Food Chemistry 123 (2010) 358–363

Contents lists available at ScienceDirect

Food Chemistry

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

Acylated anthocyanins in broccoli sprouts

Diego A. Moreno *, Santiago Pérez-Balibrea, Federico Ferreres, Ángel Gil-Izquierdo, Cristina García-Viguera

CEBAS-CSIC, Food Science and Technology Department, P.O. Box 164, E-30100 Espinardo, Murcia, Spain

ARTICLE INFO

Article history:
Received 23 December 2009
Received in revised form 5 March 2010
Accepted 21 April 2010

Keywords:
Brassica oleracea
Anthocyanins
Cyanidin
Ferulic acid
Sinapic acid
p-Coumaric acid
Malonic acid
Health
Food

ABSTRACT

Broccoli is a nutritious vegetable and a good source of vitamins and minerals. It also contains important antioxidant phytochemicals like β-carotene and α-tocopherol, indoles and isothiocyanates. On the other hand, edible sprouts are novel nutritive plant-derived foods, good source of flavonoids and other polyphenols (Moreno, Pérez-Balibrea, et al., 2006).

Anthocyanins have also been previously isolated from the Cruciferae (*Brassicaceae*) presenting unusually complex structures with one or more cinnamic acids but, as far as we are aware, never reported for broccoli sprouts. For this purpose, we studied the sprouts of commercial broccoli destined for head productions ('Marathon' and 'Nubia'), a variety produced for edible sprouts ('Intersemillas' sprouts) and a purple broccoli sprouts ('Viola').

Using HPLC–UV photo-diode array detection (PAD)–electrospray ionisation (ESI)-MS/MS (HPLC–PAD–ESI-MS/MS), acylated anthocyanins were characterised. The main peaks were cyanidin-3-*O*-diglucoside-5-*O*-glucoside acylated and double acylated with *p*-coumaric, sinapic, caffeic, ferulic or sinapic acids, with at least three predominant anthocyanins isomers of cyanidin 3-*O*-(acyl)diglucoside-5-*O*-glucoside, cyanidin 3-*O*-(acyl1)(acyl2)diglucoside-5-*O*-glucoside, and cyanidin 3-*O*-(acyl1)(acyl2)diglucoside-5-*O*-(malonyl)glucoside, within the 17 different anthocyanins characterised in broccoli sprouts for the first time. The qualitative and quantitative differences in the anthocyanin composition between the 'green head' broccoli cultivars (Marathon, Nubia), the green variety-for-sprouts (Intersemillas), and the richest variety in anthocyanins, the early purple sprouting broccoli sprouts ('Viola'), showed the dependence on cultivar to drive the phytochemical load of vegetable foods.

The broccoli sprouts could be potential naturally-healthy functional foods at the seedling stage for delivering significant levels of bioactive compounds besides other flavonoids, glucosinolate-derived isothiocyanates, together with vitamins and minerals.

1. Introduction

In recent years, increasing attention has been paid to the role of diet in human health associated with a reduced risk of a number of chronic diseases. These beneficial effects have been partially attributed to the compounds which possess antioxidant activity. The major antioxidants of vegetables are vitamins C and E, carotenoids, and phenolic compounds, especially flavonoids (Duthie et al., 2006; Singh et al., 2006).

Variation in the antioxidant contents of Brassica vegetables is caused by many factors: variety, maturity at harvest, growing condition, etc. (de Pascual-Teresa & Sánchez-Ballesta, 2008; Hale et al., 2001; Podsedek, 2007). The studies on phenolic profiles of Brassica vegetables have been focused mainly on broccoli florets. Broccoli is a source of flavonol and hydroxycinnamoyl derivatives (Lin & Harnly, 2009; Podsedek, 2007). Vallejo, García-Viguera, and Tomás-Barberán (2003), reported that quercetin and kaempferol 3-*O*-sophoroside-7-*O*-glucoside were the main flavonol glycosides, and the other minor glucosides were isoquercitrin, kaempferol 3-*O*-glucoside and kaempferol 3-*O*-diglucoside. The predominant hydroxycinnamoyl acids have been identified as 1-sinapoyl-2-feruloylgentiobiose, 1,2-diferuloylgentiobiose, 1,2,2′-trisinapoylgentiobiose, and neochlorogenic acid. In addition, 1,2′-disinapoyl-2-feruloylgentiobiose and 1-sinapoyl-2,2'-diferuloylgentiobiose, isomeric forms of 1,2,2'-trisinapoylgentiobiose and 1,2,2'-triferuloylgentiobiose, and chlorogenic acids are also present in broccoli (Vallejo, Tomás-Barberán, & Ferreres, 2004). Polyglycosylated and polyglycosylated/acylated flavonoids are common in the *Brassicaceae*, and there is considerable data on *Brassica* (Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003). However, the acylated anthocyanins, as far as we are aware, have not been previously reported in edible sprouts of broccoli.

Broccoli, belongs to the Brassica genus and is a nutritious vegetable and a good source of vitamins A and C, potassium, folic acid,

* Corresponding author. Address: CEBAS-CSIC, Food Science and Technology Department, Research Group on Quality, Safety and Bioactivity of Plant-derived Foods, P.O. Box 164, E-30100 Espinardo, Murcia, Spain. Tel.: +34 968 396369; fax: +34 968 396213.
E-mail address: dmoreno@cebas.csic.es (D.A. Moreno).

0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.04.044

riboflavin (vitamin B_2) and iron. It is high in fibre and low in calorie content and contains important antioxidant phytochemicals like, polyphenols, β-carotene and α-tocopherol, indoles and isothiocyanates (which have been shown to act as anticarcinogens by inhibition of the phase I enzymes and induction of the phase II enzymes) (Podsedek, 2007; Singh et al., 2006).

Edible sprouts are novel nutritive and phytonutrient-rich plant-derived foods, good source of flavonoids and other polyphenols, that are produced without adding extensive product development or new equipments or costly marketing efforts. On the other hand, there is an increasing awareness that multiple genetic and environmental factors affect production and accumulation of bioactive components (in the food) for the improvement of health (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Due to the increased consumption of sprouts there needs to be optimisation for quality, palatability and bioactivity.

Within the coloured flavonoids, anthocyanins are the most important group of plant pigments, also considered as multifunctional components of food due to their antioxidant activity and other beneficial biological properties (Drabent, Pliszka, & Olszewska, 1999; Sadilova, Stintzing, & Carle, 2006). Nevertheless, in certain fruits and vegetables, anthocyanins exist in smaller amounts and only some of them exist in such an amount that they can determine the proper colour. For example, in *Brassica oleracea* L. extracts, from 8 to 15 anthocyanins were found, which exist as 5-glucosindes and 3,5-glucosides with different anthocyanidins as chromophoric groups (Drabent et al., 1999).

The chemical structure of the anthocyanin determines the stability, colour intensity and potential biological activity. For example, acylation of the anthocyanin molecule improves its stability through intramolecular and/or intermolecular copigmentation, and self-association reactions. Therefore, sources of acylated anthocyanins may provide the desirable stability for food applications (de Pascual-Teresa & Sánchez-Ballesta, 2008; Giusti & Wrolstad, 2003). On the other hand, acylated anthocyanin extracts inhibited α-amylase action, indicating that acylated anthocyanins would have a potential function in suppressing the increase in postprandial glucose levels from starch (Matsui et al., 2001a), of interest in the development of a physiologically functional food (Matsui et al., 2001b). The increased stability of these pigments together with their added value due to potential beneficial effects opens a new window of opportunity for their use in a variety of food applications (Giusti & Wrolstad, 2003).

The anthocyanins that have been isolated from the Cruciferae (*Brassicaceae*) family have unusually complex structures with one or more cinnamic acids. From the chemotaxonomical point of view, two typical anthocyanidin glycoside types have been known in this family, such as anthocyanidin 3-*O*-sophoroside-5-*O*-glucosides and 3-*O*-sambubioside-5-*O*-glucosides (Honda et al., 2005). The genus *Brassica* and *Raphanus* have characteristic varieties containing acylated anthocyanidin 3-*O*-sophoroside-5-*O*-glucoside (Suzuki, Nagata, & Terahara, 1997; Tatsuzawa, Saito, Shinoda, Shigihara, & Honda, 2006).

Therefore, the aim of this work was to characterise the anthocyanin composition of edible sprouts of broccoli not described to this date using HPLC/PAD/ESI-MS*n* and also discussing the genotypical influence on the phytochemical composition of these sprouts with respect to their anthocyanin profile and contents.

2. Materials and methods

2.1. Material and sprouting conditions

Commercial broccoli and certified seeds of 'Marathon' and 'Nubia' were obtained from Ramiro Arnedo SA (Murcia, Spain). The 'Viola' seeds correspond to Early Purple Sprouting Broccoli were purchased at Thompson&Morgan UK Ltd. (Poplar Lake, Ipswich, Suffolk, UK) and the broccoli seeds from the sprouting-variety, were provided by the company "Intersemillas" (Poligono Ind. Loriguilla, Valencia, Spain). Seeds were pre-hydrated and aerated in a 0.5% NaClO solution for 2 h as in Pérez-Balibrea, Moreno, and García-Viguera (2008). Then, the decontaminated seeds were placed in a new clean Milli-Q water solution and soaked with aeration overnight. After pouring off the soaking water, the seeds were spread evenly on a tray lined with vermiculite and moisted and then placed in a controlled-environment growth chamber. The sprouting seeds were then grown in the dark for 3 days at 28 °C and then, transferred to a growth chamber with controlled photoperiod (16-h:8-h), humidity (65%:85%), and temperature (25:20 °C), and kept sprouting until day 7. The 7-day-old sprouts were collected weighed, frozen in liquid nitrogen, placed at −80 °C until freeze-dried (Christ Alpha 1-4D, Christ, Osterode am Harz, Germany), and then ground to a fine powder and stored at −20 °C for further analysis.

2.2. Separation and clean up procedure of anthocyanins for qualitative and quantitative analysis

Each sample (100 mg lipophilised and ground mass) was extracted in polypropylene-capped tubes using 2 ml of MeOH:Formic acid:H_2O (25:1:24; v:v:v) for 12 h at 4 °C, followed by centrifugation (10,000*g* at 4 °C) and recovering of the supernatant that was filtered through a 0.2 μm inorganic membrane filter (ANOTOP 10 plus, Whatman, Maidstone, UK). The extraction procedure was repeated three times. The aqueous solutions were then quantified by HPLC–PAD using cyanidin-3-*O*-β-glucopyranoside as external standard (Polyphenols, Norway).

2.3. Alkali-hydrolysed extracts

Replicates of the filtered extracts were mixed with 0.2 ml of 2 N NaOH and kept at room temperature under a N_2 atmosphere for 12 h. Then, 0.1 ml of concentrated HCl was added to the reaction mixture to bring the pH to 1.0 (Vallejo et al., 2004). The solutions were refiltered prior to the HPLC injection to provide only one deacylated anthocyanin, whose structure was identified to be cyanidin 3-*O*-diglucoside-5-*O*-glucoside by the analyses of HPLC–PAD–ESI-MS/MS.

2.4. HPLC–PAD–ESI-MS/MS for qualitative analysis

Chromatographic separations were carried out on a Luna C18 column (250 mm × 46 mm, 5 μm particle size; Phenomenex, Macclesfield, UK) with a security guard C18-ODS (4 × 30 mm) cartridge system (Phenomenex) with formic acid 1% (A) and acetonitrile (B) as solvents, starting with 5% B and using a gradient to obtain 25% B at 30 min, 50% at 35 min, 50% B at 37 min, to reach 95% B at 38 min. The flow rate was 1 ml min^{-1} and the injection volumes were 20 μl (Table 1). The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technolo-

Table 1
Gradient program for HPLC–DAD–ESI-MS/MS and quantitative analysis.

Time (min)	Solvent A (miliQ W + 1% formic acid)	Solvent B (100% ACN)
Initial	95	5
30	75	25
35	50	50
37	50	50
38	95	5
50	95	5

gies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and a G1315B photodiode array detector controlled by a ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240–600 nm, and chromatograms were recorded at 520 nm. The mass detector was a G2445A Ion-Trap Mass Spectrometer equipped with an electrospray ionisation (ESI) system and controlled by LCMSD software (Agilent, v. 4.1). Nitrogen was used as nebulising gas at a pressure of 65 psi and the flow was adjusted to 11 l min^{-1}. The heated capillary and voltage were maintained at 350 °C and 5 kV, respectively. The full scan mass covered the range from *m/z* 200 up to *m/z* 1600. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the positive ionisation mode. Total ion chromatograms were recorded as two alternating automatic scan events: full scan mass spectra (MS) and MS/MS for fragmentation of the most abundant molecular ions.

2.5. Statistical analysis

All data were subjected to Multifactor Analysis of Variance (MANOVA) using the STATGRAPHICS 7.0 version software (Manugistics, Inc.). The data shown are mean values and the significance of the differences was compared using a multiple comparison test at L.S.D. $P < 0.05$ probability level (Duncan's Multiple Range Test).

3. Results and discussion

3.1. Anthocyanin characterisation

Molecular ions (*m/z*) of common anthocyanins and typical acylating groups are presented in Table 2. The extracts, with a complex anthocyanin profile that shows different acylating patterns with only one anthocyanidin group, were evaluated. Depending on the HPLC conditions and the complexity of the anthocyanin profile, the length of the experimental run may range from several minutes to up to an hour. Usually, for practical reasons, the approach is to develop a systematic methodology that could be applied to a wide variety of different commodities under the same experimental conditions, and that implies that the use of longer HPLC programs and in many cases also a decrease in peak resolution (Giusti, Rodríguez-Saona, Griffin, & Wrolstad, 1999). Even then, a 50 min duration run as used in this work (Fig. 1) is relatively short and easily compared to former methodologies applied for the separation of anthocyanins, such as paper chromatography or thin-layer chromatography (Giusti et al., 1999).

In the case of cruciferous foods, such as red cabbage, the literature agrees on the presence of cyanidin derivatives but there is no agreement on the number of pigments present, ranging from 6 to 15 (Giusti et al., 1999) or even 23 (Wu & Prior, 2005). In the present study of edible sprouts at least 17 different peaks were detected in the majority of the samples (Table 2). The use of photo-diode array (Fig. 1), allows for the analysis of spectral characteristics and renders information regarding the acylation and glycosylation patterns (Giusti et al., 1999). However, this method will not be able to discriminate amongst pigments with similar retention times if they have similar spectral characteristics (Giusti & Wrolstad, 2003), which is the case in broccoli sprouts (Table 2). This is the reason why we used mass spectroscopic analysis as an additional tool for anthocyanin characterisation (Llorach et al., 2003).

The broccoli sprout HPLC–PDA–ESI-MS/MS data gave *m/z* ratios corresponding to the different cyanidin derivatives with the presence of acylated and non-acylated pigments (Table 2). The molecular weights of these anthocyanins varied from several hundreds to more than a thousand. In case of broccoli sprout anthocyanins (Fig. 1, Table 2), HPLC coupled with a photo-diode array detector separated major pigments but did not detect spectral differences between peaks 5 and 8, nor between 10 and 14, or 15 and 17. The ESI-MS/MS data enabled us to detect the presence of an additional acylating group on peaks 15, 16 and 17, malonic acid, as evidenced by the additional molecular weight of 86 (Table 2), also reported for red cabbage (Giusti et al., 1999). Only cyanidin was found in the studied broccoli sprouts, in accordance with previous published data on red cabbage (Giusti et al., 1999; Giusti & Wrolstad, 2003; Wu & Prior, 2005). The major acylated anthocyanins were cyanidin 3-*O*-diglucoside-5-*O*-glucoside derivatives with various acylated groups connected to the diglucoside. The MS/MS of most of the higher molecular ion acylated anthocyanins gave the fragment peak at *m/z* 449, a cyanidin 5-*O*-glucoside residue, and at *m/z* 611, a cyanidin 3-*O*-diglucoside residue. The MS–MS fragments of the acylated anthocyanins (Table 2) allow for a rough determination of the location of the acylating groups. In the case of broccoli sprouts, the fragments produced are consistent with the presence of, at least, three different aromatic groups (*p*-coumaric, sinapic or ferulic acid) attached to the C3 glycosidic substituent

Table 2
Identification of anthocyanins in broccoli sprouts.

Peak No.	*R*t (min)	$[M]^+$ (*m/z*)	$[MS2]^+$ (*m/z*)	$[MS3]^+$ (*m/z*)	Anthocyanin
	Cyanidin 3-O-diGlucoside-5-O-Glucoside				
1	11.9	773	611/449	287	Cyanidin 3-*O*-diglucoside-5-*O*-glucoside
2	13.8	611	449	287	Cyanidin 3,5-di-*O*-glucoside
	Cyanidin 3-O-(Acyl)diGlucoside-5-O-Glucoside				
3	16.5	979	817/449	449/287	Cyanidin 3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside
4	18.8	979	817/449	449/287	Cyanidin 3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside
5	23.5	949	787/449	449/287	Cyanidin 3-*O*-(feruloyl)diglucoside-5-*O*-glucoside
6	26.2	919	757/449	449/287	Cyanidin 3-*O*-(*p*-coumaroyl)diglucoside-5-*O*-glucoside
7	26.8	979	817/449	449/287	Cyanidin 3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside
8	26.8	949	787/449	449/287	Cyanidin 3-*O*-(feruloyl)diglucoside-5-*O*-glucoside
9	26.9	1141	979/817/449	449/287	Cyanidin 3-*O*-(sinapoyl)triglucoside-5-*O*-glucoside
	Cyanidin 3-O-(Acyl1)(Acyl2)diGlucoside-5-O-Glucoside				
10	29.0	1125	963/449	449/287	Cyanidin 3-*O*-(*p*-coumaroyl)(sinapoyl)diglucoside-5-*O*-glucoside
11	29.3	1155	993/449	449/287	Cyanidin 3-*O*-(sinapoyl)(feruloyl)diglucoside-5-*O*-glucoside
12	29.5	1185	1023/817/449	449/287	Cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglucoside-5-*O*-glucoside
13	30.4	1155	993/449	449/287	Cyanidin 3-*O*-(sinapoyl)(feruloyl)diglucoside-5-*O*-glucoside
14	30.6	1185	1023/817/449	449/287	Cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglucoside-5-*O*-glucoside
	Cyanidin 3-O-(Acyl1)(Acyl2)diGlucoside-5-O-(Malonyl)Glucoside				
15	31.2	1211	963/535	449/287	Cyanidin 3-*O*-(*p*-coumaroyl)(sinapoyl)diglucoside-5-*O*-(malonyl)glucoside
16	31.6	1241	993/535	449/287	Cyanidin 3-*O*-(sinapoyl)(feruloyl)diglucoside-5-*O*-(malonyl)glucoside
17	31.8	1271	1023/535/449	449/287	Cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglucoside-5-*O*-(malonyl)glucoside

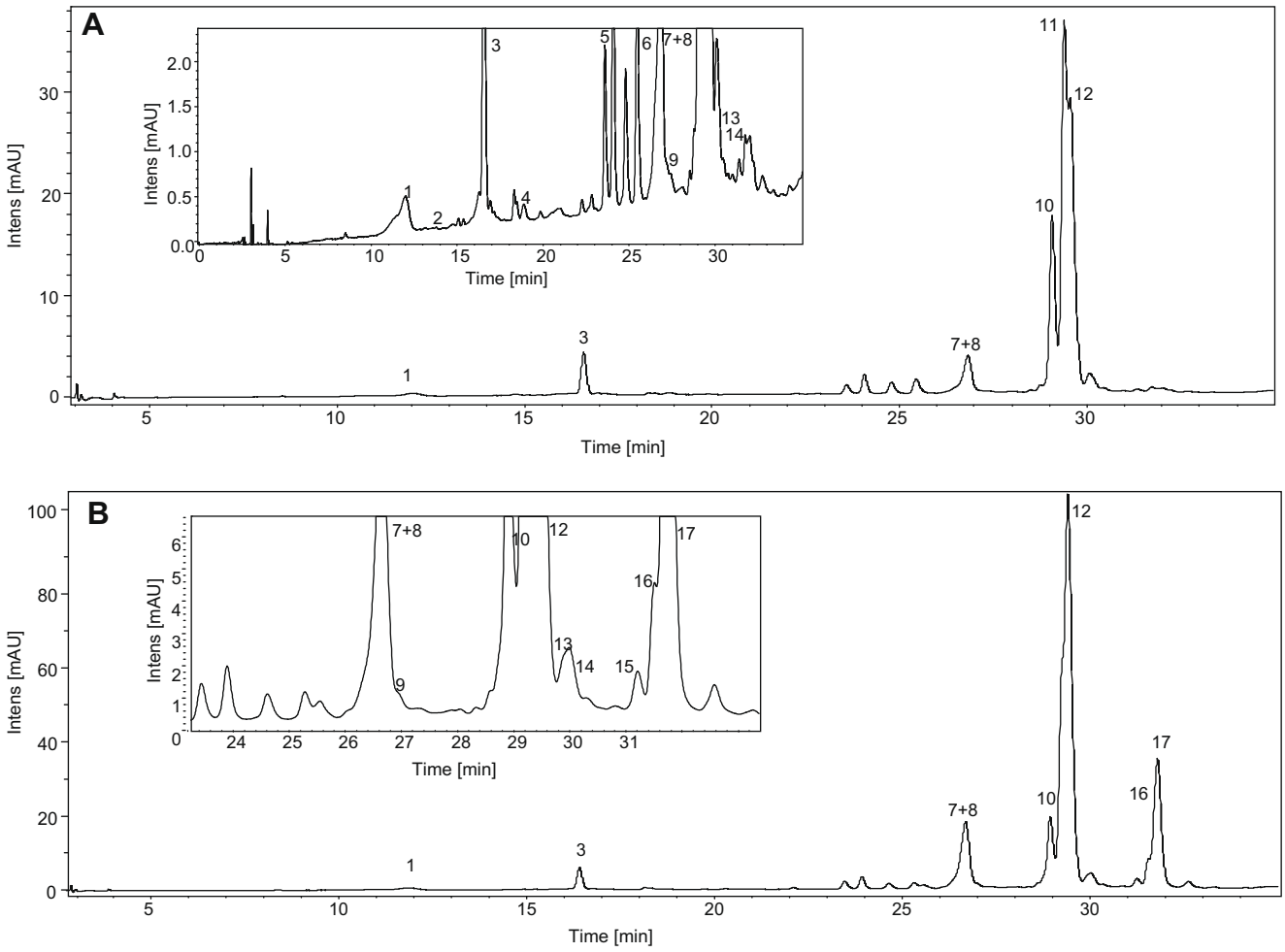

Fig. 1. Type chromatographic separation of green 'Marathon' broccolis ('Marathon', 'Nubia') (A), as compared to the (B) purple sprouting 'Viola' broccoli (at 520 nm). Tentative peak assignments: (1) Cy-3-diglc-5-glc, (2) Cy-3,5-diglc, (3) Cy-3-(sinapoyl)diglc-5-glc, (4) Cy-3(sinapoyl)diglc-5-glc, (5) Cy-3-(feruloyl)diglc-5-glc, (6) traces of Cy-3-(*p*-coumaroyl)dglc-5-glc, (7) Cy-3-(sinapoyl)diglc-5-glc, (8) Cy-3-(feruloyl)dglc-5-glc, (9) traces of Cy-3-(sinapoyl)triglc-5-glc, (10) Cy-3-(*p*-coumaroyl)(sinapoyl)dglc-5-glc, (11) Cy-3-(sinapoyl)(feruloyl)dglc-5-glc, (12) Cy-3-(sinapoyl)(sinapoyl)dglc-5-glc, (13) traces of Cy-3-(sinapoyl)(feruloyl)dglc-5-glc, (14) traces of Cy-3-(sinapoyl)(sinapoyl)dglc-5-glc, (15) Cy-3-(*p*-coumaroyl)(sinapoyl)dglc-5-(malonyl)glc, (16) Cy-3-(sinapoyl)(feruloyl)dglc-5(malonyl)glc, and (17) Cy-3-(sinapoyl)(sinapoyl)dglc-5(malonyl)glc.

whilst only one aliphatic (malonic acid) group attached to the sugars of C5. In these sprouts, the double acylating groups are attached to the sugars of C3, resulting in the formation of large fragments (*m/z* 1023, 993 and 963) with the two acylating groups and other fragments corresponding to the aglycon and the monoglucosylated cyanidin. This same pattern of fragmentation was evidenced with most anthocyanins analysed. By comparing with published data (Giusti & Wrolstad, 2003; Wu & Prior, 2005), the different peaks were identified as isomers of cyanidin 3-*O*-(acyl)diglucoside-5-*O*-glucoside, cyanidin 3-*O*-(acyl1)(acyl2)diglucoside-5-*O*-glucoside, and cyanidin 3-*O*-(acyl1)(acyl2)diglucoside-5-*O*-(malonyl)glucoside.

3.2. Dependence on the variety for anthocyanin composition

The selected peaks, in order to quantify differences in the extracts, were chosen because of their repetitive presence in different extractions and previous experiments (data not shown). By these means, the sprouts of commercial broccoli destined for head productions ('Marathon' and 'Nubia') and the variety produced for edible sprouts ('Intersemillas' sprouts) contained less total content of anthocyanins (based on the addition of the quantified amounts of the selected peaks) than the early purple sprouting broccoli sprouts ('Viola'). The influence of the cultivar on the quantity and type of anthocyanin present in these sprouts was clear (Table 3). Different abundance of peaks in different cultivars was supported by the statistical significance of the MANOVA analysis. The concentrations of the three peaks corresponding to cyanidin 3-*O*-diglc-5-*O*-gluc (1), cyanidin 3-*O*-(sinapoyl)dglc-5-*O*-glc (3), and cyanidin 3-*O*-(*p*-coumaroyl)(sinapoyl)diglc-5-*O*-glc (10) were not statistically different between cultivars. The 'Viola' sprouts presented the highest contents of acylated and double acylated peaks (7 + 8) coeluting in HPLC, but differentiated by MS/MS, cyanidin 3-*O*-(sinapoyl)diglc-5-*O*-gluc and the cyanidin 3-*O*-(feruloyl)diglc-5-*O*-glc, (12) cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglc-5-*O*-glc, (16) cyanidin 3-*O*-(sinapoyl)(feruloyl)diglc-5-*O*-(malonyl)glc, and (17) cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglc-5-*O*-(malonyl)glc (Table 3). The (11) cyanidin 3-*O*-(sinapoyl)(feruloyl)diglc-5-*O*-glc was higher in 'Marathon' than in 'Intersemillas' but not detected in the other cultivars. The sprouts obtained from the cultivars for head formation ('Marathon' and 'Nubia') did not present the anthocyanins with the acylation in C5 (malonyl group; peaks 16 and 17) that were observed in 'Viola' sprouts and also in the variety for sprouting obtained from 'Intersemillas'. The range of concentration of the three cultivars of 'green' broccolis ('Marathon', 'Nubia' and 'Intersemillas') were very similar on average (0.23–0.29 mg/100 g of fresh weight) and were clearly differentiated and smaller than in the early purple sprouting 'Viola' (0.64 mg/100 g of fresh weight), on average.

Table 3
Anthocyanins (mg 100 g^{-1} fw) in 7 days old broccoli sprouts of commercial cultivars.

Cultivars	(1)[a]	(3)	(7 + 8)	(10)	(11)	(12)	(16)	(17)	Total content (Σ)
'Marathon'	0.0032	0.0119	0.0235b[b]	0.0523	0.1116a	0.0886c	–	–	0.2912b
'Nubia'	0.0057	0.0145	0.0266b	0.0549	tr	0.1786 b	–	–	0.2804b
'Intersemillas'	0.0050	0.0142	0.0201b	0.0399	0.0634b	0.0653c	0.0048b	0.0159b	0.2285b
'Viola'	0.0042	0.0158	0.0765a	0.0516	tr	0.3716a	0.0130a	0.1035a	0.6360a
Multifactor ANOVA (*P*-value)	0.0826^{ns}	0.4609^{ns}	0.0000***	0.2670^{ns}	0.0000***	0.0000***	0.0000***	0.0000***	0.0004***
LSD ($P < 0.05$)	0.0019	0.0053	0.0134	0.0173	0.0234	0.0742	0.0019	0.0176	0.1315

*** Significant at $P < 0.001$.
tr, traces.
[a] Peak identities as in Table 1: (1) Cyanidin 3-*O*-diglucoside-5-*O*-glucoside; (3) cyanidin 3-*O*-(sinapoyl)diglucoside-5-*O*-glucoside; (7 + 8) coeluting cyanidin 3-*O*-(sinapoyl)diglucosdie-5-*O*-glucoside and cyanidin 3-*O*-(feruloyl)diglucoside-5-*O*-glucoside; (10) cyanidin 3-*O*-(*p*-coumaroyl)(sinapoyl)diglucoside-5-*O*-glucoside; (11) cyanidin 3-*O*-(sinapoyl)(feruloyl)diglucosdie-5-*O*-glucoside; (12) cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglucosdie-5-*O*-glucoside; (16) cyanidin 3-*O*-(sinapoyl)(feruloyl)diglucoside-5-*O*-(malonyl)glucoside; (17) cyanidin 3-*O*-(sinapoyl)(sinapoyl)diglucosdie-5-*O*-(malonyl)glucoside.
[b] Means ($n = 3$) within a column followed by the same lowercase letter are not significantly different at $P < 0.05$ according to Duncan's Multiple Range Test. Non-significant ($P > 0.05$).

The anthocyanin content in 7-days old sprouts is not very high (<1 mg/100 g in fresh weight; Table 3). The flavonoids in 'Marathon' broccoli sprouts of 7 days of age were in the range of 100–200 mg/100 g (Pérez-Balibrea et al., 2008). Therefore, the amount of anthocyanins in the sprouts is small at such an early sprouting stage. The significance of these compounds in the adult plant and especially in purple sprouting broccoli cultivars, richer in anthocyanins, for its contribution to health-promoting effects of new food designs deserves further attention.

4. Conclusions

There is increasing recognition of the nutritional value of the sprouts of various vegetables, beans, and crops (Moreno, Pérez-Balibrea, & García-Viguera, 2006; Pérez-Balibrea et al., 2008; Watanabe, 2007). The consumption of broccoli sprouts is limited in Europe; even though it is recognised as a healthy food in America and Asia. This study indicates that broccoli sprouts could exert health benefits due to an abundance of phytochemicals such as flavonoids, with considerable potential for functional foods at the seedling stage for delivering significant levels of bioactive compounds besides glucosinolate-derived isothiocyanates and various vitamins and minerals.

As also seen before (Giusti & Wrolstad, 2003; Llorach et al., 2003), tandem techniques such as HPLC–PAD–ESI-MS/MS could be useful tools for monitoring the authenticity of anthocyanin-containing vegetable extracts and foods. In the present study of edible sprouts, at least 17 different peaks were detected in the majority of the samples. The anthocyanin profiles demonstrated qualitative and quantitative differences between cultivars of 'green head' broccoli (Marathon, Nubia) or a green variety-for-sprouts (Intersemillas), and an anthocyanin-richer 'purple head' broccoli sprouts, as well as the first characterisation of the anthocyanins in broccoli sprouts. The major peaks identified were isomers of cyanidin 3-*O*-(acyl)diglucoside-5-*O*-glucoside, cyanidin 3-*O*-(acyl1)(acyl2)diglucoside-5-*O*-glucoside, and cyanidin 3-*O*-(acyl1)(acyl2)diglucoside-5-*O*-(malonyl)glucoside. The dependence on cultivar (purple 'Viola' *versus* green 'Marathon', 'Nubia', or 'Intersemillas') also made the case for the genotypic influence on the phytochemical load of vegetable foods and could be of interest for different fields: tracking vegetable variability (i.e., breeds, cultivars or genetic variability); seed and sprout traceability for food industry, and biochemical markers for the influence of growth conditions or processing techniques on flavonoids in foods, especially anthocyanins; etc.

Acylated anthocyanins are considered to be better candidates than non-acylated anthocyanins for food colourants because of their increased stability (de Pascual-Teresa & Sánchez-Ballesta, 2008; Wu & Prior, 2005). The potential for enriching broccoli sprouts in health-promoting compounds including anthocyanins needs further investigation.

Acknowledgements

The authors wish to thank the Fundación Seneca-Regional Agency for Science and Technology for the financial support of this work (Project ref. n# 05588/PI/07). Part of this work was also funded by M.I.C.I.N.N. (PETRI Project PET2006_0305) and CSIC I3 Programme (Project 200870I070).

The author Perez-Balibrea is funded by a grant from Murcia Regional Regional Agency for Science and Technology 'Seneca Foundation'.

References

de Pascual-Teresa, S., & Sánchez-Ballesta, M. T. (2008). Anthocyanins: from plant to health. *Phytochemistry Reviews, 7*(12), 281–299.
Drabent, R., Pliszka, B., & Olszewska, T. (1999). Fluorescence properties of plant anthocyanin pigments. I. Fluorescence of anthocyanins in *Brassica oleracea* L. extracts. *Journal of Photochemistry and Photobiology B: Biology, 50*(1), 53–58.
Duthie, S. J., Jenkinson, A. McE., Crozier, A., Mullen, W., Pirie, L., Kyle, J., et al. (2006). The effects of cranberry juice consumption on antioxidant status and biomarkers relating to heart disease and cancer in healthy human volunteers. *European Journal of Nutrition, 45*(2), 113–122.
Giusti, M. M., Rodríguez-Saona, L. E., Griffin, D., & Wrolstad, R. E. (1999). Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *Journal of Agricultural and Food Chemistry, 47*(11), 4657–4664.
Giusti, M. M., & Wrolstad, R. E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*(3), 217–225.
Hale, K. L., McGrath, S. P., Lombi, E., Stack, S. M., Terry, N., Pickering, I. J., et al. (2001). Molybdenum sequestration in *Brassica* species. A role for Anthocyanins? *Plant Physiology, 126*, 1391–1402.
Honda, T., Tatsuzawa, F., Kobayashi, N., Kasai, H., Nagumo, S., Shigihara, A., et al. (2005). Acylated anthocyanins from the violet-blue flowers of *Orychophragonus violaceus*. *Phytochemistry, 66*(15), 1844–1851.
Lin, L.-Z., & Harnly, J. M. (2009). Identification of the phenolic components of collard greens, kale, and Chinese broccoli. *Journal of Agricultural and Food Chemistry, 57*(16), 7401–7408.
Llorach, R., Gil-Izquierdo, A., Ferreres, F., & Tomás-Barberán, F. A. (2003). HPLC-DAD-MS/MS ESI characterization of unusual highly glycosylated acylated flavonoids from cauliflower (*Brassica oleracea* L. var. *botrytis*) agroindustrial byproducts. *Journal of Agricultural and Food Chemistry, 57*(13), 3895–3899.
Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., & Matsumoto, K. (2001a). α-Glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigments with potent inhibitory activity. *Journal of Agricultural and Food Chemistry, 49*(4), 1948–1951.
Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., & Matsumoto, K. (2001b). α-Glucosidase inhibitory action of natural acylated anthocyanins. 2. α-Glucosidase inhibition by isolated acylated anthocyanins. *Journal of Agricultural and Food Chemistry, 49*(4), 1952–1956.
Moreno, D. A., Carvajal, M., López-Berenguer, C., & García-Viguera, C. (2006). Chemical and biological characterisation of nutraceutical compounds of broccoli. *Journal of Pharmaceutical and Biological Analysis, 41*(5), 1508–1522.
Moreno, D. A., Pérez-Balibrea, S., & García-Viguera, C. (2006). Phytochemical quality and bioactivity of edible sprouts. *Natural Products Communications, 1*, 1037–1048.

Pérez-Balibrea, S., Moreno, D. A., & García-Viguera, C. (2008). Influence of light on health-promoting phytochemicals of broccoli sprouts. *Journal of the Science of Food and Agriculture, 88*(5), 904–910.

Podsedek, A. (2007). Natural antioxidants and antioxidant capacity of Brassica vegetables: A review. *LWT, 40*(1), 1–11.

Sadilova, E., Stintzing, F. C., & Carle, R. (2006). Anthocyanins, colour and antioxidant properties of eggplant (*Solanum melongena* L.) and violet pepper (*Capsicum annuum* L.) peel extracts. *Zeitschrift für Naturforschung C, 61*(7–8), 527–535.

Singh, J., Rai, M., Upadhyay, A. K., Bahadur, A., Chaurasia, S. N. S., & Singh, K. P. (2006). Antioxidant phytochemicals in broccoli (*Brassica oleracea* L. var. *italica* Plenck) cultivars. *Journal of Food Science and Technology, 43*(4), 391–393.

Suzuki, M., Nagata, T., & Terahara, N. (1997). New acylated anthocyanins from *Brassica campestris* var. *chinensis*. *Bioscience Biotechnology and Biochemistry, 61*(11), 1929–1930.

Tatsuzawa, F., Saito, N., Shinoda, K., Shigihara, A., & Honda, T. (2006). Acylated cyanidin 3-sambubioside-5-glucosides in three garden plants of the Cruciferae. *Phytochemistry, 67*(12), 1287–1295.

Vallejo, F., García-Viguera, C., & Tomás-Barberán, F. (2003). Changes in broccoli (*Brassica oleracea* L. var. *italica*) health-promoting compounds with inflorescence development. *Journal of Agricultural and Food Chemistry, 51*(13), 3776–3782.

Vallejo, F., Tomás-Barberán, F., & Ferreres, F. (2004). Characterisation of flavonols in broccoli (*Brassica oleracea* L. var. *italica*) by liquid chromatography–UV diode-array detection-electrospray ionisation mass spectrometry. *Journal of Chromatography A, 1054*(1–2), 181–193.

Watanabe, M. (2007). An anthocyanin compound in buckwheat sprouts and its contribution to antioxidant activity. *Bioscience Biotechnology and Biochemistry, 71*(2), 579–582.

Wu, X., & Prior, R. L. (2005). Identification and characterization of anthocyanins by high performance liquid chromatography–electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains. *Journal of Agricultural and Food Chemistry, 53*(8), 3101–3113.