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Bioactive compounds and antioxidant properties of pseudocereals-enriched water biscuits and their *in vitro* digestates

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Alpha-tocotrienol (PubChem CID: 5282347)
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Ferulic acid (PubChem CID: 445858)
Furfural (PubChem CID: 7362)
Furosine (PubChem CID: 123889)
Gamma-tocopherol (PubChem CID: 92729)
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ABSTRACT

Carotenoids, tocols, phenolic acids and antioxidant capacity were studied during *in vitro* digestion of water biscuits (WB) from bread wheat, einkorn and einkorn-pseudocereals. Antioxidant and cytotoxic activities of digestates were also measured using a 70% Caco2/30% HT-29 human intestinal co-culture layer. Antioxidant profiles differed among WB formulations. Only hydrophilic molecules were solubilised by gastric digestion. After intestinal digestion, 77% carotenoids and 67% tocols were released. Soluble-conjugated phenolic acids increased (30%) and insoluble-bound forms decreased (17%), suggesting partial conversion from bound to conjugated form. After intestinal digestion, antioxidant capacity increased regardless of type and amount of antioxidants in undigested or digested WB. All WB, especially those with quinoa flour, reduced the AAPH pro-oxidant capacity in co-culture cells. These results highlight the potential health benefits of underutilized crops and the need for *in vitro* or *in vivo* models to better address potential bioactivity at intestinal and target organs level.

1. Introduction

All over the world the human population relies on cereals as staple food; these crops, besides contributing most of our energy requirement, deliver numerous molecules possessing valuable bioactive properties. The widespread consumption of cereal-based products implies that minor changes in the concentration of specific compounds may have positive effects on human health. Some underutilized crops, such as einkorn (*Triticum monococcum* L. ssp. *monococcum*) and the pseudocereals buckwheat (*Fagopyrum esculentum*), quinoa (*Chenopodium quinoa*) and amaranth (*Amaranthus* spp.) contain relevant amounts of nutritionally valuable molecules, notably antioxidant compounds like phenolic acids, polyphenols, carotenoids and tocols (Alvarez-Jubete, Wijngaard, Arendt, & Gallagher, 2010; Hidalgo & Brandolini, 2014).

In recent years, antioxidant-rich foods have raised consumers and researchers attention, because their consumption plays an essential role in the prevention of several diseases. Among the most studied antioxidant compounds are flavonoids, phenolic acids, carotenoids and tocols, which cooperate in reducing aging-related, chronic and intestinal diseases. Antioxidants in foods can prevent oxidation and reactive oxygen species (ROS) generation, but their accessibility upon gastrointestinal digestion (GID) is a critical factor. Indeed, GID potentially affects type and rate of released antioxidant compounds, thus determining their bioavailability along the gastrointestinal system.

Antioxidant compounds release during food digestion can be studied by *in vitro* simulated GID (SGID), utilising the widely-applied

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protocol proposed by Minekus et al. (2014). Additionally, the biological properties of foods are due to the interactions of digestates with the intestinal epithelium, where food digestates can exert antioxidant capacity and possible toxicity on cells: these effects are usually studied through *in vitro* cell cultures. A physiologically important *in vitro* model of human intestinal epithelium is the co-culture of Caco2 and HT-29 cell at a 70/30 or 75/25 ratio (Mahler, Shuler, & Glahn, 2009), which boasts most features of mature small intestinal cells such as intercellular tight junctions, brush border peptidases, transport systems and mucus layer (Mahler et al., 2009; Satake et al., 2002), and permeability similar to that of human intestine (Wikman-Larhed & Artursson, 1995).

While information concerning the modifications exerted by the manufacturing process on antioxidant content and composition of polyphenols, carotenoids and tocols of diverse baked foods is available (Alvarez-Jubete, Holse, Hansen, Arendt, & Gallagher, 2009; Hidalgo & Brandolini, 2010; Hidalgo, Brandolini, & Pompei, 2010; Menga, Fares, Troccoli, Cattivelli, & Baiano, 2010), little is known about changes in antioxidant content and properties of digestates. Therefore, the aim of this study was to investigate the release and fate of carotenoids, tocols, and phenolic acids during SGID of water biscuits made with different flour formulations containing wheat, einkorn and pseudocereals. To this end, content of antioxidants and *in vitro* antioxidant capacity were measured before and at different SGID steps. Antioxidant capacity and cytotoxic activity of digestates were also measured using a 70% Caco2/30% HT-29 co-culture layer as an *in vitro* model of the human intestinal epithelium.

2. Materials and methods

2.1. Materials

The analyses were performed on water biscuits (WB) from einkorn wheat whole meal flour enriched with quinoa, amaranth or buckwheat and, as a control, on water biscuits from einkorn as well as wheat whole meal flour. Additionally, the analyses were performed on *in vitro* digested samples. To this end, seeds of einkorn wheat (*Triticum monococcum* L. ssp. *monococcum* cv. Monlis), common wheat for biscuits (*Triticum aestivum* L. ssp. *aestivum* cv. Bramante), amaranth (*Amaranthus cruentus* L. cv. MT-3), and buckwheat (*Fagopyrum esculentum* Moench local population Seis) were produced in 2014 in the fields of the Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA) – Research Unit for the Selection of Cereals (Sant'Angelo Lodigiano, LO, Italy), while the quinoa (*Chenopodium quinoa* Willd) sample came from the market.

After harvesting, the seeds were stored at 5 °C. Immediately before milling the hulled kernels (Monlis) were cleaned with an Otake FC4S thresher (Satake, Japan). Whole meal flour of all samples was prepared using a Cyclotec 1093 lab mill (FOSS Tecator, Denmark).

2.2. Sample preparation

2.2.1. Water biscuits

The WB were prepared using only whole meal flour and deionized water. In particular, for each sample 80 g of whole meal flour (or combinations of flours) at 14% humidity and 35 mL of water were blended for 90 s using a Hobart C-100 electric mixer (National MFG CO, USA). Subsequently the dough was rolled to 7 mm height and cut with a die cutter (internal diameter 60 mm) to obtain two identical disks. The biscuits were immediately baked in an Ovenlab rotary oven (National MFG CO, U.S.A.) at 205 °C for 30 min, cooled at room temperature for 30 min and stored at −20 °C.

Five different types of WB were prepared: 100% einkorn flour, 70% einkorn – 30% amaranth, 70% einkorn – 30% quinoa, 70% einkorn – 30% buckwheat, 100% bread wheat. Before analysis the WB were ground with a lab mill (Braun, Germany).

2.2.2. In vitro digestion

Water biscuits digestion was carried out on ground samples using the *in vitro* simulated gastro-intestinal digestion protocol reported in Cattaneo et al. (2015). Supernatant (soluble fraction) and sediment (insoluble fraction) after the gastric and intestinal steps of *in vitro* digestion were obtained by centrifuging the digestates at 4000 g for 20 min. Reference blanks were obtained by performing the digestions without the addition of WB sample. All the gastric and intestinal digestates were freeze-dried and stored at −18 °C.

2.3. Chemical analyses

The following analyses were performed twice on WB and digested samples.

2.3.1. Dry matter

Dry matter (DM) was determined by oven-drying (130 °C, six hours).

2.3.2. Carotenoids and tocols

WB (2 g), supernatants (5 g) and sediments (0.5 g) from the gastric and intestinal digestions were tested for carotenoid and tocol content. Carotenoids were extracted and quantified by NP-HPLC as described in Hidalgo et al. (2010). The total carotenoids were computed as the sum of the different compounds. Tocols were extracted and quantified by NP-HPLC as detailed in Hidalgo and Brandolini (2010). The total tocols were computed as the sum of tocopherols and tocotrienols. The results are expressed as mg/kg DM.

2.3.3. Phenolic acids

Soluble conjugated and insoluble bound phenolic acid extraction was performed as described by Yilmaz, Brandolini, and Hidalgo (2015) with minor modifications. Exactly 0.5 g of WB underwent a first phase of solvent extraction with methanol:acetone:water (7:7:6); after centrifugation, supernatant and sediment were hydrolysed with 4 M NaOH. The preparation of the digested samples (2.5 g supernatant or 0.5 g sediment) started with the hydrolysis step. All samples were prepared under dark and stored at −20 °C before injection. The samples were analysed by RP-HPLC as described by Brandolini, Castoldi, Plizzari, and Hidalgo (2013). The total conjugated and total bound phenolic acid contents were computed as the sums of the compounds identified. The results are expressed as mg/kg DM.

2.3.4. Total polyphenols (TPP)

Exactly 0.5 g of ground WB, gastric and intestinal sediments were weighted and extracted twice with 5 mL of methanol acidified with an aqueous solution of HCl 1% (80:20, methanol:HCl) as described by Yilmaz et al. (2015) under dark and refrigeration. Gastric and intestinal surnatants instead were directly analysed. Total polyphenols quantification was achieved with the Folin-Ciocalteu method and expressed as mg ferulic acid equivalent (FAE)/kg DM.

2.3.5. Ferric reducing antioxidant capacity (FRAP)

FRAP was determined as described by Benzie and Strain (1996), with the modifications of Yilmaz et al. (2015) on the same samples used for TPP quantification. The antioxidant capacity was expressed as mmol of Trolox equivalent (TE)/kg DM.

2.3.6. Heat damage

For the assessment of heat damage in WB, the following analyses were performed: furosine as described by Cattaneo et al. (2015), glycosylisomaltol (GLI), hydroxymethylfurfural (HMF) and furfural as described by Rufián-Henares, Delgado-Andrade, and Morales (2006).