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Bioaccessibility of phenolic compounds following *in vitro* large intestine fermentation of nuts for human consumption

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Sesamin (PubChem CID: 72307)
Matairesinol (PubChem CID: 119205)
2-Hydroxybenzoic acid (PubChem CID: 338)
Cyanidin 3-O-glucoside (PubChem CID: 12303203)
5-Pentadecylresorcinol (PubChem CID: 76617)
Tyrosol (PubChem CID: 10393)
4-Hydroxyphenylacetic acid (PubChem CID: 127)

ABSTRACT

A bioaccessibility study of polyphenols after *in vitro* simulated large intestine fermentation was carried out on edible nuts. Raw nuts were also analysed for total phenolic content and antioxidant potential, considering both bound and free phenolics. The highest phenolic content was found in walnuts, followed by pistachios extracts (596.9 and 410.1 mg gallic acid equivalents 100 g^{-1}, respectively). Consistently, the total antioxidant capacity was highest in walnuts (3689.7 μM trolox equivalents 100 g^{-1}) followed by peanuts and pistachios (3169.6 and 2990.1 μM trolox equivalents 100 g^{-1}, respectively). Data showed high correlations between total phenolics and both antioxidant activities. The metabolomics-based phenolic profile depicted during *in vitro* fermentation showed a degradation of higher-molecular-weight phenolics over 48 hours of faecal fermentation, with a concurrent increase in low-molecular-weight compounds (hydroxybenzoic and hydroxycinnamic acids, alkylphenols, and tyrosols). Our findings indicate that nuts deliver polyphenols into the colon, with bioaccessibility values not negligible for alkylphenols, tyrosols and phenolic acids.

1. Introduction

Edible tree nuts are nutrient dense foods widely consumed all over the world, not only for their taste and sensorial attributes, but also for health benefits (Alasalvar & Shahidi, 2008). In Western countries nuts are consumed as snacks, desserts or part of a meal, and are commonly eaten as whole (fresh or roasted), in spreads, as oils or included in several commercial products.

Indications suggested that a healthy diet supplemented with one daily serving of nuts (from 30 to about 40 g of mixed nuts) is associated with favourable plasma lipid profiles, reduced risk of coronary heart disease, certain types of cancer, atherosclerosis, type-2 diabetes, inflammation, and several other chronic diseases (Afshin, Micha, Khatibzadeh, & Mozaffarian, 2014; Ros, 2010; Grosso et al., 2015). Accordingly, as reviewed by Mukuddem-Petersen, Oosthuizen, and Jerling (2005), people who ate nuts 5 times/week experienced an ~50% reduction in risk of coronary heart disease compared with those eating nuts < 1 time/week. In addition, evidence from epidemiological studies and clinical trials suggested that their regular consumption neither contributes to obesity nor increases the risk of developing diabetes (Ros, 2015).

The mechanism for these beneficial effects is probably linked to the synergistic interaction of several bioactive constituents of nuts, which may favourably influence human physiology and health. In particular, nuts have a desirable nutritional composition, being low in sugars, rich in vegetable proteins, dietary fibre, vitamins (e.g., folic acid, niacin, vitamin B6, and vitamin E) minerals (e.g., calcium, potassium and magnesium) and fat-soluble bioactive compounds (e.g., unsaturated fatty acids, phytosterols, essential oils, and terpenoids). In addition, they are a good source of phytochemicals including phenolic compounds and phytates (Schlörmann et al., 2015; Venkatachalam & Sathe, 2006). However, some of them (e.g., antioxidants) may degrade or get

Abbreviations: ANOVA, analysis of variance; AAPH, 2,2′-Azobis(2-amidinopropane) dihydrochloride; DM, dry matter; DNA, deoxyribonucleic acid; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; MS, mass spectrometry; ORAC, oxygen radical absorbance capacity; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; rRNA, ribosomal ribonucleic acid; RT-PCR, real time polymerase chain reaction; TE, trolox equivalents; TPC, total phenolic content; TPTZ, tripyridyltriazine; UHPLC-ESI-QTOF-MS, ultra-high-pressure liquid chromatography electrospray ionization quadrupole-time-of-flight mass spectrometer; VIP, variable importance in projection

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lost after technological treatments (Chang, Alasalvar, Bolling, & Shahidi, 2016). This is because, even if walnuts can be consumed raw with their intact skins, most other nuts are usually roasted or subject to different technological treatments (stir-fried, oil-fried or boiled). The roasting process involves microstructural and chemical changes, such as the decrease in moisture content, lipid modifications, and changes in colour as well as the formation of compounds responsible for the typical roasted flavour, mainly due to Maillard reaction products (Alamprese, Ratti, & Rossi, 2009; Amaral, Casal, Seabra, & Oliveira, 2006).

Among phytochemicals, phenolic compounds are gaining a substantial interest. Phenolics can be considered the largest group of natural antioxidants in the diet and are reducing agents (Shahidi & Ambigaipalan, 2015). The diversity in their chemical structure determines the biological properties, such as bioavailability, antioxidant capacity, and specific interactions with cell receptors. These phytochemicals include flavonoids, tannins (hydrolysable and condensed), phenolic acids, stilbenes, lignans, and phenolic aldehydes. Besides their direct role as antioxidants, they can be covalently bound to indigestible components of the food matrix (mainly dietary fibre), thus failing to be absorbed into the small intestine of healthy individuals, and reaching the colon intact, where they become available for bacterial microflora thus promoting an antioxidant environment (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005; Quirós-Sauceda et al., 2014). Phenolic compounds are phytochemicals extensively metabolised after consumption; thus, the bioavailability of nut polyphenols should be considered when evaluating the potential health benefits of nuts and nut co-products. However, bioavailability is influenced by bioaccessibility, which is defined as the relative amount of nutrients or phytochemicals released from a complex food matrix in the lumen of the gastrointestinal tract, becoming available for absorption into the body (Kamiloglu, Pasli, Ozcelik, & Capanoglu, 2014; Mandalari et al., 2010). Even though simulated *in vitro* gastrointestinal digestion models cannot directly mimic *in vivo* conditions, these models could be helpful for investigating the effect of the food matrix on polyphenol bioaccessibility, in order to obtain essential data to support claims of the biological relevance of these nut compounds in the context of nutrition and human health (Chang, Alasalvar, & Shahidi, 2016).

In particular, when reaching the large intestine, microbial enzymes may release antioxidant compounds from complex macromolecules, thus enhancing their direct absorption rather than allowing further microbial metabolism, which may be responsible for the beneficial health effects of consuming antioxidant-rich food (Pérez-Jiménez, Serrano, Tabernero, & Arranz, 2009). Several authors have focussed on the potential application of *in vitro* models (gastro-intestinal digestion and colonic fermentation) in order to mimic human *in vivo* conditions (Chen et al., 2016; Juániz et al., 2017; Mosele, Macià, Romero, Motilva, & Rubió, 2015).

Regarding nuts, Mandalari et al. (2010) evaluated the prebiotic potential of almond skin by an *in vitro* digestion model followed by colonic fermentation using faecal bacterial cultures, showing that almond skin exhibited the potential to be used as a novel source of prebiotics, increasing the "beneficial" bacterial population into the colon. In addition, it has been demonstrated that polyphenols and other phytochemicals are bioaccessible in pistachios during simulated human gastric digestion and therefore available for absorption in the gastrointestinal tract (Mandalari et al., 2013).

On this basis, the current work aimed to profile the phenolic compounds, as well as assess the antioxidant capacity, of natural and roasted nuts largely consumed by humans, through *in vitro* large intestine fermentation. Peanuts, hazelnuts (peeled and unpeeled), pistachios, almonds and walnuts were specifically targeted, and the different phenolics prior and after *in vitro* gastrointestinal digestion and large intestine fermentation were profiled and quantified according to their phenolic class. To the best of our knowledge, information regarding the screening of phytochemicals such as phenolic compounds in these food matrices after *in vitro* large intestine fermentation using an untargeted UHPLC-ESI-QTOF-MS approach is still scarce. In particular, the use of a metabolomic approach is expected to provide a much deeper investigation of the actual phenolic composition of these food matrices, as compared to classical targeted approaches.

Table 1
Label indications on chemical composition (g) of six different edible nut samples are reported. Nutritional values are expressed per 100 g of raw product.

Nuts	Carbohydrates	Proteins	Lipids	TDF	SDF	IDF
Peanuts	21.5	23.7	49.7	5.1	1.0	4.1
Peeled Hazelnuts	15.6	14.0	61.0	9.4	1.5	7.9
Pistachios	24.0	20.0	45.0	11.0	2.7	8.3
Almonds	19.2	21.0	49.9	9.9	1.0	8.9
Unpeeled Hazelnuts	15.3	14.4	60.7	9.6	1.4	8.2
Walnuts	11.8	14.0	68.0	6.2	1.9	4.3

TDF = total dietary fibre; SDF = soluble dietary fibre; IDF = insoluble dietary fibre.

2. Materials and methods

2.1. Samples

Six different edible nuts, including roasted peanuts (*Arachis hypogaea* L.), roasted and peeled hazelnuts (*Corylus avellana* L.), roasted pistachios (*Pistacia vera* L.), roasted almonds (*Prunus dulcis* Mill.), roasted and unpeeled hazelnuts (*Corylus avellana* L.) and natural walnuts (*Juglans regia* L.) were included in this study. Samples were gratefully donated by Nicola Scala S.r.l. (Liveri, Italy). For each sample, the chemical composition in accordance with the label indications, is presented in Table 1. In addition, the insoluble and soluble dietary fibre contents were enzymatically quantified (Megazyme assay kit K-INTDF 02/15) following manufacturer's procedure, and included in Table 1. All samples were freshly prepared prior to the experiment.

2.2. Extraction of phenolic compounds (free and bound)

From each nut, six individual sample replicates (1 g each), were milled in a laboratory mill equipped with a 1 mm screen (Retsch grinder model ZM1; Brinkman Instruments, Rexdale, ON, Canada) and were extracted in 10 ml of 1% formic acid in 70% methanol (LCMS grade, VWR, Milan, Italy) using Ultra-turrax (Ika T25, Staufen, Germany). The extracts were then centrifuged at 7000 rpm for 10 min at 4 °C, and 5% trichloroacetic acid (BioUltra from Sigma Aldrich, Milan, Italy) was added in order to promote overnight protein precipitation in the freezer, at −18 °C. The resulting solutions, representative of free phenolic compounds, were then filtered using 0.22 µm cellulose syringe filters and collected in amber vials for further use.

Bound phenolics were extracted from the residue obtained from the previous extractions, following the method reported by Zaupa et al. (2014). The residue was further hydrolysed with 3 ml of 2 M sodium hydroxide and kept at room temperature for 1 hour. After alkaline hydrolysis, the pH of the mixture was adjusted to pH 3 by adding 2.7 ml of 3 M citric acid. The bound phenolics were then extracted with 8 ml of ethyl acetate. After 15 min of 6500 rpm centrifugation, 4 ml of the ethyl acetate supernatant was dried under nitrogen flow (55 °C) and the residue was dissolved in 1 ml of 1% formic acid in 80% methanol, vortexed and centrifuged at 6500 rpm for 10 min. The resulting solutions were filtered using 0.22 µm cellulose syringe filters and aliquots of 200 µl were then transferred to amber vials for further analysis.

2.3. Evaluation of phenolic profile

An aliquot of each extract was retained to measure the total phenolic composition using a slightly modified Folin-Ciocalteu assay (Rocchetti et al., 2017), and expressed as gallic acid equivalents (GAE).