and tumoral suppression, while it down-regulated genes involved in inflammation, lipid biosynthesis as well as proto-oncogenes (Lizarraga *et al*., 2011).

But the most relevant result of the effect of NEPP in gastrointestinal health was observed in a study with APCMin/+ (the validated animal model for the study of colon cancer). The authors reported a significant decrease (higher than 50%) in total polyps after GADF supplementation as compared to a control group (Sánchez-Tena *et al*., 2013). Additionally, another animal study explored other potential mechanisms of action of NEPP in the modulation of gastrointestinal health and particularly cancer, reporting a reduction of cleaved caspase-3 and a minor release of cytochrome c from mitochondria to the cytosol (López-Oliva *et al*., 2013). Overall, it may be expected that the action of NEPP in the colon, together with other agents such as non-absorbed EPP and dietary fibre contributes to providing a flow of substrates needed to downregulate cancer pathways (Ricciardiello *et al*., 2011).

Additionally, it may happen that NEPP contribute to the effects observed in some studies providing whole materials rich in both EPP and NEPP, although the study did not specifically measure NEPP content. For instance, a recent animal experiment found that incorporating the red seaweed *Pyropia columbina* to the diet of Wistar rats improved the endogenous antioxidant system in the colon as well as the intestinal mucosal barrier function (Cian *et al*., 2018). Since edible seaweeds have been reported to show relevant amounts of NEPP (Sanz-Pintos *et al*., 2017) and, based on the results with NEPP-rich materials, it may be hypothesized that these constituents contributed to the observed effects.

2.5.3. Effects on cardiometabolic health

Although research on the effects of NEPP on cardiometabolic regulation is still scarce, the results have been rather promising. Again, grape products and, particularly, grape pomace and GADF have been the most studied products for the above reasons. Thus, supplementation with GADF (7.5 g/day) to normo- and hyper-cholesterolemic subjects for 16 weeks did not have any effect on the lipid profile of normocholesterolemic subjects (neither in the control group), but it caused a significant

decrease in total cholesterol, LD-cholesterol, and triglycerides in the hypercholesterolemic subjects. Moreover, a significant decrease in blood pressure was observed in the supplemented group as a whole (Pérez-Jiménez and Saura-Calixto, 2008).

Interestingly, the authors reported that the observed effects were higher than those previously reported separately for dietary fibre or polyphenols, suggesting a synergy among all the beneficial compounds of the product (both EPP and NEPP in the case of phenolic compounds). Indeed, some authors have highlighted that certain physiological effects attributed to insoluble dietary fibre may be partially due to associated phenolic compounds (Macho-González *et al*., 2017; Macho-González *et al*., 2018). A potential mechanism for the hypocholesterolemic effect of NEPP is their bile acid-binding capacity. This effect, widely known for dietary fibre, was reported specifically for NEPP in an *in vitro* experiment where the alcohol-insoluble solids (including removal of EPP) were prepared from different fruits. Interestingly, although cellulose, pectin, or lignin alone also provided this effect, it was much higher when NEPP were also present (Hamauzu and Mizuno, 2011).

Some clinical studies with grape pomace have been published in a few years. Thus, a supplementation to males with at least one factor of metabolic syndrome with 20 g/day of grape pomace as an ingredient in different food products significantly decreased systolic and diastolic blood pressure, being the antihypertensive effect related to the role of polyphenols in the activation of endothelial nitric oxide synthase (eNOS) (Urquiaga *et al*., 2015). But the most interesting aspect of this study was a significant decrease in plasma fasting glucose, which agrees with a study in healthy rats where one of the metabolic pathways observed to be modified in colonic mucosal was that of gene G6PC2, related to the maintenance of plasma blood glucose as well as with the inhibitory effects of NEPP on digestive enzymes (Yan et a., 2018). Therefore, there may be connections between the local effects in colon and the systemic outcomes. Moreover, another recent study also reported the potential of grape pomace in the regulation of carbohydrate metabolism, since a significant decrease in fasting plasma insulin and an improvement in insulin sensitivity indexes was observed after supplementing subjects with at least two factors of Metabolic Syndrome with grape pomace (8 g/day) for six weeks (Martínez-Maqueda *et al*., 2018).

Nevertheless, due to the differences in subject characteristics, study design, products doses, etc., these results should be confirmed in more clinical trials. At the same time, to better understand the potential systemic effects of NEPP, these clinical trials should be concomitant with preclinical studies that may provide very useful information, such as the potential accumulation in target tissues of NEPP metabolites after their intake. For instance, the accumulation of metabolites in brown adipose tissue after supplementation with extractable proanthocyanidins has been reported (Serra *et al*., 2013); similar research on NEPP would help to better understand the potential biological effects of these compounds.

All the studies mentioned were mostly focused on the role of NEPA. But HPP may also play an important role in cardiometabolic regulation. Indeed, the widely known beneficial effects of whole-grain cereals are due to their high dietary fibre content as compared to that of refined cereals, but it should not be disregarded that they also contain a very important fraction of HPP associated with dietary fibre. Indeed, a study in obese subjects supplemented with whole-grain wheat where a significant decrease in several inflammation markers was observed, also reported a significant association between modifications in gut microbiota populations and the circulating levels of ferulic acid- mostly present in wholegrain wheat as HPP (Vitaglione *et al*., 2014).

2.6. Perspectives

This chapter has summarized current knowledge on NEPP or macromolecular antioxidants. As has been shown, the interest in this fraction of dietary phytochemicals has slowly increased during the last two decades, and at the moment there is some level of evidence that provides certain knowledge of NEPP: they are relevant contributors to total polyphenol content, in many cases equivalent to EPP; in consequence, they also present a high contribution of total daily polyphenol intake; they are at least partially bioavailable after intake, with specific features as compared to the metabolic fate of EPP (longer circulation times of derived metabolites, interactions with dietary fibre); they may exhibit either local effects through the digestive tube or systemic effects after absorption of derived metabolites, with several health implications.

Nevertheless, at the same time, the information of NEPP is still limited in many aspects, with some important gaps that need to be completed, as this chapter also tried to show. Thus, standard methodologies that allowed the analysis of NEPP and, if possible, without affecting the original structures in food samples, should try to be adopted. Moreover, maybe two parallel methodologies should be considered: one for those laboratories not completely familiar with the topic but which may run some of these analyses in a routine way, and another one for a much more detailed comprehensive characterization. Indeed, increasing available data on a wide number of food items is a key aspect for improving the estimations performed up to this moment on NEPP intake in different populations. This kind of estimations should be extended to more countries or populations, as the first step to finally establish potential associations with health outcomes. At the same time, studies on biological aspects of NEPP (including both metabolic fate and *in vivo* effects) are essential to finally understand the potential relevance of these compounds. For this, clinical trials should be chosen whenever possible to obtain results that may be translated into common clinical/nutritional practice. In this context, it is especially relevant to keep in mind that nutritional doses, i.e., doses that may be achieved through a common diet, are tested. In this sense, the role of NEPP as food constituents and not as drugs should never be disregarded; they, as EPPP, will not have drastic effects in the measured markers or by a specific mechanistic action, but it will be the combination of different molecules and though life time, which may ultimately have a health effect, as suggested for EPP (de Pascual-Teresa and Clifford, 2017).

Finally, it should not be disregarded that, since foods are consumed as a whole, and due to the strong association between NEPP and dietary fibre, both food constituents will actually behave as a whole complex, and their isolated effects can be only partially studied. So this complex as a whole will be ultimately the responsible of the potential observed health effects and even in combination with other constituents associated with it in certain food products, such as melanoidins in maillardized dietary fibres, or the recently reported insoluble methylxanthines in coca products (Mudenuti *et al*., 2018). In the same way, since plant foods are not rich either only in EPP or NEPP, a whole approach to total food polyphenols should be considered, because both classes of phenolic compounds, with specific characteristics, may finally behave together

improving the overall health status of a subject; moreover, studies in model systems have reported synergies between both classes of phenolic compounds (Çelik *et al*., 2015). Thus, in the same way of the suggested concept of "the three Ps" (Marchesi *et al*., 2016) (probiotics, prebiotics, and polyphenols) for microbiota regulation, where mutual interactions take place among them, the same perspective should be used for approaching total dietary polyphenols, as the combination and interaction between EPP and NEPP.

Figure captions

Figure 2.1. Contribution of non-extractable polyphenols to total polyphenol content in different common fruits and vegetables. EPP, extractable polyphenols; HPP, hydrolysable polyphenols; NEPA, non-extractable proanthocyanidins.

Figure 2.2. Metabolic fate of non-extractable polyphenols (NEPP). NEPP, non-extractable polyphenols; HPP, hydrolysable polyphenols; NEPA, non-extractable proanthocyanidins.

Figure 2.3. Percentage of daily antioxidant capacity per capita intake in the Spanish diet by FRAP assay (μmolTrolox), expressed per food group. FRAP, ferric-reducing antioxidant power assay

Figure 2.4. Local and systemic effects of NEPP. HPP, hydrolysable polyphenols; NEPA, non-extractable proanthocyanidins.

Table 2.1. Strategies for the extraction of extractable and non-extractable polyphenols

Sample	Pretreatment of sample	Procedure for EPP removal	Procedure for NEPP release	Reference
Apples (Golden delicious), peach (Royal) and nectarine (Royal)	Freeze-dried and milled to a particle size of <0.5 mm of edible part from each fruit	Extraction of EPP and EPA with methanol/water (50:50, v/v; pH 2) acidified with 2 N HCl and acetone/water (70:30, v/v)	Release of HPP with methanol/H2SO4 90:10 (v/v), 85°C for 20 h. Release of NEPA with 10 mL of butanol/HCl (97.5:2.5 v/v) and 0.7 g of FeCl3 at 100°C for 1 h.	Arranz *et al*., 2009.
Apple (Golden delicious), Banana (Cavendish) Cooked pinto beans and Red grape pomace (Cencibel)	Freeze-dried and milled to a particle size of <0.5 mm of edible part from each fruit	Extraction of EPP with methanol/water (50:50, v/v; pH 2) and acetone/water (70:30, v/v).	Release of NEPA with HCl/butanol (5:95, v/v) containing 0.7 g of FeCl3/l at 100°C for 1 h.	Zurita *et al*., 2012.
Mixtures of five types of plant foods: cereals, vegetables, legumes, nuts, and fruits.	Freeze dried and milled to a particle size of 0.5 mm	Extraction of EPP with methanol/water (50:50 v/v at pH 2) followed by acetone/water extraction (70:30, v/v).	Release of HPP with methanol/H_2SO_4 90:10 v/v, 85 °C during 20 h. Release of NEPA with butanol/HCl 97.5:2.5 v/v, 100 °C during 60 min.	Arranz *et al*., 2010.
Pomegranate peel	Freeze-dried	Extraction of EPP with Methanol/DMSO/H2O	Release of ellagitannins with 4 M HCl in water at 90 °C for 24 h followed by	García-Villalba *et*

		(40:40:20, v/v/v) containing 0.1% HCl	extraction with dimethyl sulfoxide/methanol (50:50, v/v)	*al*., 2015.
Cranberry beans (*Phaseolus vulgaris* L.)	Milled	Extraction of EPP with 70% Methanol + 1% HCl (v/v) + sonication for 15 min, rotational mixing at room temperature for 2 h	Release of HPP with 2 N NaOH followed by sonication for 15 min, rotational mixing at room temperature for 16 h	Chen *et al*., 2015.
Rice (*Oryza sativa* L.)	Milled	Extraction of EPP with 80% methanol and hexane	Release of HPP with 4 M NaOH acidified with 6 M HCl at room temperature for 4 h and release with ethyl acetate	de Mira *et al*., 2009.
Winemaking by-products	Freeze dried and defatted with hexane	Extraction of EPP with 70% (v/v) acetone at 30 °C for 20 min	Release of HPP with Viscozyme solution (2% in 0.1 M phosphate buffer, pH 4) at 37 °C for 12 h or pronase solution (1 mg/mL in 0.1 M phosphate buffer, pH 8) and stirred for 1 h	de Camargo *et al*., 2016.

Table 2.2. Contribution total intake of EPP and NEPA from Spanish diet*.

Food group		EPP		NEPP		Total
	Consumption[a]	Content[b]	Intake[c]	Content[b]	Intake[c]	Intake[c]
Fruits	192.8	58.4	110.4	168.8	439	549.5
Vegetables	230.1	32.1	73.6	53.4	122.9	196.5
Cereals	139.8	43.8	61.1	171.1	291.7	352.8
Nuts	4.3	125.6	6.8	314.4	28.8	35.3
Legumes	12.1	51.7	6.2	497.7	66.3	72.5
Total	579.1	311.6	258.1	1205.4	948.7	1206.6

EPP, extractable polyphenols; NEPP, non-extractable polyphenols; fw, fresh weight.

[a]g fresh edible portion/day/person

[b] mg/100g fw

[c]mg/day/person

* Data from Arranz *et al*., 2010.

Table 2.3. Summary of biological activities reported for phenolic microbial metabolites

Compound	Effect	Model	Reference
Gluc-Urolithin A	Inhibition of monocytes adhesion and endothelial cell migration	HAEC stimulated with TNF-α	Giménez-Bastida *et al.,* 2012
Urolithin B, Gluc-urolithin B	Reduction of MCP-1, fractalkine and VEGF expresion	Ventricular cardiomyocytes from neonatal rats exposed to high glucose concentration	Sala *et al.,* 2015
Urolithin A and its analogue	Enhancement of gut barrier integrity and decreased systemic inflammation	C57BL/6, AhR−/−, Nrf2−/− mice and human colon epithelial carcinoma cell lines, HT29 Mouse bone marrow, derived macrophages	Singh *et al*., 2019
4-hydroxybenzoic acid	Reduction of TNF-α concentration and IL-1β	THP-1 stimulated with LPS	di Gesso *et al*., 2015
Isovanillic acid, Gluc-isovanillic acid, Gluc-vanillic acid, Sulf-protocatechuic acid-3 and Sulf-benzoic acid	Reduction of TNF-α secretion	THP-1 stimulated with LPS	di Gesso *et al*., 2015
Protocatechuic, vanillic, ferulic and hippuric acid	Reduction of the adhesion of monocytes to endothelial cells	THP-1 human monocytic cells to HUVEC stimulated with TNF-α	Krga *et al*., 2016
5-(3′,4′-Dihydroxyphenyl-γ-valerolactone)	Reduction of the adhesion of monocytes to endothelial cells	THP-1 human monocytic cells to HUVEC stimulated with TNF-α	Lee *et al*., 2017

Hippuric acid, α-hydroxyhippuric acid and Sulf-3-(3,4-dihydroxyphenyl)propionic acid	Decrease of adhesive activity against uropathogenic bacteria	Uropathogenic bacteria (E. coli ATCC 53503, E. coli DSM 10791, and E. faecalis 04-1) and T24 bladder cells (ATCC HTB4)	González de Llano *et al*., 2019
Dihydrocaffeic	Decrease of oxidative stress	HepG2 and human vascular endothelium cells (EA.hy926) stimulated with TNF-α	Wang *et al*., 2018
Dihydroferulic acid	Decrease of ENOS concentrations	HepG2 and human vascular endothelium cells (EA. hy926) stimulated with TNF-α	Wang *et al*., 2018
3,4-dihydroxyphenylacetic acid and 4-methylcatechol	Vasorelaxant effects on mesenteric artery Hypotensive effect	Isolated rat thoracic aorta and mesenteric artery Normotensive Wistar: Han rats and spontaneously hypertensive rats	Pourová *et al*., 2018
Sulf-isovanillic acid	Stimulation of glucose transport	Human skeletal muscle myoblast line, LHCN-M2,	Houghton *et al*., 2018
p-coumaric acid, 3-(4-OH-phenyl)-propionate and caffeic acid	Inhibition of HDAC activity	HT-29 human colon carcinoma cells	Waldecker *et al*., 2008
3-(3,4-dihydroxyphenyl) propionic acid, 3-(4-hydroxy-3-methoxyphenyl) propionic acid and 3-(3-hydroxyphenyl) propionic acid	Inhibition of prostanoid production	Fibroblast (CCD-Co18) cells stimulated with IL-1β	Russell *et al*., 2008

3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic acids and Gluc-salicylic acid	Protection against neuronal death	SH-SY5Y neuroblastoma cells induced with SIN-1	Esteban-Fernández *et al*., 2017
Dihydroxyphenyl valerolactone	Prevention of memory impairment	NonTg mice	Dal-Pan *et al*., 2017
Urolithin A and B, 3-methoxy-4-hydroxyphenylaceticacid, pyrogallol, dihydrocaffeic acid, feruloylglycine, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid	Reduction of AGEs formation Protection of oxidative stress	Albumin glycation *in vitro* Human neuroblastoma SK-N-MC clonal cell line	Verzelloni *et al*., 2011

Gluc, glucuronide; Sulf, sufate; HAEC, Human aortic endothelial cells; AGE, advanced glycation end products; TNF-α, Tumor necrosis factor alpha; HUVEC, Human umbilical vein endothelial cell; LPS, lipopolysaccharide; AGEs, glycation end products; HDAC, histone deacetylase

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