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Food Chemistry 119 (2010) 1257–1261

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Quali-quantitative analysis of flavonoids of *Cornus mas* L. (Cornaceae) fruits

Agata Maria Pawlowska [a], Fabiano Camangi [b], Alessandra Braca [a,*]

[a] Dipartimento di Scienze Farmaceutiche, Università di Pisa, Via Bonanno 33, 56126 Pisa, Italy
[b] Scuola Superiore S. Anna di Studi, Universitari e di Perfezionamento di Pisa, Piazza Martiri della, Libertà 33, 56127 Pisa, Italy

ARTICLE INFO

Article history:
Received 2 April 2009
Received in revised form 10 June 2009
Accepted 31 July 2009

Keywords:
Cornus mas
Flavonoids
Anthocyanins
HPLC
ESI-MS/MS

ABSTRACT

The methanol extract obtained from the ripe fruits of *Cornus mas* L. (Cornaceae) have been phytochemically studied. On the basis of HPLC–PDA–MS/MS^n analysis eight compounds have been identified as quercetin, kaempferol, and aromadendrin glycosilated derivatives. Three major compounds have been also isolated by Sephadex LH-20 column chromatography followed by HPLC and characterised by NMR spectroscopy. Moreover, LC–PDA–MS analysis of the red pigment revealed the presence of three anthocyanins. The quantitative analysis of all compounds was reported.

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1. Introduction

Cornus mas L. (Cornaceae family) known as the European and Asiatic cornelian cherry is a species of dogwood native to Southern Europe and Southwest Asia. It is a medium to large deciduous shrub or small tree growing to 5–12 m tall, with dark brown branches and greenish twigs. The fruit is an oblong, red drupe 2 cm long and 1.5 cm in diameter, containing a single seed. The fruit is edible, but when unripe it is astringent. The most common use of cornelian cherry fruits, is to produce different drinks, sweets, gels, and jams (Millspaugh, 1974). Extract from the fruits is also used in Europe for cosmetic purposes, replacing synthetic astringent substances, and are claimed to exert a favourable action on the human complexion (Polinicencu, Popescu, & Nistor, 1980). As ornamental, cornelian cherry, with its brilliant leaves and abundant, attractive flowers, is employed with very interesting effect in parks and small gardens (Slimestad & Andersen, 1998).

Fruits and vegetables are a good source of natural antioxidants, which containing many different radical scavenger components that provide protection against harmful-free radicals and therefore associated with lower incidence and mortality rates of cancer and heart diseases in addition to a number of other health benefits (Shui & Leong, 2006; Wang, Cao, & Prior, 1996). Among natural compounds, phenolics and in particular flavonoids were found to be an important part of human diet and are considered as active principles in many medicinal plants. In addition, flavonoids with their subclass anthocyanins, contribute the yellow, orange, red, and blue colour to flowers, fruits, and vegetables (Cooper-Driver, 2001), and could become important in the replacing of the synthetic pigments by the natural ones.

There are some investigations regarding the physical and chemical properties of cornelian cherry fruits, their antioxidant capacity, phenol, ascorbic acid, as well as anthocyanin contents (Demir & Kalyoncu, 2003; Didin, Kızılaslan, & Fenercioğlu, 2000; Guleryuz, Bolat, & Pirlak, 1998; Klimenko, 2004; Marinova, Ribarova, & Atanassova, 2005; Seeram, Schutzki, Chandra, & Nair, 2002; Tural & Koca, 2008; Vareed, Reddy, Schutzki, & Nair, 2006). Previous studies showed that the berries of *C. mas* contain five anthocyanins, identified by paper chromatography, spectrophotometric, and peroxide oxidation analysis as delphinidin 3-galactoside, cyanidin 3-galactoside, cyanidin 3-rhamnosylgalactoside, pelargonidin 3-galactoside, and pelargonidin 3-rhamnosylgalactoside (Du & Francis, 1973a, 1973b). The later work (Seeram et al., 2002) showed that anthocyanins of cornelian cherry are the mixture of three compounds: delphinidin 3-*O*-galactoside, cyanidin 3-*O*-galactoside, and pelargonidin 3-*O*-galactoside. Recent studies (Tural & Koca, 2008) indicated that three main *C. mas* anthocyanins were cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, and pelargonidin 3-*O*-glucoside. However, a survey of the previous literature revealed that the flavonoids content of the fruits has not been reported before.

The objectives of this study were to identify individual flavonoids of the fruits, evaluate their profile, and compare the content of anthocyanins, in order to understand the vast potential value of these fruits as food.

* Corresponding author. Tel.: +39 050 2219688; fax: +39 050 2219660.
E-mail address: braca@farm.unipi.it (A. Braca).

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doi:10.1016/j.foodchem.2009.07.063

2. Materials and methods

2.1. Plant material

C. mas L. fruits were collected in Riparbella (PI), Italy, in September 2006. The specimen was further identified and authenticated by Dr. Fabiano Camangi, Scuola Superiore S. Anna di Studi Universitari e di Perfezionamento di Pisa, Pisa, Italy.

2.2. Reagents

Standards of quercetin 3-*O*-rutinoside were purchased from Merck (E. Merck, Darmstadt, Germany). Standards of cyanidin 3-*O*-galactoside and pelargonidin 3-*O*-glucoside were obtained from Extrasynthese (Extrasynthese, France). Standard of pelargonidin 3-*O*-rutinoside were kindly provided by Indena, Italy. HPLC grade acetonitrile (CH_3CN), formic (HCOOH), and acetic (CH_3COOH) acids were purchased from J.T. Baker (Baker Mallinckrodt, Phillisburg, NJ, USA). HPLC grade water (18 mΩ) was prepared by a Mill-Ω^{50} purification system (Millipore Corp., Beddford, MA, USA).

2.3. General methods

An Avance Bruker 250 NMR spectrometer was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referenced to the solvents peaks δ_H 3.34 and δ_C 49.0 for CD_3OD. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden); HPLC separations were conducted on a Shimadzu LC-8A (Shimadzu Corp., Kyoto, Japan) series pumping system equipped with a Waters R401 refractive index detector and a Shimadzu injector with a Waters μ-Bondapak C_{18} column (7.8 × 300 mm, 10 μm, Waters, Milford, MA, USA). Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (E. Merck, Darmstad, Germany). HPLC–PDA–ESI-MS analyses were performed using a Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Flavonoid analyses were performed using a 4.6 × 250 mm, 5.0 μm, X Terra C_{18} column (Waters, Milford, MA) and the eluent was a mixture of 0.1% acetonitrile solution of CH_3COOH (solvent A) and 0.1% aqueous solution of CH_3COOH (solvent B). The solvent gradient was as follows: 0–50 min, 8–45% (A). Elution was performed at a flow rate of 1.0 ml/min with a splitting system of 2:8 to MS detector (200 μl/min) and PDA detector (800 μl/min), respectively. Analyses were performed with an ESI interface in the negative mode. The ionisation conditions were optimised, and the parameters were as follows: capillary temperature, 260 °C; capillary voltage, 18 V; tube lens offset, 5 V; sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 6 arbitrary units; spray voltage, 4.5 kV; scan range of *m/z* 200–700. PDA data were recorded with 220–500 nm range with the preferential channels 254 and 324 nm as the detection wavelengths. Anthocyanin analyses were performed using a 4.6 × 250 mm, 4 μm, Synergi Polar-RP 80 A column (Phenomenex Corp., Torrance, CA) and the eluent was a mixture of 1% acetonitrile solution of HCOOH (solvent A) and 1% aqueous solution of HCOOH (solvent B). The solvent gradient was as follows 0–20 min, 8–25% (A). Elution was performed at a flow rate of 1 ml/min with the splitting system of 2:8 to MS detector, respectively. Analyses were conducted with an ESI interface in the positive mode. Cyanidin 3-*O*-galactoside was used to optimise the ionisation and the fragmentation conditions; parameters were as follows: capillary temperature, 280 °C; capillary voltage, 11 V; tube lens offset, 15 V; sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 6 arbitrary units; spray voltage, 3.5 kV; scan range of *m/z* 200–700. PDA data were recorded with 220–600 nm range with a preferential channel of 515 nm as the detection wavelength. In both cases N_2 was used as the sheath and auxiliary gas. The volumes of injections were 20 μl. HPLC–PDA quantitative analyses were performed using a Waters 600E multisolvent delivery system, a Waters 717plus autosampler, and a Waters 996 PDA detector (Waters, Milford, MA) equipped with Millenium^{32} Chromatography Manager Software. The experimental conditions (solvent gradient, PDA channels, and columns) were the same as described above. The volume of injection was 25 μl. Quantitative determination was carried using calibration curves of standards. Quercetin 3-*O*-rutinoside, aromadendrin 7-*O*-glucoside, and cyanidin 3-*O*-galactoside were selected as the external standards of calibration for flavonols, dihydroflavonol, and anthocyanins, respectively. Standard calibration curves were prepared in a concentration range 0.0005–0.05 mg/ml with five different concentration levels (0.0005, 0.001, 0.005, 0.01, and 0.05 mg/ml) for quercetin 3-*O*-rutinoside and in a range of 0.001–0.05 mg/ml with four concentration levels (0.001, 0.005, 0.01, and 0.05 mg/ml) for aromadendrin 7-*O*-glucoside and cyanidin 3-*O*-galactoside. Triplicate injections were made for each level, and a weight linear regression was generated. The calibration curves with the external standards were obtained using concentration (mg/ml) with respect to the area obtained from the integration of the PDA peaks at a wavelength of 254 nm for flavonoids, 285 nm for dihydroflavonol, and 515 nm for anthocyanins. The relationship between variables was investigated using linear simple correlation. For the linear regression of the external standard, R^2 was 0.999 for quercetin 3-*O*-rutinoside, 1.000 for aromadendrin 7-*O*-glucoside, and 1.000 for cyanidin 3-*O*-galactoside, respectively. For the quantification of the compounds, a GraphPad Software Prism 3.0 was used. The amount of the compound was finally expressed in mg/10 g of fresh fruits.

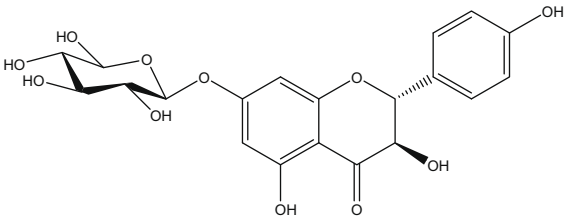
2.4. Extraction, isolation, and identification of flavonoids

Lyophilized fruits of *C. mas* (280 g) were defatted at room temperature with *n*-hexane, and extracted with MeOH by exhaustive maceration (5 × 500 ml) to yield 178 g of residue, which was dissolved in water and partitioned firstly with EtOAc and then with *n*-BuOH. The dried *n*-butanol extract (5 g) was subjected to fractionation on a Sephadex LH-20 column, using MeOH as eluent at a flow rate of 0.8 ml/min. Fractions of 8 ml were collected and grouped into 10 (A–J) fractions by TLC analyses on silica 60 F_{254} gel-coated glass sheets developed with *n*-BuOH–AcOH–H_2O (60:15:25) as the eluent. Fractions G (55.9 mg), H (36.1 mg), I (21.3 mg), and J (31.4 mg) were separately purified by RP-HPLC on a 7.8 × 300 mm i.d., C_{18} μ-Bondapak column at a flow rate of 2.0 ml/min with MeOH–H_2O (45:55) for fraction G, MeOH–H_2O (35:65) for fraction H, MeOH–H_2O (3:7) for fraction I, and MeOH–H_2O (4:6) for fraction J, to afford compounds **1** (6.1 mg, t_R = 12 min) and **7** (2.5 mg, t_R = 30 min) from fraction H, **4** (12.0 mg, t_R = 20 min) from fraction G, and **7** from fraction I (1.5 mg, t_R = 16 min) and J (3.3 mg, t_R = 9 min), respectively. Compounds were identified by spectroscopic methods and HPLC–PDA–ESI-MS analysis with authentic standards (Fig. 1):

Aromadendrin 7-*O*-β-D-glucoside (**1**): yellow amorphous powder. Negative ESI-MS: *m/z* 449 $[M-H]^-$. ^1H and ^{13}C data are consistent with previously published data (Slimestad, Andersen, & Francis, 1994).

Quercetin 3-*O*-β-D-xyloside (**2**): ESI-MS: *m/z* 433 $[M-H]^-$. The compound was identified by HPLC–PDA–ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

Quercetin 3-*O*-*a*-L-rhamnoside (**3**): ESI-MS: *m/z* 447 $[M-H]^-$. The compound was identified by HPLC–PDA–ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.



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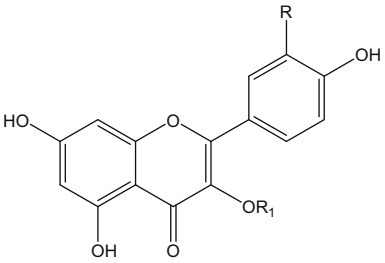
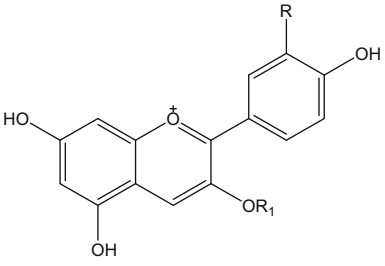


	R	R_1
2	OH	xyl
3	OH	rha
4	OH	rha-(1→6)-glc
5	OH	gal
6	OH	glc
7	OH	glu
8	H	gal



	R	R_1
9	OH	gal
10	H	glc
11	H	rha-(1→6)-glc

Fig. 1. Chemical structures of flavonoids identified in *C. mas* fruits. xyl = β-D-xylose, rha = α-L-rhamnose, glc = β-D-glucose, gal = β-D-galactose, glu = β-D-glucuronic acid.

Quercetin 3-*O*-rutinoside (**4**): yellow amorphous powder. Negative ESI-MS: *m/z* 609 $[M-H]^-$. ^1H and ^{13}C data are consistent with previously published data (Rastrelli, Saturnino, Schettino, & Dini, 1995).

Quercetin 3-*O*-β-D-galactoside (**5**): ESI-MS: *m/z* 463 $[M-H]^-$. The compound was identified by HPLC–PDA–ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

Quercetin 3-*O*-β-D-glucoside (**6**): ESI-MS: *m/z* 463 $[M-H]^-$. The compound was identified by HPLC–PDA–ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) with authentic standard.

Quercetin 3-*O*-β-D-glucuronide (**7**): yellow amorphous powder. ESI-MS: *m/z* 477 $[M-H]^-$. ^1H and ^{13}C data are consistent with previously published data (Moon, Tsushida, Nakahara, & Terao, 2001).

Kaempferol 3-*O*-β-D-galactoside (**8**): ESI-MS: *m/z* 447 $[M-H]^-$. The compound was identified by HPLC–PDA–ESI-MS analysis (retention time, UV spectrum, and ESI-MS spectrometric data) and comparison with authentic standard.

2.5. Extraction and identification of anthocyanins

Fresh fruits (10 g) of *C. mas* were homogenised in 60 ml of 2% HCl methanol solution. The solution was filtered using a Büchner funnel and the filtrate was used for HPLC analysis. Identifications were made by comparison of MS, PDA/UV, and retention data recorded for each anthocyanins standard.

3. Results and discussion

The methanol extract of the fruits of *C. mas* was partitioned with *n*-hexane, EtOAc, and *n*-BuOH. The butanol extract was subjected to fractionation with an initial separation by Sephadex LH-20 column chromatography. Subsequent purification of the fractions by semipreparative HPLC led to the isolation of three compounds: aromadendrin 7-*O*-β-D-glucoside (**1**), quercetin 3-*O*-rutinoside (**4**), and quercetin 3-*O*-β-D-glucuronide (**7**) (Fig. 1). The structure of each isolated compound was established by ^1H and ^{13}C NMR data and confirmed by comparison with those reported in the literature. The presence of not isolated compounds, quercetin 3-*O*-β-D-xyloside (**2**), quercetin 3-*O*-α-L-rahmnoside (**3**), quercetin 3-*O*-β-D-galactoside (**5**), quercetin 3-*O*-β-D-glucoside (**6**), and kaempferol 3-*O*-β-D-galactoside (**8**), was revealed by evaluation of the flavonoid profile throughout HPLC–PDA–ESI-MS analyses. The components were identified by comparison of obtained data (retention times, UV spectra, MS spectrometric data) with those of authentic standards. The LC-MS Base Peak chromatogram of the methanol extract and the chromatographic, spectroscopic, and spectrometric data, as well as, the quantitative amounts of individual compounds are shown in Fig. 2 and Table 1, respectively. Results obtained from quantitative analyses demonstrated that the flavonoids content in fruit was 221.3 mg/10 g. Methanol extract of cornelian cherries presented a rich flavonoid glycosides composition, that included eight compounds. Except for compound **1**, which belongs to the dihydroflavonols, they were all *O*-flavonol glycosides with quercetin and kaempferol as aglycones and oligosaccharide moieties as mono- or disaccharides linked at the 3-OH position. Quercetin 3-*O*-β-D-glucuronide (**7**) was the major constituent (69.9 mg), followed by kaempferol 3-*O*-β-D-galactoside (**8**) (41.3 mg).

The anthocyanin profile of cornelian cherry berries was carried out by means of HPLC–PDA–ESI-MS analyses. The chromatogram of the anthocyanins extract, recorded at 515 nm, is shown in Fig. 3. All the compounds were identified by comparison of their HPLC retention times, elution orders, ESI-MS spectrometric data, and photodiode array PDA/UV–vis with anthocyanin standards

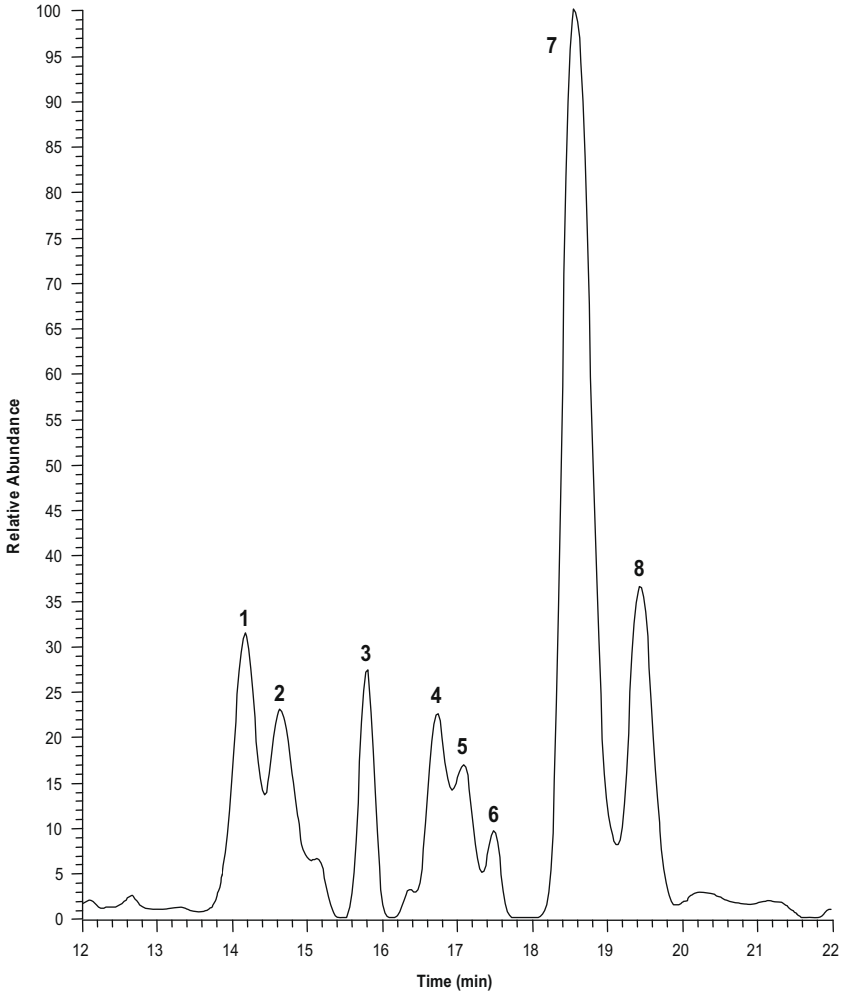


Fig. 2. Qualitative HPLC–ESI-MS chromatogram of flavonoid glycosides of *C. mas* fruits.

Table 1
Chromatographic, spectroscopic, and spectrometric data and quantitative amount (mg/10 g) of flavonoids found in *C. mas* fruits.

Peak	Compound	t_R (min)	$[M-H]^-$ (m/z)	MS/MS fragments (m/z)	λ_{max}	Quantitative amount[a]
1	Aromadendrin 7-*O*-glucoside	14.2	449	287	285	12.1
2	Quercetin 3-*O*-xyloside	14.6	433	301	255, 355	26.1
3	Quercetin 3-*O*-rhamnoside	15.8	447	301	255, 355	27.4
4	Quercetin 3-*O*-rutinoside	16.7	609	463, 301	255, 355	24.8
5	Quercetin 3-*O*-galactoside	17.1	463	301	255, 355	14.6
6	Quercetin 3-*O*-glucoside	17.5	463	301	255, 355	5.1
7	Quercetin 3-*O*-glucuronide	18.6	477	301	255, 355	69.9
8	Kaempferol 3-*O*-galactoside	19.4	447	285	265, 340	41.3

[a] Values are means ($n = 3$): the relative standard deviations for all compounds were <1%.

(Table 2). Compound **9** was recognised as cyanidin 3-*O*-galactoside, **10** as pelargonidin 3-*O*-glucoside, and **11** as pelargonidin 3-*O*-rutinoside, respectively (Fig. 1). Our results of the anthocyanin qualitative composition were not completely in agreement with those previously mentioned (Du & Francis, 1973a, 1973b; Seeram et al., 2002; Tural & Koca, 2008). The total amount of anthocyanins (Table 2) in *C. mas* fruits, determined on the cyanidin 3-*O*-galactoside basis, was 11.7 mg/10 g of fresh fruits. This result is comparable with those reported by Tural and Koca (2008) and Pantelidis, Vasilakakis, Manganaris, and Diamantidis (2007). Pelargonidin 3-*O*-glucoside (**10**) was the predominant anthocyanin, followed by cyanidin 3-*O*-galactoside (**9**). Pelargonidin 3-*O*-rutinoside was the least abundant anthocyanin and present only in trace amounts. The differences in the composition of the fruits, could be due to the growing conditions, such as soil, geographical and environmental conditions during the fruit development, degree of maturity at harvest, and/or genetic differences.

Considering that the epidemiological and experimental studies are correct in suggesting that higher intake of phenolics from food are associated with reduced risk of cancer, heart disease, and stroke, the immediate challenge is to increase the level of these beneficial phytochemicals in major food plants and find their potential new sources. The fruits of cornelian cherry revealed the presence of considerable amounts of flavonoids. Thus, results of the present study supported the antioxidant and nutraceutical potential of this plant species.

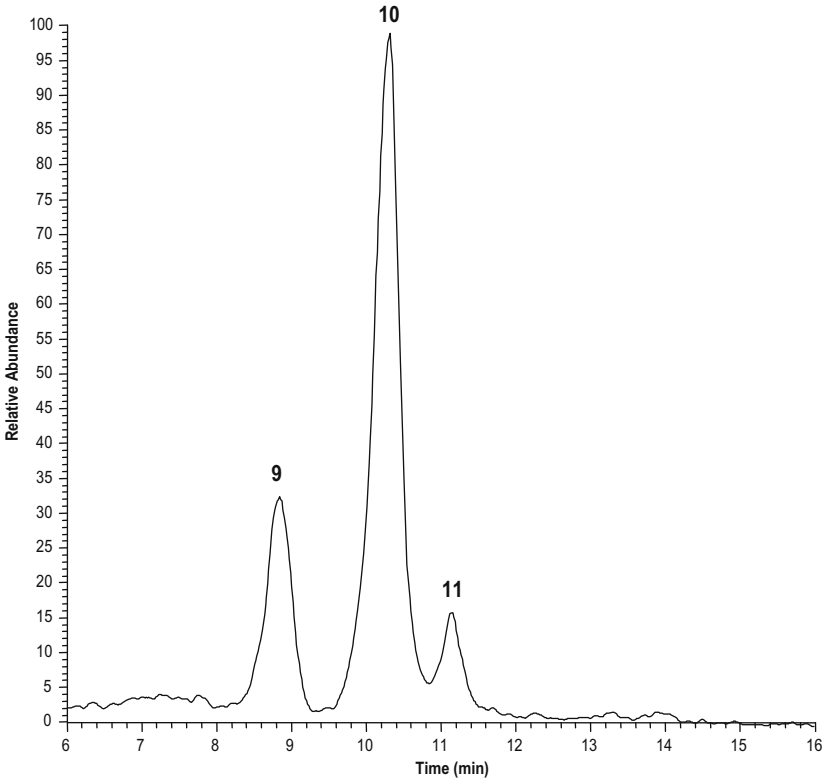


Fig. 3. HPLC–PDA chromatogram recorded at 515 nm corresponding to the anthocyanin profile of *C. mas* fruits.

Table 2
Chromatographic, spectroscopic, and spectrometric data and quantitative amount (mg/10 g) of anthocyanins found in *C. mas* fruits.

Peak	Compound	t_R (min)	$[M]^+$ (m/z)	MS/MS fragments (m/z)	λ_{max}	Amount[a]
9	Cyanidin 3-*O*-galactoside	8.8	449	287	280, 525	2.8
10	Pelargonidin 3-*O*-glucoside	10.3	433	271	280, 500	8.7
11	Pelargonidin 3-*O*-rutinoside	11.1	579	433, 271	280, 505	0.2

[a] Values are means ($n = 3$): the relative standard deviations for all compounds were <1%.

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