Food Chemistry 166 (2015) 380–388

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Characterisation of phenolics, betanins and antioxidant activities in seeds of three *Chenopodium quinoa* Willd. genotypes

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ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 4 June 2014
Accepted 5 June 2014
Available online 14 June 2014

Keywords:
Quinoa
Betacyanin
Betanin
Free phenolics
Conjugated phenolics
Polyphenols
Antioxidant activity

ABSTRACT

Quinoa (*Chenopodium quinoa* Willd.) is known for its exceptional nutritional value and potential health benefits. The present study identified the composition of different forms of extractable phenolics and betacyanins of quinoa cultivars in white, red and black, and how they contribute to antioxidant activities. Results showed that at least 23 phenolic compounds were found in either free or conjugated forms (liberated by alkaline and/or acid hydrolysis); the majority of which were phenolic acids, mainly vanillic acid, ferulic acid and their derivatives as well as main flavonoids quercetin, kaempferol and their glycosides. Betacyanins, mainly betanin and isobetanin, were confirmed for the first time to be the pigments of the red and black quinoa seeds, instead of anthocyanins. Darker quinoa seeds had higher phenolic concentration and antioxidant activity. Findings of these phenolics, along with betacyanins in this study add new knowledge to the functional components of quinoa seeds of different cultivar background.

1. Introduction

Quinoa (*Chenopodium quinoa* Willd.) has received much attention in most recent years because of its exceptional nutritional value and potential health benefits. Although quinoa originated from the Andean region in South America, its high genetic variability has allowed for cultivars to be adapted for growth in many parts of the world, including drought and high salinity regions (Jacobsen, 2003). Production of quinoa seeds is also currently being explored in low climate regions such as in Ontario, Canada. Quinoa seeds are the main edible parts of the crop and are available in at least three different colours including white, red and black (Vaughan, Geissler, & Nicholson, 2009). Quinoa and quinoa products are rich in not only macronutrients such as protein, polysaccharide and fats, but also micronutrients such as polyphenols, vitamins and minerals (Repo-Carrasco-Valencia, Hellström, Pihlava, & Mattila, 2010; Vega-Gálvez et al., 2010). Polyphenols, including phenolic acids, flavonoids and tannins make up the bioactive secondary plant metabolites that contribute to diverse physiological properties such as antimicrobial, antioxidant, anti-inflammatory, antitumor and anti-carcinogenic effects (Benavente-García & Castillo, 2008). These beneficial phytochemicals may significantly differ in different cultivars of the same plant. Meanwhile, highly pigmented fruits, vegetables and grains are of particular interest due to strong antioxidant activities exerted by compounds such as anthocyanins and carotenoids. Dark coloured quinoa seeds have been shown to contain anthocyanins (Pasko et al., 2009). However, pigmentation in plants of the *Amaranthaceae* family such as amaranth, are well known to be governed by betalains in both the leaf and seed (Cai, Sun, & Corke, 2001; Repo-Carrasco-Valencia et al., 2010). However, no betalains have been reported in quinoa seeds. Betalains and anthocyanins may have similar physicochemical properties e.g. water-soluble, purple colour (absorbance at 520 nm), and are distributed and produced in similar tissues of vegetative plants, they are however, structurally and biosynthetically distinct. More importantly, it is well established that betalains and anthocyanins in plants are mutually exclusive, i.e. plants producing anthocyanins will not synthesise betalains and vice versa (Stafford, 1994). Betalains contain nitrogen and are synthesised from tyrosine then DOPA, whereas anthocyanins have no nitrogen and are from phenylalanine then cinnamates (Tanaka, Sasaki, & Ohmiya, 2008). Betalains are divided into two subgroups: the red–violet betacyanins and the yellow–orange betaxanthins (Strack, Vogt, & Schliemann,

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http://dx.doi.org/10.1016/j.foodchem.2014.06.018
0308-8146/

2003). For these reasons, there is a need to clarify and confirm the identities of the pigments in coloured quinoa seeds, i.e. whether they are anthocyanins or betalains.

Phenolics and saponins have been studied for quinoa seed, but only the free forms of phenolics in the polar extract were identified (Gómez-Caravaca, Segura-Carretero, Fernández-Gutiérrez, & Caboni, 2011). However, it is well known that phenolics exist in conjugated forms, even in the polar extractable fraction (Kim, Tsao, Yang, & Cui, 2006). These extractable phenolics in conjugated form are different from the bound phenolics that are attached to cell wall structures such as lignins. Extractable phenolics, both free and conjugated, can often be released by acid and alkaline hydrolyses which are commonly employed to aid the quantification and identification of free and bound polyphenols (Li, Deng, Liu, Loewen, and Tsao, 2012; Li, Deng, Zhu, et al., 2012; Ross, Beta, & Arntfield, 2009; Sani, Iqbal, Chan, & Ismail, 2012). These may not only be the glycosides or derivatives that can be separated and detected in a common HPLC method, in fact our preliminary study showed that the phenolic profile of the crude extract was similar to that of the free phenolics fraction which was partitioned into the organic layer (discussed later). As the consumption of quinoa becomes increasingly popular, there is a need to systematically examine the bioactive components such as phenolics and betalains in quinoa, in order to improve and maximise the nutritional quality of this super food. The objectives of this study were to characterise and identify the free and conjugated phenolic compounds in extracts of three typical coloured quinoa genotypes, to assess the potential health benefits by antioxidant assays, and to find out whether the pigments of coloured quinoa seeds are in fact betalains instead of anthocyanins. Information from this study will add new knowledge to the nutritional composition and help in the development of high quality quinoa cultivars that can be adapted to different climates such as Canadian environment, and functional foods with optimal nutritional values.

2. Materials and methods

2.1. Quinoa seeds

White, red and black quinoa seeds were purchased locally in Guelph, Ontario (July 2013). The seeds (500 g) were ground separately with a commercial coffee blender into fine powder and stored in polyethylene tubes at −80 °C prior to extraction.

2.2. Chemicals and reagents

All standard reference materials including *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, kaempferol, catechin, epicatechin, *p*-coumaric acid, ferulic acid, caffeic acid, vanillic acid, quercetin-3-rutinoside (rutin), 5-hydroxymethyl-2-furan-2-carbaldehyde (HMF), 1,3,5-tri(2-pyridyl)-2,4,6-triazine (TPTZ), *L*-ascorbic acid, gallic acid, Folin–Ciocalteu's phenol reagent, fluorescein, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox and 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma (St. Louis, MO, USA). 5-Methoxymethylfuran-2-carbaldehyde (MMF) standard was obtained from Enamine LLC (Monmouth Jct, NJ). Sodium acetate, ferric chloride hexahydrate, sodium phosphate monobasic, sodium phosphate dibasic and HPLC-grade solvents, including methanol, formic acid and hydrochloric acid (HCl) were purchased from Caledon Laboratories (Georgetown, ON, Canada).

2.3. Sample extraction and fractionation

Hydrophilic phytochemicals, mainly phenolic compounds, were extracted with acidified aqueous methanol. Quinoa seed powder (1.5 g) was accurately weighed and transferred into a 50 mL polypropylene tube (Mississauga, Ontario) and mixed with 15 mL 70% methanol containing 0.1% HCl (*v*/*v*). The extraction was carried out on a rotary shaker (Scientific Industries Inc., USA) at 400 rpm for 2 h at room temperature followed by 15 min ultra-sonication (VWR, Mississauga, Ontario, Canada). The mixture was centrifuged at 6000 rpm for 15 min (Eppendorf centrifuge 5810R, Brinkman Instruments Inc., Westbury, NY). The supernatant was collected, and the residue re-extracted twice more. The combined supernatant was topped up to 45 mL, and used as crude extract for further studies on the different bioactive components.

To obtain the free and conjugated phenolic fractions, a 1.5 mL aliquot of the crude extract (containing all extractable compounds) was evaporated to dryness using a Savant Speed Vac Concentrator attached to a Labconco CentriVap Cold Trap, and re-suspended in 0.5 mL acidified water (pH 2 with HCl). This was partitioned with 1 mL of diethyl ether/ethyl acetate (DE/EA, 1:1, *v*/*v*), repeated three times, and the combined organic layer, the free phenolics (FP) fraction, was evaporated under N_2 and reconstituted in 70% methanol, and filtered using Phenex-NY 4 mm 0.2 μm syringe filters (Sigma–Aldrich Co., St. Louis, MO) prior to HPLC analysis.

Conjugated phenolics remaining in the aqueous layer was first treated with 250 μL of 10 M NaOH (final NaOH concentration of 2 M) and rotate mixed at room temperature overnight. The hydrolysate was acidified with HCl to pH 2 and the liberated phenolics were extracted three times with DE/EA as mentioned above. The resulting organic layer contained base-hydrolysable phenolics (BHP). The remaining aqueous extract was subsequently hydrolysed with HCl (final HCl concentration of 2 M) and heated at 85 °C for 1 h. The solution was again partitioned with DE/EA three times, combined and dried under N_2 and reconstituted in 70% methanol and filtered similarly before analysis. This fraction contained acid-hydrolysable phenolics (AHP).

To extract betalains, 1.5 g of ground quinoa seeds were mixed with 15 mL of 50% methanol containing HCl (pH 3) in a 50 mL polypropylene tube similar to that described above, and sonicated for 15 min. The homogenate was centrifuged at 6000 rpm for 15 min. The supernatant was collected and the residue re-extracted the same way for two more times. The combined supernatant was subjected HPLC analysis after filtered through a 0.2-μm PTFE membrane filter (VWR International, ON, Canada). Beetroots (*Beta vulgaris* L.), purchased from a local supermarket (Guelph, Ontario, Canada), were freeze-dried and ground into powder and extracted following the same protocol as quinoa samples. Beetroot extract was used as a standard reference material along with authenticated betanin standards (Nemzer et al., 2011).

2.4. Determination of total phenolic content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu assay with modifications (Li, Deng, Liu, et al., 2012; Li, Deng, Zhu, et al., 2012; Singleton & Rossi, 1965). Briefly, 25 μL of each sample and standards were transferred into appropriate wells of a 96-well plate in triplicate, mixed with 125 μL of 10-fold freshly diluted Folin–Ciocalteu phenol reagent, allowed to react for 10 min, and then added 125 μL of 7.5% sodium carbonate (w/v), incubated for 60 min prior to measuring the absorbance at 765 nm using a UV/Vis microplate reader against a reagent blank (EL 340, Bio-Tek Instruments Inc., Winooski, VT, USA). The TPC was expressed as milligrams of GA equivalents per gram of quinoa seed (mg GAE/g) (r^2 = 0.997).

2.5. Determination of total flavonoid content

The total flavonoids content (TFC) was measured according to established method by Zou, Chang, Gu, and Qian (2011). A 25 μL

aliquot of appropriately diluted sample solution (with methanol) or standard solution of catechin (0, 15.625, 31.25, 61.25, 125, 250 and 500 mg/L) was mixed with 110 μL of a 0.066 M $NaNO_2$ solution in a well of 96-well plate. After 5 min, 15 μL of a 0.75 M $AlCl_3$ solution was added and allowed to stand for additional 5 min, and then finally 100 μL of 0.5 M NaOH solution was added to the mixture and let react for another 6 min. Absorbance of the mixture was determined at 510 nm versus a prepared water blank using the EL 340 UV/Vis microplate reader. The TFC was expressed as milligrams of catechin equivalents per g quinoa sample (mg CE/g) (r^2 = 0.999).

2.6. Radical scavenging activity assays

The radical scavenging activity of the extracts and fractions was assessed using a slightly modified DPPH method of Li, Deng, Liu, et al. (2012) and Li, Deng, Zhu, et al. (2012). Briefly, 25 μL of appropriately diluted samples or trolox solutions (62.5, 125, 250, 500, 750, and 1000 μM) were added to 200 μL of DPPH solution (350 μM, dissolved in methanol) in a well of 96-well plate. The mixture was allowed to react at room temperature in the dark for 4 h before the absorbance was measured at 517 nm against a methanol blank using the same microplate reader as described above. The DPPH radical scavenging activity was expressed as micromoles of trolox equivalents (TE) per gram of sample (μmol TE/g) (r^2 = 0.999).

2.7. Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power (FRAP) assay was determined according to the method of Li, Deng, Liu, et al. (2012); Li, Deng, Zhu, et al. (2012) with a few modifications. Briefly, the FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6) with 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ at a ratio of 10:1:1 (v/v/v). Ten microlitre of a properly diluted sample or a series of *L*-ascorbic acid solutions (62.5, 125, 250, 500, 750, and 1000 μM) and 300 μL of freshly prepared FRAP reagent were added in a well and incubated at room temperature for 120 min, after which the absorbance was read 593 nm against the reagent blank. The FRAP antioxidant activity was expressed as μmol ascorbic acid equivalent (AAE) per gram quinoa (μmol AAE/g) (r^2 = 0.997).

2.8. Oxygen radical absorption capacity (ORAC) assay

The ORAC assay was conducted according to reported protocols with slight modifications (Li, Deng, Liu, et al., 2012; Li, Deng, Zhu, et al., 2012). To each well of a polystyrene 96-well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany), 25 μL of an appropriately diluted sample, blank or a series of standards of trolox solutions (6.25, 12.5, 25, 50 and 100 μmol/L) were added and mixed with 150 μL of working fluorescein solution (8.68×10^{-5} mM in phosphate buffer, pH 7.4), and incubated for 30 min at 37 °C. Subsequently, 25 μL of AAPH (153 mM in phosphate buffer) was added to each well to initiate the reaction. The fluorescence (excitation/emission wavelength = 485/528 nm) was read every minute for 120 min or until it reached zero in a fluorescence plate reader equipped with an automatic thermostatic holder (PLX 800, Bio-Tek Instruments, Inc., Winooski, VT, USA). A calibration curve was constructed daily by plotting the calculated differences of area under the fluorescein decay curve between the blank and the sample. The ORAC values were expressed as μmol Trolox equivalent per gram quinoa sample (μmol TE/g) (r^2 = 0.998).

2.9. Quantification of individual compounds by HPLC-DAD

Individual phenolics and betalains were analysed using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) system consisted of a degasser, a binary gradient pump, a thermoautosampler and a diode array detector (DAD), and the ChemStation software. Separation was in a Kinetex XB-C18 column (100 mm × 4.6 mm, 2.6 μm) (Phenomenex Inc., Torrance, CA). The binary mobile phase consisted of 5% formic acid in water (*v*/*v*) (solvent A) and 95% methanol mixed with 5% acetonitrile (*v*/*v*) (solvent B). The solvent gradient was as follows: 0–40 min, 0–80% B; 40–42 min, 80–100% B; 42–44 min, 100% B; 44–44.5 min, 100–0% B. Injection volume was 7 μL and flow rate was kept at 0.7 mL/min for a total run time of 50 min. Peaks were monitored at 280 nm, 360 nm, 520 nm and 536 nm for the different phenolic compounds and betalains. Quantification was performed with external standards using standard curves generated between 50 and 0.25 mg/L ($r^2 \geqslant 0.9999$).

2.10. Mass spectrometry analysis

Identification and structural confirmation of the polyphenols were done using a Dionex UHPLC UltiMate 3000 liquid chromatograph interfaced to an amaZon SL ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). An Agilent Poroshell 120 column (150 mm × 4.6 mm, 2.7 μm) was used for chromatographic separation. The mass spectrometer electrospray capillary voltage was maintained at 4.5 kV and the drying temperature at 220 °C with a flow rate of 10 L/min. Nebulizer pressure was 40 psi. Nitrogen was used as both nebulizing and drying gas. Helium was used as collision gas at a pressure of 60 psi. The mass-to-charge ratio (*m*/*z*) was scanned across the range 50–1200 *m*/*z* in enhanced resolution negative-ion auto MS/MS mode. The Smart Parameter Setting (SPS) was used to automatically optimise the trap drive level for precursor ions. The instrument was externally calibrated with the ESI TuneMix (Agilent). The initial mobile phase conditions for the LC were 98% solvent A consisting of 0.1% formic acid in water and 2% solvent B consisting of 0.1% formic acid in acetonitrile (*v*/*v*). The gradient went to 98% solvent B in 30 min. The flow rate was maintained at 0.4 mL/min. UV monitoring was at 280 nm for the phenolic compounds. Data acquisition and processing were performed using Esquire Control software.

Identification and structural confirmation of betalains were carried out using a Shimadzu HPLC system (Kyoto, Japan) coupled with a triple quadrupole IONICS 3Q Molecular Analyzer 220 (IONICS, Bolton, Canada). An Agilent ZORBAX SB-C18 column (2.1 × 100 mm, 3.5 μm) was used for separation together with a binary mobile phase consisted of solvent A (99.9% H_2O + 0.1% formic acid) and solvent B (99.9% methanol + 0.1% formic acid) (*v*/*v*), with the following gradient program: 0–10 min, gradient 0–25% B; 10–11 min, gradient 25–100% B; 11–14 min, isocratic 100% B; 14–15 min, gradient 100–0% B; 15–21 min, isocratic 0% B. The column was kept at 25 °C. The flow rate was set at 0.4 mL/min and the injection volume was 10 μL. ESI in positive mode was used for the multiple reactions monitoring (MRM) data collection. The drying gas, heating gas and nebulizer gas were set at 100, 375 and 350 (arbitrary unit), respectively. The hot source induced desolvation (HSID) and ESI probe temperatures were controlled at 250 °C and 300 °C, respectively. The ion spray voltage was set at 5200 V. The dwell time for MRM data collection was 100 ms.

2.11. Statistical analysis

Results were expressed as mean value ± standard deviation of three independent replicates. One-way analysis of variance (ANOVA) was used to compare the means. Differences were considered significant at $p \leqslant 0.05$. All statistical analyses were performed using SPSS (Version 18.0, Chicago, IL).

3. Results and discussion

3.1. Total phenolic and flavonoid contents

The TPC of the FP fraction were significantly higher in darker coloured quinoa seeds with the black quinoa having the highest concentration at 5.18 mg GAE/g quinoa samples. Similar trend was found for TPC of the BHP and AHP fractions of all three quinoa seeds (Fig. 1(A)). Higher TPC in the BHP and AHP fractions suggest that many phenolics exist in conjugated forms soluble in the crude extract by acidified 70% methanol extraction. The total phenolic index (TPI), sum of all individual concentrations, of the FP fractions also increased with colour intensity at 467, 635 and 682 mg/kg, respectively in the white, red and black quinoa (Table 1). Flavonoids represented a small portion of the total phenolics as determined in both TFC and in individually identified compounds. TFC in the FP and BHP fractions in red and black quinoa were 1–2-folds higher than that in the white quinoa (Fig. 1B). The TPI of the BHP fraction were also higher than those in the FP except for the black quinoa, whereas the TPI in AHP were similar in all quinoas (Tables 1 and 2). Assessment of the TPC of AHP needs to be cautious as when the aqueous layer was hydrolysed with 2 M HCl at 85 °C for 1 h, it produced very high concentrations of furan derivatives (HMF, furfural and MMF). These are degradation products of free sugar molecules that were either from the original crude extract or produced from the alkaline hydrolysis prior to the acid hydrolysis. Although these compounds did not seem to significantly affect the TPC value at low concentrations in our previous studies (data not shown), we found that if the concentration of these compounds were >3000 mg/kg, as was found in the AHP in our current study (Table 2), they can react with the Folin–Ciocalteu reagent and lead to significant overestimation of TPC (Chen et al., 2014). This means that a portion of the TPC in the AHP reported in Table 1 might be derived from furan derivatives. It needs to be pointed out that the acid hydrolysis condition adopted in this study is widely used in polyphenol research (Ross et al., 2009; Sani et al., 2012).

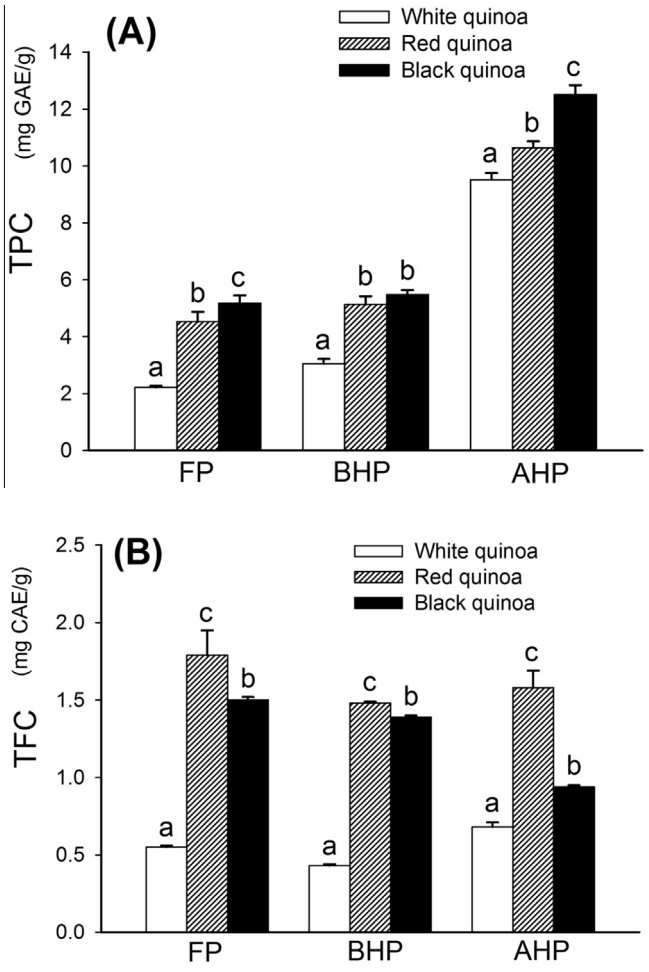

Fig. 1. Total phenolic (TPC, panel A) and total flavonoid contents (TFC, panel B) of the hydrophilic extract of the white, red and black quinoa seeds. TPC is in mg gallic acid equivalent/g seed, and TFC in mg catechin equivalent/g seed. Values with different letters are significantly different from each other (values are mean ± SD, $n = 3$, $p \leqslant 0.05$).

3.2. Characterisation of free phenolics

The retention time, UV/Vis and mass spectral data of all extractable phenolic compounds, including the free phenolics in the FP fraction, and the conjugated phenolics released upon alkaline (BHP) or acid (AHP) hydrolysis are summarised in Table 1. A total of 23 phenolic compounds were identified in the FP fraction based on matching retention time and UV/Vis spectral data with the standards and molecular and fragmentation ions information from the LC/ESI-MS/MS experiments (Table 1, Fig. 2). Peaks 3–4, 7, 11–14 and 16–20 are the major phenolics detected in all three fractions (Fig. 3A). Peaks were positively identified and confirmed when all data matched standard reference compounds. Those with no available standards are tentatively identified according to the UV/Vis and mass data (Table 1).

Peaks 1 and 2 were only found in the red and black quinoa seeds. The molecular ion of peak 1 was m/z 153 $[M-H]^-$, and based on the retention time and UV spectrum, it was confirmed to be protocatechuic acid. Peak 2 had a molecular ion of m/z 325 $[M-H]^-$ and a fragment ion at 163 $[M-H]^-$ suggesting loss of a glucose moiety. Peak 2 was tentatively identified as *p*-coumaric acid-glucoside, which has been found in flaxseed previously (Johnsson et al., 2002). The molecular ion of peak 3 was m/z 137 $[M-H]^-$, and its retention time and other spectral data matched with those of *p*-hydroxybenzoic acid. Peaks 3 and 4 were found in all three quinoa samples. The MS/MS ion m/z 167 $[M-glucose]^-$ of peak 4 (m/z 329 $[M-H]^-$) suggests it is likely a phenolic acid glucoside. The UV spectrum of the peak was almost identical to that of vanillic acid, peak 4 was therefore tentatively identified as vanillic acid 4-glucoside, which has been reported by others (Dini, Carlo Tenore, & Dini, 2004). Peak 6 was mainly found in black quinoa and positively identified as caffeic acid. Peak 7 (m/z 167 $[M-H]^-$) was confirmed to be vanillic acid and it was one of the major phenolic compounds common to all quinoas. Peak 11 showed a molecular ion $[M-H]^-$ of m/z 283. The definite identity of this minor component is yet to be determined, however, this peak could be one of the three compounds (acacetin, questin or phenylethyl caffeate) postulated before by Gómez-Caravaca et al. (2011) due to identical molecular weight and similar structural feature. Three cinnamic acids, *p*-coumaric acid (peak 12), ferulic acid (peak 13) and isoferulic acid (peak 15) were positively identified by comparing all spectral data with the standards. Peak 14 was the most prominent peak detected at 280 nm. While it had the same retention time as one of the standard (sinapic acid) (Fig. 3A, top), it was not sinapic acid. Rather, based on the similarity of UV/Vis data to ferulic acid and its MS and MS/MS daughter ions, it is highly likely to be ferulic acid-glucoside (Gómez-Caravaca et al., 2011).

Peaks 16–21 were flavonoid aglycones or their glycosides whose peak intensities were higher at 360 nm (data not shown). Other than peak 16, which was tentatively identified as kaempferol 3, 7-dirhamnoside due to the unavailability of a commercial standard, all others were positively identified (Fig. 3A, Table 1). Peak 16 has been reported by others along with kaempferol 3-glucoside

Table 1
HPLC-DAD-MS identification and concentration (mg/kg) of individual free phenolic compounds in white, red and black *quinoa* species.[A].

Free phenolic compounds			UV/VIS (nm)	Molecular weight	*m/z*	MS/MS		WQ	RQ	BQ
Peak#	Time	Name								
1	5.665	3,4-Dihydroxybenzoic acid	260, 295	154	153	109	Both	ND	29.82 ± 0.67^{b}	47.38 ± 1.39^{a}
2	8.344	*p*-Coumaric acid 4-glucoside	280, 320	326	325	163	MS	ND	19.34 ± 1.21^{b}	31.31 ± 1.88^{a}
3	8.650	*p*-Hydroxybenzoic acid	260, 300	138	137	137, 113	Both	15.84 ± 0.72^{b}	17.24 ± 0.49^{a}	16.97 ± 0.31^{a}
4	9.484	Vanillic acid 4-glucoside	262, 290	330	329	167	MS	23.09 ± 1.75^{b}	24.62 ± 1.12^{b}	27.39 ± 1.09^{a}
5	9.708	2,5-Dihydroxybenzoic acid	260, 295	154	153	153	Both	0.59 ± 0.06^{a}	0.73 ± 0.13^{a}	0.28 ± 0.04^{b}
6	11.432	Caffeic acid	270	180	179	179, 135	Both	4.39 ± 0.02^{c}	4.94 ± 0.03^{b}	19.61 ± 0.02^{a}
7	11.759	Vanillic acid	260, 295	168	167		Both	63.45 ± 2.22^{b}	70.02 ± 1.71^{a}	39.03 ± 2.04^{c}
8	12.891	Epigallocatechin	279	305	304	233	MS	1.55 ± 0.03^{c}	2.71 ± 0.04^{b}	3.21 ± 0.04^{a}
9	14.036	Epicatechin	279	290	289	245	Both	4.62 ± 0.12^{a}	3.89 ± 0.11^{c}	4.23 ± 0.18^{b}
10	14.827	Vanillin	262, 290	152	151		Both	4.19 ± 0.09^{c}	6.65 ± 0.24^{b}	8.39 ± 0.39^{a}
11	15.349	Acacetin/questin/apigenin-7-methylether	290, 350	284	283.1		MS	10.08 ± 0.73^{b}	13.33 ± 0.48^{b}	16.56 ± 0.55^{a}
12	15.560	*p*-Coumaric acid	320	164	163		Both	13.01 ± 0.58^{c}	22.73 ± 0.54^{b}	29.52 ± 1.06^{a}
13	17.584	Ferulic acid	330, 290	194	193	149	Both	37.52 ± 2.61^{c}	58.41 ± 1.82^{a}	47.21 ± 1.77^{b}
14	18.591	Ferulic acid 4-glucoside	330, 290	356	355	355, 193	MS	131.97 ± 2.26^{c}	151.65 ± 3.17^{b}	161.39 ± 0.64^{a}
15	19.385	Isoferulic acid	330, 290	194	193	149	Both	8.21 ± 0.26^{c}	19.44 ± 0.62^{a}	12.35 ± 0.49^{b}
16	20.614	Kaempferol 3,7-dirhamnoside	260, 355	578	577	285	MS	20.61 ± 0.94^{c}	27.00 ± 1.14^{b}	29.41 ± 0.52^{a}
17	21.198	Kaempferol 3-galactoside	256, 355	448	447	285	Both	24.01 ± 1.42^{b}	28.78 ± 1.77^{a}	23.32 ± 0.81^{b}
18	21.371	Quercetin-3-rutinoside	256, 355	610	609	301	Both	57.10 ± 2.76^{b}	71.04 ± 1.99^{a}	57.63 ± 1.47^{b}
19	23.470	Kaempferol 3-glucoside	256, 355	448	447	285	Both	13.29 ± 1.33^{c}	16.42 ± 1.58^{b}	24.08 ± 1.69^{a}
20	23.813	Quercetin 3-arabinoside	266, 350	434	433	301, 179	Both	24.97 ± 1.19^{b}	26.46 ± 1.28^{b}	65.79 ± 0.71^{a}
21	26.585	Quercetin	260, 365	302	301	301	Both	5.27 ± 0.82^{c}	11.82 ± 0.41^{b}	12.99 ± 0.11^{a}
22	29.740	Kaempferol	265, 365	286	285	193	Both	2.56 ± 0.08^{a}	1.18 ± 0.08^{c}	1.58 ± 0.07^{b}
23	35.140	Biochanin A	262	284	283	283	Both	0.67 ± 0.27^{c}	6.44 ± 0.45^{a}	2.42 ± 0.79^{b}
Total phenols index (TPI)								466.99 ± 3.27^{c}	634.66 ± 5.87^{b}	682.05 ± 4.73^{a}

TPI: total phenolic index (sum of individual phenolic concentrations, mg/kg quinoa); WQ: white quinoa; RQ: red quinoa; BQ: black quinoa; Both: identified by retention time; UV/Vis and LC–MS/MS data; MS: tentatively identified by LC–MS/MS only.
[A] Values are mean ± SD, $n = 3$. ND = not detected. Values followed by the same letter in the same row are not significantly different ($p < 0.05$).

Table 2
HPLC-DAD-MS identification and concentration (mg/kg) of alkaline and acid hydrolysable phenolics in white, red and black *quinoa* species.[A].

Peak#	Time	Name	BHP			AHP		
			WQ[C]	RQ	BQ	WQ	RQ	BQ
1	6.076	3,4-Dihydroxybenzoic acid	ND	45.82 ± 0.67^{a}	31.38 ± 1.39^{b}			
A1	6.113	HMF				2444.09 ± 213.55^{a}	2327.86 ± 188.29^{a}	2237.01 ± 344.75^{a}
B1	7.470	2,4-Dihydroxybenzoic acid	5.35 ± 0.36^{c}	21.06 ± 0.15^{a}	9.11 ± 0.23^{b}			
3	8.663	*p*-Hydroxybenzoic acid	51.84 ± 0.72^{a}	45.24 ± 0.49^{b}	31.97 ± 0.31^{c}	85.95 ± 1.04^{a}	49.38 ± 2.38^{b}	47.95 ± 1.89^{b}
B2	9.146	Naringin	ND	29.83 ± 1.97^{a}	18.37 ± 1.39^{b}			
4	9.492	Vanillic acid 4-glucoside	37.83 ± 2.46^{a}	35.94 ± 3.42^{a}	22.00 ± 2.31^{b}			
7	11.773	Vanillic acid	224.39 ± 6.02^{a}	234.94 ± 4.03^{a}	207.61 ± 0.02^{b}	229.51 ± 6.46^{b}	176.61 ± 3.17^{c}	257.50 ± 2.58^{a}
A2	9.890	Furfural				197.44 ± 3.29^{b}	237.77 ± 6.74^{a}	197.54 ± 8.35^{b}
10	14.827	Vanillin	5.62 ± 0.12^{c}	23.89 ± 0.11^{a}	8.23 ± 0.18^{b}	12.32 ± 1.64^{ab}	13.91 ± 1.35^{a}	10.24 ± 1.07^{b}
11	15.349	Acacetin/questin/apigenin-7-methylether	18.19 ± 0.09^{a}	5.65 ± 0.24^{b}	4.39 ± 0.39^{c}			
12	15.603	*p*-Coumaric acid	34.08 ± 0.73^{a}	23.33 ± 0.48^{b}	21.56 ± 0.55^{c}	51.11 ± 3.95^{b}	95.19 ± 5.67^{a}	53.32 ± 7.67^{b}
A3	15.685	MMF				655.53 ± 8.54^{c}	1102.49 ± 8.97^{a}	746.11 ± 5.47^{b}
13	17.610	Ferulic acid	207.52 ± 2.61^{a}	238.41 ± 1.82^{b}	257.21 ± 1.77^{c}	28.77 ± 1.78^{b}	30.35 ± 3.02^{a}	22.84 ± 1.15^{c}
14	18.591	Ferulic acid 4-glucoside	25.97 ± 2.26^{a}	19.65 ± 3.17^{b}	12.39 ± 0.64^{c}			
16	20.614	Kaempferol 3,7-dirhamnoside	4.61 ± 0.94^{c}	14.00 ± 1.14^{a}	7.41 ± 0.52^{b}			
17	21.198	Kaempferol 3-galactoside	8.01 ± 1.42^{b}	12.78 ± 1.77^{a}	10.32 ± 0.81^{ab}			
18	21.400	Quercetin-3-rutinoside	17.10 ± 2.76^{a}	11.04 ± 1.99^{b}	7.63 ± 1.47^{c}			
19	23.470	Kaempferol 3-glucoside	3.29 ± 1.33^{a}	6.42 ± 1.58^{a}	4.08 ± 1.69^{a}			
20	23.764	Quercetin 3-arabinoside	18.97 ± 1.19^{c}	8.46 ± 1.28^{b}	5.79 ± 0.71^{a}			
B3	24.856	Puerarin	15.27 ± 0.82^{c}	6.82 ± 0.41^{b}	9.99 ± 0.11^{a}			
21	26.603	Quercetin	18.56 ± 0.08^{a}	16.18 ± 0.08^{b}	15.58 ± 0.07^{b}	11.55 ± 0.86^{c}	28.18 ± 0.42^{a}	17.26 ± 0.47^{b}
22	29.353	Kaempferol	4.67 ± 0.27^{a}	4.44 ± 0.45^{ab}	3.42 ± 0.79^{b}	31.86 ± 0.82^{a}	18.29 ± 0.93^{c}	20.69 ± 0.32^{b}
Total phenols index (TPI)			701.27 ± 5.04^{b}	758.08 ± 3.76^{a}	657.06 ± 3.13^{b}	685.17 ± 4.23^{a}	663.79 ± 4.36^{b}	666.72 ± 3.65^{b}

TPI: total phenolic index (sum of individual phenolic concentrations, mg/kg quinoa); WQ: white quinoa; RQ: red quinoa; BQ: black quinoa; HMF: 5-hydroxymethyl-2-furan-2-carbaldehyde; MMF: 5-methoxymethylfuran-2-carbaldehyde.
[A] Values are mean ± SD, $n = 3$. ND = not detected. Values followed by the same letter in the same row are not significantly different ($p < 0.05$).

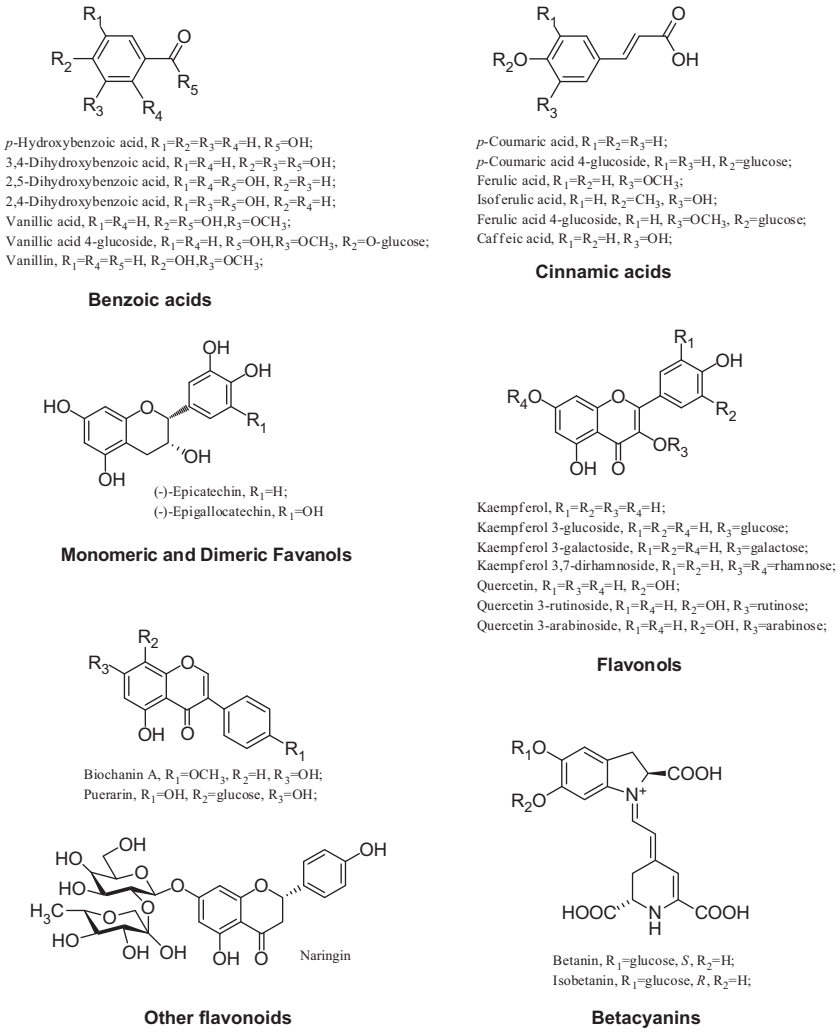

Fig. 2. Chemical structures of identified phenolic compounds and betacyanins found in quinoa seeds.

and quercetin 3-β-arabinoside (Dini et al., 2004; Gómez-Caravaca et al., 2011). Black quinoa had higher kaempferol 3-galactoside (Peak 20) than the other two quinoa samples. The major phenolic acids (both benzoic acids and cinnamic acids) and flavonoids were found to exist simultaneously in aglycone and glycoside forms (Fig. 3A, Table 1).

Peaks 8, 9 and 10 were minor components but positively identified as epigallocatechin, epicatechin and vanillin, respectively based on matching data with the standards and those reported by others (Gómez-Caravaca et al., 2011).

All phenolic compounds were quantified according to individual calibration curves of the standards. Those tentatively identified compounds, all of which were glucose or glycoside derivatives of known phenolic compounds were quantified using the calibration curve of the respective aglycones. Among the individual compounds of significant amount as listed in Table 1, phenolic acids (benzoic acid and cinnamic acid) and their derivatives were the main phenolics. Flavonoids were mainly quercetin and kaempferol and their glycosides. Not all compounds were found in all three quinoa samples.

All identified phenolic compounds, except protocatechuic acid and *p*-coumaric acid-glucoside, were found in all three cultivars. These two compounds (peaks 1 and 2) were only identified in the red and black quinoa samples. Ferulic acid-4-glucoside was the predominant phenolic compound at 132, 152 and 161 mg/kg, in the white, red and black quinoa, respectively. Other major phenolics include vanillic acid, ferulic acid and the flavonoid rutin, all of which were interestingly at the highest concentration in red quinoa (Table 1).

3.3. Characterisation of conjugated phenolics

The aqueous layer after partitioning to obtain the FP fraction contained extractable, but conjugated phenolics that are only water soluble. These phenolics may be associated with water soluble peptides or oligosaccharides as reported by others (Saulnier et al., 1999; Yokotsuka & Singleton, 1995). A total of 19 phenolic compounds were released after alkaline hydrolysis in all three quinoa samples, except peaks 1 and B1 (Fig. 3B) which were not detected in the BHP of the white quinoa (Table 2). Three new peaks B-1, B-2 and B-3 with t_R at 7.470, 9.492 and 24.856 min, showed their respective molecular ions $[M-H]^-$ at *m*/*z* 153, *m*/*z* 579 and *m*/*z* 415, however their identities are yet to be confirmed. All other phenolic compounds were the same as those identified in the FP fraction, but had significantly higher concentrations (Tables 1 and 2), suggesting a significant amount of phenolics in quinoa are in alkaline hydrolysable conjugation with other components. The major phenolic acids and flavonoids of BHP that were released in both aglycone and glycoside forms were also similar to the major peaks of FP. These phenolic compounds were identified by matching the retention time and spectral data with those of the standards and with data reported by others (Gómez-Caravaca

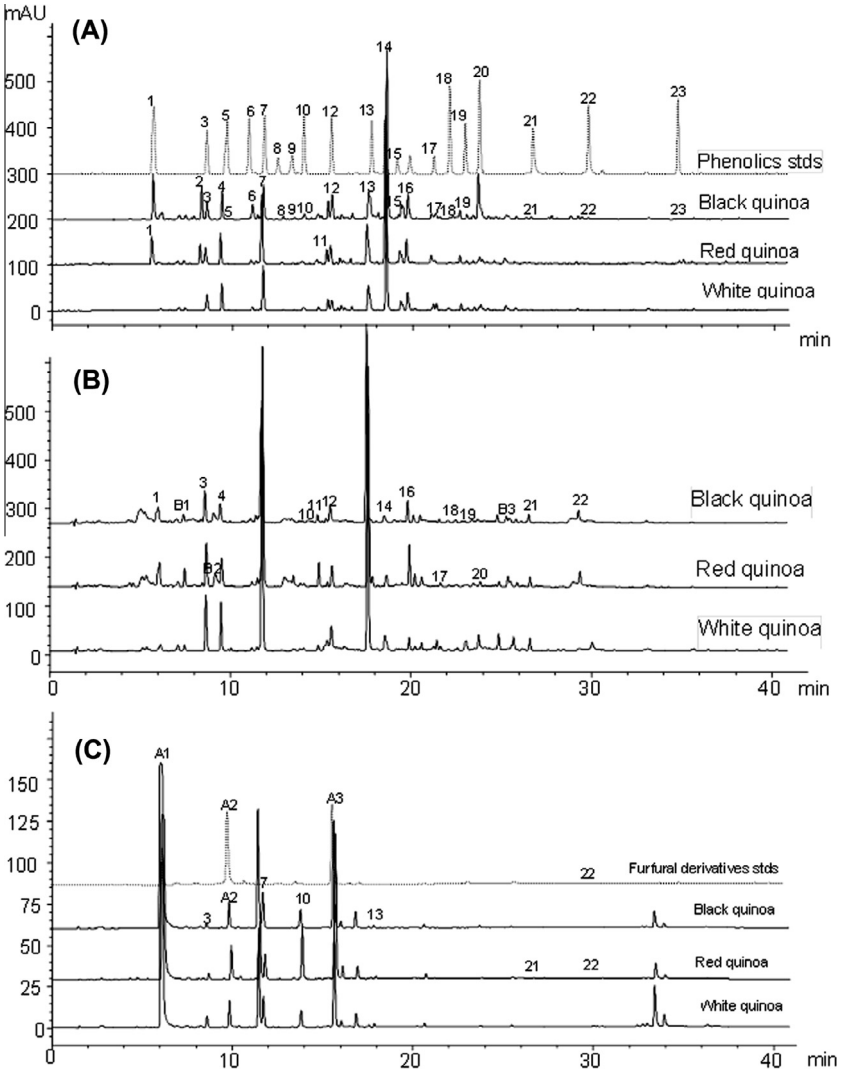

Fig. 3. HPLC profiles of the free (panel A), alkaline (panel B) and acid (panel C) hydrolysable phenolic compounds of the white, red and black quinoa. Peaks were detected at 280 nm. Refer to Table 1 for peak identities. Peaks B1, B2 and B3 were compounds only detected in the alkaline hydrolysable fraction (panel B), and peaks A1, A2 and A3 were sugar degradation products or derivatives HMF, furfural and MMF, respectively.

et al., 2011; Kuljanabhagavad & Wink, 2009; Madl, Sterk, Mittelbach, & Rechberger, 2006). Vanillic acid and ferulic acid again were the two overwhelmingly dominant extractable phenolics in BHP. Similar results were found in other cereal grains (Kim et al., 2006).

Sequential acid hydrolysis of the aqueous layer following BHP extraction led to positive identification of 7 AHP compounds, peaks 3, 7, 10, 12, 13, 21 and 22, all of which were identified in both the FP and BHP fractions of all three quinoas, however, different from the previous fractions, only aglycones of phenolic acids and flavonols were identified (Fig. 3, Table 2). Again a large amount of vanillic acid was further released by acid hydrolysis (176–257 mg/kg) in all three quinoas. *p*-Hydroxybenzoic acid (peak 3) instead of ferulic acid (peak 13) was the second dominant AHP followed by *p*-coumaric acid. Quercetin and kaempferol were the two flavonol aglycones identified in the AHP (Table 2). Eight phenolic compounds and three furfural derivatives have been found in treated samples after acid hydrolysis of these fractions.

As mentioned earlier, a commonly used acid hydrolysis method with 2 M HCl at 85 °C for 1 h produced a large amount of the sugar degradation products HMF (peak A1) and furfural (peak A2), and because of the presence of methanol, MMF (peak A3). These three peaks were positively identified by matching the retention time and HPLC-DAD/ESI-MS data of the standards. Because of the fact that these compounds absorb UV light at 280 nm, they are often misidentified as phenolic compounds (Ross et al., 2009). As our data show (Table 2), the concentrations of these sugar degradation products can be as much as 2444.09 mg/kg, extremely high compared with the phenolic compounds. The total of the three furan derivatives was >3000 mg/kg. For this reason, caution must be taken in characterising phenolic compounds resulting from acid hydrolysis.

3.4. Antioxidant activities

The antioxidant activities of the phenolic fractions were evaluated using three common chemical model systems, i.e. FRAP, DPPH, ORAC assays (Fig. 4). The antioxidant activities of the phenolic fractions showed highly positive correlation with TPC, especially in the FRAP and ORAC assays, with the correlation coefficient r^2 values ranging from 0.9865 to 0.9956; TPC and DPPH was also positive with slightly lower r^2 value between 0.8421 and 0.9076. FRAP values displayed significantly higher activity in AHP than FP and BHP fractions and was found to be comparable to values obtained in common beans (Zhang et al., 2014). ORAC values of the FP, BHP and AHP fractions were on the lower end when compared to

common beans and fruits and vegetables (Li, Deng, Liu, et al., 2012; Li, Deng, Zhu, et al., 2012; Zhang et al., 2014).

The structure of HMF and its derivatives have been shown by others to exert certain degree of antioxidant activities in the DPPH, FRAP and ORAC assays in a time- and dose-dependent (Li, Li, Qian, Kim, & Kim, 2009; Zhao et al., 2013). As shown in Table 2, the concentrations of HMF, MMF and furfural in the acid hydrolysate were collectively as high as >3000 mg/kg, and according to our research, concentrations at this level could contribute significantly to the TPC, FRAP and ORAC, but not DPPH. Considering the influence of HMF and its derivatives on antioxidant activities, we suggest the use of solid phase extraction (SPE) to remove free sugars from samples prior to acid hydrolysis (Sani et al., 2012; You et al., 2011).

3.5. Identification of betacyanins

The amaranthine type betacyanins are commonly found in the *Amaranthaceae* family of plants such as amaranth, in both the leaf and seed (Cai et al., 2001; Repo-Carrasco-Valencia et al., 2010). Quinoa seeds which belong to the same family but different genus as amaranth, have not been reported to contain betacyanins, but rather anthocyanins have been reported for coloured quinoa (Pasko et al., 2009), which is proven to be misidentification as a result of the present study.

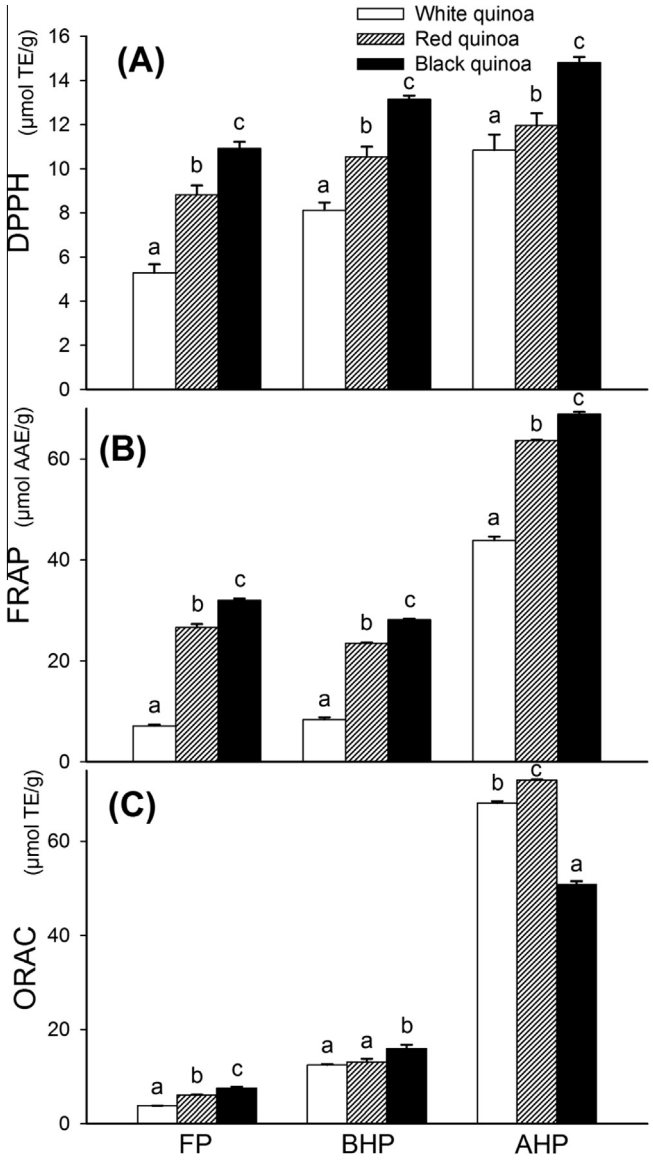

Fig. 4. Antioxidant activities as measure by DPPH (μmol Trolox equivalent TE/g), FRAP (μmol ascorbic acid equivalent AAE/g), and ORAC (μmol Trolox equivalent TE/g) assays. Values with different letters are significantly different from each other (values are mean ± SD, $n = 3$, $p \leqslant 0.05$).

The UV/Vis absorption spectra of betacyanins are similar to those of anthocyanins, however, betacyanins absorb at slightly longer wavelength at 536–538 nm instead of 520 nm, and the absorption is not affected as much by pH as anthocyanins. In the present study, only extracts of the red and black quinoa seeds showed peaks at 538 nm. Two peaks were detected in both coloured seeds at 8.5 min (peak 1) and 9.6 min (peak 2), both having the same molecular ion of m/z 551 $[M+H]^+$, and a fragment ion m/z 389 $[M-\text{glucose}+H]^+$ when analysed by positive ESI-MS (Supplemental data). Peak 1 was overwhelmingly dominant among the two. The UV/Vis spectra and MS data of the two peaks matched with those of betanin and isobetanin. Because of the lack of the standard reference materials for individual betanin and isobetanin (the reference material from Sigma was not valid and did not show any signals of betanin and isobetanin at 538 nm), and the fact that these two betacyanins are well known and characterised in beetroot, extract of beetroot was therefore used as a reference to confirm the identity of the two peaks in quinoa seed extracts (Nemzer et al., 2011). Two peaks at 8.6 and 9.6 min were detected at 538 nm in the beetroot extract, and further study by LC–MS showed exactly the same mass spectral data as those found in coloured quinoa seeds. Reversed-phase chromatography of beetroot suggests the early eluting peak 1 to be betanin, and peak 2 to be isobetanin (Nemzer et al., 2011). Based on the retention time, UV/Vis and MS data, and those reported in the literature on beets, we therefore conclude that peaks 1 and 2 in the extracts of the red and black quinoa seeds were betanin and isobetanin, respectively (Fig. 2 and Supplemental data). As far as we are aware of, this is the first report on the positive identification of betacyanins in coloured quinoa seeds. Our result also proved that the pigments of coloured quinoa seeds were not anthocyanins as previously reported by others. Betacyanins have been shown to have unique health promoting properties (Stintzing & Carle, 2004). Discovery of betacyanins in coloured quinoa seeds will certainly add more value to this increasingly popular functional grain.

4. Conclusion

Quinoa is an emerging pseudo cereal grain that has drawn much attention from consumers to industry and to scientists due to its richness of different nutrients and gluten free nature. The present study into the phenolic composition of the hydrophilic extract of quinoa seeds of three typical colours suggests that the phenolic compounds not only existed in free, but also in conjugated forms that can only be liberated by alkaline and/or acid hydrolysis. Our results also showed that the majority of the extractable phenolics were in conjugated forms. Betacyanins, mainly betanin and isobetanin, were confirmed for the first time to be the pigments of the red and black quinoa seeds, instead of anthocyanins. The total phenolic contents and betacyanins were dependent on quinoa seed colour. Darker quinoa seeds had higher phenolic concentration and antioxidant activity. Further analysis of the individual phenolic compounds revealed that at least 23 phenolic compounds were found in either free or conjugated forms, among them vanillic acid and ferulic acid and their derivatives were the main phenolic acids, and quercetin and kaempferol and their glycosides the main flavonoids. These compounds along with betacyanins have been known for many health promoting effects. Findings from this study provide much needed information

for adaptation and growing of quinoa cultivars with optimal nutritional values. The strong positive correlations between the phenolics and the antioxidant activities warrant further studies on the bioaccessibility, bioavailability and mechanism of action of these compounds and betacyanins on biomarkers related to health risks. Investigation into the bound phenolics, i.e. the non-extractable phenolics and their role *in vivo* is also a future research direction.

Acknowledgements

We would like to thank Dr. Dyanne Brewer and Dr. Armen Charchoglyan, Advanced Analysis Center, University of Guelph for providing mass spectra reports. This project is supported by the A-Base Research (#1004) of Agriculture & Agri-Food Canada (AAFC), and partially by the Ontario Soil and Crop Improvement Association and Katan Kitchens through Canadian Agricultural Adaptation Program (CAAP) (AAFC RBPI #2882).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.06.018.

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