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Polyphenol composition and *in vitro* antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking

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ABSTRACT

This study examined the polyphenol composition and antioxidant properties of methanolic extracts from amaranth, quinoa, buckwheat and wheat, and evaluated how these properties were affected following two types of processing: sprouting and baking. The total phenol content amongst the seed extracts were significantly higher in buckwheat (323.4 mgGAE/100 g) and decreased in the following order: buckwheat > quinoa > wheat > amaranth. Antioxidant capacity, measured by the radical 2,2-diphenyl-1-picylhydrazyl scavenging capacity and the ferric ion reducing antioxidant power assays was also highest for buckwheat seed extract ($p < 0.01$). Total phenol content and antioxidant activity was generally found to increase with sprouting, and a decrease in levels was observed following breadmaking. Analysis by liquid chromatography coupled with diode array detector revealed the presence of phenolic acids, catechins, flavanol, flavone and flavonol glycosides. Overall, quinoa and buckwheat seeds and sprouts represent potential rich sources of polyphenol compounds for enhancing the nutritive properties of foods such as gluten-free breads.

1. Introduction

1.1. General

The pseudocereals amaranth, quinoa and buckwheat have attracted much interest in recent years. One of the reasons for this renewed interest is their excellent nutrient profile. In addition to being one of the important energy sources due to their starch content, these pseudocereals provide good quality protein, dietary fibre and lipids rich in unsaturated fats (Alvarez-Jubete, Arendt, & Gallagher, in press). Moreover, they contain adequate levels of important micronutrients such as minerals and vitamins and significant amounts of other bioactive components such as saponins, phytosterols, squalene, fagopyritols and polyphenols (Berghofer & Schoenlechner, 2002; Taylor & Parker, 2002; Wijngaard & Arendt, 2006). In addition, amaranth, quinoa and buckwheat seeds are also naturally gluten-free and thus, they are currently emerging as healthy alternatives to gluten-containing grains in the gluten-free diet (Kupper, 2005).

Much research has been conducted over the past ten years on the polyphenol composition of foods, and also on polyphenol bioavailability, metabolism and biological effects (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). The increased interest in polyphenols in the past decade has been brought about by results from epidemiological studies linking the consumption of diets rich in plant foods with decreased risk of diseases associated with oxidative stress, such as cancer and cardiovascular disease (Scalbert, Manach, Morand, Remesy, & Jimenez, 2005).

Polyphenols are secondary plant metabolites that play a role in the protection of plants against ultraviolet radiation, pathogens and herbivores (Harborne & Williams, 2000). Several hundred molecules with polyphenol structure (i.e., benzene rings with one or more hydroxyl groups) have been identified in edible plants (Manach et al., 2004). Fruit and beverages, such as tea, red wine, and coffee, are the main sources of polyphenols, however, vegetables, cereals and leguminous plants are also good sources (Manach et al., 2004). Current estimated intake of polyphenols is >100 mg d^{-1}. This is in contrast with the intake of important vitamins such as vitamin E (8.5 mg d^{-1}), vitamin C (80 mg d^{-1}) and β-carotene (1.9 mg d^{-1}). This suggests that these compounds

Abbreviations: A, amaranth; Q, quinoa; B, buckwheat; 50% pseudobreads = A, Q and B breads, breads containing 50% pseudocereal flour; 100% quinoa bread = 100%Q bread, bread containing 100% quinoa flour; sprouted buckwheat bread = SpB bread, bread containing 50% buckwheat flour and 50% sprouted buckwheat flour; GF, gluten-free; GFC, gluten-free control; WC, wheat control.

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represent an important part of the total dietary intake of biologically active compounds (Hooper & Cassidy, 2006).

Published data on the antioxidant properties and polyphenol composition of the pseudocereals amaranth and quinoa is limited, however, substantially more has been reported on the antioxidant properties of buckwheat. However, to properly assess their relevance as potential sources of dietary antioxidants, information on the impact of processing on these compounds is also essential, as they are generally processed before consumption.

Processing can modify the polyphenol content of foods in several ways (Manach et al., 2004). In particular, sprouting has been reported as a means of increasing the polyphenol content of buckwheat seeds (Kim, Kim, & Park, 2004). Conversely, studies have shown that thermal processing of fruits, vegetables and cereals can have a detrimental effect on flavonoid compounds (Dietrych-Szostak & Oleszek, 1999). The extent of which flavonoid loss was due to processing has been shown to be highly dependent on factors such as the type of substrate and the processing conditions, mainly the length and temperature of the process (Sensoy, Rosen, Ho, & Karwe, 2006). For example, in a recent study, extrusion did not affect the antioxidant activity of buckwheat, whereas roasting caused a slight decrease in antioxidant activity (Sensoy et al., 2006). Thermal processing of cereals, such as baking, can also result in the synthesis of substances with antioxidant properties, such as some Maillard reaction products in bread crust (Lindenmeier & Hofmann, 2004).

Baking still represents one of the most common ways of processing cereals; however, to date no studies have been published on the impact of baking on the antioxidant properties and polyphenol composition of amaranth, quinoa and buckwheat. Identifying foods rich in antioxidants, as well as processing methods for preserving or enhancing these high levels may ultimately result in the manufacture of food products rich in these compounds, with their associated potential health protective properties.

In a previous study (Alvarez-Jubete et al., in press), the use of amaranth, quinoa and buckwheat as potential healthy ingredients for improving the nutrient content of gluten-free breads was evaluated. Results showed that these pseudocereals represent feasible ingredients in the manufacture of nutrient-rich gluten-free products. The nutritional benefits of pseudocereals were also highlighted, not only for celiac patients, but also for the general population. In the present study, another aspect of amaranth, quinoa and buckwheat seeds nutritional quality is examined: their *in vitro* antioxidant properties and polyphenol composition, and how these properties are affected by the sprouting and baking processes.

The objectives of the present study were the following:

(1) To determine the antioxidant capacity, total phenol content and polyphenol composition of amaranth, quinoa, buckwheat and wheat seeds.
(2) To determine the effects of sprouting and breadmaking on these properties.
(3) To aid in contributing to the formulation of nutritionally enhanced gluten-free breads.

2. Materials and methods

2.1. Seed materials

Amaranth seeds (*Amarantus caudatus*, harvested in Peru) and quinoa seeds (*Chenopodium quinoa*, grown in Bolivia) were obtained from Ziegler & Co., Germany. Buckwheat seeds (*Fagopyrum esculentum* Möench, grown in China) were sourced by Munster Wholefoods, Ireland. Wheat grains (*Triticum aestivum* L.), variety Raffles, grown in Ireland were provided by Gold Crop, Ireland.

All seeds were delivered cleaned from dust and any other contaminants.

Quinoa seeds were pre-processed by the manufacturer to partially remove the saponins and dust off the grains by washing, centrifuging and drying.

The samples were kept in paper bags inside drums in a dry and cool room until analysis. Prior to bread preparation and/or chemical analysis the samples were ground using a Cemotec 1090 sample mill (FOSS Tecator, Sweden).

2.2. Sprouting method

Steeping and germination of the seeds was completed using a Micro Malting Machine (Joe White Malting Systems, Perth, Australia). During the steeping step, 1 kg of each of the seeds was subjected to alternating wet and dry cycles of 3 h duration over a 24 h period, and the temperature was kept at 15 °C. The steeped grains were then germinated at 18 °C for buckwheat and wheat, and at 10 °C for amaranth and quinoa. Germination time was 96, 110, 98 and 82 h for buckwheat, wheat, amaranth and quinoa, respectively. During steeping and germinating, all samples were turned every 30 min. The germination time for each of the grains was based on preliminary studies. Shorter germination periods resulted in sprouts that were not developed sufficiently and longer times resulted in overgrowth. The bud from the germinated seeds was approximately 4 cm for buckwheat, 1 cm for wheat and 0.5 cm in the case of quinoa and amaranth. Germinated seeds were then freeze-dried (Ima Edwards, Dongen, The Netherlands) and kept frozen at −20 °C until analysis.

2.3. Chemicals

Ethyl gallate, 3-coumaric acid, 4-hydroxybenzoic acid, protocatechuic acid, sinapic acid, vanillic acid, apigenin, apigenin-6-*C*-glucoside, apigenin-8-*C*-glucoside, apigenin-7-*O*-glucoside, luteolin, luteolin-6-*C*-glucoside, luteolin-8-*C*-glucoside, luteolin-7-*O*-glucoside, luteolin-3,7′-di-*O*-glucoside and kaempferol-3-*O*-glucoside were purchased from Extrasynthèse (Lyon, France). (+)-Catechin, (−)-catechin, cinnamic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ellagic acid, ferulic acid, gallic acid, (−)-epicatechin, kaempferol, myricetin, quercetin, rutin, and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and Folin Ciocalteu Reagent (FCR) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, USA).

2.4. Bread ingredients

Pseudocereal and wheat seeds (as above), sprouted buckwheat seeds (as above), rice flour (S&B Herba, Kent, UK), potato starch (Healy Chemicals Ltd., Ireland), wheat flour (Odlum Group, Dublin, Ireland), 100% vegetable oil (Homestead, Dublin, Ireland), bakers fat (Irish Bakels Ltd., Ireland), xanthan gum (All In All Ingredients, Dublin, Ireland), fresh yeast (Yeast Products, Dublin, Ireland), salt (Imeos Enterprises, Cheshire, UK) and caster cane sugar (Tate & Lyle, UK).

2.5. Preparation of breads

The bread formulations used for the baking trials are presented in Table 1. In addition to two control formulations (GFC and WC), three types of pseudocereal-containing breads were produced: (i) breads containing 50% of each of the pseudocereal flours (A, Q and B breads); (ii) 100% quinoa bread (100%Q) (100% amaranth bread was not produced, due to its functional attributes in preliminary trials were not satisfactory) and (iii) sprouted buckwheat

Table 1
Bread formulations.

Ingredient (%)	Gluten-free control (GFC)	Wheat control (W)	50% pseudobreads			100% pseudobreads	
			Amaranth (A)	Quinoa (Q)	Buckwheat (B)	100% quinoa (100%Q)	Sprouted buckwheat (SpB)
Wheat flour	–	✓	–	–	–	–	–
Rice flour	✓	–	✓	✓	✓	–	–
Potato starch	✓	–	–	–	–	–	–
Amaranth seeds	–	–	✓	–	–	–	–
Quinoa seeds	–	–	–	✓	–	✓	–
Buckwheat seeds	–	–	–	–	✓	–	✓
Wheat grain	–	✓	–	–	–	–	–
Buckwheat sprouts	–	–	–	–	–	–	✓
Yeast	✓	✓	✓	✓	✓	✓	✓
Sugar	✓	–	✓	✓	✓	✓	✓
Salt	✓	✓	✓	✓	✓	✓	✓
Xanthan gum	✓	–	✓	✓	✓	✓	✓
Vegetable oil	✓	–	✓	✓	✓	✓	✓
Bakers fat	–	✓	–	–	–	–	–
Water	✓	✓	✓	✓	✓	✓	✓

bread, a bread containing buckwheat and sprouted buckwheat flour to maximize content of polyphenol compounds.

The batters/doughs were prepared as follows:

(i) Gluten-free batter: dry ingredients were mixed together for 1 min using an A120 Hobart mixer (Hobart, UK) at speed one, yeast was dissolved in the water and added to the dry ingredients together with the oil and the batter formed was mixed for a further minute. After scraping the base of the bowl, the batter was further mixed for 2 min at speed two.
(ii) Wheat dough: the yeast was dissolved in water, added to the rest of the ingredients and mixed (Hobart, UK) for 3 min at speed three. Optimal mixing time was recorded from the Farinograph (Brabender, Germany).

Batter or dough (for gluten-free breads and wheat breads, respectively) was scaled into pup loaves tins (65 g) and placed in a proofer (Koma, UK) for 45 min at 35 °C and 80% relative humidity. The loaves were baked in a deck oven (Tom Chandley Ovens, Manchester, UK) at 220–225 °C for 20 min. They were cooled to room temperature, sliced and freeze-dried (Ima Edwards, Dongen, The Netherlands).

2.6. Analytical methods

2.6.1. Sample extraction

Sample extracts, for the measurement of antioxidant activity and phenols content and composition, were prepared from 1.25 g freshly ground sample in 25 ml methanol. Samples were homogenised for 2 min at 12,000 rpm using an Ultra-Turrax T-25 tissue homogeniser (IKA-group, Saufen, Germany), vortexed with a V400 Multituve Vortexer (Alpha laboratories, North York, Canada) for 20 min at 1050 rpm and centrifuged for 10 min at 2,000 rpm (MSE Mistral 3000i, Sanyo Gallenkamp, Leicestershire, UK). The final extracts were obtained by filtering 10 ml of the supernatant through 0.22 μm PTFE syringe filters (Phenomenex, Macclesfield Cheshire, UK) and were stored at −20 °C until analysis.

2.6.2. Total phenols assay by Folin–Ciocalteau reagent

The total phenolic content of methanolic grain extracts were evaluated using a modified version of the Folin–Ciocalteu assay as described by Singelton and Rossi (Singleton & Rossi, 1965). Briefly, 100 μl of methanolic grain extract or standard, 100 μl of MeOH, 100 μl of Fiolin–Ciocalteu reagent and 700 μl of Na_2CO_3 were added into a 1.5 ml micro-centrifuge tube. The samples were vortexed immediately and the tubes were incubated in the dark for 20 min at room temperature. After incubation all samples were centrifuged at 13,000 rpm for three min. The absorbance of the supernatant was then measured at 735 nm in 1 ml plastic cuvettes using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Japan). Gallic acid was used as a standard and a calibration curve was prepared with a range of concentrations from 10 to 200 mg l^{-1}. The results are expressed in mg gallic acid equivalent per 100 g dry-weight basis (mgGAE/100 g dwb).

2.6.3. Antioxidant capacity determined by using the radical DPPH· scavenging capacity assay

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical was used to measure the free radical scavenging capacity of the sample extracts as described by Goupy, Hugues, Boivin, and Amiot (1999), with some modifications. The reaction mixture consisted of 500 μl of diluted sample (serial dilutions of the grain extracts were prepared prior analysis) and 500 μl of a freshly made DPPH· methanolic solution (0.05 mg/ml) and was prepared in 1.5 ml microcentrifuge tubes. The absorbance of the freshly prepared DPPH· solution was measured prior to analysis and absorbance values were in the range 1.2–1.3. After vortexing, the tubes were left in the dark for 30 min at room temperature. The absorbance was then measured against methanol at 515 nm in 1 ml cuvettes using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Milton Keynes). As the DPPH· was reduced by the amount of antioxidants present in the sample, the colour of the solution faded in a proportional correlation to the antioxidant concentration. The sample concentration that caused a decrease in the initial DPPH· concentration by 50% was defined as the IC_{50} and was used to calculate the antioxidant capacity. The IC_{50} of Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxilic acid), a synthetic hydrophilic vitamin E analogue, was also calculated in all the experiments, and the antioxidant capacity of the sample was then expressed as Trolox equivalent antioxidant capacity values (TEAC) using the formula $TEAC = (IC50_{Trolox}/IC50_{Sample}) \times 10^5$, as previously outlined by Hagen et al. (2007). To facilitate the comparison of results with those derived from the ferric reducing antioxidant power (FRAP) assay, antioxidant capacity was also expressed as mg Trolox equivalent (TE) per 100 g sample dry-weight basis (mgTE/100 g dwb) (Stratil, Klejdus, & Kubánhacek, 2006).

2.6.4. Antioxidant capacity determined by using theferric ion reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to Stratil et al. (2006), with slight modifications. The oxidant in the FRAP assay consisted

of acetate buffer (pH 3.6), ferric chloride solution (20 mM) and TPTZ solution (10 mM TPTZ in 40 mM HCl) in a proportion of 10:1:1, respectively, and was freshly prepared on the day of analysis. To obtain a FRAP value for antioxidant activity 900 μl of FRAP solution warmed to 37 °C were added to 100 μl of appropriately diluted sample, blank or standard in a micro-centrifuge tube. The tubes were vortexed and left at 37 °C for exactly 40 min and the absorbance was measured at 593 nm. A Trolox standard curve prepared from a 0.2 mM Trolox methanolic stock solution was used to calculate the antioxidant capacity of the samples, which was expressed in mg Trolox equivalent per 100 g dry-weight basis (mgTE/100 g dwb).

2.6.5. Analysis of the polyphenol composition by using HPLC–DAD

The HPLC-analysis was performed on a SPD-M10A vp Shimadzu chromatographic system (Shimadzu UK Ltd, UK) equipped with pump, degasser and diode array detector (DAD) and controlled through EZ Start 7.3 software (Shimadzu UK Ltd., UK). Separations were conducted on a Zorbax SB C_{18} column (Agilent Technologies, Dublin, Ireland) with dimensions 150 × 4.6 mm and 5 μm particle size. The mobile phase consisted of 6% acetic acid in 2 mM sodium acetate (final pH 2.55, v/v) as eluent A and 100% acetonitrile as eluent B, and was based in the method described by Tsao and Yang (2003). The solvent gradient program was set as follows: initial conditions 95% A, 5% B; 0–45 min, 0–15% B; 45–60 min, 15–30% B; 60–65 min, 30–50% B; 65–70 min, 50–100% B. Column temperature was set at 37 °C, flow rate was 1 ml min^{-1} and the injection volume was 10 μl.

For identification purposes, a spectral library was constructed comprising the retention times and spectra of the above listed standards under the chromatographic conditions specified above. Standard calibration curves were also prepared and used for quantitative analysis and the results expressed as μmoles of aglycon/100 g dry sample. Detected peaks with same spectra as a library standard, but with different retention times, were referred to as derivatives of the standard and quantified using the respective standard calibration curve. For example, detected peaks with the same spectra as rutin but with different retention times, were identified as quercetin glycosides, quantified using rutin calibration curve and the results were expressed as μmoles of quercetin/100 g dry sample. Hydroxybenzoic acids and flavanols were detected at a wavelength of 280 nm, hydroxycinnamic acid derivatives at 320 nm, flavones and flavonols at 360 nm.

2.7. Statistical analysis

All analysis were performed in triplicate. Statistical analysis was conducted, where appropriate, by using one way analysis of variance (ANOVA), followed by the Tukey–Kramer test to assess any differences between group means using the Statistics Toolbox of the software Matlab 7.6 R2008a (Mathworks, US). Pearson correlation coefficient (R^2) and p-value were used to show correlations and their significance using the Basic Statistics of the software MINITAB version 15 (MINITAB Ltd., UK). Differences of $p < 0.01$ were considered significant.

3. Results and discussion

3.1. Seeds

3.1.1. Total phenols and antioxidant capacity of the seeds

The results for the total phenol content assay (by FCR) and antioxidant capacity assays (DPPH· and FRAP assays) of amaranth, quinoa, buckwheat and wheat seeds are presented in Table 2.

Total phenol content among the pseudocereal seed methanolic extracts differed greatly and was highest in buckwheat (323.4 mgGAE/100 g), followed by quinoa and amaranth ($p < 0.01$). The total phenol content of wheat was significantly higher than amaranth, lower than buckwheat ($p < 0.01$), and statistically not different from quinoa.

Similarly, the antioxidant capacity of the pseudocereal seed extracts, measured by both DPPH· and FRAP assays, was highest in buckwheat seed extracts ($p < 0.01$). Antioxidant capacity of wheat was significantly lower than buckwheat but statistically not different from quinoa. In the FRAP assay, wheat showed significantly higher antioxidant capacity compared with amaranth, whereas no significant differences were observed in the DPPH· assay.

In a recent study (Gorinstein et al., 2007), the total phenol content (TPH) of amaranth, quinoa and buckwheat were 15.5, 25 and 29 mgGAE/100 g dwb, respectively. Similar buckwheat TPH content to that in the present study was reported by Holasova et al. (2002) who also measured TPH levels in oats and barley. The TPH of wheat in the present study (53.1 mgGAE/100 g dwb) is comparable to the values found in the literature and the levels are significantly lower compared to other grains such as barley, millet, rye and sorghum (Ragaee, Abdel-Aal, & Noaman, 2006). From the literature, only two studies were found to report higher

Table 2
Total phenol content and antioxidant capacity of seeds, sprouts and breads.

	Total phenol as gallic acid equivalent (mgGAE/100 g dwb)	DPPH scavenging capacity		FRAP assay (mgTE/100 g dwb)
		TEAC ($IC50_{Trolox}/IC50_s$) × 10^5	mgTE/100 g dwb	
Seeds				
Amaranth	21.2 ± 2.3	16.2 ± 0.4	28.4 ± 1.3	55.3 ± 1.6
Quinoa	71.7 ± 5.5	34.8 ± 1.0	57.7 ± 1.7	92.1 ± 1.7
Buckwheat	323 ± 14.1	465 ± 22.7	620 ± 28.1	436 ± 12.8
Wheat	53.1 ± 2.8	13.6 ± 0.2	44.1 ± 0.4	110 ± 4.7
Sprouts				
Amaranth	82.2 ± 4.6	22.4 ± 1.2	27.1 ± 2.7	122 ± 11.1
Quinoa	147 ± 3.7	33.7 ± 2.2	50.4 ± 2.4	164 ± 0.6
Buckwheat	670 ± 12.3	606 ± 52.5	666 ± 62.6	739 ± 8.4
Wheat	110 ± 7.9	30.0 ± 4.4	73.7 ± 11.5	210 ± 12.7
Breads				
Amaranth (A)	13.8 ± 0.0	ND	10.3 ± 0.2	60.6 ± 6.2
Quinoa (Q)	30.7 ± 0.3	12.3 ± 0.1	16.8 ± 0.7	71.4 ± 2.8
Buckwheat (B)	64.5 ± 3.1	50.4 ± 2.7	58.8 ± 3.9	148 ± 4.6
Wheat control (WC)	29.1 ± 0.6	9.9 ± 0.2	14.1 ± 0.4	81.7 ± 1.6
GF control (GFC)	8.8 ± 1.0	ND	5.6 ± 0.2	47.6 ± 3.3
100% quinoa (100%Q)	55.2 ± 0.9	17.4 ± 0.5	22.3 ± 0.5	87.0 ± 5.2
Sp buckwheat (SpB)	116 ± 1.8	69.6 ± 1.8	76.8 ± 2.5	264 ± 3.6

TPH values for a cereal/pseudocereal than the reported values for buckwheat in the present study. The samples in question are the less frequently researched canihua (*Chenopodium pallidicaule*), a pseudocereal growing in Bolivia at 3600–4400 m altitude, and sorghum (*Sorghum bicolor* L.), having TPH contents of 430 and 413 mgGAE/100 g, respectively (Peñarrieta, Alvarado, Akesson, & Bergenståhl, 2008; Ragaee et al., 2006).

Referring to the antioxidant capacity, amaranth and quinoa, FRAP values are higher than values previously reported by Gorinstein et al. (2008) but nevertheless comparable, whereas buckwheat FRAP values were over four times higher in the present study compared to data by Gorinstein et al. (2008). Canihua FRAP values, measured by Peñarrieta et al. (2008), were lower than buckwheat FRAP values in the present study, despite the higher TPH content mentioned above.

Data variation in the antioxidant capacity of cereals is to be expected, as many factors such as genetics, agrotechnical processes and environmental conditions can influence the presence of phenolic compounds (Nsimba, Kikuzaki, & Konishi, 2008; Yu, Perret, Harris, Wilson, & Haley, 2003). In addition, a comparison of results from different studies can be difficult due to variability in the experimental conditions amongst the methods used (Huang, Ou, & Prior, 2005; Stratil et al., 2006).

Despite these variations, however, buckwheat has been consistently reported as one of the greatest sources of antioxidant activity amongst cereals and pseudocereals (Gallardo, Jimenez, & Garcia-Conesa, 2006; Gorinstein et al., 2007, 2008; Holasova et al., 2002; Zielinski & Kozlowska, 2000). The present study was in agreement with the findings of these previously-reported studies and shows that buckwheat represents an exceptional source of antioxidant activity.

Total phenol content by FCR and *in vitro* antioxidant capacity assays, such as the DPPH· and FRAP assays (which were used in this study), represent convenient methods for the identification of potential sources of antioxidant compounds (Stratil et al., 2006). As already mentioned, antioxidants, such as polyphenols, have significant potential health benefits; they may protect cell constituents against oxidative damage and therefore limit the risk of various degenerative diseases associated to oxidative stress such as cancer, cardiovascular disease and osteoporosis. However, the value of *in vitro* antioxidant capacity assays for assessing the health-related implications of a food extract has been limited for a number of reasons, mainly due to the lack of standardization amongst these methods, the changes in the antioxidant activity of polyphenols following extensive metabolization in the body and the large variation in bioavailability existent among the different types of polyphenols (Scalbert et al., 2005). Nevertheless, this does not exclude antioxidant properties (redox properties) of polyphenols from being one of the key parameters in determining their biological effects (Scalbert et al., 2005).

3.2. Polyphenol composition of the pseudocereal seeds

Polyphenol composition analysis by chromatographic methods, such as HPLC–DAD, provided more in depth information when compared with spectrophotometric methods (such as total phenol by FCR), as this provided information on the exact type and quantity of the polyphenols present. However, the identification of all the phenolic compounds present in a sample by using HPLC–DAD, may prove difficult and time consuming due to the complexity of the composition of foods (Huang et al., 2005). Nevertheless, in this study, between 80% and 90% of total peak area was identified and quantified by building a spectral library consisting of 30 polyphenol standards. Analysis was carried out directly after extraction, without hydrolysis, so that aglycons and glycosides, which differ greatly in bioavailability (Manach et al., 2004), could be determined separately, and to avoid potential decomposition of polyphenols (Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003).

Several polyphenol groups were detected and identified in the different seed methanolic extracts (Table 3). Both simple polyphenols and flavonoids were present in quinoa and buckwheat seeds, however, no flavonoids were detected in wheat and amaranth seeds. In amaranth seeds, the smallest peak (20% total peak area) of the only two peaks detected was identified as protocatechuic acid. The second and largest peak, with a spectrum suggestive of a hydroxybenzoic acid, remains to be identified. A high-performance liquid chromatogram of quinoa seeds is presented in Fig. 1. In quinoa seeds, the flavonols quercetin and kaempferol glycosides were the most abundant polyphenols. Two benzoic acids, protocatechuic acid and a vanillic acid derivative, were also present in quinoa seeds together with two other unknown compounds (10.3% total peak area) with a spectrum indicating the presence of hydroxybenzoic acids. The polyphenol content of quinoa seeds, determined in this study, was in agreement with published work by Dini, Tenore, and Dini (2004). These authors reported kaempferol and quercetin glycosides as the predominant polyphenols in a sweet variety of quinoa seeds and also detected a vanillic acid glucoside. In buckwheat seeds, glycosides of the flavonol quercetin were the predominant polyphenols, followed by a catechin. Flavone glycosides (luteolin and apigenin glycosides) were also present together with two caffeic acids, a syringic acid derivative and two unknown compounds (10.1% total peak area) with spectra suggestive of hydroxybenzoic and cinnamic acid, respectively. Dietrych-Szostak et al. (1999) isolated and identified the following flavonoids in buckwheat seeds: luteolin and apigenin glycosides as well as rutin and quercetin, which was in general agreement with our results. Watanabe (1998) detected four catechins and rutin in buckwheat methanolic extracts. The one peak detected in wheat seeds did not match any of the standards in the spectral library, however, it did have a spectrum suggestive of a hydroxybenzoic acid.

Catechins and quercetin glucosides were amongst the best absorbed classes of polyphenols, after isoflavones and gallic acid (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Catechins have also been extensively studied for their biological effects and have shown in human intervention studies to have positive effects on plasma antioxidant biomarkers and on energy metabolism (Williamson & Manach, 2005). In the flavonol class, quercetin has also been widely studied, and it has shown some *in vivo* effects on carcinogenesis markers, and small effects on plasma antioxidant biomarkers (Williamson & Manach, 2005).

In comparison with wide variety of common fruits and vegetables (Sakakibara et al., 2003), quinoa constitutes a rich source of quercetin and kaempferol glycosides, and buckwheat represents a good source of luteolin and apigenin glycosides, catechins (buckwheat seeds) and quercetin glycosides.

3.3. Impact of processing: sprouting and baking

3.3.1. Total phenol content and antioxidant capacity of sprouted seeds

The results for the total phenol content assay (by FCR) and antioxidant capacity assays (DPPH· and FRAP assays) of sprouted amaranth, quinoa, buckwheat and wheat seeds are summarised in Table 2.

Total phenol content was doubled following sprouting of quinoa, buckwheat and wheat and quadrupled in the case of amaranth. Among the pseudocereal sprouts, buckwheat had the highest total phenol content (670.2 mgGAE/100 g), followed by quinoa (147.2 mgGAE/100 g) and amaranth (82.2 mgGAE/100 g). Total phenol content of sprouted wheat was significantly higher than in amaranth but lower than in quinoa and buckwheat ($p < 0.01$).

Table 3
Polyphenol content (μmol/100 g dwb ± SD) of the different methanolic extracts (seeds, sprouts and breads).

	Simple polyphenols		Flavonoids	
Seeds				
Amaranth	Protocatechuic acid	13.6 ± 9.4	No peaks detected	
Quinoa	Protocatechuic acid	9.7 ± 1.0	Quercetin glycosides	43.4 ± 2.5
	Vanillic acid derivative	4.9 ± 0.6	Kaempferol glycosides	36.7 ± 3.7
Buckwheat	Syringic acid derivative	10.5 ± 0.7	Catequin	40.2 ± 3.5
	Caffeic acid	15.1 ± 3.1	Luteolin glycoside	3.5 ± 1.1
	Caffeic acid derivative	4.6 ± 1.2	Apigenin glycoside	3.9 ± 0.5
			Quercetin glycosides	30.1 ± 6.4
Wheat	No identified peaks		No peaks detected	
Sprouts				
Amaranth	Protocatechuic acid	14.0 ± 2.1	No peaks detected	
Quinoa	Protocatechuic acid	9.0 ± 0.4	Quercetin glycosides	66.6 ± 1.7
	Vanillic acid derivative	11.7 ± 0.3	Kaempferol glycosides	56.0 ± 2.1
	Vanillic acid	6.7 ± 0.6		
	Caffeic acid derivative	9.5 ± 0.1		
Buckwheat	Syringic acid derivative	12.4 ± 1.1	Catechin	68.2 ± 0.2
	Caffeic acid	8.8 ± 0.2	Luteolin glycoside	30.2 ± 0.4
	Caffeic acid derivative	5.5 ± 0.4	Apigenin glycoside	20.2 ± 0.3
	3-Coumaric acid derivative	58.6 ± 1.1	Quercetin glycosides	42.6 ± 1.0
Wheat	Ethyl gallate	200 ± 34.2	No peaks detected	
Breads				
Amaranth	No identified peaks		No peaks detected	
Quinoa	No identified peaks		Quercetin glycosides	7.1 ± 1.3
			Kaempferol glycosides	7.7 ± 1.3
Buckwheat	Protocatechuic acid	5.8 ± 0.7	Quercetin glycosides	8.6 ± 1.4
			Quercetin	7.2 ± 0.4
GF control	No peaks detected		No peaks detected	
Wheat control	No peaks detected		No peaks detected	
100% Quinoa	No identified peaks		Quercetin glycosides	17.1 ± 0.4
			Kaempferol glycosides	19.2 ± 0.3
Sprouted Buckwheat	Protocatechuic acid	5.2 ± 0.8	Luteolin glycoside	9.1 ± 1.4
			Apigenin glycoside	3.0 ± 0.4
			Quercetin glycoside	3.2 ± 0.4
			Kaempferol glycoside	4.9 ± 0.6
			Quercetin	15.4 ± 1.9

Antioxidant capacity (DPPH· method) also increased following sprouting, although interestingly, the difference was only significant in the case of wheat seeds. Again, buckwheat sprouted seeds showed the highest antioxidant capacity of all sprouted seeds tested ($p < 0.01$). No statistical differences in antioxidant capacity were found between amaranth, quinoa and wheat sprouts. Similar results were observed when the FRAP assay was used.

The increase in antioxidant activity with sprouting is one of the many metabolic changes that take places upon sprouting of seeds, mainly due to an increase in the activity of the endogenous hydrolytic enzymes. Other common metabolic changes include improved protein and starch digestion, increased sugar and B vitamin content and decreased levels of phytates and proteases inhibitors (Chavan & Kadam, 1989).

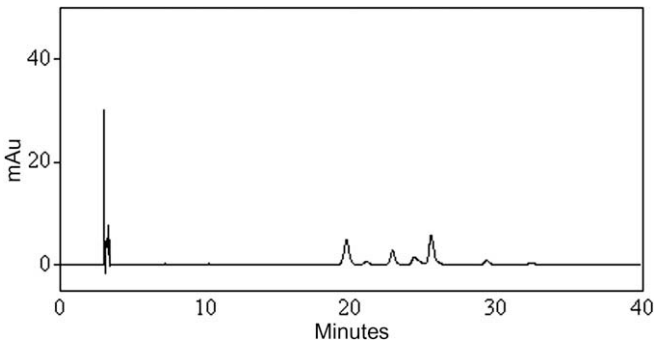

Fig. 1. High-performance liquid chromatogram of a methanolic extract of quinoa seeds (360 nm).

Overall, such changes due to sprouting, are desirable from a nutritional point of view, and the pseudocereal sprouted seeds are nutritionally superior compared with the non-sprouted seeds (Colmenares de Ruiz & Bressani, 1990; Khattak, Zeb, Bibi, Khalil, & Khattak, 2007; Kim et al., 2004). Thus, they represent attractive ingredients in the formulation of foods with an increased nutrient and antioxidant profile. Amongst all of the sprouted seeds tested, buckwheat sprouts showed the highest antioxidant activity, and therefore exhibit the highest potential as a source of compounds with antioxidant activity.

3.3.2. Total phenol content and antioxidant capacity of baked breads

The results for the total phenol content (by FCR) and antioxidant capacity (DPPH· and FRAP assays) of the baked breads produced in this study are presented in Table 2.

Total phenol contents of the 50% pseudobreads were significantly higher compared with the gluten-free control bread, with highest values found in breads containing buckwheat ($p < 0.01$). The total phenol content in wheat bread was significantly higher than for the gluten-free control and amaranth breads but lower compared with quinoa and buckwheat breads ($p < 0.01$).

The antioxidant capacity, measured by both DPPH· and FRAP assays, was also higher in the 50% pseudobreads compared with the GFC bread, with the buckwheat bread again having the highest overall result ($p < 0.01$). The antioxidant capacity of wheat bread was significantly lower compared with buckwheat bread. In the FRAP method, WC bread antioxidant capacity was significantly higher than in Q and A breads whereas in the DPPH· method it was only significantly higher compared with A bread but statistically not different from Q bread.

The replacement of potato starch by quinoa and sprouted buckwheat flour resulted in breads (100% quinoa and sprouted buckwheat breads) with a significantly increased total phenol content and higher antioxidant capacity ($p < 0.01$). In particular, total phenol content and antioxidant capacity was higher in sprouted buckwheat bread compared with 100% quinoa bread.

A comparison of the measured total phenol content in breads with the expected values (calculated using the approximation that the pseudocereal flour is the only ingredient contributing to TPH content in bread) suggests that some degradation may have occurred. This effect was particularly pronounced in the case of buckwheat, where total phenol content reduction from buckwheat seeds to buckwheat bread was 323–64.5 mgGAE/100 g dwb. Degradation of antioxidant compounds during quinoa breadmaking appears to have occurred also, however, to a smaller extent. Similar reductions were observed following breadmaking with wheat, whereby TPH content decreased from 53.1 to 29.1 mgGAE/100 g dwb.

However, despite the loss of total phenol content and antioxidant activity following breadmaking, all of the breads containing pseudocereals showed significantly higher antioxidant capacity when compared with the gluten-free control.

3.3.3. Polyphenol composition of the sprouted seeds and baked breads

The polyphenol content of the different sprouted seeds and baked breads methanolic extracts is summarised in Table 3. In general, sprouting resulted in an increase in the polyphenol content. In particular, kaempferol and quercetin glycosides in quinoa sprouts reached 56.0 and 66.6 μmol/100 g compared with 36.7 and 43.4 μmol/100 g in quinoa seeds (a high-performance liquid chromatogram of sprouted quinoa seeds is presented in Fig. 2). In the case of buckwheat, the main increases due to sprouting were observed in the levels of catechin, 3-coumaric acid, and luteolin and apigenin glycosides ($p < 0.01$). Sprouting of wheat also appeared to significantly increase its polyphenol content, e.g. seven peaks were detected in wheat sprouts compared with one peak in wheat seeds. However, only one of these peaks was identified and corresponded to ethyl gallate (48.9% total peak area). The spectra of these unknown compounds are suggestive of hydroxybenzoic acids.

The increased polyphenol content with sprouting reported in this study, is also in agreement with the literature. Kim et al. (2004) found that in buckwheat seeds, the content of two quercetin glycosides, rutin and quercitrin, and that of two other unknown compounds, increased notably as sprouting day progressed, whereas content of chlorogenic acid was found to increase only moderately.

The opposite effect to sprouting was observed following breadmaking, and polyphenol content was generally found to be reduced in the bread samples when compared with the original seeds. High-performance liquid chromatograms of quinoa bread and 100% quinoa bread are presented in Figs. 3 and 4, respectively. In particular, quercetin and kaempferol glycosides content in 100% quinoa breads was 17.1 and 19.2 μmol/100 g, compared with 43.4 and 36.7 μmol/100 g in quinoa seeds (Figs. 3 and 4). In the case of buckwheat, quercetin glycosides content decreased significantly with breadmaking, resulting in an increase in quercetin content through hydrolysis. The observed degradation of flavonoids during heat processing in this study has also been previously reported by other authors (Dietrych-Szostak et al., 1999; Im, Huff, & Hsieh, 2003; Kreft, Fabjan, & Yasumoto, 2006).

There were no polyphenols detected in the gluten-free control bread and therefore the introduction of pseudocereals served to enhance the polyphenol content of these products. Despite the negative impact of baking on the polyphenol content of pseudocereals, the breads made using quinoa and buckwheat flour still contained flavonoids in significant quantities, in particular the 100% quinoa and sprouted buckwheat breads. These characteristics are highly desirable in gluten-free products as their nutritional quality has been reported to be of concern (Thompson, 2000). Moreover, these breads also represent healthy alternatives for the general population alike.

3.4. Correlations

In this study, significant correlations were found between TPH content (Folin–Ciocalteau method) and antioxidant activity (DPPH· and FRAP assays) in all of the extracts studied (seeds, sprouts and breads) (Table 4). This is due to the fact that the chemistry behind these methods is based on the same principles (redox properties), therefore a high correlation among values determined by all these

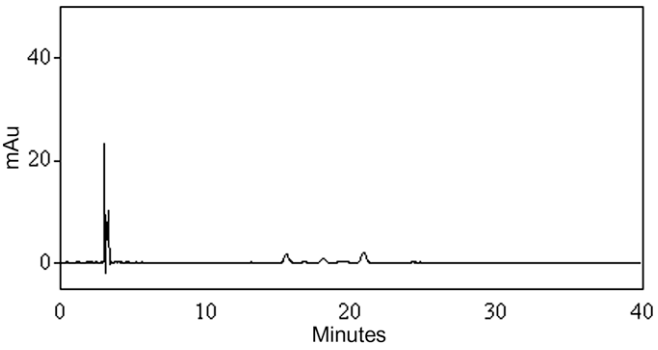

Fig. 3. High-performance liquid chromatogram of a methanolic extract of quinoa bread (360 nm).

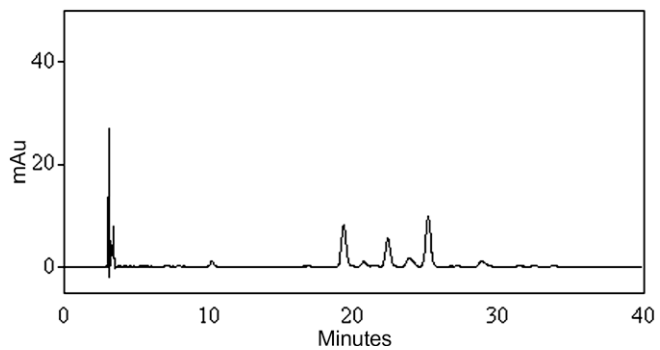

Fig. 2. High-performance liquid chromatogram of a methanolic extract of quinoa sprouted seeds (360 nm).

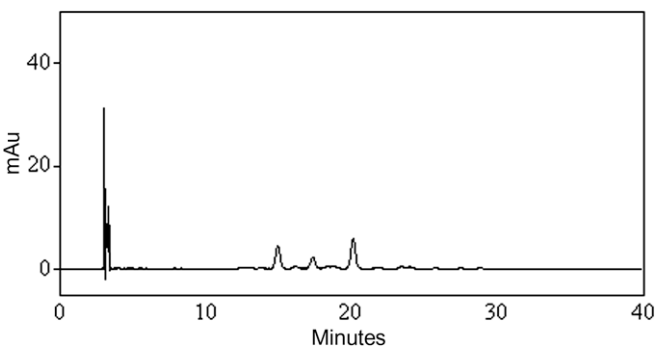

Fig. 4. High-performance liquid chromatogram of a methanolic extract of 100% quinoa bread (360 nm).

Table 4
Correlations of TPH content and antioxidant activity of the different methanolic extracts (seeds, sprouts and breads). All correlations were statistically significant.

Methanolic extract	TPH vs. DPPH (R^2)	TPH vs. FRAP (R^2)	DPPH vs. FRAP (R^2)
Seeds	0.99	0.99	0.99
Sprouts	0.99	0.99	0.99
Breads	0.93	0.97	0.96

methods is expected (Huang et al., 2005; Stratil et al., 2006). Sun and Ho (2005) also found a significant correlation ($R^2 = 0.96$) between TPH content and antioxidant activity in buckwheat extract. On the other hand, Nsimba et al. (2008) reported weak correlations between TPH content and antioxidant activity (DPPH· and FRAP assays) in amaranth and quinoa extracts.

The correlation discrepancies found in the literature, could be explained, on the basis of differences in the interpretation of the results, by individual methods and/or presence and evaluation of interfering substances (such as ascorbic acid, saccharides and carotenoids) (Gallardo et al., 2006; Stratil et al., 2006). Furthermore, the antioxidant activity of a substance can vary from method to method depending on factors such as antioxidant solubility, oxidation state, medium pH and type of oxidation-prone substrate (Stratil et al., 2006).

The correlation between total phenol content by FCR and by HPLC–DAD was also studied. The correlation coefficient for the seed extracts was 0.63. However, significantly better correlations between these two methods were obtained for the sprout and bread extracts ($R^2 = 0.99$, and $R^2 = 0.78$, respectively). Amaranth and wheat extracts results were not included in correlation analysis due to the low level of identified polyphenols by HPLC. The lower correlation obtained for quinoa and buckwheat seeds extracts could be due to the presence of non-phenolic compounds with antioxidant capacity in the seed extracts. However, in the sprouted extracts, it appears that most of the antioxidant activities were due to the presence of polyphenols ($R^2 = 0.99$). These results suggest that total phenol content assay (by FCR), although less informative than the chromatographic determination, can be used as a quick screening method for total phenol levels (Stratil et al., 2006). However, chromatographic determination is still necessary when information on the type and quantity of the polyphenols present is required.

4. Conclusions

The total phenol content and antioxidant activity amongst the pseudocereal seed extracts was highest in buckwheat ($p < 0.01$) and decreased in the order buckwheat > quinoa > amaranth. Analysis by using HPLC–DAD showed that quinoa and buckwheat represent the best sources of polyphenols among the studied seeds and consist predominantly of quercetin and kaempferol glycosides in quinoa, and catechin and quercetin glycosides in buckwheat.

Sprouting resulted in an overall increase in the total phenol content and antioxidant capacity and highest values were obtained for the buckwheat sprout extracts. In particular, kaempferol and quercetin glycosides in quinoa seeds increased significantly upon sprouting and reached 56.0 and 66.6 μmol/100 g, respectively. Conversely, bread baking (i.e. high temperatures) had a negative impact on antioxidant properties. Yet, the pseudocereal-containing breads made using quinoa and buckwheat flour still contained polyphenols in significant quantities, particularly the 100% quinoa and sprouted buckwheat breads.

All of the pseudocereals-containing gluten-free breads showed significantly higher antioxidant capacity and total phenol content compared to the gluten-free control. Also, buckwheat, 100% quinoa and sprouted buckwheat breads had significantly higher antioxidant capacity and total phenol content than wheat bread. Therefore, these pseudocereal seeds represent feasible ingredients in gluten-free baking for increasing the antioxidant properties and phenolic content of gluten-free breads, and improving their overall nutritional quality. As previously discussed, improving the nutritional quality of gluten-free products is essential, as the presently available gluten-free products in the market have been shown to be of poor nutritional quality.

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