

Short Communication

Anthocyanin profile and antioxidant capacity of black carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) from Cuevas Bajas, Spain



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ARTICLE INFO

Article history:

Received 30 October 2012

Received in revised form 1 October 2013

Accepted 12 November 2013

Keywords:

Anthocyanins

Polyphenols

Black carrot

Daucus carota

Antioxidant

FRAP

DPPH

Bioactive non-nutrients

Food analysis

Food composition

ABSTRACT

The present work deals with the study of the anthocyanin profile of two different black carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) cultivars, associated with Antonina and Purple Haze varieties, from Cuevas Bajas (Málaga, Spain) and some of their antioxidant features. The main anthocyanins detected by LC–MS were found to correspond to five cyanidin-based anthocyanins: cyanidin 3-xylosylglucosylgalactoside, cyanidin 3-xylosylgalactoside and the sinapic, ferulic and coumaric acids derivative of cyanidin 3-xylosylglucosylgalactoside. The anthocyanins present in the black carrots were essentially acylated and their levels were found to correspond to 25% and 50% of the total phenolic content for the Purple Haze and Antonina varieties, respectively. Moreover, the reducing capacity of the two black carrots extracts (86.4 ± 8.0 and $182.0 \pm 27 \mu\text{M TE}/100 \text{ g fw}$) and the radical scavenging ability (17.6 ± 9.0 and $240.0 \pm 54.0 \mu\text{M TE}/100 \text{ g fw}$) expressed in Trolox equivalents units were determined. The antioxidant features of the black carrot extracts were shown to be significantly higher than those of orange carrots used herein for comparison. Overall, this work highlights the Cuevas Bajas black carrots as rich sources of anthocyanins with significant antioxidant capacities and good nutritional value.

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1. Introduction

Carrots are the most popular vegetables after potatoes (Hadley and Fordham, 2003). Although orange carrot varieties are more common, consumption of black or purple carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) is currently increasing in Western Europe. These latter originated in Middle Asia and were introduced and cultivated in Europe by the Dutch. Although the cultivar of black carrots is widespread in several countries, studies focusing on these vegetables have been reported mainly in Germany (Sadilova et al., 2009; Montilla et al., 2011) Turkey (Turker et al., 2004; Kirca et al., 2006; Ünal and Bellur, 2009) and Australia (Netzel et al., 2007).

Besides the presence of known antioxidants such as vitamins C and E, black carrots have attracted the attention of the scientific community due to their phenolic compounds content (Alasalvar et al., 2001; Kirca et al., 2006; Singh et al., 2011), which contribute

significantly to the antioxidant capacity. The main particularity of this vegetable lies in its intense purple colour from the presence of anthocyanins in the outer parts of the vegetable (Wrolstad, 2004). Anthocyanins are phenolic compounds which constitute the largest group of water-soluble pigments and they occur frequently in the plant kingdom (Kammerer et al., 2004a,b), usually associated with red fruits, but also occurring in vegetables, roots and cereals (Mazza and Miniati, 1993). Besides their colour features (Khandare et al., 2011), anthocyanins may also contribute some health benefits, such as the reduction in the risk of coronary heart disease, reduced risk of stroke, antitumor properties, anti-inflammatory effects and improved cognitive behaviour (Cao et al., 1996; Wang et al., 1997; Clifford, 2000; Scalbert and Williamson, 2000; Prior, 2003; Netzel et al., 2007).

The scientific community has become aware of the importance of anthocyanins in the diet as different experiments have been performed to support and explain some of the biological and health-promoting effects that are attributed to these molecules. The more commonly known anthocyanins are based on six anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin, but 539 anthocyanins isolated

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from plants have been reported so far (Andersen and Jordheim, 2006). The main anthocyanins present in fruits are glycosylated at the 3-OH position (3-O-monoglycosides) and to a lesser extent, in positions 3-OH and 5-OH (3,5-O-diglycosides). In the case of black carrots, the major anthocyanins are derived from cyanidin being essentially acylated (Schwarz et al., 2004).

Although there are some works published in the literature dealing with black carrot anthocyanins, there is no report of any detailed anthocyanin characterization of black carrots from Spain. Therefore, the aim of this study was to perform a characterization of anthocyanins of two cultivars of black carrots which were associated to Antonina and Purple Haze varieties, grown in the same region of southern Spain, Cuevas Bajas in northern Málaga. Additionally, the study was designed to evaluate some of their antioxidant features in comparison with the regular orange carrot harvested in the same region.

2. Materials and methods

2.1. Sample materials

Black carrots (*Daucus carota* L. ssp. *sativus* var. *atrorubens* Alef.) and orange carrots (*Daucus carota* L. ssp. *sativus*) used in this study were cultivated in Cuevas Bajas, (Málaga, Spain), harvested in autumn of both 2010 and 2011. Both varieties of black and orange carrots were obtained from alluvial soils, deep and fairly uniform silty-sandy loam texture rich in organic materials. These soils permit infiltration drainage, allowing frequent watering. The fertilizers used were mainly green manure. The planting season began in late August with harvest in mid-November to late December. Random sampling was used to obtain different carrots (3 independent samples of each variety during harvest time) of every cultivar. After harvest, the samples were washed and stored at 4 °C until analysis; the experiments were performed in the following week in order to obtain the most representative results from fresh carrots without affecting food composition (Greenfield and Southgate, 2003).

2.2. Solvents and reagents

Chloroform, pentane and cyclohexane were analytical grade and purchased from Merck® (Darmstadt, Germany). Acetonitrile (CH₃CN) was HPLC grade and purchased from Merck (Darmstadt, Germany). Formic acid (HCOOH) p.a. > 98%, DPPH, FeCl₃ and Trolox, were purchased from Sigma–Aldrich® (Madrid, Spain). 2,4,6-Tripyridyl-s-triazine (TPTZ) was purchased from Fluka® (Madrid, Spain). Cyanidin-3-glucoside (Assay (HPLC) ≥ 96%) was purchased from Extrasynthese® (Genay, France).

2.3. Sample extraction

100 g of black and orange fresh carrots were peeled and finely chopped, to avoid the traces of carotenes in the black carrots. These samples were subjected to extraction with 100 mL EtOH:H₂O (1:1, v/v), containing 0.01% HCl (37% v/v) and left with stirring at room temperature (25 °C) for a period of 2 h. Then, the extracts were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane, and the ethanol was evaporated under vacuum. The resulting extracts were purified by liquid–liquid extraction with chloroform (4 × 50 mL), pentane (4 × 50 mL) and cyclohexane (4 × 50 mL). The resulting aqueous fractions were concentrated in a vacuum rotary evaporator until 2 mL at a temperature of 30 °C and stored at 4 °C in the absence of light. The extraction was carried out in triplicate for each sample, and the further analyses were performed within 3 days.

2.4. Anthocyanin analysis by HPLC

All extracts were analyzed by HPLC (Knauer K-1001) on a 250 mm × 4.6 mm i.d. reversed-phase C₁₈ column (Merck); detection was performed at 520 nm using a diode array detector (Knauer K-2800). The solvents were (A) H₂O/HCOOH (9:1) and (B) H₂O/CH₃CN/HCOOH (6:3:1). The gradient was 20–85% B for 70 min at a flow rate of 1.0 mL/min. The column was washed with 100% B for 20 min and then stabilized in the initial conditions for another 20 min. The quantification was performed using calibration curves of cyanidin-3-glucoside, and the results were expressed as cyanidin-3-glucoside equivalents. The range of the linear calibration curves (r² > 0.98) was 0.05 (limit of detection) to 0.5 mg/L for the lower concentration compounds and 0.5–1000 mg/L for the higher concentration compounds. The reproducibility of this method from extraction to HPLC analysis for four samples of the same sample gave a coefficient of variation < 5%.

2.5. LC–MS

A liquid chromatograph (Hewlett-Packard 1100 series) equipped with an AQUA (Phenomenex, Torrance, CA) reversed phase column (150 mm × 4.6 mm, 5 µm, C18) with thermostat at 35 °C was used. The solvents were (A) aqueous 0.1% TFA and (B) CH₃CN, at a flow rate of 0.5 mL/min with the gradient as reported elsewhere (Oliveira et al., 2006). Double-online detection was performed on a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corp., San Jose, CA) equipped with an API source using electrospray ionization (ESI) interface. Both the auxiliary and the sheath gas was a mixture of N₂/He. The capillary voltage was 3 V and the capillary temperature at 190 °C. The spectra were recorded in positive ion mode from *m/z* 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and an MS–MS of the most intense ion using relative collision energies of 30 and 60 eV.

2.6. Total phenolic content

The total phenolic content of the extracts was determined according to the Folin–Ciocalteu method adjusted to a microscale (Arnous et al., 2001). In a micro-tube, 1100 µL of distilled water, 15 µL of sample dissolved in methanol, and 75 µL of Folin–Ciocalteu reagent were mixed. After 1 min, 310 µL of 20% aqueous of Na₂CO₃ was added, and the mixture was mixed and allowed to stand at room temperature in the dark for 120 min. The absorbance was read at 750 nm, and the total phenolic concentration was calculated from a calibration curve using gallic acid as standard. The results are expressed as milligrams per litre of gallic acid equivalents (mg/L GAE).

2.7. Ferric reducing/antioxidant power (FRAP)

The FRAP assay (Benzie and Strain, 1996) was performed with some modifications. The reaction was carried out in a microplate reader of 96 well plates (Biotek Powerwave XS with software KC4). The reaction was conducted in the wells of the plate with a temperature of 37 °C. In short, FRAP reagent (10 vol of 300 mM acetate buffer (pH 3.6) + 1 vol of 10 mM TPTZ in 40 mM HCl + 1 vol of 20 mM FeCl₃) was diluted to one-third with acetate buffer. 270 µL of this solution was added in each well together with 30 µL of extract. The control assay was performed with 270 µL of FRAP reagent and 30 µL of methanol. The absorbance at 593 nm was measured at 0 and 4 min. The reducing power is expressed in µM Trolox equivalents determined using a calibration curve of Trolox.

2.8. Radical DPPH scavenging activity

Following the method described in the literature (Bondet et al., 1997) with minor modifications, radical scavenging activities were determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl) as a free radical. The tested extracts reacted with DPPH and the decreased in absorbance at 515 nm, indicating the potential scavenging of the extracts. Control assays were performed with extracts of the black carrots to subtract its contribution in this absorbance. The reaction for scavenging DPPH radicals was performed in a micro-plate reader of 96 well plates (Biotek Powerwave XS with software KC4). The reaction was carried out on the plate wells with a temperature of 25 °C. A solution of 60 μM DPPH was prepared in methanol. 270 μL of this solution was added to each well together with 30 μL of antioxidant. The compounds tested were a final concentration of 10 μM. The decrease in absorbance was measured at 515 nm, at $t = 0$ and every 5 min, for 20 min. For the final analysis, the 0–20 min reaction time range was used. Antiradical activity was expressed as Trolox equivalents. The antiradical activity was calculated from the equation determined from the linear regression after plotting known solutions with different concentrations of Trolox.

2.9. Statistical analysis

Prepared samples of black carrot macerates were divided into various experimental units of 100 g each in 500 mL beakers. All tests were performed in triplicate. Values are expressed as means ± standard deviation. The statistical significance of difference between various groups was evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni test, using GraphPad Prism 5 software, v.5.01, GraphPad Software, Inc. (supplied by the University of Porto, Portugal, www.graphpad.com). Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Anthocyanin profile of black carrots

Anthocyanins are responsible for the unlimited diversity of colours from orange and red through purple and blue hues of several fruits, vegetables and plants. The most commonly known anthocyanins are based on six anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin but there are almost 600 anthocyanins reported to be isolated from plants (Andersen and Jordheim, 2006). These compounds differ in the hydroxylation and methoxylation patterns of ring B. Anthocyanins can be glycosylated in the 3-OH, 5-OH and less commonly in the 7-OH position and, to a lesser extent, in both positions 3-OH and 5-OH (3,5-O-diglycosides). The sugar moieties vary but are usually a mono or disaccharide unit, frequently glucose, galactose, rhamnose, arabinose, rutinose or xylose (Francis, 1989). The sugar moiety may also be attached to aliphatic or aromatic acids.

Black carrots have been reported as very good sources of anthocyanins including acylated forms (Kammerer et al., 2003, 2004a), but there is no detailed report on the anthocyanin profiles of black carrot cultivars from Spain.

The chromatograms recorded at 520 nm corresponding to the anthocyanin profiles of the black carrot extracts from Cuevas Bajas (Spain) are displayed in Fig. 1. Table 1 shows the mass of the individual anthocyanins identified by LC–MS, as well as the λ_{\max} values obtained from the respective UV–visible spectra recorded from the HPLC–DAD detector. The respective chemical structures are shown in Figs. 2 and 3. It can be seen from Fig. 1 and Table 1 that both black carrots contain essentially acylated anthocyanins. The major anthocyanins detected are cyanidin-based containing different sugar moieties non-acylated (compound 1 and 2), or

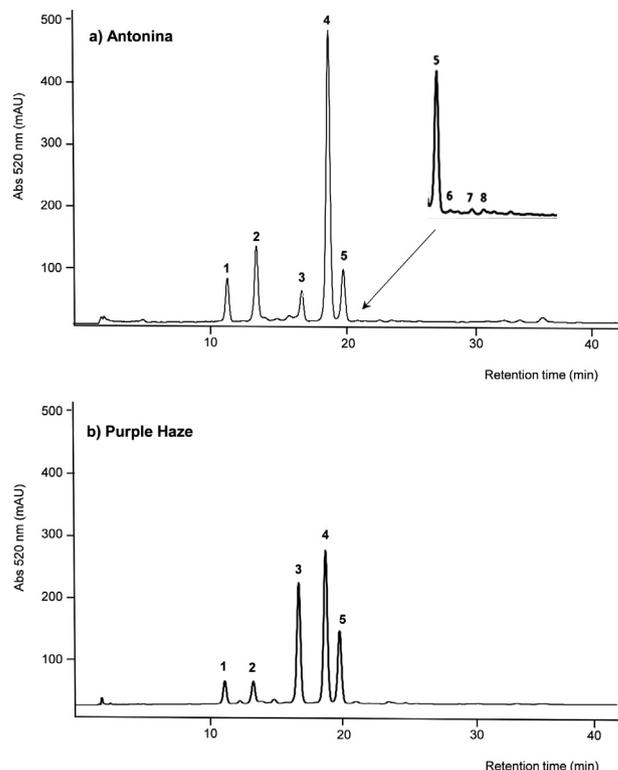


Fig. 1. HPLC chromatogram of the black carrot extracts recorded at 520 nm. The numbers correspond to the compounds indicated in Table 1.

acylated with sinapic acid (compound 3), ferulic acid (compound 4) or coumaric acid (compound 5). The identification of each anthocyanin was made based on the UV–visible features, MS and fragmentation pattern, all in comparison with the data already reported in the literature. The anthocyanin profile was found to agree with those reported elsewhere (Kammerer et al., 2003, Netzel et al., 2007; Montilla et al., 2011; Türkyılmaz et al., 2012). The predominant peak 4 (m/z 919) in the two varieties corresponds to cyanidin 3-*O*-feruloyl-(xylosyl-galactosyl-galactoside), which represents approximately 80% and 40% of the total anthocyanin content, which is typical of the black carrot varieties Antonina (Fig. 1a) and Purple Haze (Fig. 1b), respectively. This data is consistent with previous reports (Kammerer et al., 2003). The MS² fragmentation pattern of these five anthocyanins showed a product ion at $m/z = 287$ corresponding to the respective cyanidin aglycone.

Three other anthocyanins were detected in very small amounts: peak 6 (m/z 757), peak 7 (m/z 903) and peak 8 (m/z 933), which were found to correspond to peonidin 3-xylosylglucosylgalactoside, ferulic acid derivative of pelargonidin 3-xylosylglucosylgalactoside and ferulic acid derivative of peonidin 3-xylosylglucosylgalactoside, respectively. Once again, this elution profile agrees with others already reported in the literature. The MS² fragmentation pattern products showed ions at $m/z = 301$ and 271, which correspond to peonidin and pelargonidin aglycones. This profile was not found in the Purple Haze cultivar (Fig. 1b). Although stability studies were not performed, some conclusions can be yielded from this composition of anthocyanins as acylation of anthocyanidin-*O*-glycosides with an aromatic acid has been shown to improve colour stability (Malien-Aubert et al., 2001; Hernández-Herrero and Frutos, 2011).

3.2. Total phenolic compounds

A quantification of total phenolic compounds was performed by using the Folin–Ciocalteu method. This method has for many years

Table 1
Mass spectrometric data and identification of the anthocyanins detected in the black carrot extracts by LC–MS in the positive ion mode. The λ_{\max} values were obtained from the UV–visible spectra recorded from the HPLC–DAD detector.

Retention time (min)	Peak	λ_{\max} (nm)	Anthocyanin	Mass (m/z)	MS ² main fragment (m/z)
11.20	1	517	Cyanidin 3-xylosylglucosylgalactoside	743	287
13.39	2	518	Cyanidin 3-xylosylgalactoside	581	287
16.63	3	530	Sinapic acid derivative of cyanidin 3-xylosylglucosylgalactoside	949	287
18.53	4	528	Ferulic acid derivative of cyanidin 3-xylosylglucosylgalactoside	919	287
19.67	5	527	Coumaric acid derivative of cyanidin 3-xylosylglucosylgalactoside	889	287
20.80	6	530	Peonidin 3-xylosylglucosylgalactoside	757	301
21.76	7	527	Ferulic acid derivative of pelargonidin 3-xylosylglucosylgalactoside	903	271
23.22	8	530	Ferulic acid derivative of peonidin 3-xylosylglucosylgalactoside	933	301

been used to measure total phenolics in natural matrices but since its mechanism is an oxidation/reduction reaction, it may also be considered as an antioxidant method (Prior et al., 2005). A skin extract of regular orange carrot (*Daucus carota* L. ssp. *sativus*) grown in the same location was also tested aiming to make a comparison with the black carrot extracts. In order to avoid the influence of carotenoids in black carrots analysis, extracts obtained without the liquid–liquid extraction steps with chloroform, pentane and cyclohexane were also analysed and the differences observed between the relative amounts of the two carrot extracts were similar (data not shown). The levels of phenolic compounds

in the carrot extracts tested were significantly higher in the black carrot extracts: 187.8 and 492 mg GAE/100 g fw respectively for Antonina and Purple Haze cultivar, and 9.4 mg GAE/100 g fw obtained for the orange carrot extract (Table 2). This difference is partly due to the presence of anthocyanins that occur in large amounts in black carrot extracts. Indeed, the anthocyanins present in the black carrots were found to correspond to 25% and 50% of the total phenolic content for cultivar Purple Haze and Antonina, respectively. However, these black carrots contain a much higher content in other phenolic compounds and are therefore a much richer source of phenolic compounds than the orange carrot

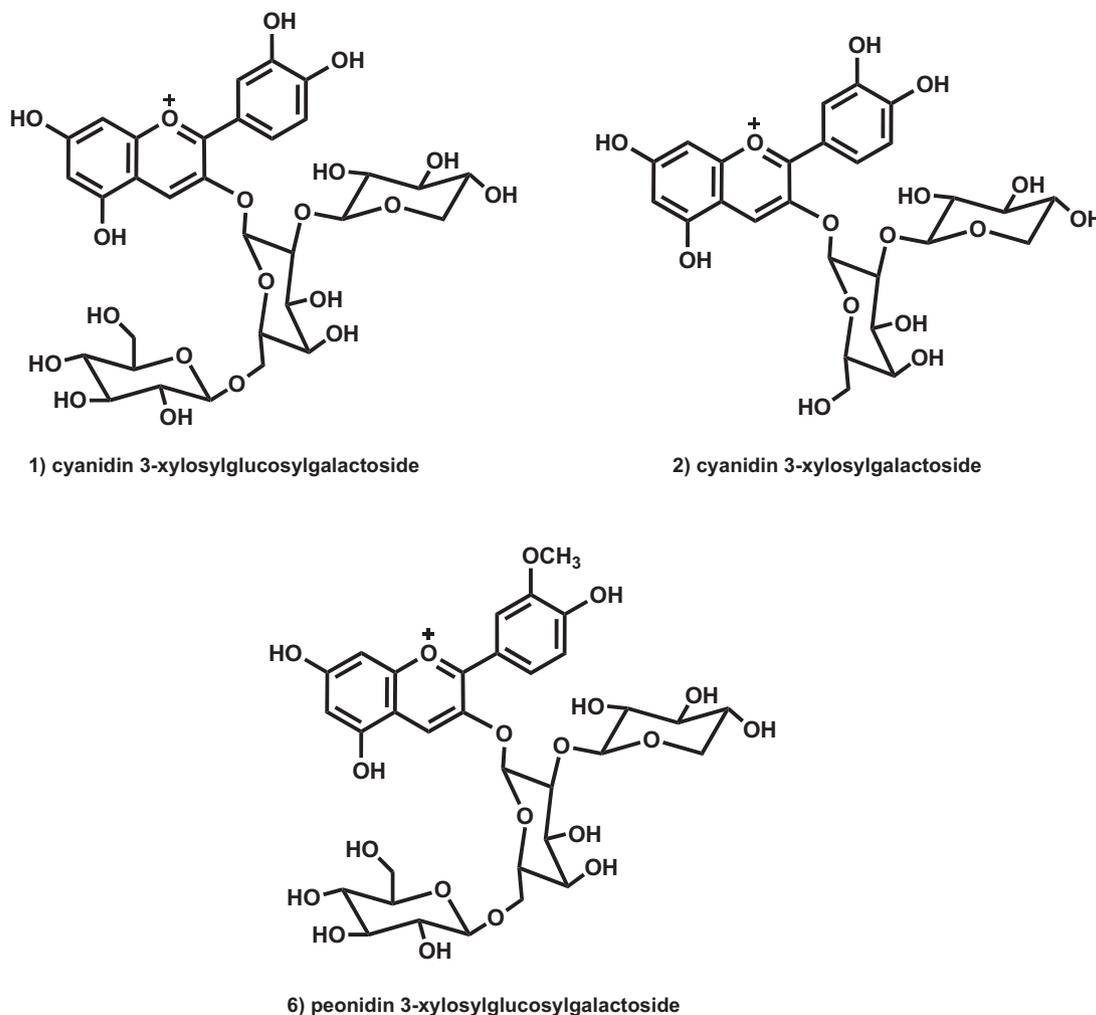


Fig. 2. Chemical structures of the non-acylated anthocyanins detected in the black carrot extracts: (1) cyanidin 3-xylosylglucosylgalactoside, (2) cyanidin 3-xylosylgalactoside, (6) peonidin 3-xylosylglucosylgalactoside.

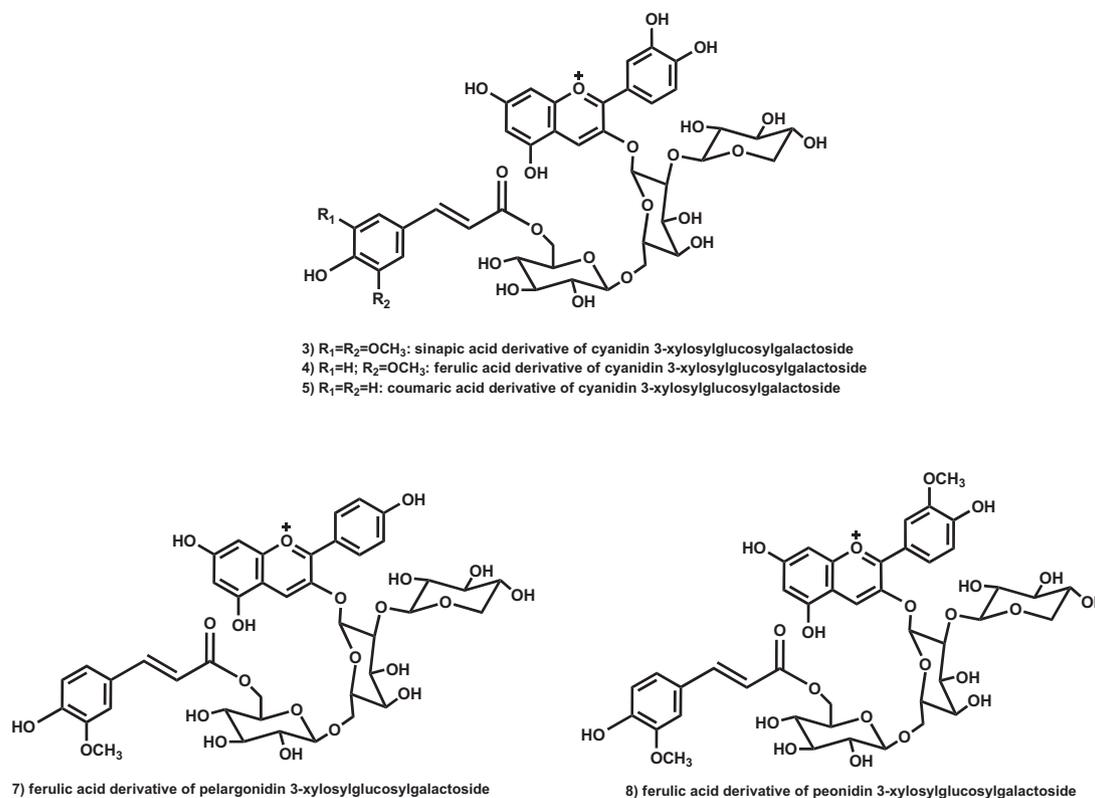


Fig. 3. Chemical structures of the acylated anthocyanins detected in the black carrot extracts: (3) sinapic acid derivative of cyanidin 3-xylosylglucosylgalactoside, (4) ferulic acid derivative of cyanidin 3-xylosylglucosylgalactoside, (5) coumaric acid derivative of cyanidin 3-xylosylglucosylgalactoside, (7) ferulic acid derivative of pelargonidin 3-xylosylglucosylgalactoside, and (8) ferulic acid derivative of peonidin 3-xylosylglucosylgalactoside.

Table 2

Contents of total phenols and anthocyanins of the orange and black carrot extracts. Antiradical activity and reducing power assessed by DPPH and FRAP methods.

	Total Phenols (mg GAE/100 g fw)	Anthocyanins (mg/100 g fw)	DPPH ($\mu\text{M TE}/100\text{ g fw}$)	FRAP ($\mu\text{M TE}/100\text{ g fw}$)
Orange carrot	9.4 \pm 0.8	–	1.4 \pm 0.4	1.3 \pm 0.5
Black carrot (Antonina, 2010)	187.8 \pm 9.0	93.8 \pm 3.0	17.6 \pm 9.0	86.4 \pm 8.0
Black carrot (Purple Haze, 2011)	492.0 \pm 63	126.4 \pm 6.0	240.0 \pm 54.0	182.0 \pm 27.0

Values are mean \pm standard deviation of at least 3 determinations of 3 independent samples; fw, fresh weight.

extract. Previous data reported in the literature showed that black carrots contain more phenolic compounds than orange variety, including also a higher lycopene content (Grassman et al., 2007).

3.3. Antioxidant features

Two antioxidant features of the black carrot extract were assayed: the reducing capacity and the free radical scavenging ability. The results obtained were compared with those of the orange carrot extract. The free radical scavenging activity of both carrot extracts was assayed using the DPPH method as described in the experimental section. The antiradical capacities of the black carrot extracts were much higher than that of the orange carrot extract (Table 2). This result was already anticipated attending to the significantly higher amounts of total phenolics present in the black carrot extracts including anthocyanins that have been widely reported to have good free radical scavenging capacities (Bors et al., 1990; Williams et al., 2004; Azevedo et al., 2010).

Moreover, the reducing capacity of the black carrot extracts was assayed using the FRAP method. Again, the reducing capacity was much higher for the black carrot extract (Table 2). However, bearing the difference in phenolic levels between the black carrot extracts and the orange carrot extract, the reducing capacities of the black carrot extracts appear to be even higher than expected.

This result could be explained by the fact that polyphenols such as anthocyanins are more prone to this characteristic than other compounds.

Among the two black carrot varieties, Purple Haze seemed to display higher antioxidant features than Antonina variety, which is positively correlated with its higher levels of phenolic compounds.

4. Conclusions

The present work reports for the first time the anthocyanin profile of two varieties of black carrots cultivated in the Cuevas Bajas region of southern Spain. A good correlation between the concentration of phenolic compounds and the total antioxidant capacity was reported. High levels of acylated anthocyanins tend to improve the total antioxidant capacity compared to non-acylated anthocyanins, as recently reported. The HPLC profiles of both black carrots were associated to Antonina and Haze Purple varieties according to previous published research work. Altogether, these results clearly show that Cuevas Bajas black carrots are rich sources of anthocyanins. Considering the health benefits that have been associated with the consumption of anthocyanin-rich foods and diets, black carrot appears as an important food vegetable with good nutritional value.

Acknowledgments

The authors would like to thank the Fundação para a Ciência e Tecnologia (FCT, Portugal) under the frame of the Ciência 2007 program. Thanks are also due to the City Council of the town of Cuevas Bajas, Málaga (Spain) to promote this investigation in the context of the program for characterization of black carrots.

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