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Macromolecular antioxidants or non-extractable polyphenols in fruit and vegetables: Intake in four European countries

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ABSTRACT

Macromolecular antioxidants or non-extractable polyphenols are an emerging and ignored fraction of dietary antioxidants that present differential structural and physiological features as compared with the well-known extractable polyphenols. In this study, the macromolecular antioxidant content and profile were determined by the first time for the 24 most consumed fruit and vegetables in four European countries (France, Germany, The Netherlands and Spain). Results showed that macromolecular antioxidants, made up of hydolysable polyphenols and polymeric proanthocyanidins, are major contributors (mean value 57%) to the total polyphenol content of fruit and vegetables. Macromolecular antioxidant intake from fruit and vegetable determined in the four countries selected was about 200 mg. Spain had the highest daily per capita macromolecular antioxidant intake from fruit, while The Netherlands had the highest intake derived from vegetables. Future studies of plant food macromolecular antioxidant analysis and of dietary intakes in different populations are needed to elucidate the contribution of these macromolecular antioxidants to the health effects associated with fruit and vegetable consumption, and with dietary antioxidant intake.

1. Introduction

A clear association between the consumption of fruit and vegetables (F&V) and the prevention of several chronic diseases has been established over recent decades, based on many clinical and observational studies. In this way, an increased consumption of F&V has been related, with different levels of evidence, to reductions in the risk of CVD, certain kinds of cancer and some cognitive diseases (Estruch et al., 2013; Pavia, Pileggi, Nobile, & Angelillo, 2006; Pitsavos et al., 2005; Polidori et al., 2009). Amongst the different constituents responsible for these beneficial effects, phytochemicals and in particular polyphenols are considered to play an important role.

F&V polyphenols are a family of hundreds of antioxidant compounds made up of different subclasses (flavonoids, phenolic acids, stilbenes, lignans and others), for which several health effects related to the prevention of different chronic diseases have been widely reported (Del Rio et al., 2013; González et al., 2011). However, most studies of polyphenols are based on underestimated polyphenol contents, because they include only those polyphenols that are found in the supernatants derived from aqueous-organic extractions from foods, i.e., extractable polyphenols (EPP), which are considered in those studies to be total polyphenols. Nevertheless, it has been reported that a significant fraction of polyphenols remains in the residues of the polyphenol extractions, the so-called non-extractable polyphenols (NEPP) or macromolecular antioxidants (MACAN) (Arranz, Saura-Calixto, Shaha, & Kroon, 2009; Bravo, Abia, & Saura-Calixto, 1994; Hellström, Törrönen, & Mattila, 2009). They are named MACAN due to some specific differential features they have: they are either polymeric polyphenols or single polyphenols linked to macromolecular food constituents; they are not extracted by common aqueous-organic procedures; they are accessible and bioavailable only in the large intestine. In contrast, soluble polyphenols or EPP are mainly low molecular weight structures, soluble in aqueous-organic mixtures and they are potentially bioavailable in the small intestine (Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013; Saura-Calixto, 2012). Both classes present significant antioxidant capacity.

MACAN can mostly be divided into two fractions: hydrolysable polyphenols (HPP), which are low molecular weight phenolic compounds strongly associated with polysaccharides or proteins; and non-extractable proanthocyanidins (NEPA), which are high molecular weight structures. Despite MACAN having been commonly ignored in chemical analysis, when plant foods are consumed both EPP and MACAN are ingested. This implies that the reported health effects of dietary polyphenols may actually be due, at least partially, to MACAN. Indeed, they reach the colon intact where they yield

Abbreviations: *AAPH*, 2,2′-azobi(2-amidinopropane) dihydrochloride; *ABTS*, 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulphonic acid); *EPP*, extractable polyphenols; *FRAP*, ferric reducing/antioxidant power; F&V, fruit and vegetables; *GAE*, gallic acid equivalents; *HPP*, hydrolysable polyphenols; MACAN, macromolecular antioxidants; *NEPA*, non-extractable proanthocyanidins; *NEPP*, non-extractable polyphenols; *ORAC*, oxygen radical absorbance capacity; *TPTZ*, 2,4,6-tris(2-pyridyl)-*s*-triazine.

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different bioavailable metabolites through the action of the microbiota (Choy, Jaggers, Oteiza, & Waterhouse, 2013; Mateos-Martín, Pérez-Jiménez, Fuguet, & Torres, 2012). Although studies of the health effects of NEPP are scarce, they have shown promising results in relation to gastrointestinal health (including colorectal cancer) and CVD (Pérez-Jiménez et al., 2013).

There are still very few data available on MACAN content in specific individual plant foods; most of what there is corresponds to cereals (Chandrasekara & Shahidi, 2010; Holtekjolen, Kinitz, & Knutsen, 2006; Pérez-Jiménez & Torres, 2011). Similarly, data on dietary intake of MACAN are also limited to a few studies (Arranz, Silván, & Saura-Calixto, 2010; Hervert-Hernández, García, Rosado, & Goñi, 2011; Saura-Calixto, Serrano, & Goñi, 2007) and they are based on MACAN content data that correspond to pools for different food groups, not to individual foods. Thus, they do not allow us to obtain information on the contribution of the different foods to MACAN intake.

The aim of this work is to determine MACAN content (including both HPP and NEPA) in the most consumed F&V in different European countries, in order to estimate MACAN intake from F&V intake. Additionally, the antioxidant capacity associated with NEPP was evaluated by ORAC assay (oxygen-radical absorbance capacity), as a parameter related to potential health-related properties of MACAN.

2. Materials & methods

2.1. Chemical reagents

The Folin–Ciocalteu reagent was from Panreac, Castellar del Vallés, Barcelona, Spain. AAPH (2,2′-azobi(2-amidinopropane)dihydrochlorid), fluorescein (3,6′-dihydroxy-spiro[isobenzofuran-1 [3H], 9′[9H]-xanthen]-3-one), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, rutin, vanillic acid, ferulic acid and naringenin were from Sigma-Aldrich Química, S.A., Madrid, Spain. All the reagents used were of analytical grade.

Condensed tannin concentrate from Mediterranean carob pods (*Ceratonia siliqua* L) was supplied by Nestlé Ltd. (Vers-chez-les Blancs, Switzerland).

2.1. Sample selection

Four European countries (Spain, France, Germany and The Netherlands) were selected as representative of the different European diets. For each of these countries, consumption data for individual F&V in each of the two categories were retrieved. Since it was not possible to find a common source of food consumption data by food item for all the European countries, different recent sources were used. For Spain, the data was from household expenditure surveys, covering 12,000 households (Ministerio de Agricultura, 2013). For The Netherlands, data from food questionnaires were used, based on 3000 households for fruit (Kistemarkers, 1998) and 2000 subjects for vegetables (Voorrips et al., 2000). For Germany, data on total consumption of F&V came from a national nutrition food survey based on food questionnaires given to 20,000 subjects (MRI, 2008); while data for individual food items were retrieved from official agricultural statistics (BMELV, 2012; ZMP, 2006). For F&V consumption in France, since detailed data both from household expenditure surveys (2000 households) and food questionnaires (5000 subjects) were available (Brat et al., 2006), a mean value was obtained, in order to balance the pros and cons of each method. The strategy we adopted to select the individual samples in each case was to take the consumption data per individual item in decreasing order until at least 75% of the total consumption was covered; calculations were based on edible fractions. Potato was not included in the vegetable group, due to its specific composition which is more closely related to that of cereals, as reported in previous studies (Tennant, Davidson, & Day, 2014).

Samples were acquired in local supermarkets as fresh products with variety properly indicated, except for red beetroot, which was canned (El Corte Inglés S.A., Madrid, Spain) and Brussels sprouts, which were frozen (Findus España S.L.U., Tajona, Navarra, Spain)—although small differences in polyphenol content may appear in processed vegetables they would mostly affect to EPP and not to NEPP, which are more stable due to their macromolecular structure. Similarly, although some differences may be found in polyphenol content depending on the variety, those selected here were the most representative ones. Varieties are listed as Supplementary material (Table S1) together with their moisture content.

2.2. Preparation and analysis of polyphenol fractions

After freeze-drying and milling the edible parts of the samples to a particle size of less than 0.5 mm, polyphenol fractions were obtained as previously described (Arranz et al., 2009).

2.2.1. Extractable polyphenols

Briefly, 0.5 g of sample was placed in a capped centrifuge tube, 20 mL of acidic methanol/water/HCl (50:50, v/v; pH 2) was added and the tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500 *g* in a Thermo Heraeus Megafuge 11 (Thermo Fisher, Waltham, MA, USA) for 10 min and the supernatant was recovered. Twenty millilitres of acetone/water (70:30, v/v) was added to the residue, and the shaking and centrifugation were repeated. The methanolic and acetonic extracts were combined, corresponding to EPP solutions.

EPP content was determined in these solutions according to the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1998). A test sample (0.5 mL) was mixed with 1 mL of Folin–Ciocalteu reagent and swirled. After 3 min, 10 mL of sodium carbonate solution (75 g/L) was added and mixed. Additional distilled water was mixed in thoroughly by inverting the tubes several times. After 1 h, the absorbance at 750 nm was recorded. The results were expressed as mg/100 g gallic acid equivalents (GAE).

2.2.2. Non-extractable polyphenols or MACAN

Independent residues from EPP extraction were subjected to different treatments in order to obtain HPP and NEPA solutions. Total MACAN content corresponded either to the sum of HPP plus NEPA contents, both obtained by spectrophotometry (Method 1), or to the sum of HPP content determined by HPLC (Method 2) plus the NEPA content.

2.2.2.1. Hydrolyzable polyphenols. The residues from EPP extractions were subjected to hydrolysis with methanol and sulphuric acid for 20 h at 85 °C (Hartzfeld, Forkner, Hunter, & Hagerman, 2002) the pH was later adjusted to 5.5. These hydrolysates were then subjected to SPE treatment with Oasis HLB cartridges (5400 mg, 3 cc, ref. 30 μm) from Waters (Milford, MA, USA) in order to remove salts that may have damaged the MS system: after activation with methanol (5 mL) and methanol 50% (5 mL), 1 mL of the sample was loaded and it was eluted successively with methanol (1 mL) and methanol 80% (1 mL). The eluates were combined and concentrated under nitrogen.

The HPP content was determined spectrophotometrically (Method 1) via the Folin–Ciocalteu assay, carried out as above for EPP; and also by HPLC-DAD (Method 2). For Method 2, an Agilent 1200 series LC (Santa Clara, CA, USA) was used. A 20 μL sample was separated in a Luna C18 (50 × 2.1 mm i.d.) 3.5-μm particle size column and a Phenomenex Securityguard C18 (4 × 3 mm i.d.) column (Torrance, CA, USA). Gradient elution was performed with a binary system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in acetonitrile. The following increasing linear gradient (v/v) of [B] was used (t, %B): (0, 6), (10, 23), (15, 50), (20, 50), (23, 100), (25, 100), (27, 8) and (30, 8), followed by a re-equilibration step. The flow was set at 0.4 mL/min. Signals were collected at the maximum wavelengths