

Prunus mahaleb L. fruit extracts: a novel source for natural food pigments

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Abstract There is an increasing consumer and retailer demand for new natural colorants. Anthocyanins are a class of molecules already used as water-soluble natural colorant and for their health effects. *Prunus mahaleb* L. is a tree native to the Mediterranean area, producing highly pigmented small-stone fruits currently not used for fresh consumption due to their astringent and sour taste. In the present study, we set up a protocol to obtain a “mahaleb fruit concentrated extract” (*mfce*) and characterized it chemically by high-performance liquid chromatography/diode array detection. Anthocyanins, flavonols, flavanols, coumarin, sugars, ascorbic acid and organic acids were identified and quantified. After characterization, the antioxidant capacity of the *mfce* was assayed in vitro by three different methods: Trolox equivalent antioxidant capacity, Oxygen Radical Absorbance Capacity and Folin–Ciocalteu assays. The concentrated extract produced and characterized in this study showed a high content of anthocyanins, flavonols and coumarin and a strong antioxidant capacity. Therefore, *mfce* may be of interest as a natural food colorant and as an ingredient in the formulation of functional foods.

Keywords *Prunus mahaleb* L. · Anthocyanins · Sugars · Organic acids · Antioxidant activity · Food pigments

Introduction

The use of food colorants as additives in the food industry is important for both food companies and consumers in determining their expectations and perceptions of processed food. Although artificial colorants are easy to produce, stable, less expensive and have better coloring properties than natural colorants, in recent years, consumers have increasingly begun to prefer natural colorants, as synthetic colorants are associated with allergenic effects, intolerance reactions and hyperactivity [1]. Furthermore, evidence is increasingly appearing in the scientific literature about the health-promoting capacity of natural colorants [2]. For these reasons, demand for natural colorants has increased both from retailers and from consumers, leading to greater efforts on the part of the food industry to discover new natural colorants with appealing features [3]. At present, the European Union has authorized about 43 colorants as food additives [4], several of which are derived from natural source by physical and/or chemical extraction. The ban on using certain synthetic colorants has above all affected red pigments; prohibition of the use of FD&C Red No.2 in the USA by the Food and Drug Administration, scarlet GN and Ponceau GR in France and Orange RN in the UK has led to an increased demand for and use of natural new sources of red pigments [5]. Natural red colorants used to date in food are betacyanins, carmic acid, carotenoids and, especially, anthocyanins. Anthocyanins constitute the largest group of water-soluble natural pigments including more than 635 anthocyanins identified in nature and responsible for the red, orange, purple and blue colors of many fruits, vegetables and flowers [6]. Anthocyanins are easily dissolved in aqueous media and used as natural water-soluble colorants. In the USA, anthocyanins are classified as color additives that are exempt from certification [7], whereas in the European Union

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anthocyanin colorants are categorized under classification number E163. Anthocyanins are also used as food colorant in Asian, Central American and South American countries [2]. Another significant property of anthocyanins is their antioxidant activity, which plays an important role in their possible health benefits. There is much evidence that dietary consumption of anthocyanins may have anti-inflammatory, anti-carcinogenic and neuroprotective activities, reduce the incidence of cardiovascular disease and help in obesity and metabolic syndrome control [8]. Like most of the current commercial, naturally derived food colorants, anthocyanins have several drawbacks. Anthocyanins, betanins, carotenoids and chlorophyll pigments are decolorized by oxidation, making them sensitive to light, heat and oxygen [9]. For this reason, the use of a natural colorant in food requires knowledge of its stability and the conditions governing its degradation, so that food industries can find right conditions to optimize the industrial processing and storage of colored products. Anthocyanin chemical stability is the main focus of many studies performed in beverage, juice extract and jam [10]. It would appear from these studies that the color and stability of anthocyanins in food products are dependent on several factors, including structure and concentration of the pigment, pH, temperature, light intensity, presence of copigments, metallic ions, enzymes, oxygen, ascorbic acid and sugars. By contrast, it is still unclear whether the degradation of anthocyanins lowers antioxidant activity of the final colored products [11]. As result of anthocyanin instability, efforts are made to optimize thermal processes in combination with nonthermal technologies (high pressure or ultrasound) and to discover new natural sources of more stable anthocyanins. *Prunus mahaleb* L. (*P. mahaleb*) is a deciduous tree native to Mediterranean region, Iran and Central Asia. The tree prefers a warm dry climate and well-drained soils and is robust and resistant to several diseases. Due to these characteristics, the mahaleb cherry tree is used as a rootstock in grafting sweet cherry, especially in Italy and above all in Apulia. *P. mahaleb* produces highly pigmented small-stone fruits that have an astringent and sour taste. Mahaleb seed and its powder are fragrant and have a taste comparable to bitter almonds with cherry notes. They are used in small quantities to sharpen sweet foods in Turkey, Armenia and Greece. Moreover, mahaleb fruit is used in liqueur, wine and vinegar production in the food industry [12, 13]. In our previous studies [14, 15] and in a few other works [12, 13], these fruits had been preliminary characterized revealing a high content in anthocyanins. Mahaleb fruit anthocyanins or fruit extracts could be used as a new natural colorant for food formulations. Although many of the currently authorized natural food colorants have been accepted on the basis of tradition, for anthocyanins extracted from new sources and/or using non-aqueous extraction methods, the absence of extract qualitative and quantitative chemical

characterization means that the presumption of safety, prescribed for food additives derived from natural sources in the 1975 Scientific Committee for Food (SCF) opinion [16], cannot be verified. The aims of this work were to set up ideal conditions for producing a mahaleb fruit concentrated extract with high total soluble solid content (TSS) exploitable as a food additive, as well as its chemical characterization. The evaluation of phenolic compounds, sugar, organic acid content and antioxidant capacity is the basis for verifying the safety of the extract as a food colorant and its possible use as a health-promoting food additive.

Materials and methods

Plant materials

Organic *P. mahaleb* fruits were purchased from a local nursery at fully ripened stage; any leaves, unripe fruits and other contaminating material were removed, and the fruits were then stored at -20°C until extraction.

Standards and reagents

All the solvents and reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA). Cyanidin 3-*O*-rutinoside ($\geq 96\%$ HPLC), cyanidin 3-*O*-glucoside ($\geq 96\%$ HPLC), cyanidin 3-*O*-sambubioside (cyanidin 3-*O*-xylosyl glucoside) ($\geq 95\%$ HPLC) and cyanidin 3,5-*O*-diglucoside ($\geq 97\%$ HPLC) were purchased from Extrasynthèse (Genay Cedex-France). The other standards and ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). All extraction solvents and reagents were of food grade, while all chromatographic solvents were of HPLC grade.

Preparation of mahaleb fruit concentrated extract (mfce)

The mahaleb fruits were thawed and homogenized using a household fruit centrifuge obtaining a very thick fruit juice and fruit peel, pulp and seeds. Obtained fruit juice was mixed with peel, pulp and seeds, and the mix was extracted using ethanol or water as solvents in a ratio of 1 g/1 mL; moreover, stock solutions of citric acid (1 M) or acetic acid (1 M) were added in different percentages (1, 2 and 5 %) to obtain different levels of solvent acidity. The extraction was carried out at room temperature, overnight and in the dark without stirring. Afterward, the extract was filtered through paper and concentrated using a rotary evaporator (Buchi, Rotavapor R205) at 35°C , to obtain a concentrated extract with a total soluble solid content of 60 °Brix, measured at 20°C using an Optech refractometer (München, Germany).

Quantification of anthocyanins

Total monomeric anthocyanins were quantified following the spectrophotometric pH differential method described by Giusti et al. [17]: the extracts were mixed using the appropriate dilution factor, with two different solutions to obtain different pH values. For pH 1.0, the extract was mixed with a 0.025 M KCl solution, and for pH 4.5, the extract was mixed with CH₃COONa 0.4 M. After 15 min of incubation at room temperature, the absorbance of the samples was measured at $\lambda_{\text{vis-max}}$ and 700 nm (Beckman DU640 spectrophotometer, USA). Absorbance of the diluted sample (A) was calculated as follows:

$$A = (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH}1.0} - (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH}4.5}$$

The absorbance value A was used to calculate the monomeric anthocyanin pigment concentration (C) in the original extract using the following formula:

$$C \text{ (mg/litre)} = (A \times MW \times DF \times 1000) / \varepsilon$$

where MW and ε are, respectively, the molecular weight and the extinction coefficient of cyanidin 3-*O*-rutinoside (keracyanin), DF the dilution factor, A the absorbance and C the concentration of anthocyanin expressed as mg of cyanidin 3-*O*-rutinoside per liter.

Acid hydrolysis of mfce

The *mfce* was diluted in water and mixed with HCl 1 M (final concentration), heated in a capped tube at 95 °C for 2 h and then cooled in an ice bath. The hydrolyzate was filtered and analyzed by High-Performance Liquid Chromatography (HPLC) analysis.

High-Performance Liquid Chromatography analysis

Identification and quantification of phenolic compounds

To quantify and characterize the phenolic compounds in *mfce*, we performed an HPLC analysis using an Agilent-1100 liquid chromatograph equipped with a DAD detector. The column was a C18 Luna (Phenomenex, 250 × 46 mm, 5 μ m) in conjunction with a C18 guard cartridge column, both thermostated at 30 °C. The mobile phase was (A) H₂O/formic acid = 95/5 and (B) acetonitrile/formic acid = 95/5. The samples were eluted following the multi-step linear gradient proposed by Obon et al. [18] with slight modifications: 1 min of isocratic elution with 6.7 % B, 25 min of linear gradient from 6.7 to 16.7 % B, 9 min of linear gradient from 16.7 to 55.6 % B, 5 min of isocratic elution with 55.6 % B, 3 min of linear gradient from 55.6 B to 80 % B and 8 min of isocratic elution with 80 % B. Equilibration time: 10 min before the next injection. Flow rate: 0.7 mL/min.

UV–Vis spectra were recorded in the 200–800 nm range, and the chromatograms were acquired at 280, 350 and 520 nm. The identity of the phenolic compounds was ascertained by comparing their retention times and UV–Vis spectra with that of authentic standards. Quantification of individual polyphenolic compounds was directly performed by HPLC/DAD using a five-point regression curve ($r^2 \geq 0.99$) generated through the use of authentic standards. Compounds that did not have their own external standard were quantified using the most similar standard available. Catechin, coumarin and phenolic acids were determined at 280 nm, flavonols at 350 nm and anthocyanins at 520 nm. Total anthocyanins were quantified using cyanidin 3-*O*-rutinoside (keracyanin) as reference compounds.

Identification and quantification of sugars and organic acids

Sugars and organic acids were identified and quantified in *mfce* by HPLC analysis using an HPLC system (Agilent-1100 series) equipped with a pump system, a refractive index detector (RID) for sugar analysis, and a UV/Vis detector monitored at 210 nm for the analysis of organic acids. Sugars and organic acids were simultaneously analyzed onto an Aminex HPX-87H column (Bio-Rad, 300 × 7.8 mm, 9 μ m) kept at 55 °C. The analytical conditions used were as follows: flow 0.3 mL min^{−1}, eluent 0.045 N H₂SO₄ with 6 % acetonitrile (v/v) [19]. The identities of both sugars and organic acids were ascertained by comparison and combination of their retention times and UV–Vis spectra with those of authentic standards. Quantification of individual sugars or organic acids was performed directly by HPLC/DAD/RID using a five-point regression curve ($r^2 \geq 0.99$) on the basis of authentic standards.

Analysis of vitamin C

HPLC analysis of ascorbic acid was performed using a C18 column (Phenomenex Luna C18, 250 × 4.60 mm, 5 μ m) and the HPLC system described above (Agilent-1100 series). Column temperature was set at 25 °C and the mobile phase was distilled water acidified to pH 2.2 using H₂SO₄, at a flow rate of 1 mL/min. Quantification was performed with a DAD detector at a wavelength of 254 nm using a nine-point regression curve ($r^2 \geq 0.99$) of L-ascorbic acid [20].

Antioxidant activity assay

Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is based on the ability of antioxidants to scavenge the long-life radical cation 2,2'-azino-bis(3-ethylbenzothiazolone 6-sulfonate) (ABTS^{•+}). The assay was

performed as previously described by Re et al. [21] with minor modifications. ABTS was dissolved in water at a concentration of 7 mM. ABTS radical cation (ABTS^+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study, the ABTS^+ solution was diluted with H_2O to an absorbance of $0.70 (\pm 0.02)$ at 734 nm and equilibrated at 30 °C. The assay was adapted to a 96-well format (Costar, 96-well clear round bottom plate, Corning). In each well, 180 μL of diluted ABTS^+ solution was placed and mixed with 20 μL of diluted extract or Trolox standard solutions (from 0 to 25 μM). The absorbance at 734 nm was read 10 min after the addition of the radical solution using a microplate reader (Tecan, Infinite M200). Appropriate solvent blanks were run in each assay. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of Trolox for the standard reference data.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC procedure was carried out following the procedure established by Dávalos et al. [22]. The reaction was carried out using a 96-well plate (Optiplate 96 F, black, Perkin Elmer) in a 75 mM phosphate buffer (pH 7.4), and the final reaction volume was 200 μL . Antioxidant (20 μL) and fluorescein (120 μL ; 70 nM, final concentration) solutions were placed in the well of the microplate. The mixture was preincubated for 15 min at 37 °C. 2,2'-Azobis-(2-methylpropionamide) dihydrochloride (AAPH) solution (60 μL ; 12 mM, final concentration) was rapidly added using a multichannel pipet. The microplate was immediately placed in a microplate reader (Tecan, Infinite M200), and the fluorescence recorded (excitation and emission wavelengths of 485 and 527 nm, respectively) every minute for 60 min. Immediately following the addition of AAPH, the plate was shaken for 6 sec. A blank (fluorescein + AAPH) using phosphate buffer instead of the antioxidant solution was carried out in each assay. All the reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. Decay curves (fluorescence intensity vs. time) were recorded and the area between the two decay curves (with or without antioxidant) was calculated. The net area under the curve was obtained by subtracting the blank value from that of the sample or standard. Subsequently, the degree of antioxidant-mediated protection was quantified using the antioxidant Trolox as a standard. Different Trolox concentrations (1–6 μM) were used to make a standard curve. Final ORAC values were expressed as μmol Trolox equivalents (TE)/mL of *mfce*.

Folin–Ciocalteu (F–C) assay A rapid microplate methodology [23] was used to assess F–C reducing capacity in *mfce*. The assay was performed in microtiter 96-well plates (Costar, 96-well clear round bottom plate, Corning) using a microplate reader (Tecan, Infinite M200). Gallic acid standard solution or *mfce* sample (50 μL) and Folin–Ciocalteu Reagent (FCR) (1:5, v/v) (50 μL) were placed in each well, and then, 100 μL of sodium hydroxide solution (0.35 M) was added. The absorbance at 760 nm of the blue complex formed was monitored after 5 min. To evaluate the intrinsic absorption of sample, 50 μL of 0.4 M of acid solution was added instead of FCR. The reagent blank was evaluated by the addition of 50 μL of water instead of standard compound or sample. All experiments were performed in quadruplicate at room temperature. A calibration curve relating the absorbance and the concentration of gallic acid in the range from 2.5 to 40.0 mgL^{-1} was established ($R \geq 0.9997$). The absorbance values obtained for samples were related to those of the gallic acid standard curve, and the F–C reducing capacity was expressed as gallic acid equivalents (mgGAE/mL).

Statistics

All the trials were performed at least in triplicate, and all the data were reported as mean $\pm$ standard deviation (SD). The statistical significance was evaluated using Student's *t* test, and $p < 0.05$, 0.01 or 0.001 were taken as significant (Microsoft Excel 2003 Software). Analysis of variance was performed using the free software package PAST [24]. Tukey's HSD post hoc tests were applied to establish significant differences between means ($\alpha = 0.05$).

Results and discussion

Optimized method for preparation of an anthocyanin-rich mahaleb fruit concentrated extract (*mfce*)

One of the aims of this work was to identify optimal extraction conditions for a *P. mahaleb* L. fruit extract rich in bio-functional compounds that could be used as a natural pigment in the food industry. Optimization of the extraction procedure for anthocyanins from mahaleb fruits was carried out using two solvents: food-grade ethanol and water. Both solvents may be used during the processing of raw materials, food components or food ingredients as indicated in Directive 2009/32/EC of the European Parliament [25]. Moreover, stock solutions of citric acid (1 M) and acetic acid (1 M) were selected to obtain different levels of solvent acidity (1, 2 and 5 %). The acidity of solvents is important to prevent degradation of anthocyanins during extraction

Table 1 Anthocyanin yield expressed as mg of keracyanin equivalents (KE)/mg of fruit fresh weight (FW), obtained from *P.mahaleb* fruits using twelve solvent combinations

Solvent	pH	Anthocyanins (mgKE/gFW)
EtOH + 1 % citric acid 1 M	3.68	5.542 ± 0.171 ^a
EtOH + 2 % citric acid 1 M	3.58	4.947 ± 0.376 ^a
EtOH + 5 % citric acid 1 M	3.34	4.830 ± 0.502 ^a
EtOH + 1 % acetic acid 1 M	4.87	5.019 ± 0.102 ^a
EtOH + 2 % acetic acid 1 M	4.77	5.184 ± 0.031 ^a
EtOH + 5 % acetic acid 1 M	4.50	5.504 ± 0.277 ^a
H ₂ O + 1 % citric acid 1 M	2.63	3.380 ± 0.154 ^b
H ₂ O + 2 % citric acid 1 M	2.41	3.158 ± 0.059 ^b
H ₂ O + 5 % citric acid 1 M	2.21	2.445 ± 0.127 ^c
H ₂ O + 1 % acetic acid 1 M	3.29	3.332 ± 0.254 ^b
H ₂ O + 2 % acetic acid 1 M	3.14	3.380 ± 0.201 ^b
H ₂ O + 5 % acetic acid 1 M	2.93	3.501 ± 0.074 ^b

Data are the mean ± SD ($n = 3$). Different letters indicate significantly different values at $p < 0.001$

from fruits and storage of extracts, whereas excess acid can lead to hydrolysis of acylated anthocyanins during extraction. For this reason, the use of weak acids, such as citric or acetic acid, is advisable [26]. Table 1 lists the twelve combinations of solvent and acid used for fruit extraction, corresponding pH values and anthocyanin yields. To evaluate the efficiency of each combination in the extraction process, the anthocyanin content of extracts was quantified, before solvent evaporation, by the pH differential method [17], and the results were expressed as mg of anthocyanin/g of fruit fresh weight (FW). As can be observed in Table 1, the use of water or ethanol as extracting solvents showed a significant ($p < 0.001$) effect on final anthocyanin yield. Lower yields of anthocyanins were obtained in water when compared to ethanol. The type of acid used did not significantly affect the anthocyanin yield. Maximum anthocyanin yield (5.542 ± 0.171 mgKE/g fresh weight) was obtained using ethanol in combination with 1 % (v/v) citric acid, and this solvent was successively used to obtain an anthocyanin-rich extract. Previous reports on *Vaccinium* species [27] and sour cherry cultivars [28] indicated a large variation in anthocyanin content. Blueberry was reported to have an anthocyanin content of 62–300 mg/100 g FW, while the total anthocyanin content of sour cherries was between 21 and 285 mg/100 g FW. Both these fruits, considered to be among the most anthocyanin rich, showed lower or similar anthocyanin content than that obtained from mahaleb fruits using the extraction protocol developed in this study. After extraction, the majority of the solvent was evaporated to obtain a very concentrated extract with a high content of soluble solids (60.00 ± 1.47 °Brix), similar to the values reported for industrial fruit juice concentrate (60–65 °Brix)

Table 2 Sugar, organic acid and phenolic compositions of *mfce*

Compounds	
<i>Sugars</i> (g/mL)	
Glucose	0.297 ± 0.045
Fructose	0.170 ± 0.022
Sorbitol	0.145 ± 0.029
<i>Organic acids</i> (g/mL)	
Tartaric acid	0.193 ± 0.018
Malic acid	0.067 ± 0.007
Succinic acid	0.0041 ± 0.004
citric acid	0.131 ± 0.012
<i>Ascorbic acid</i> (μg/mL)	78.040 ± 6.080
<i>Anthocyanins</i> (mgKE/mL)	24.395 ± 2.613
<i>Coumarin</i> (μg/mL)	901.818 ± 18.026
<i>Flavanols</i> (μg/mL)	
Catechin	932.122 ± 32.156
<i>Flavonols</i> (μg/mL)	
Quercetin	55.905 ± 1.555
Quercetin-3- <i>O</i> -glucoside	772.751 ± 8.295
Rutin	572.562 ± 9.080

Results are the mean ± SD ($n = 4$)

KE keracyanin equivalents

[29], a pH value of 5.31 ± 0.21 and a density equal to 1.26 ± 0.03 kg/L. Using this method, we obtained a product with high anthocyanin content and good potential for storage, transport and preservation.

High-performance liquid chromatography analysis of *mfce*

The characterization of *mfce* phenolic compounds and sugars/organic acids was performed by reversed-phase HPLC and by ion-moderated partition HPLC, respectively. Amounts of sugars, organic acids and phenolic compounds identified in *mfce* are indicated in Table 2.

Phenolic compounds

In *mfce*, three classes of phenolics were characterized: anthocyanins, flavonols and flavanols (catechin). Coumarin (benzo-2-pyrone) and its precursors were also identified and quantified.

Anthocyanins. Identification and peak assignment of anthocyanins in *mfce* was based on comparison of their retention times and UV–Vis spectra with those of standards and on published data on *P. mahaleb* L. fruit anthocyanin characterization by MS analysis [12–14]. Figure 1A shows the HPLC anthocyanin profile of *mfce*. The four peaks in Fig. 1A were identified as cyanidin 3-*O*-xylosyl glucoside (cyanidin 3-*O*-sambubioside) (peak 1), cyanidin

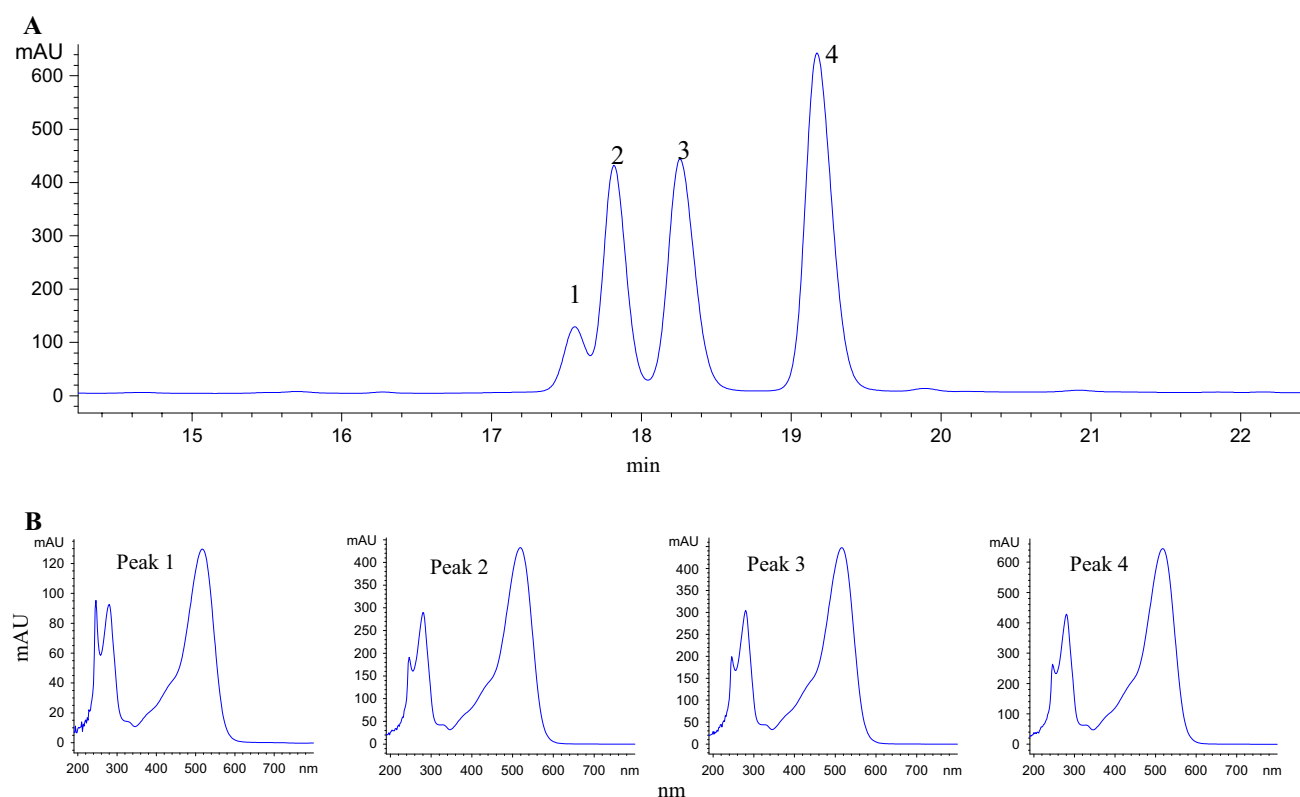


Fig. 1 **A:** anthocyanin profile in *mfce* ($\lambda = 520$ nm) analyzed using HPLC. Peaks: 1 cyanidin 3-*O*-xylosyl-glucoside; 2 cyanidin 3-*O*-xylosyl-rutinoside; 3 cyanidin 3-*O*-glucoside; 4 cyanidin 3-*O*-rutinoside. **B:** UV–Vis spectra of the HPLC peaks recorded in chromatogram shown in A

Table 3 Chromatographic and spectral characteristics of peaks in Figs. 1 (A, B) and 2

Peak no.	Compounds	RT (min)	λ_{vis} (nm)	λ_{UV} (nm)	E_{440}/E_{vis} (%)	E_{440}/E_{vis} indicating	Acyl peak (310–360 nm)
1	Cyanidin 3-xylosyl glucoside	17.55	518	280	31.81	Monoside	N
2	Cyanidin 3-xylosyl-rutinoside	17.82	518	280	31.11	Monoside	N
3	Cyanidin 3-glucoside	18.25	516	280	32.10	Monoside	N
4	Cyanidin 3-rutinoside	19.16	518	280	31.60	Monoside	N
5	Cyanidin	29.60	524	280	26.23	–	N

RT Retention time, E_{440} absorptivity at 440 nm, E_{vis} absorptivity at the corresponding maxima λ_{vis} , N no acylation with hydroxy aromatic acid detected

3-*O*-xylosyl-rutinoside (peak 2), cyanidin 3-*O*-glucoside (kuromanin) (peak 3) and cyanidin 3-*O*-rutinoside (kercyanin) (peak 4). Due to the lack of the relative commercial standard, peak 2 was tentatively identified as cyanidin 3-*O*-xylosyl-rutinoside on the basis of our previous results from LC–MS analysis of anthocyanins in *P. mahaleb* L. fruits [14]. UV–Vis spectra were also carried out to confirm peak identification (Fig. 1B) as spectral properties are useful for characterizing anthocyanins [30]. Data (retention time, λ_{UV} and λ_{vis} , acylation peak) obtained when analyzing the UV–Vis spectra of anthocyanins peaks are

reported in Table 3. The visible λ_{max} of our spectra corresponded exactly to those already reported in the literature for the same molecules [30, 31]. Information about the glycosidation substitution pattern of the anthocyanins can be obtained using peak spectral characteristics λ_{UV} and λ_{vis} , and their corresponding absorptivities (Table 3). The 3-glycosides and the 3,5-diglycosides show differences in their 400–460 nm regions. These differences have been expressed as $E_{440}/E_{\lambda_{max}}$ ratio, a ratio of 29–35 % indicating a monoside and a ratio of 15–24 % indicating a bioside [32]. As shown in Table 3, the anthocyanins isolated from

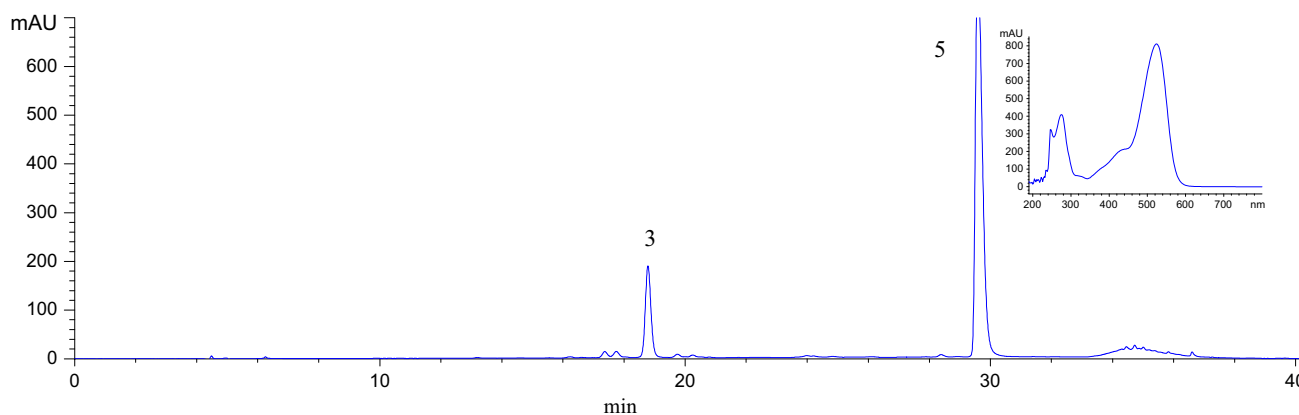


Fig. 2 Anthocyanin profile in hydrolyzed *mfce* ($\lambda = 520$ nm) analyzed using HPLC. Peaks: 3 cyanidin 3-*O*-glucoside; 5 cyanidin. Inset is UV-Vis spectra of peak 5

mfce are all monosides. In a previous study on *P. mahaleb* fruit, Ieri et al. [12] identified a very low content of cyanidin 3,5-diglucoside. The absence of cyanidin 3,5-diglucoside in *mfce* could be a result of degradation due to the pH of the extract (pH 5–6) as also suggested by Hou et al. [30]. Information regarding the presence of acylation by hydroxylated aromatic organic acids can also be obtained from anthocyanin spectra; this is pointed out by the presence of a peak in the 310–360 nm range [32]. As shown in Table 3, none of the identified anthocyanins showed a peak in this area.

In order to determine which aglycones were present in the extract, a comparison of an acid hydrolysate of *mfce* with the standard anthocyanidins was performed. HPLC retention time and spectral characteristics of the peaks demonstrated that cyanidin was the only aglycone in *mfce*. Figure 2 shows the HPLC chromatogram of the extract after 2 h of acid hydrolysis; comparison of the chromatograms in Figs. 1A and 2 shows the almost complete disappearance (84 %) of the anthocyanin peaks with the exception of a residual kuromanin peak (peak 3). Moreover, the appearance of an anthocyanidin peak with a retention time of 29.60 min was observed. Giusti et al. [33] reported that acid hydrolysis of anthocyanin diglycosides in red radish resulted in the formation of aglycon and intermediate pigments, some of which were monoglycosides. Therefore, the presence of the cyanidin 3-glucoside peak after 2 h of hydrolysis is unlikely to be due to the stability of the molecule [34], but could be an intermediate derived from the hydrolysis of keracyanin. Retention time, λ_{UV} and λ_{Vis} obtained for peak no. 5 (Table 3), confirmed that it corresponds to cyanidin. It is known from the literature that anthocyanins elute at shorter retention times than their parent anthocyanidins [35] when analyzed by reversed-phase (RP) HPLC as shown in Table 3. Anthocyanidins exhibit a 6 to 10 nm higher visible λ_{max} than the corresponding anthocyanins, and indeed,

Table 4 Single anthocyanin HPLC quantification in *mfce*

Anthocyanin	mg/mL $\pm$ SD
Cyanidin 3-glucoside	4.080 $\pm$ 0.079
Cyanidin 3-rutinoside	7.110 $\pm$ 0.079
Cyanidin 3-xylosyl glucoside	2.284 $\pm$ 0.078
Cyanidin 3-xylosyl-rutinoside	4.024 $\pm$ 0.046

Quantification was performed on the basis of authentic standard except cyanidin 3-xylosyl-rutinoside that was quantified using the most similar standard available (cyanidin 3-xylosyl glucoside). Data are the mean $\pm$ SD ($n = 4$)

the λ_{max} of peak 5 corresponds perfectly to that reported by Hong et al. [31] for cyanidin (523–525 nm). Lastly for the aglycon molecules, the $E_{440}/E_{\lambda_{max}}$ ratio is intermediate compared to the bioside and monoside ratio [33] exactly as reported in Table 3 for peak 5.

In Table 4, the HPLC quantification of the individual anthocyanins identified in *mfce* is reported. Cyanidin was the only anthocyanidin identified in *mfce*, this features being common to anthocyanidin profile of many fruits belonging to the *Prunus* genus such as *P. cerasus*, *P. avium*, *P. domestica* and *P. persica* fruits as well as blackberry, elderberry and red currant in which the only or predominant anthocyanidin is indeed cyaniding [31, 36, 37]. The anthocyanin profile of *mfce* is similar to that of sweet cherry and tart cherry fruits, in which cyanidin 3-*O*-rutinoside is among the most abundant anthocyanins identified [38–40]. In conclusion, anthocyanins are the main polyphenol compounds in *mfce* and their concentration is much higher when compared to cherry juice concentrate [41]. Moreover, the anthocyanin content is even higher than in red fruit juice concentrates (elderberry, blackcurrant, chokeberry, etc.), well known to be the most important dietary sources of anthocyanins [42].

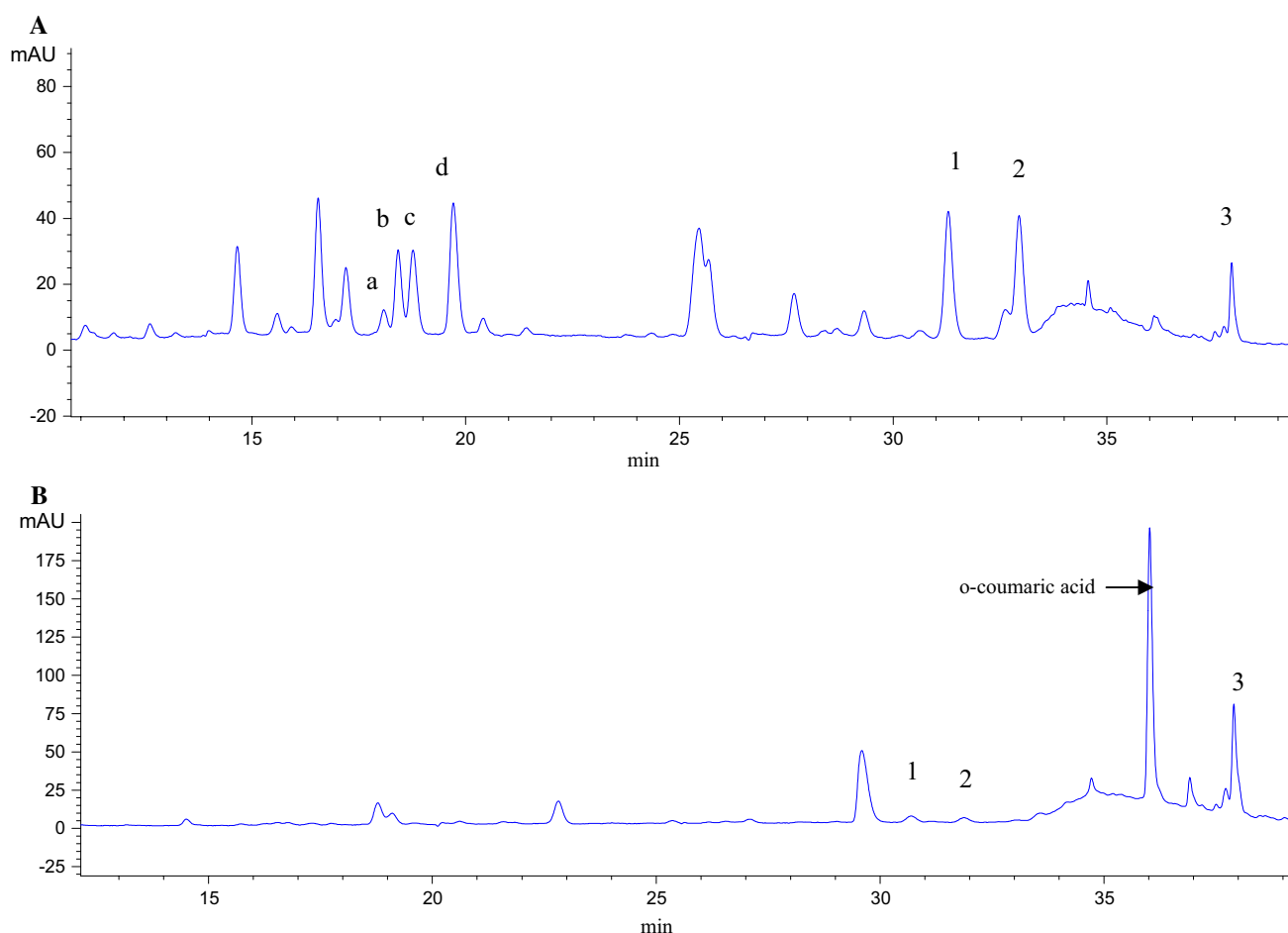


Fig. 3 HPLC chromatographic profile ($\lambda = 350$) nm of *mfce* before (A) and after (B) acid hydrolysis. Peaks *a*, *b*, *c* and *d* show the absorption of anthocyanins at $\lambda = 350$ nm. Peaks: 1 rutin; 2 quercetin 3-*O*-glucoside; 3 quercetin

Flavonols

HPLC/DAD analysis of *mfce* at 350 nm (Fig. 3A) indicates the presence of rutin (quercetin-3-rutinoside), quercetin-3-glucoside and quercetin. An HPLC chromatogram of extract after acid hydrolysis (Fig. 3B) showed the disappearance of rutin and quercetin-3-glucoside and an increase in quercetin. The presence of rutin and/or quercetin has been reported by several authors in *P. avium*, *P. cerasus* and *P. mahaleb* fruits [12, 20, 40, 43, 44]. The amounts of rutin, quercetin-3-glucoside and quercetin (Table 2) in *mfce* are higher than reported by Bermúdez-Soto et al. [42] for blackcurrant juice concentrate and similar to those found in chokeberry juice concentrate.

Catechin, phenolic acids and coumarin

As shown in Table 2, catechin was the only flavanol identified (peak 1, Fig. 4) and quantified in *mfce* (Table 2). Catechin has also been identified in white mahaleb seeds

by Mariod et al. [45] and in sweet cherry fruit extracts by Gündoğdu et al. [20]. The amount of catechin in *mfce* was much lower than the anthocyanin amount; nevertheless, it was comparable with the total flavan-3-ol amount in elderberry concentrate [42].

The identification of main peaks observed in the HPLC chromatogram at 280 nm (Fig. 4A) was based on the study of the HPLC chromatogram of *mfce* after HCl hydrolysis (Fig. 4B) and comparison with MS data on *P. mahaleb* fruit polyphenol characterization from Ieri et al. [12]. Comparison of the chromatograms in Fig. 4A and B shows the complete disappearance of peaks 2, 3 and 4 and the appearance of two new peaks (peaks 7 and 8). Moreover, as discussed above, acid hydrolysis resulted in the disappearance of the anthocyanin peaks. Peak 7 was identified as melilotic acid (or dihydro-*o*-coumaric acid) and peak 8 as *o*-coumaric acid on the basis of comparison of their retention times and UV–Vis spectra with those of authentic standards. Peak 6 is still unknown. From these results and from the analysis of UV–Vis spectra, we tentatively identified peak 3 as

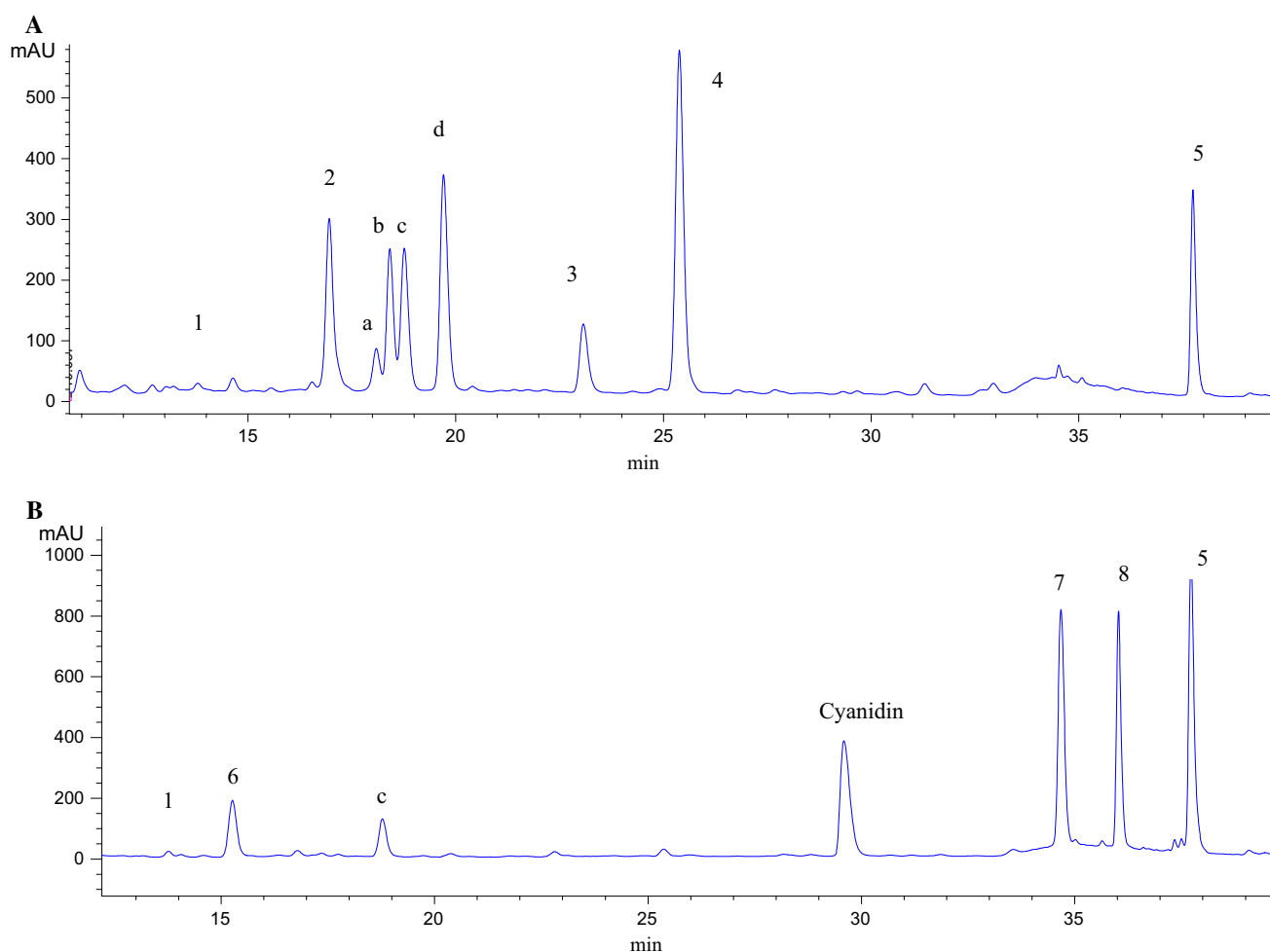


Fig. 4 HPLC chromatographic profile ($\lambda = 280$ nm) of *mfce* before (A) and after (B) acid hydrolysis. Peaks a, b, c and d correspond to anthocyanin absorption at $\lambda = 280$ nm. Peaks: 1 catechin; 2 unknown

compound; 3 dihydro-*o*-coumaric acid 2-*O*-glucoside; 4 *o*-coumaric acid 2-*O*-glucoside; 5 coumarin; 6 unknown compound; 7 dihydro-*o*-coumaric acid; 8 *o*-coumaric acid

dihydro-*o*-coumaric acid 2-*O*-glucoside. Peak 4 was identified as *o*-coumaric acid 2-*O*-glucoside by comparison of its retention time with the retention time of melilotoside (glucoside of trans-2-coumaric acid) standard. Melilotoside content was calculated as 3.60 ± 0.02 mg/mL of *mfce*. As far as peak 2 is concerned, we do not have sufficient information to definitely identify this compound. Dihydro-*o*-coumaric acid 2-*O*-glucoside and *o*-coumaric acid 2-*O*-glucoside are metabolites related to the synthesis and conversion of coumarin in higher plants [46, 47]. Coumarin is a secondary plant product found in many plants such as tonka seeds, sweet clover, *Mikania glomerata* and *M. laevigata*, lavender, strawberry, cherries, liquorice and mostly in *Cinnamomum cassia* [47, 48]. Coumarin belongs to a group of ingredients listed by the European Council as “active principles” because in foods they have a strong flavor but also a toxicological relevance. Coumarin has been reported to cause hepatic damage in laboratory animals

and to have carcinogenic effects in long-term, high-dose animal experiments [49]. However, in 2008, the European Food Safety Authority (EFSA) found no evidence of a coumarin’s genotoxic mechanism, and so a tolerable daily intake (TDI) of 0.1 mg/kg of body weight was agreed, based on human data experiments [50]. Moreover, in the same document, EFSA stated that exposure to coumarin exceeding the TDI by threefold for 1–2 weeks would not raise any safety concerns. The presence of coumarin in the leaves, fruits and seeds of *P. mahaleb* is known from the literature [12, 51, 52]. Because of the TDI set by EFSA, we quantified coumarin in *mfce*. Peak 5 in Fig. 4A–B was identified as coumarin on the basis of a comparison of its retention time and UV–Vis spectrum with those of the standard and was quantified on the basis of authentic standard (Table 2). Coumarin content in *mfce* is very high, and this should be taken into account when suggesting its use as a colorant or additive in food. In many of the published

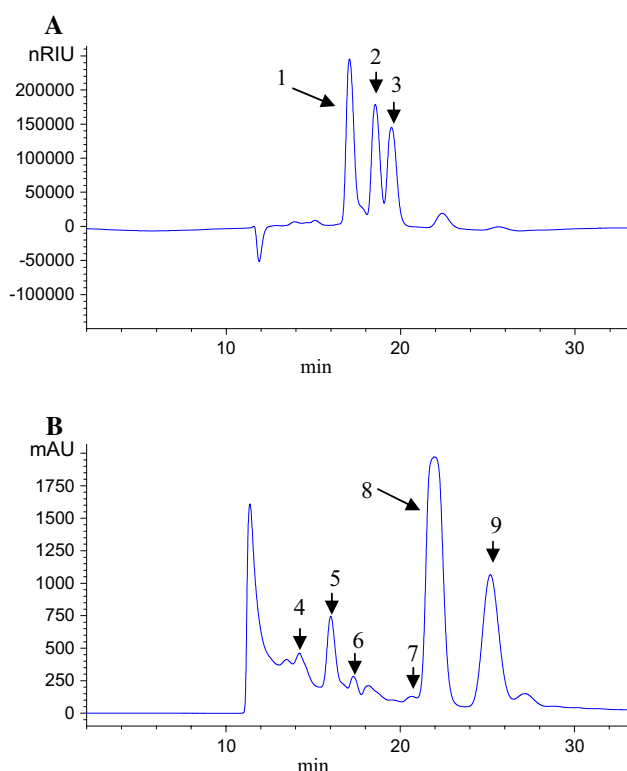


Fig. 5 HPLC chromatogram of sugars (A) and organic acids (B) in *mfce*. Peaks: 1 glucose; 2 fructose; 3 sorbitol; 4 citric acid; 5 tartaric acid; 6 malic acid; 7 succinic acid; 8 not identified; 9 not identified

studies, coumarin and its derivatives are attributed to a range of pharmacological effects ranging from antioxidant to anti-inflammatory [53, 54] with special emphasis on its intestinal anti-inflammatory activity [55].

Sugars, organic acids and ascorbic acid

The main sugars found in *mfce* were glucose and fructose; moreover, the extract contains the sugar alcohol sorbitol (Table 2; Fig. 5A). Ozturk et al. [13] found that the major sugars in *P. mahaleb* fruits were glucose, fructose and sucrose. Our results are in agreement with several studies reporting that sweetness in cherry fruits is mainly due to glucose and fructose, followed by sorbitol, while sucrose was identified in very low amounts [38, 40, 56–58]. Moreover, it is known that the presence of sorbitol is a taxonomic criterion within the Rosaceae family, to which *P. mahaleb* belongs [59]. Tartaric acid was found to be the major organic acid in *mfce* followed by citric acid, malic acid and succinic acid (Table 2; Fig. 5B). A high content of tartaric acid was determined in the fruits of Ericaceae family members and is more common in wild than in cultivated fruits [59]; Mahmood et al. [57] indicated tartaric acid as one of the major organic acids in sweet cherry fruits

collected in Pakistan. However, several references on the organic acid content of *Prunus* fruit indicated malic acid as the dominant organic acid contributing to the acidity of cherry, peach and apricot fruits [40, 58]. It is known that tartaric acid is effective for treatment of dry skin and that fruit rich in tartaric acid is of value for skin health, while organic acids have a protective function against fruit disease due to their antioxidant activity [60]. In addition, it is known that organic acids and sugars are associated with the organoleptic properties of fruits and that the sugar/acid ratio influences the perception of sweetness. Fruit such as blackcurrant, redcurrant and other Grossulariaceae species have a low sugar/organic acid ratio (about 2) and are perceived as sour fruits [59]. *Mfce* shows a sugar/organic acids ratio of 1.5 which explains its extremely sour and bitter taste. For this reason, this concentrated extract is intended to be used not for fresh consumption but as a food colorant or as source of phytochemicals. Ascorbic acid content in *mfce* was evaluated by HPLC assay (Table 2). Results were much lower than reported by Burdurlu et al. [61] for citrus juice concentrates and by Vaillant et al. [62] for a passion fruit juice concentrate. This low value could be explained by taking into account the high anthocyanin concentration of *mfce*; indeed, it is known that anthocyanins promote the oxidation of ascorbic acid in pomegranate juice [63].

Antioxidant activity of *mfce*

In order to test the potential antioxidant activity of the mahaleb fruit concentrate extract, three in vitro assays were performed. The ORAC assay measures hydrophilic antioxidant capacity toward peroxy radicals and is a hydrogen atom transfer (HAT) reaction-based assay, while TEAC is a single electron transfer (ET) reaction-based assay and measures the extract's scavenging activity against the ABTS^{•+} radical. Moreover, to complete the evaluation of *mfce*'s antioxidant capacity, the Folin–Ciocalteu assay was also used. This method has been applied for many years to measure the total phenolic content in natural products, but the reaction mechanism is an oxidation/reduction reaction not specific to phenolic compounds. In fact, the F–C assay measures the ability of both phenolic and nonphenolic compounds in alkaline medium to reduce the phosphomolybdic/phosphotungstic acid reagent to blue complexes that are detected spectrophotometrically. For this reason, the parameter assessed was reported as “F–C reducing capacity” [64] and the results were correlated with antioxidant activity determined by other widely used antioxidant methods (TEAC and ORAC). Table 5 reports *mfce* antioxidant activity expressed as μmol of Trolox equivalents (TE) per mL of extract for ORAC and TEAC assays and as mg of gallic acid equivalents per mL of extract for the Folin–Ciocalteu assay. The values in Table 5 show that *mfce* has a

Table 5 Antioxidant capacity of *mfce* assayed by TEAC, ORAC and F–C reducing capacity

ORAC value	450.550 ± 18.447 μ mol TE/mL extract
TEAC value	446.613 ± 30.540 μ mol TE/mL extract
F–C reducing capacity	22.734 ± 0.253 mg GAE/mL extract

Data are the mean ± SD ($n = 3$)

strong free-radical scavenging activity, when compared with the few reports on commercial fruit juice concentrate [41, 42]. The Folin–Ciocalteu assay gave a value of 22.734 mg of gallic acid equivalents per mL of extract; this is in the range of phenolic content values for red fruit juice concentrates reported by Bermúdez-Soto et al. [42] and is similar to the value indicated for blackcurrant. Blackcurrant is a berry fruit well known as a rich source of anthocyanin and phenolic compounds and popular as a colorant, flavoring and preserver in food and beverages for human consumption. Moreover, recent studies suggested blackcurrant exerts antioxidant and anti-inflammatory activity and has potential health benefits against hypertension, cardiovascular disease, neurodegenerative disease and ocular disease [65, 66]. *Mfce*'s free-radical scavenging activity against ABTS⁺ radical (Table 5), when expressed as mg TE/mL of extract, resulted 111.782 ± 7.5 mg of TE per mL, in the same range as those reported by Bermúdez-Soto et al. [42] for chokeberry, elderberry and blackcurrant fruit juice concentrates. The ORAC assay value reported in Table 5 for *mfce* was much higher than the one reported by Ou et al. [41] for a *P. cerasus* fruit juice concentrate (68 °Brix). This finding is of particular commercial value because it validates the exploitation of processed *P. mahaleb* as an ingredient for the production of functional food.

Conclusion

Optimization studies showed that the most effective solvent mixture for extracting phenolic compounds from *P. mahaleb* fruits was ethanol supplemented with 1 % citric acid 1 M. The concentrated extract (60 °Brix) had a higher anthocyanin content than and a similar flavonol content to red berry fruit concentrate juices reported in the literature. Moreover, a high coumarin content was found in *mfce*. These compounds together with the high content of organic acids found in this extract may explain its high antioxidant capacity. TEAC, ORAC and Folin–Ciocalteu assays gave results similar to those reported in the literature for berry juice concentrates already used for coloring and for their anti-inflammatory and health-promoting activities. In addition, these results suggest that mahaleb fruits, currently not used for fresh consumption, may be processed to obtain a concentrated fruit extract exploitable as a new source

of natural colorants and of phenolic compounds with biological activities. Further studies on the stability and functional attributes of *mfce* are needed to assess its potential application as a food colorant and/or functional food.

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Compliance with Ethical Standards

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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