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Fractioned High Pressure Extraction of Anthocyanins from Elderberry (*Sambucus nigra* L.) Pomace

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Abstract Fractionated high pressure extractions from dry and *in natura* elderberry pomace were performed in order to obtain anthocyanin rich extracts. Experiments were carried out using CO_2 supercritical fluid extraction followed by enhanced solvent extraction (ESE) with CO_2/EtOH–H_2O mixtures (1–100%, *v*/*v*), to obtain anthocyanin rich fractions in the second step, at 313 K and ~20 MPa. Higher extract yields, anthocyanin contents and antioxidant activities occurred by the presence of water, both in the raw material and in the solvent mixture. The CO_2 dissolved in the ESE solvent mixture favored either anthocyanin contents or antioxidant activities, which were not directly related. Comparing to the literature data for elderberries and grapes, these fractions had higher anthocyanins contents. From these results, an added economical value to this agroindustrial residue is proposed, using solvents and techniques "generally regarded as safe" in the food and pharmaceutical industries.

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Introduction

Presently, one of the most important trends in food and pharmaceutical industries is the growing demand for valuable natural sources of antioxidant compounds. Among common fruits and vegetables, elderberry (*Sambucus nigra* L.) is one of the richest in total phenolics and anthocyanins and, consequently, in antioxidant capacity (Bermúdez-Soto and Tomás-Barberan 2004; Wu et al. 2004). Cyanidin 3-glucoside (CyG) and cyanidin 3-sambubioside (CyS) are the main anthocyanins present (Braga et al. 2002) and quercetin 3-rutinoside (rutin) is the most representative flavonol (Bermúdez-Soto and Tomás-Barberan 2004; Fig. 1).

Most part of the literature focus essentially on elderberry fruit studies and a few work has been devoted to elderberry pomace regardless of its high anthocyanin content (75–98% of total berry anthocyanins) when compared to its weight (25–40% of total berry weight; Brønnum-Hansen et al. 1985). Despite its significant potential value, elderberry pomace does not own an important economic high value as it is traditionally used as animal feed or as an organic fertilizer. However, with some additional and proper processing, this byproduct could be easily transformed, from a residual low-value status into a very interesting high-value one, for consumer-accepted uses in food, cosmetic, and pharmaceutical industries.

Anthocyanins are polar molecules which are normally extracted from raw plant tissues by conventional solvent extraction (CSE) methodologies, using polar solvents slightly acidified with acids or sulfites. For some applications, high pressure extraction processes, like supercritical fluid extraction

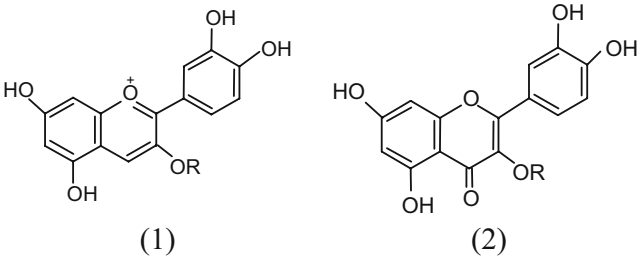

Fig. 1 Main flavonoids present in elderberries: (*1*) cyanidin 3-glucoside (R-glucose) and cyanidin 3-sambubioside (R-xylose-glucose); (*2*) quercetin 3-rutinoside (R-rutinose)

(SFE) and enhanced solvent extraction (ESE), already proved to represent valuable alternatives to CSE. SFE is an attractive "tunable" technique which may remove selectively various active constituents from plant matrices. Moreover, it can be particularly attractive for the extraction of antioxidants and anthocyanins because it may avoid their thermal degradation, and due to the absence of light and oxygen, it can prevent oxidation reactions (Díaz-Reinoso et al. 2006). In food and pharmaceutical applications, CO_2 is the most employed supercritical solvent. The main drawbacks of CO_2 are its nonpolar nature and its inability to extract high molecular weight compounds which might limit the extraction performance. Even though the addition of suitable cosolvents is the most frequent employed strategy to overcome these problems, a more recent employed approach is its association with other methodologies, such as ESE. ESE involves the use of H_2O or organic solvents at considerable elevated temperatures (313–473 K) and pressures (3.3–20.3 MPa). It offers the possibility to perform efficient and "enhanced" extractions due to its improved characteristics in terms of mass transfer and of solvating properties, which can even be improved by the utilization of gas-expanded liquids, obtained upon the dissolution of CO_2 in H_2O or in an organic solvent. This method takes advantage of the beneficial combination between typical liquids solvation properties and the advantageous transport properties of supercritical fluids (Chamblee et al. 2004), and it was already applied to the extraction of polar solutes (Yuan and Olesik 1997). Another unique and potentially useful property of CO_2-aqueous and CO_2-alcohol gas-expanded liquids is the generation of in situ carbonic acid and alkyl carbonic acid, respectively (Towes et al. 1995; West et al. 2001). In anthocyanin extraction, this fact can be an important advantage: there will be a temporary reduction in the extraction medium pH value, and this will increase anthocyanin stability and cell membrane permeability, leading to higher diffusivities (Türker and Erdoğdu 2006; Norton and Sun 2008). Moreover, it also can inactivate unwanted enzymes (Kamat et al. 1995; Norton and Sun 2008) and microorganisms (Foster et al. 2003) that may destroy these pigments (Delgado-Vargas and Paredes-López 2003; Garcia-Palazon et al. 2004). On the other hand, the presence of undesired compounds in several vegetal matrixes, which may be coextracted or which may interfere negatively with the extraction of desired substances, decreasing extraction yield and selectivity, is a typical situation in natural products extraction methodologies (conventional and supercritical). A common way to overcome this situation is to employ extraction fractionated procedures (Pasquel et al. 2000; Reverchon 1997; Reverchon et al. 1999), using different solvent mixtures at different stages of the extraction process.

In this work, we explored the use of high-pressure fractioned extraction of elderberry pomace, using environmental friendly and food- or pharmaceutical-accepted solvents and techniques, in order to obtain anthocyanin-rich extracts which can be used as natural colorants, dietary supplements, or phytochemical products. For all extraction experiments, a CO_2 SFE was employed in a first extraction step and, subsequently, different CO_2–EtOH–H_2O mixtures were employed in an ESE step. The influence of the polar ESE solvent mixture composition and of the raw material humidity were studied concerning extract yield, anthocyanin content, and antioxidant activity and compared to the results obtained by CSE.

Materials and Methods

Raw Material

In natura elderberries, provided by Cooperativa Agrícola do Vale do Varosa (Tarouca, Portugal), were collected in August 2004 according to Neto and Monteiro (2002) and stored under vacuum at 255 K. Elderberry pomace was obtained by mechanical pressing (Hafico, Neuss, Germany) and dehydrated in a fluidized bed dryer (MK II, Sherwood Scientific, Cambridge, England) at 308 K, in the absence of light, for 12 h. Pomace was milled in a grinder (Braun, KSM 2, Kronberg, Germany) for 2 min, conditioned under vacuum at 255 K, and its H_2O content was determined gravimetrically (triplicate assays).

Chemicals

Carbon dioxide (99.998%, Praxair, Madrid, Spain), EtOH (99.5%, Panreac Quimica SA, Barcelona, Spain), and distilled H_2O were used for the extraction experiments. Chemicals or solvents employed for extract analyses were: EtOH (P.A.), MeOH (Lichrosolv), formic acid (98–100%), hydrochloric acid (P.A.), glacial acetic acid (P.A.), *n*-hexane (96%), and ethyl acetate (99.5%) purchased from Merck (Darmstadt, Germany), *p*-anysaldehyde (Sigma–Aldrich Inc., St. Louis, MO, USA), 2-aminoethyl diphenylborinate (97%, Fluka, Steinheim, Germany), 2,2-diphenyl-1-picrylhydrazyl (~90%, Sigma–Aldrich Inc., Steinheim, Germany), and

Ultra pure Milli Q water. Standards used for Thin Layer Chromatography (TLC) analyses were quercetin dehydrate (≥98%), rutin hydrate (≥95%), D-(+)-catechin hydrate (98%), and gallic acid (≥98%), purchased from Sigma–Aldrich Inc. (Steinheim, Germany) and (−)-epicatechin (≥90%, Fluka, Buchs, Switzerland). Standards used for High-Performance Liquid Chromatography (HPLC) analysis were cyanidin 3-glucoside chloride (analytical grade, Extrasynthèse, Genay, France) and rutin (extra pure, Merck, Darmstadt, Germany).

Conventional Solvent Extractions

These extractions were carried out using either an EtOH–H_2O (8:2, *v*/*v*) mixture or H_2O, at 313 K. Dried elderberry pomace was homogenized using an Ultra-turrax (Ystral, D79282, Ballrechten-Dottingen, Germany) slowly increasing the rotational velocity from 8000 to 24,000 rpm, during the ~3 min extraction time, and employing a 1:10 (*w*/*v*) solid/solvent ratio. The resulting slurry was then centrifuged (3,000 rpm, 15 min) and filtered. The filter cake was reextracted for four times, until depletion of its anthocyanin characteristic color, and filtrates were combined and concentrated using a rotary evaporator (BÜCHI Rotavapor R-114, Flawil, Switzerland) at 313 K. Later, extracts were lyophilized (FTS Systems Inc., N.Y., USA) and kept at 255 K. All procedures were made in the absence of light. The experimental conditions employed are reported in Table 1.

Fractioned High Pressure Extractions

These extractions were carried out using the apparatus presented in Fig. 2. Liquid CO_2 was delivered to the extraction cell using a high pressure liquid compressor (maximum pressure of 30 MPa) and EtOH, EtOH–H_2O (8:2, *v*/*v*) or H_2O were delivered by a high pressure liquid pump (L-6200A, Hitachi, Merck, Darmstadt, Germany); solid/solvent ratios utilized are reported in Table 1. A stainless steel extraction cell (~30 mL) was filled with elderberry pomace in three layers separated by glass beads (4 mm diameter), in order to achieve a uniform distribution of the solvent flow and a reduction of the dead space in the cell. Cotton wool was placed on both endings of the cell, to prevent line obstructions. Extraction cell was placed into a water bath with temperature controlled by an immersion circulator (±0.1 K, DC30, Thermo Haake, Karlsruhe, Germany) and pressure was maintained by a back-pressure regulator (26-1762-24-090, Tescom, Selmsdorf, Germany) and measured by a pressure transducer (C204, Setra, Boxborough, MA, USA). Extracts were recovered in a recovering flask and a trap, placed in an ice bath, and the expanded CO_2 flow was measured by a wet gas meter (DM3C ZE 1411, G.H. Zeal Ltd., London, England). A two-step fractioned high-pressure extraction methodology was employed, comprising: (1) a first CO_2 SFE step in order to remove the low polarity CO_2-soluble compounds (15 min static+40 min dynamic period); tubing line was cleaned with EtOH; (2) a second ESE step (45 min), in order to extract polar compounds like anthocyanins, and wherein CO_2 and/or EtOH, EtOH–H_2O (8:2, *v*/*v*) or H_2O were introduced into the system; different amounts of EtOH and EtOH–H_2O (90% and 100%, *v*/*v*) were assayed for *in natura* and for dried elderberry pomace. These solvent mixtures will be named ethanolic solvents. CO_2 and H_2O mixtures were also assayed with dried elderberry pomace, employing different percentages of H_2O (10–100%, *v*/*v*). Operational conditions of both steps (313 K and 20 MPa) were selected based on the literature information concerning the solubility (in supercritical CO_2) of the main components of vegetable oils, triglycerides (Bamberger et al. 1988), and on the anthocyanin stability (Jackman et al. 1987). Extracts were concentrated at 313 K, under vacuum and in the absence of light, and kept at 255 K. For the first SFE step, extraction curve was determined in order to define the extraction time that assured a high lipophilic material removal from the plant material. Some ESE assays were duplicated in order to determine the experimental error of the yield values; the others were single. Employed experimental conditions are reported in Table 1.

Thin Layer Chromatography

TLC analyses were performed using silica gel plates with a 254 nm fluorescent indicator (20×20 cm×0.2 mm; Fluka, Steinheim, Germany). Same extract concentrations were chromatographed. For low polarity compounds analysis, hexane-ethyl acetate (8:2, *v*/*v*) and an anisaldehyde solution (Wagner et al. 1984) were used as the mobile phase and the spray reagent, respectively. For the analysis of phenolic compounds, the mobile phase was ethyl acetate–formic acid–glacial acetic acid–H_2O (100:11:11:27, *v*/*v*) solution and a NP solution (MeOH-2-aminoethyl diphenylborinate, 99:1, *v*/*w*) was used for detection of phenolic compounds, at 365 nm.

High Performance Liquid Chromatography

Quantification of anthocyanins and rutin was performed in a Gilson apparatus equipped with a diode-array detector. An ODS-2 column (250×4.6 mm i.d., 5 μm, Spherisorb S5, Waters, MA, USA) at 298 K and a C18 guard cartridge (30×4 mm i.d., 4 μm, Hichrom, Berkshire, UK) were used. A mobile phase, constituted by aqueous formic acid (5%, *v*/*v*) (A) and MeOH (B), was used with a discontinuous gradient of 5–15% B (0–10 min), 15–25% B (10–15 min), 25–50% B (15–40 min) and 50–80% B (40–50 min), followed by an isocratic elution during 10 min, at a flow rate of 1 mL/min.

Table 1 Elderberry pomace extractions at 313 K: experimental conditions, global yield, composition, and antioxidant activity of obtained extracts

	Operational conditions			Global Yield (%, db)	Global Yield ± SD (%, db)	Phenolic[a] (mg g^{-1}, db)				Phenolic[a] ± SD (mg g^{-1}, db)				Ant. activity (IC_{50}, μg)	Ant. activity ± SD (IC_{50}, μg)
	Solvent Mixture[b] (%)	Solid/ Solvent ratio	Pomace humidity			CyS	CyG	TA	R	CyS	CyG	TA	R		
Conventional solvent extraction at Patm															
CSE (~15 min)	EtOH–H_2O (100)	1:33	8.5%	15.9		61	78	153	13					48	
	H_2O (100)	1:50		33.3		30	31	67	6					86	
Fractioned high pressure extraction at 20±0.6 MPa and 313±0.1 K															
SFE (55 min)	CO_2 (100)	1:200	61%		2.8					–	–	–	–		-
ESE (45 min)	EtOH (90) + CO_2(10)	1:140			13.1					24	38	68	8		122
	EtOH–H_2O (90) + CO_2(10)				14.4±3.2					59	72	149	14		63±16
	EtOH (100)				14.2±1.8					33	43	85	8		110±7
	EtOH–H_2O (100)				15.4±2.1					44	48	105	9		77±10
SFE (55 min)	CO_2 (100)	1:200	8.5%		17.2					–	–	–	–		-
ESE (45 min)	EtOH (90) + CO_2 (10)	1:100			6.4					33	24	62	9		150
	EtOH–H_2O (90) + CO_2(10)				16.8±0.3					52±1.1	54±0.5	120±1.1	10±0.1		57±5
	EtOH (100)				12.7±1.3					24	35	66	8		171±30
	EtOH–H_2O (100)				18.2±0.5					45	55	111	10		57±7
SFE (55 min)	CO_2 (100)	1:200	6.1%		17.4±1.2					–	–	–	–		-
ESE (45 min)	H_2O (10) + CO_2 (90)	1:167			7.5					18	16	46	10		46
	H_2O (20) + CO_2 (80)				10.5					25	24	65	12		40
	H_2O (50) + CO_2 (50)				15.1					15	14	39	9		41
	H_2O (80) + CO_2 (20)				16.3					20	22	52	8		44
	H_2O (100)				17.9					37	41	89	9		52

[a] *CyS* Cyanidin-3-sambubioside, *CyG* cyanidin-3-glucoside, *TA* total anthocyanins, *R* rutin, *SD* standard deviation. HPLC evaluation of polyphenol contents using external standards (CyG and R). The correlation peak area or concentration was assessed by least-squares regression model.

[b] EtOH–H_2O mixture had always a fixed proportion of 8:2 (*v*/*v*).

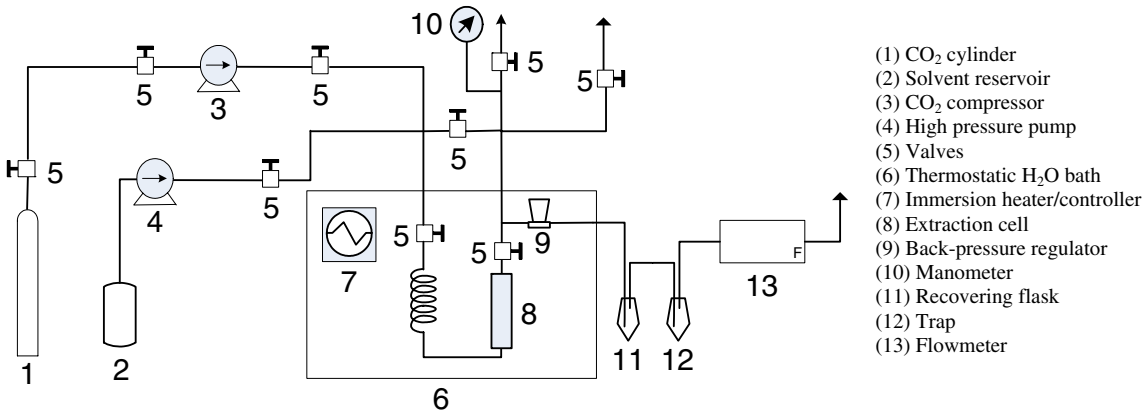

Fig. 2 Schematic diagram of the employed high pressure extraction apparatus

Samples were adjusted to pH ~2 with HCl and microfiltered (0.20 μm) before HPLC injection. Anthocyanins were identified from their chromatographic and ultraviolet (UV) spectral properties and the major anthocyanins, CyG and CyS, by comparing with the CyG standard and according to literature data (Brønnum-Hansen and Hansen 1983; Wu et al. 2004). Quantification of anthocyanins (CyS, CyG and total anthocyanins, TA) and rutin (R) was carried out using external standards (CyG and R), at 520 and 360 nm, respectively. The correlation between peak area or concentration was assessed by the least-squares regression model. One of the extracts was injected three times to determine the standard deviation of the assay.

Antioxidant Activity (2,2-diphenyl-1-picrylhydrazyl Assay)

The antioxidant activity of the extracts was evaluated by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Blois 1958). Aliquots (100 μL) of extracts were added to 500 μL of a MeOH solution (500 μM) of DPPH radical in the presence of 100 mM acetate buffer, pH 6.0 (1 mL), and MeOH (1.4 mL). After mixing for 30 s, the reaction mixtures were kept in the dark at room temperature for 30 min, and the absorbance was measured at 517 nm, on a UV-visible spectrophotometer (U-2000, Hitachi, Tokyo, Japan). Triplicate assays were performed. The reducing capacities of the samples were estimated from the observed absorbance decrease and expressed as IC_{50} values, defined as the amount of elderberry extract [dry basis (db)] that decreased by 50%, the initial absorbance of the DPPH radical solution, at the referred wavelength.

Calculation Procedures

Global yields were obtained considering the total extracted mass divided by feed mass in a db. For both high-pressure extraction steps, the total extract mass was determined by the sum of the extract obtained in the recovering flask and trap; for first step, the extract recovered from tubing was also considered. The overall SFE curve was constructed using the accumulated mass of extract, collected at a given extraction time interval. The data was fitted by a curve formed by two lines. The fitting was done by minimizing the least regression error in the least squares sense, using the fmin search function of Matlab (R2007a). The first line was identified with the constant extraction rate period (CER) and the corresponding kinetic parameters were calculated (M_{CER}, t_{CER}, and Y_{CER}), according to Rodrigues et al. (2002).

Results and Discussion

Elderberry pomace represented around 25% (*w*/*w*, db) of the total fruit weight. Two lots of dry and one lot of *in natura* pomace were employed, with humidity [± standard deviation (SD)] of 8.5±0.1, 6.1±0.1, and 61±2% (*w*/*w*, db), respectively (Table 1).

The elderberry pomace SFE (first step) exhibited a typical overall extraction curve profile (Fig. 3), with 5.3×10^{-7} kg/s for M_{CER}, 12.1 min for t_{CER}, and Y_{CER} of 2.0×10^{-3}. For all SFE experiments, extraction was prolonged for 40 min to guarantee the diffusional period. The mass of extract recovered from tubing cleaning represented ~9% of the total obtained extract mass in this step. Global yields standard deviation (1.2%) was calculated from three SFE assays. For ESE (second step) duplicated assays, higher standard deviations (Table 1) were obtained for *in natura* pomace, probably due to the nonhomogeneity of the solvent and the H_2O from the raw material.

The obtained global yields are represented in Fig. 4. For fractioned high pressure extractions with ethanolic mixtures in the second step (ESE), higher yields of first + second steps were always obtained for dried elderberry pomace (~24–35%) when compared to those using *in natura* pomace (~16–18%). The main contribution to this difference was

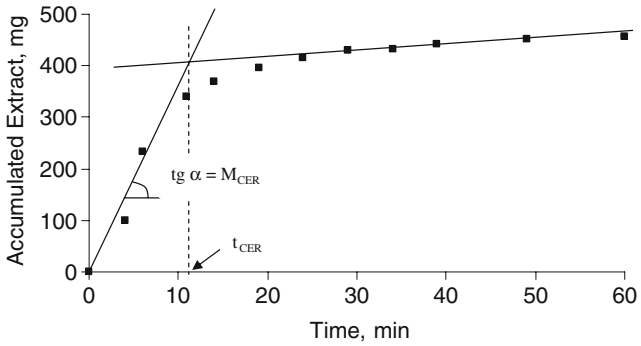

Fig. 3 Kinetics of the first step (SFE) elderberry pomace extraction, at 20.6±0.6 MPa and 313±0.1 K, and at a flow rate of 27.3×10^{-5} kg s^{-1}

essentially due to the first step, wherein the less polar substances were extracted. This could have happened because for *in natura* pomace, the seeds were not efficiently comminuted, and H_2O may have acted as a barrier to diffusion and may have increased the overall polarity of the solvent (Temelli 2000; Reverchon and Marrone 2001). Therefore, drying the byproduct seems to permit a more efficient SFE of low polarity compounds. Since anthocyanins are mainly located in outermost layer of elderberry skin cell walls (Manach et al. 2004) wherein the fatty acids and waxes exist (Pinelo et al. 2006), an effective lipophilic substances removal, in a first-step SFE, can make polar material more available for extraction.

For the second-step ethanolic ESE, the solvent mixture, which was in a single homogeneous phase according to Durling et al. (2007), did not significantly affect global yields for the *in natura* pomace (~13–15%), while they had a greater variation for the dry pomace (~6–18%; Fig. 4). The initial high *in natura* material H_2O content improved the extraction ability of the solvent mixture, suggesting that the presence of H_2O in the extraction medium could also provide higher yields of more polar compounds, namely anthocyanins, by increasing the solvent mixture density and polarity. In fact, for both raw materials (*in natura* and dried), the presence of H_2O in the enhanced ethanolic mixture resulted in an increment in polyphenol contents (anthocyanins and rutin) and in the antioxidant activity of the extracts (Table 1).

The dissolved CO_2 negatively affected ESE yields (decrease of 1–7%); and so, the possible positive influence of the enhanced transport properties of gas-expanded liquids (Chamblee et al. 2004) and the decrease in the solvent mixture pH were not so significant as the negative influence of the decrease in the solvent polarity that happened when CO_2 was added to the solvent (Weikel et al. 2006). However, an increase of TA contents, from 105 to 149 mg g^{-1} for in natura pomace and from 111 to 120 mg g^{-1} for dried pomace (Table 1), occurred for the CO_2-EtOH–H_2O gas-expanded mixtures; so, not only the higher solvent mixture density and polarity but also the solvent's pH drop can have important roles in the extraction of these polar substances (West et al. 2001). Therefore, it seems that, for this ternary solvent mixture, CO_2 played a similar role as sulfites and weak acids in the conventional solid–liquid extraction of anthocyanins from natural matrixes. In fact, for both raw materials, the highest TA contents and antioxidant activities were obtained when the solvent was the CO_2-EtOH–H_2O gas-expanded mixture, presenting *in natura* pomace similar TA content (149 mg g^{-1}) as the CSE ethanolic extract (153 mg g^{-1}) that was the highest obtained. Similar antioxidant activities were verified for the *in natura* and the dried pomaces: IC_{50} of 63± 16 and 57±5 μg, respectively.

When EtOH–H_2O was used as the extracting solvent, a 2% increase in yield was obtained for the ESE relative to the CSE; so, high pressure was more favorable to the extraction yield than simultaneous milling, stirring, and extraction. The lower solid/solvent ratio used in ESE (1:100) when compared to the one used in CSE (1:33) also favored extraction yield. For this solvent mixture (EtOH–H_2O, 8:2 *v*/*v*), and at these particular conditions of pressure

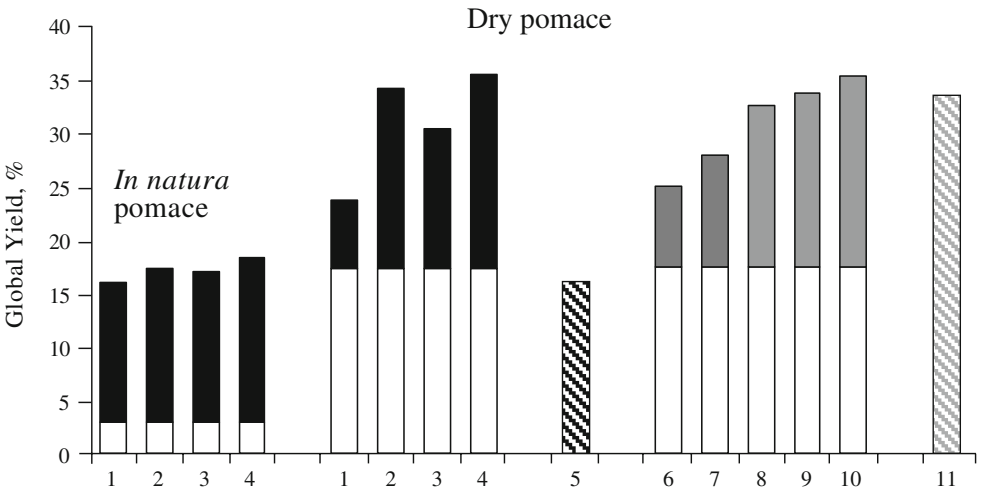

Fig. 4 Obtained global yields (db) for elderberry pomace extractions. *Empty bar* SFE-CO_2; Ethanolic Extracts (*filled bar*) ESE: 1 EtOH+CO_2 (10%), 2 EtOH–H_2O+CO_2 (10%), 3 EtOH, 4 EtOH–H_2O; (*dark diagonally striped bar*) CSE: 5 EtOH–H_2O; Aqueous Extracts (*grey bar*) ESE: 6 H_2O+CO_2 (90%), 7 H_2O+CO_2 (80%), 8 H_2O+CO_2 (50%), 9 H_2O+CO_2 (20%), 10 H_2O; (*light diagonally striped bar*) CSE: 11 H_2O

and temperature, ESE seems to be not an advantageous alternative to CSE if the objective is to obtain anthocyanin-rich fractions having high antioxidant activities.

Dawidowicz et al. (2006) performed pressurized liquid extraction of *S. nigra* berries, using an EtOH–H_2O (8:2, *v*/*v*) mixture as the extracting solvent at 6 MPa and 293 K and obtained an extract with ~1.1 mg g^{-1} of rutin and ~5 mg g^{-1} of CyS + CyG. The difference between the phenolic compositions in that extract and the ones obtained in this study from elderberry pomace can be related to the different extraction methodologies (no fractionation, lower operational pressure, and temperature) and the raw materials nature.

Because H_2O seemed to have a positive influence on the observed ESE global yields, this solvent was tested for dry pomace, just employing CO_2 and H_2O (10–100%) on the 2nd extraction step. Higher global yields occurred when the H_2O content was incremented in the ESE solvent mixture (Fig. 4) that increased the relative amount of the H_2O rich liquid phase (a gas-expanded liquid) but did not change the composition of the two phases present, which remained constant (Perakis et al. 2006). Therefore, the observed raise in ESE yields was just due to an increment in the amount of the high density and polarity phase, which presented a better capacity to dissolve polar substances like anthocyanins. This raise is similar to the one that occurs in conventional solid–liquid extraction when solvent/solid ratio increases (Cacace and Mazza 2002). As happened with the ethanolic solvent, when no CO_2 was added, a

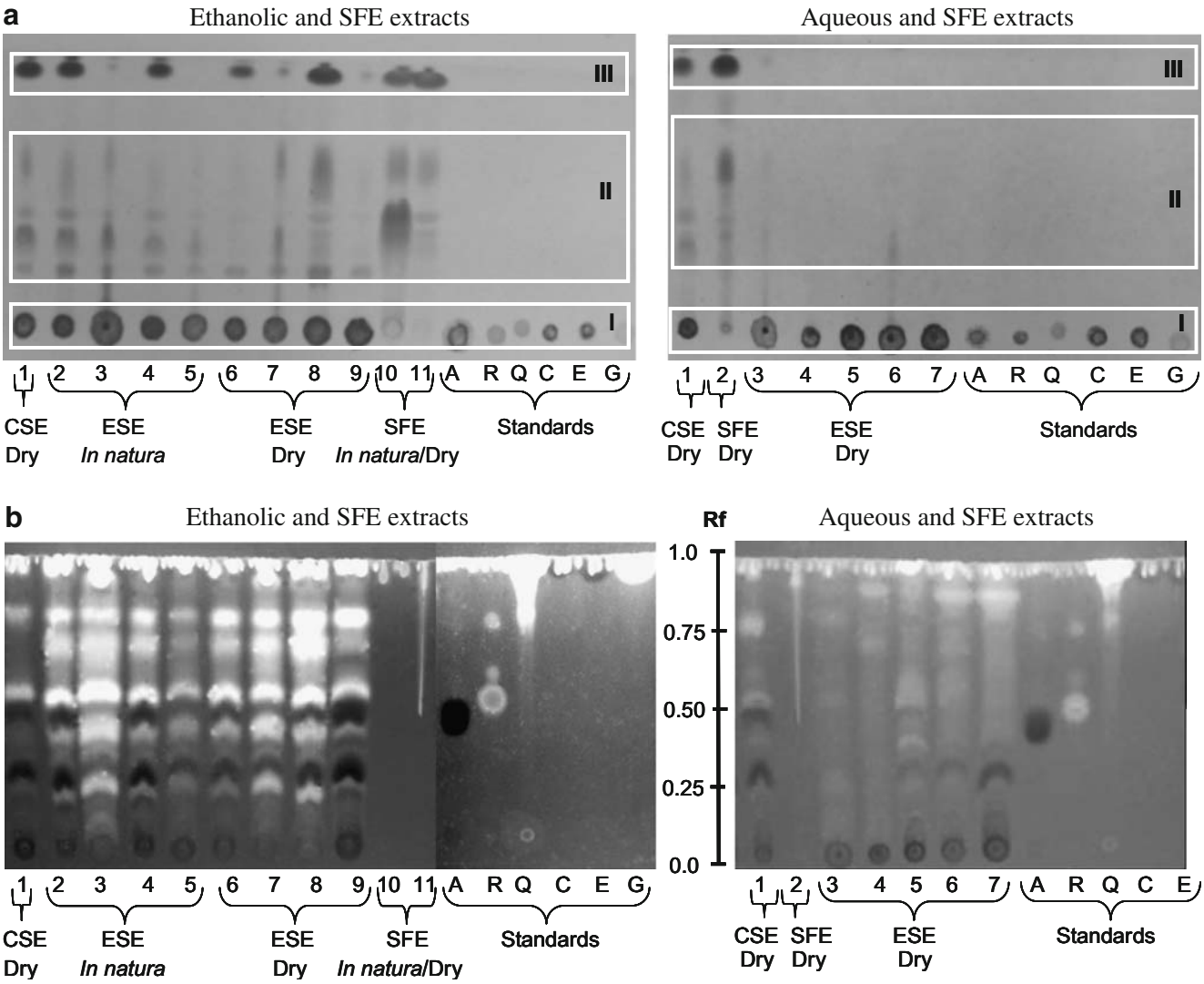

Fig. 5 Analysis of low polarity compounds by anisaldehyde sprayed TLC plates (**a**) and of high polarity compounds by NP sprayed TLC plates, observed at 365 nm (**b**). Conventional extracts at 313 K and high pressure extracts at 20.6±0.6 MPa and 313±0.1 K. Ethanolic and SFE extracts: *1* CSE—EtOH–H_2O; *2*, *6* ESE—EtOH+CO_2; *3*, *7* ESE—EtOH–H_2O+CO_2; *4*, *8* ESE—EtOH; *5*, *9* ESE—EtOH–H_2O; *10*, *11* SFE—CO_2. Aqueous and SFE extracts: *1* CSE—H_2O, *2* SFE—CO_2, *3* ESE—H_2O (10%), *4* ESE—H_2O (20%), *5* ESE—H_2O (50%), *6* ESE—H_2O (80%), *7* ESE—H_2O (100%). *Standards*: *A* Cyanidin 3-glucoside, *R* Rutin, *Q* Quercetin, *C* Catechin, *E* Epicatechin, *G* Gallic acid

maximum ESE yield was attained (~18%), showing a negative CO_2 influence on extraction yield.

In contrast, there was not a direct relationship between the H_2O content in the enhanced solvent mixture and the TA and rutin extract contents or antioxidant activities. Even though the highest amount of TA (89 mg g^{-1}) was obtained when no CO_2 was added to the solvent mixture, the extracts obtained with the CO_2–H_2O gas-expanded liquid showed higher antioxidant activities (IC_{50} 40–46 μg). These results suggest that the pH drop that followed the possible carbonic acid formation [pH may decrease down to 2.8, according to Towes et al. (1995)] did not influence positively the amount of extracted anthocyanins but possibly influenced their stability, once these pigments are known to be at the most stable state at pH 1 to 3 (Delgado-Vargas and Paredes-López 2003). In fact, ESE aqueous extracts IC_{50} values were close to the ethanolic CSE value (48 μg), despite having around three times less anthocyanins. However, rutin contents and other eventually extracted substances, like proanthocyanidins, may have also contributed to the extracts antioxidant activity.

Comparing the aqueous extraction methodologies, and in opposite to what happened with the ethanolic experiments, extraction from a nondefatted raw material and simultaneous milling and extraction was more favorable to extraction yield than high pressure (Table 1). Although, the obtained high CSE yield was due to the coextraction of compounds that did not contribute to the extract antioxidant activity as is confirmed when TA and rutin contents (67 and 6 mg g^{-1} respectively), and antioxidant activity (IC_{50}~ 86 μg) are compared to ethanolic CSE ones (153 and 13 mg g^{-1}, respectively, and IC_{50}~48 μg).

The presence of low polarity compounds in extracts was monitored by using anisaldehyde sprayed TLC plates (Fig. 5a). For ESE, the presence of zones that migrated with the solvent (II and III) in some extracts indicates that lipophilic compounds were not completely extracted during SFE. The lipophilic composition of these extracts varied with the nature of the employed solvent, being almost absent in aqueous extracts (Fig. 5a). Strong zones at the solvent front (III) appeared in those ESE ethanolic extracts in which H_2O was not used in the solvent mixture, which means that the presence of H_2O avoided the coextraction of high lipophilic compounds, playing an anti-solvent role. Despite the fact that the low polarity substances were not completely extracted, TLC analysis was an indication that CO_2 SFE was capable of extracting lipophilic or low polarity compounds, which resulted in the concentration of phenolic compounds and other polar substances in the vegetal matrix for the subsequent extraction step.

The overall phenolic composition of the extracts was monitored by using NP sprayed TLC plates (Fig. 5b). Several zones appeared in both ethanolic and aqueous CSE and ESE extracts, with higher intensity for the ethanolic ones: two main dark blue zones with Rf 0.28 and 0.48 (CyG), five weaker light blue zones (corresponding to non identified anthocyanins), two orange fluorescent zones at Rf 0.55 (R) and at Rf 0.8 that may correspond to quercetin glycosides, and two light blue fluorescents that may be assigned to phenol carboxylic acids (Wagner et al. 1984), among others. The other standards used in the analysis appeared at the solvent front and were not identified in extracts.

For most extracts, the major anthocyanin was found to be CyG, and the sum of CyS and CyG contents represented around 90% of the TA contents (Table 1, Fig. 6). These results are similar to those already reported in literature for elderberries (Wu et al. 2004). In general, there was not a direct relationship between TA contents and antioxidant activity of the extracts, which is in agreement with the results obtained by Kähkönen et al. (2001) and Nakajima et al. (2004) for berry extracts obtained by CSE, using different solvents. There was not an evident and direct relationship between rutin contents and the antioxidant activity of the extracts, either, although extracts with higher

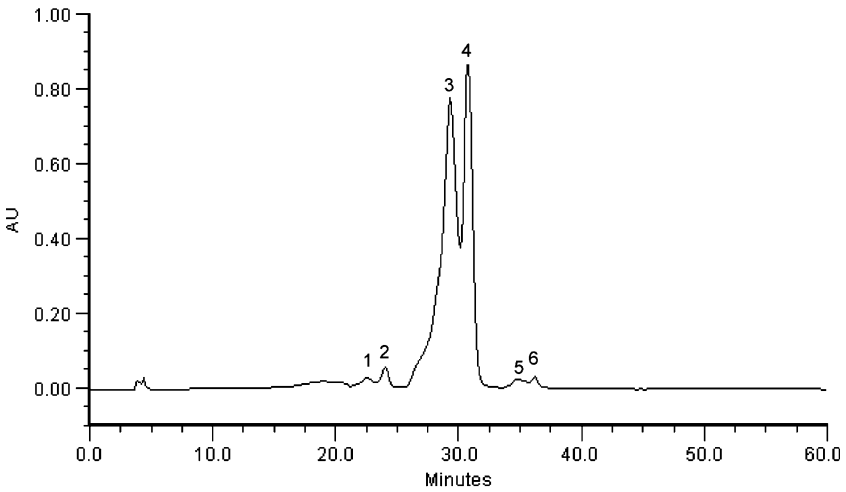

Fig. 6 HPLC profile of *in natura* elderberry pomace extract obtained by ESE using EtOH (90%) + CO_2 (10%), at 313 K and ~20 MPa recorded at 520 nm. Peaks: *1* and *2* anthocyanins; *3* cyanidin 3-sambubioside; *4* cyanidin 3-glucoside; *5* quercetin glycoside; *6* rutin

antioxidant activity (aqueous ESE (20% H_2O)—IC_{50} of 40 μg, and ethanolic CSE—IC_{50} of 48 μg) were among the ones with higher rutin contents. These results are in accordance with the fact that the relationship between antioxidant activity of berry extracts and their phenolic composition is complex (Kähkönen et al. 2001) and, in addition, synergism can have a significant effect on the antioxidant response of plant extracts.

Total anthocyanin content in the elderberry pomace extracts obtained in this work (39–153 mg g^{-1}) were considerably higher than total anthocyanin content in the dry red grape skin extracts (41–57 mg g^{-1}), obtained by Ju and Howard (2003) and using different solvents (acidified alcohols, H_2O and acetone mixtures) at 323 K and 10.1 MPa. This is probably the best anthocyanin content that can be obtained using grape pomace as raw material, since the authors claim that the employed grape variety was exceptionally rich in TA and, like in elderberry pomace, these were more concentrated in grape skin than in the whole grape. These results show that elderberry pomace is a very good source of anthocyanins when compared to wine industry byproducts.

Conclusions

Anthocyanin rich elderberry pomace extracts were obtained, employing a fractioned high pressure methodology. During the first step CO_2 SFE that was more efficient for the dry raw material, essentially lipophilic compounds were extracted. For ethanolic ESE assays, the presence of H_2O in the solvent mixture resulted in an increment in global yields and in TA contents with a consequent improvement in the extracts antioxidant activity. Moreover, for the ESE assays using EtOH–H_2O in the solvent mixture, the dissolved CO_2 favored TA contents and antioxidant properties. For the aqueous ESE assays, even though global yields increased with the H_2O content increase in the solvent mixture, there was not a direct relationship with TA contents or antioxidant capacities. Antioxidant activity of these aqueous ESE extracts were high and comparable to the ethanolic CSE extract, despite their low TA and phenolic (observed by TLC) contents.

Relatively to the methodology used, ESE has several additional advantages over CSE, such as the possibility of extract fractionation and a higher extraction flexibility, which is offered by the possibility of modifying solvent dissolution capability just by changing operational conditions as dissolved CO_2, and temperature and pressure, which can also be explored.

The presented results clearly indicate that it is possible to obtain anthocyanin rich extracts from elderberry pomace possessing high antioxidant activity and, in this way, adding economical value to this agroindustrial residue. These was done using solvents and techniques considered as "acceptable" and "generally regarded as safe" in the food and pharmaceutical industries.

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