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Selective Recovery of Anthocyanins and Hydroxycinnamates from a Byproduct of Citrus Processing

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The adsorption−desorption performances of commercial resins (two polystyrene−divinylbenzene copolymers and one methacrylic) were tested in column studies for the selective recovery of anthocyanins and hydroxycinnamates from pigmented pulp wash (PW), a byproduct of blood orange juice processing. Methanol, ethanol, and their mixtures with different percentages of water as eluents were tested in order to investigate the selective desorption of these natural antioxidants with the goal of minimizing the presence of other compounds, mostly flavanones and limonoids, in the concentrated eluates. The results indicated that polystyrene−divinylbenzene resins were able to adsorb a major amount of anthocyanins and hydroxycinnamates and to desorb them in more-concentrated fractions. No selectivity was observed using pure methanol and ethanol which resulted in concentrates with no further separation of components from the starting raw PW. The ethanol/water concentrates showed higher yields than the fractions attained using methanol/water; however, a lower selectivity toward anthocyanin pigments was observed. The best performing resin, EXA-118, together with the mixture methanol/water 50:50 (v/v) as best eluent, appears to be the most suitable system to obtain highly concentrated extracts. Thus, it was chosen to perform a larger experiment, to analyze the selectivity of the removal upon an increase in elution volume.

KEYWORDS: Adsorption; anthocyanins; desorption; hydroxycinnamates; pigmented oranges; pulp wash; resins

INTRODUCTION

The popularity of orange juices and orange-based drinks has grown worldwide among the consumers during the past decade for their sensory and health-promoting qualities. On the other hand, the increasing production has enhanced the disposal problem under economic and environmental points of view because of the energetic cost of treatments, drying, storage, and transport (*1*). In recent years, interest in management methods for improving the competitiveness of the food industries has increased. Areas of focus have not only included processes for obtaining superior quality (*2*) and product recognition but also in the application of waste reduction policies by full exploitation of the byproducts with the creation of new value-added segments of production (*3*−*7*). Over the past five years, our research group has published papers on the recovery of flavonoids with striking biological and antioxidative properties (*8*) from byproduct streams of pigmented orange juice processing using the methodology of concentration on resins. New opportunities are coming, in fact, for these natural antioxidants in the growing segments of dietary supplements and functional foods production (*9*) and because of their possible utilization by pharmaceutical (*10*) and cosmetic (*11*) industries. We have demonstrated the applicability of the resin concentration methodology to recover 97% pure hesperidin from the peel (*12*) and from the wastewater of the essential-oil-recovery line (*13*), while highly concentrated alcoholic extracts of anthocyanins, hesperidin, and hydroxycinnamates were obtained from pigmented pulp wash (*14*). Moreover, we have also investigated the behavior of 13 commercial copolymers in batch solution at different pH's and temperatures in the presence of hesperidin (*15*) and/or cyanidin 3-glucoside (*16*) to compare adsorption capacity and to predict resin performances by their Freundlich and Langmuir parameters. The tested resins are nonpolar polystyrene−divinylbenzene (PS-DVB) or slightly polar acrylic copolymers widely used because of their high adsorption capacity, possible recovery of the adsorbed compounds, easy regeneration, and high durability. The results demonstrated a greater binding affinity of these matrixes for hydrophobic molecules, like hesperidin, rather than the more valuable hydrophilic anthocyanin pigments (*14*, *16*). Anthocyanins and hydroxycinnamates, together with ascorbic acid, are in fact among the major contributors to the total antioxidant activity of blood orange juices (*17*, *18*) and, differently from carotenoids, they cannot be synthesized and

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must be extracted from natural sources (*1*). The goal of the present work was to investigate the possibility to obtain highly concentrated and suitably purified extracts of anthocyanins and hydroxycinnamates, reducing desorption of flavanones and limonoids. For these reasons, natural antioxidants from pigmented orange pulp wash (PW) were adsorbed in columns filled with three selected resins, accurately chosen on the basis of the previous works' results, and eluted with methanol, ethanol, and their mixtures with different percentages of water. The best performing resin/eluent system was further investigated in an additional experiment on an enlarged scale with the aim to evaluate the concentrate composition upon increasing the volume of the eluate.

MATERIALS AND METHODS

Sample Preparation, Resins, Eluents, and Standards. A sample of pigmented orange PW (50 L) was provided by a local citrus-processing plant (Ruby Co., Catania, Italy). To remove undissolved solids, the sample was centrifuged at 9000 rpm × 20 min at 4 °C (Braun Biotech GMBH DR 15, Melsungen, Germany), the supernatant was then poured out in plastic containers of 1.5 L and immediately stored at −18 °C to avoid degradation of ascorbic acid and anthocyanins but also to avoid fermentation of sugars until column experiments and analyses were conducted.

Three commercial adsorbent resins of different matrix types (Resindion, Mitsubishi Chem. Co., Milan, Italy) were tested: EXA-90 and EXA-118 are PS-DVB copolymers, while EXA-31 has a methacrylic structure. The resins are authorized by the FDA for food processing (*15, 16*); they were activated by an overnight treatment with 96% ethanol (Sigma Aldrich, Milan, Italy) and rinsed with 5 bed volumes (BV) of distilled water before use.

The used eluents were 100% methanol and ethanol (Sigma Aldrich) and their mixtures with different percentage of distilled water (25%, 50%, and 75%, v/v), respectively.

Standard compounds for spectrophotometry and HPLC were used without further purification: ascorbic acid (Carlo Erba, Milan, Italy), galacturonic acid and phloroglucinol (Fluka, Milan, Italy), cyanidin 3-glucoside chloride and narirutin (Extrasynthèse, Genày, France), hesperidin, glucose, fructose, sucrose, caffeic acid, sinapic acid, ferulic acid, *p*-coumaric acid, and limonin (Sigma Aldrich). HPLC-grade solvents were from Sigma Aldrich.

Operating Conditions. The clarified PW was loaded with an outflow of 10 mL/min onto columns (26.0 cm × 2.5 cm i.d.) packed with 25 mL of activated resin until the outflowed PW remained colorless, that is up to resin saturation of anthocyanins. After washing with distilled water (4 BV) to remove the sugars, acids, and other water-soluble compounds, the compounds of interest were desorbed with a flow of 10 mL/min using a fixed volume of different alcohol/water mixtures. This volume was determined for each resin as the amount of methanol required to remove all the adsorbed anthocyanins. Each experiment was carried out in duplicate without re-using the resin.

A preparative lab-scale experiment was performed using the EXA-118 resin and 50% aqueous methanol as eluent. The apparatus included a glass column (52.5 cm × 4 cm i.d.) filled with 250 mL of resin, a membrane pump having controlled flow rate equipped with an auto blocking valve (Laboport FM 30, TT 18, KNF Italia, Milan, Italy), and a UV detector (Knauer, Germany) for in-line monitoring of the eluent's absorbance. It also includes independent reservoirs and lines in a Rilsan tube for loading of PW, eluent and regeneration mixtures, and washing water, and for collecting eluted fractions and exhausted PW. The resin was then saturated of anthocyanins using a 6 L of PW and washed with 4 BV of distilled water. The elution was performed after discarding the first colorless 150 mL of eluate, collecting seven fractions (100 mL each) in separate graduated cylinders. The experiment was replicated after overnight purification of the resin with 25% aqueous ethanol, rinsing with 4 BV of 1 N NaOH, 3 BV of 50% aqueous ethanol to remove impurities, and washing with 5 BV of distilled water to eliminate ethanol traces.

Table 1. Composition of Clarified PW before and after the Adsorption on Resins[a]

	loaded PW	eluted PW[b]
pH	3.4	3.4
total soluble solids (Brix)	9.4	9.0
titrable acidity (g/100 mL)[c]	1.1	1.0
total sugars (g/100 mL)	7.45	6.68
glucose (g/100 mL)	2.36	2.15
fructose (g/100 mL)	2.41	2.08
sucrose (g/100 mL)	2.68	2.45
ascorbic acid (mg/100 mL)	178.2	70.7
pectins (mg/L)[d]	671.3	671
phlorin (mg/L)[e]	19.2	14.2
total anthocyanins (mg/L)[f]	21.6	–
hesperidin (mg/L)	53.7	–
narirutin (mg/L)	41.2	–
total hydroxycinnamic acids (mg/L)[g]	104.5	–
sinapic (mg/L)	14.6	–
caffeic (mg/L)	6.3	–
ferulic (mg/L)	56.4	–
p-coumaric (mg/L)	27.1	–
limonin (mg/L)	18.5	–

[a] Mean value of two determinations. [b] Mean values from EXA-118, EXA-90, and EXA-31 resin. The deviation among the three outflowed PW does not exceed ±6%. [c] As anhydrous citric acid. [d] As galacturonic acid. [e] As phloroglucinol after enzymatic hydrolysis. [f] As cyanidin 3-glucoside chloride. [g] After alkaline hydrolysis of hydroxycinnamates.

Analytical Measurements of Clarified PW, Outflowed PW, and Alcoholic Eluates. The content of total soluble solids (Brix) was measured using a Zeiss refractometer at 20 °C. Acidity was determined by titration of 10 mL of PW with 0.1 N NaOH until pH 8.1 and expressed as percent (%) of anhydrous citric acid. The sugars (glucose, fructose, and sucrose) were quantified by an HPLC Thermo LC System (Thermoquest) equipped with a Perkin-Elmer LC-25 refractive index detector, using a μBondapak NH_2 column (Waters, 10 μm 300 mm × 3.9 mm i.d.) and an isocratic CH_3CN/H_2O 85/15 (v/v) as mobile phase at a flow of 1.2 mL/min at 30 °C in 30 min. Calibration curves of standard sugars were previously determined. The content of phlorin (phloroglucinol−glucoside) was quantified by HPLC/PDA as phloroglucinol aglycon after enzymatic hydrolysis, following Scordino et al. (*19*). The pectin content was determined using a colorimetric method and expressed as galacturonic acid (*20*); the sample was first purified from the phenolic compounds which were adsorbed into a C_{18} cartridge (Supelco), and the galacturonic acid of the soluble fraction was fixed in a weakly basic anion-exchange resin (Nekrolith RAM 1) and desorbed with sodium carbonate solution (*21*). Total anthocyanins were quantified by a spectrophotometric method following Rapisarda et al. (*22*) and expressed as cyanidin 3-glucoside; their distribution profile was determined by HPLC (*22*). Hydroxycinnamates were determined by HPLC/PDA as free hydroxycinnamic acids after alkaline hydrolysis (*23*). Ascorbic acid, hesperidin and narirutin, and limonin were quantified by HPLC/PDA, following Scordino et al. (*19*), Di Mauro et al. (*13*), and Shaw et al. (*24*), respectively. All analyses were performed in duplicate.

RESULTS AND DISCUSSION

Pulp Wash Characterization. PW is a water extract obtained by a countercurrent multistep washing of the pulp separated from the prime juice (*25*). It is characterized by a reduced amount of pigments and flavor but a higher level of pectins, hesperidin, and bitter compounds (*26*). Because of the high content of hesperidin, the original sample was centrifuged before use with the purpose of avoiding the blocking of the resin bed and to minimize the flavanone presence, which tends to associate with suspended solids (*13*). Hesperidin strongly competes against the anthocyanins for the adsorbing sites of the resin (*14*); in fact, the tested copolymers have always shown a very high

Table 2. Physical Properties of Resins and Adsorption of PW Components on Resins

	EXA-31	EXA-90	EXA-118
structure	methacrylic	PS-DVB	PS-DVB
pore radius (Å)	170	105	90
surface area (m^2/g)	470	630	1200
porosity (mL/g)	1.20	1.18	1.04
particle size (mm)	0.35	0.25	0.25
density (g/mL)	1.09	1.18	1.04
loaded resin volume (mL)	25	25	25
loaded PW volume (mL)[a]	360	500	600
Adsorbed PW Components (mg)[b]			
total anthocyanins[c]	8.0	10.8	13.0
total hydroxycinnamic acids[d]	38.7	52.2	62.7
hesperidin	19.9	26.8	32.2
narirutin	15.2	20.6	24.7
limonin	6.9	9.3	11.1
total	88.7	119.7	143.7

[a] Limit value for saturation of resin by anthocyanins. [b] Mean value of two experiments. [c] As cyanidin 3-glucoside chloride. [d] After alkaline hydrolysis of hydroxycinnamates.

binding capacity toward this flavanone (*12*, *13*, *15*). Table 1 reports the chemical composition of clarified PW used for all experiments. The amount of hesperidin is considerably reduced (53.7 mg/L), but it is still 2.5 times higher than the anthocyanins content (21.6 mg/L) and is associated with a relatively high amount of narirutin (41.2 mg/L). In fact, it was observed that centrifugation plays an important role in reducing the hesperidin content; however, the rate of the narirutin reduction was lower; thus, the narirutin-to-hesperidin ratio in byproducts increases with respect to the pure juice (*27*). The HPLC determinations of the anthocyanins showed the typical profile of blood orange juices (*28*), where among a dozen pigments not all identified yet, cyanidin 3-*β*-*O*-glucoside and cyanidin 3-(6″-malonyl)-*β*-*O*-glucoside predominate, representing about the 80% of the total anthocyanins (*28*−*30*). The analyzed PW has shown also a considerable content of hydroxycinnamic derivatives (104.5 mg/L), about two times higher in comparison with the juice (*31*); after alkaline hydrolysis, they are distributed as follows: 54% of ferulic, 26% of *p*-coumaric, 14% of sinapic, and 6% of caffeic acids, respectively. With regard to limonin, the major contributor to bitterness in orange juice, a large quantity (18.5 mg/L) is found in the analyzed sample; although limonin is not always present in fresh fruit in its bitter form, its precursors are converted during processing, which results in a significant defect in strongly processed byproducts (*25*). The used PW is also characterized by an amount of soluble solids (9.4 Brix) slightly lower than in the juice and by higher acidity (1.1%). Most of the soluble solids are sugars (7.45 g/100 mL), but the ratio between sucrose and reducing sugars is lower than in orange juice due to the partial inversion of sucrose to fructose and glucose. Great amounts of ascorbic and pectins are also observed together with a high content of phlorin, a marker of the addition of byproduct extracts to the pure juice and for which a fast and reliable protocol of analysis has been recently proposed by our group (*19*).

Adsorption Capacity of the Resins. Two of the commercial resins tested in this study were based on a PS-DVB structure:

Table 3. Desorption of PW Components from EXA-31 Resin Using Alcohol/Water Mixtures as Eluents[a]

	loaded[b]	eluted[c]							
		MeOH (%)				EtOH (%)			
component		100	75	50	25	100	75	50	25
				Anthocyanins[d]					
mg/L	21.6	159.9	153.5	91.7	7.3	160.0	160.0	109.6	45.7
mg	8.0	8.0	7.7	4.6	0.4	8.0	8.0	5.5	2.3
%[e]	9	10	12	16	12	11	10	9	24
recovery (%)	–	100	96	57	5	100	100	68	29
C. F.	–	7.4	7.1	4.2	0.3	7.4	7.4	5.1	2.1
				Hydroxycinnamates[f]					
mg/L	104.5	640.0	610.5	366.9	48.0	589.2	717.3	510.9	120.0
mg	38.7	32.0	30.5	18.3	2.4	29.5	35.9	25.5	6.0
%[e]	44	41	46	64	75	42	46	44	65
recovery (%)	–	85	81	49	6	78	95	68	16
C. F.	–	6.1	5.8	3.5	0.5	5.6	6.9	4.9	1.1
				Hesperidin					
mg/L	53.7	384.0	314.1	72.0	8.0	350.0	389.5	320.5	14.0
mg	19.9	19.2	15.7	3.6	0.4	17.5	19.5	16.0	0.7
%[e]	22	24	24	13	13	25	25	27	7
recovery (%)	–	97	79	18	2	88	98	81	4
C. F.	–	7.2	5.9	1.3	0.1	6.5	7.3	6.0	0.3
				Narirutin					
mg/L	41.2	298.0	237.2	38.4	n.d.	302.9	290.0	237.4	8.0
mg	15.2	14.9	11.9	1.9	–	15.1	14.5	11.9	0.4
%[e]	17	19	18	7	–	21	19	20	4
recovery (%)	–	98	78	13	–	99	95	78	3
C. F.	–	7.2	5.8	0.9	–	7.4	7.0	5.8	0.2
				Limonin					
mg/L	18.5	88.0	n.d.	n.d.	n.d.	12.0	n.d.	n.d.	n.d.
mg	6.9	4.4	–	–	–	0.6	–	–	–
%[e]	8	6	–	–	–	1	–	–	–
recovery (%)	–	66	–	–	–	9	–	–	–
C. F.	–	4.7	–	–	–	0.6	–	–	–

[a] Mean value of two observations of duplicate experiments on 25 mL of resin. [b] Loaded volume: 360 mL. [c] Eluate volume: 50 mL. [d] As cyanidin 3-glucoside chloride. [e] With respect to the adsorbed compounds. [f] As free hydroxycinnamic acids after alkaline hydrolysis of hydroxycinnamates. C. F.: concentration factor. n.d.: not detected.

Table 4. Desorption of PW Components from EXA-118 Resin Using Alcohol/Water Mixtures as Eluents[a]

	loaded[b]	eluted[c]							
		MeOH (%)				EtOH (%)			
component		100	75	50	25	100	75	50	25
				Anthocyanins[d]					
mg/L	21.6	423.3	406.7	222.4	13.6	423.3	426.7	366.0	90.8
mg	13.0	12.7	12.2	6.7	0.4	12.7	12.8	11.0	2.7
%[e]	9	9	14	21	24	10	10	12	23
recovery (%)		98	94	51	3	98	99	85	21
C. F.	–	19.6	18.8	10.3	0.6	19.6	19.7	16.9	4.2
				Hydroxycinnamates[f]					
mg/L	104.5	2019.0	1532.2	787.7	43.3	1734.2	2002.7	1572.8	290.0
mg	62.7	60.6	46.0	23.6	1.3	52.0	60.1	47.2	8.7
%[e]	44	45	53	74	76	40	46	49	72
recovery (%)		97	73	38	2	83	96	75	14
C. F.	–	19.3	14.7	7.5	0.4	16.6	19.2	15.1	2.8
				Hesperidin					
mg/L	53.7	993.3	482.4	32.0	n.d.	1090.0	1107.5	698.0	13.3
mg	32.2	29.8	14.5	1.0	–	32.7	33.2	20.9	0.4
%[e]	22	22	17	3	–	25	25	22	3
recovery (%)		93	45	3	–	102	103	65	1
C. F.	–	18.5	9.0	0.6	–	20.3	20.6	13.0	0.2
				Narirutin					
mg/L	41.2	836.7	449.9	25.5	n.d.	856.7	856.2	526.3	6.7
mg	24.7	25.1	13.5	0.8	–	25.7	25.7	15.8	0.2
%[e]	17	18	16	2	–	20	19	17	2
recovery (%)		102	55	3	–	104	104	64	1
C. F.	–	20.3	10.9	0.6	–	20.8	20.8	12.8	0.2
				Limonin					
mg/L	18.5	253.3	n.d.	n.d.	n.d.	211.5	n.d.	n.d.	n.d.
mg	11.1	7.6	–	–	–	6.3	–	–	–
%[e]	8	6	–	–	–	5	–	–	–
recovery (%)		68	–	–	–	57	–	–	–
C. F.	–	13.7	–	–	–	11.4	–	–	–

[a] Mean value of two observations of duplicate experiments on 25 mL of resin. [b] Loaded volume: 600 mL. [c] Eluate volume: 30 mL. [d] As cyanidin 3-glucoside chloride. [e] With respect to the adsorbed compounds. [f] As free hydroxycinnamic acids after alkaline hydrolysis of hydroxycinnamates. C. F.: concentration factor. n.d.: not detected.

EXA-118 and EXA-90. Both of them were particularly suitable for the adsorption of anthocyanins because of their ideal pore radii and high surface areas, thus strongly increasing their adsorption capacity (*16*). In particular, in the case of EXA 118, the value of the surface area is double. The acrylic resin EXA-31 instead, even though highly recommended by the manufacturers as ideal for anthocyanins recovery because of its partly hydrophilic nature, has shown a minor efficiency (*14*, *16*). EXA-31 was included in this study in order to verify any different cleanup behavior in terms of selectivity for adsorbed compounds and volume of eluent required for their desorption with the respect of the two PS-DVB copolymers. The physical properties of the resins are reported in Table 2. The analysis of the eluate outflowed from the columns has evidenced the presence of sugars, phlorin, citric, ascorbic, and pectins which are not retained by the resin during the loading of PW (Table 1). The recovery of the water-soluble components in the outflowed PW, except ascorbic acid, was almost quantitative, varying from 74% for phlorin to 100% for pectins. The literature reported no adsorption of ascorbic acid for this kind of adsorbents (*2*); thus, its remarkable loss (about 60%) was ascribed to oxidative degradation during the loading and desorbing operations and during storage before analysis; it was confirmed by the results of subsequent analyses that showed decreasing concentration values. Any residual trace of water-soluble compounds was further eliminated from the column by washing the saturated resin with 4 BV of distilled water before the desorption step. The other PW components (anthocyanins, hydroxycinnamates, hesperidin, narirutin, and limonin) were adsorbed by the polymeric matrix. The PW volume required to saturate 25 mL of resin of anthocyanins and the corresponding amount of total compound adsorbed are reported in Table 2. The results have confirmed the highest adsorption capacity of EXA-118 with respect to EXA-90 and EXA-31. The EXA-118 resin in fact adsorbed 600 mL of PW before the saturation, with total amount of retained compounds of about 144 mg; EXA-90 and EXA-31 were saturated after 500 and 360 mL, respectively. The amount of anthocyanins and hydroxycinnamates adsorbed on the resin amounted to 9% and 44% of all adsorbed compounds, respectively.

Recovery of the Adsorbed Compounds. To compare the efficiency of the tested eluents, the adsorbed compounds were desorbed with a fixed eluent volume, defined as the methanol volume required to recover 100% of the anthocyanins adsorbed. The desorption experiences indicate a better cleanup capability of PS-DVB resins, which needed a minor volume of eluent (30 mL) to desorb all anthocyanins with respect to the methacrylic one (50 mL). The efficiency of the selective desorption of the different eluent mixtures employed for the removal of the natural antioxidants adsorbed on EXA-31, EXA-118, and EXA-90 resins are shown in Table 3, Table 4, and Table 5, respectively. The extracts were highly concentrated solutions of anthocyanins, hydroxycinnamates, flavanone glycosides, and limonin without sugars and other water-soluble constituents. Under the same conditions of desorption, the concentration of these compounds depended on the selectivity of the eluent mixture. In all cases, the adsorption/desorption systems were able to reach acceptable anthocyanin concentrations from about 7 (EXA-31) to 20 (EXA-118) times considering 100% recovery. Moreover, the distribution profiles of anthocyanins and hydroxycinnamates are

Table 5. Desorption of PW Components from EXA-90 Resin Using Alcohol/Water Mixtures as Eluents[a]

	loaded[b]	eluted[c]							
		MeOH (%)				EtOH(%)			
component		100	75	50	25	100	75	50	25
				Anthocyanins[d]					
mg/L	21.6	353.3	343.3	163.3	3.8	350.0	363.3	216.7	50.3
mg	10.8	10.6	10.3	4.9	0.1	10.5	10.9	6.5	1.5
%[e]	9	11	14	24	9	10	10	9	21
recovery (%)		98	95	45	1	97	101	60	14
C. F.	–	16.3	15.9	7.5	0.2	16.2	16.8	10.0	2.3
				Hydroxycinnamates[f]					
mg/L	104.5	1505.8	1285.0	423.0	33.3	1417.7	1583.1	1240.2	183.3
mg	52.2	45.2	38.6	12.7	1.0	42.5	47.5	37.2	5.5
%[e]	44	44	51	61	82	42	45	49	76
recovery (%)		86	74	24	2	81	91	71	11
C. F.	–	14.4	12.3	4.0	0.3	13.6	15.2	11.9	1.8
				Hesperidin					
mg/L	53.7	836.8	476.8	71.4	3.6	869.6	854.8	595.7	6.7
mg	26.8	25.1	14.3	2.1	0.1	26.1	25.6	17.9	0.2
%[e]	22	25	19	10	9	25	25	24	3
recovery (%)		94	53	8	0.4	97	96	67	1
C. F.	–	15.6	8.9	1.3	0.1	16.2	15.9	11.1	0.1
				Narirutin					
mg/L	41.2	565.9	407.6	34.2	n.d.	714.6	683.0	447.0	n.d.
mg	20.6	17.0	12.2	1.0	–	21.4	20.5	13.4	–
%[e]	17	17	16	5	–	21	20	18	–
recovery (%)		82	59	5	–	194	99	65	–
C. F.	–	13.7	9.9	0.8	–	17.3	16.6	10.8	–
				Limonin					
mg/L	18.5	103.2	n.d.	n.d.	n.d.	71.6	n.d.	n.d.	n.d.
mg	9.3	3.1	–	–	–	2.1	–	–	–
%[e]	8	3	–	–	–	2	–	–	–
recovery (%)		33	–	–	–	23	–	–	–
C. F.	–	5.6	–	–	–	3.9	–	–	–

[a] Mean value of two observations of duplicate experiments on 25 mL of resin. [b] Loaded volume:500 mL. [c] Eluate volume: 30 mL. [d] As cyanidin 3-glucoside chloride. [e] With respect to the adsorbed compounds. [f] As free hydroxycinnamic acids after alkaline hydrolysis of hydroxycinnamates. C. F.: concentration factor. n.d.: not detected.

identical to that of the loaded sample, as assessed by HPLC (data not shown).

Both 100% methanol and ethanol totally remove the loaded anthocyanins; however, a considerable amount of other desorbed compounds were present in the eluates, particularly hesperidin and narirutin which tended to precipitate as white specks in the concentrated alcoholic solution. With respect to the pure solvent, the 75% ethanol had determined the total desorption of anthocyanins but a major presence of hesperidin, narirutin and hydroxycinnamates. The 75% methanol instead determined a general decrease of yield with regard to 100% methanol. The higher desorption capacity of the ethanol/water mixtures in comparison with the methanol/water blends was observed at all ratios investigated but revealed scarce selectivity toward the compounds of interest. As shown by the percentage distribution of anthocyanins (about 9−10%) with respect to the other eluted compounds, no variation was observed between the loaded PW and the obtained extracts in 100% methanol and 100%, 75%, and 50% ethanol for all resins. This result particularly evidenced the lack of selectivity toward the pigments of those eluents which desorptions performed alcoholic concentrates with a similar composition of the loaded sample; a little decrement is shown only in the case of limonin, which removal did not improve upon changing the water proportion. A little increase in anthocyanins content with respect to the other desorbed compounds could be observed in the 75% methanolic extracts, but a real enrichment, together with a decrement of hesperidin and narirutin, was registered only in 50% aqueous methanolic extracts. Obviously, the increase of the percentage of anthocyanins from 9% to 16−24% and the minimization of the presence of the flavanones from 20% to 5% involves a relative decrement of the pigments concentration factors (from 7.0 to 4.2 for EXA-31 and from 20 to 10.3 for EXA-118) because of the minor recovery of the loaded compounds; the resins, in fact, retained more color. In general, a better recovery could be reached, increasing the collected volumes. The 25% alcoholic mixtures are not discussed because of the smallest recovery that makes these eluents unsuitable.

The best results were reached with the EXA-118 and the 50% aqueous methanol (Table 4), in which the 51% of the loaded pigments (6.7 mg) were recovered with a concentration factor of 10.3 times and a selectivity of 21%. This amount of anthocyanins corresponds to the maximum adsorption capacity of the same volume of EXA-31 resin. The presence of narirutin, hesperidin, and limonin was practically negligible. Moreover, the extract shows a considerable content of hydroxycinnamates (23.6 mg) with a concentration factor of 7.5 times and a selectivity of 74%. The presence of hydroxycinnamates is useful because they are co-pigments able to stabilize anthocyanins through the formation of intermolecular hydrophobic complexes, which are more resistant than the free anthocyanins toward the degradation factors (*32*−*35*).

Lab-Scale Experiment. The most effective resin/eluent system was tested in a lab-scale apparatus by increasing both the volume of the resin (250 mL) and of the loaded PW (6 L) 10 times. Seven fractions (100 mL each) were collected with the aim of investigating the changes of the concentrate composition (recovery and selectivity) upon increasing the eluted

Table 6. Desorption of PW Components from EXA-118 Resin Using 50% Aqueous Methanol as Eluent in a Lab Experiment[a]

component	loaded[b]	eluted fractions[c]						
		1	2	3	4	5	6	7
				Anthocyanins[d]				
mg/L	21.6	173.3	277.9	213.9	151.7	106.4	71.4	69.3
mg	129.8	17.3	27.8	21.4	15.2	10.6	7.1	6.9
%[e]	9	29	21	21	16	13	13	15
recovery (%)	–	13	21	16	12	8	6	5
C. F.	–	8.0	12.8	9.9	7.0	4.9	3.3	3.2
				Hydroxycinnamates[f]				
mg/L	104.5	391.1	973.9	729.5	530.7	415.1	307.9	267.5
mg	626.9	39.1	97.4	73.0	53.1	41.5	30.8	26.8
%[e]	44	66	72	72	54	49	55	59
recovery (%)	–	6	16	12	8	7	5	4
C. F.	–	3.7	9.3	7.0	5.1	4.0	2.9	2.6
				Hesperidin				
mg/L	53.7	30.9	74.8	45.8	41.8	38.8	41.7	38.6
mg	322.0	3.1	7.5	4.6	4.2	3.9	4.2	3.9
%[e]	22	5	6	5	4	5	8	9
recovery (%)	–	1	2	1	1	1	1	1
C. F.	–	0.6	1.4	0.9	0.8	0.7	0.8	0.7
				Narirutin				
mg/L	41.2	n.d.	18.8	23.4	31.2	45.4	49.5	39.6
mg	247.2	–	1.9	2.3	3.1	4.5	5.0	4.0
%[e]	17	–	1	2	3	5	9	9
recovery (%)	–	–	1	1	1	2	2	2
C. F.	–	–	0.5	0.6	0.8	1.1	1.2	1.0
				Limonin				
mg/L	18.5	n.d.	n.d.	n.d	221.6	237.8	83.7	34.5
mg	111.2	–	–	–	22.16	23.78	8.37	3.45
%[e]	8	–	–	–	23	28	15	8
recovery (%)	–	–	–	–	20	21	8	3
C. F.	–	–	–	–	12.0	12.8	4.5	1.9

[a] Mean value of two observations of duplicate experiments on 250 mL of resin. [b] Loaded volume: 6 L. [c] Fractions volume: 100 mL. [d] As cyanidin 3-glucoside chloride. [e] With respect to the adsorbed compounds. [f] As free hydroxycinnamic acids after alkaline hydrolysis of hydroxycinnamates. C. F.: concentration factor. n.d.: not detected.

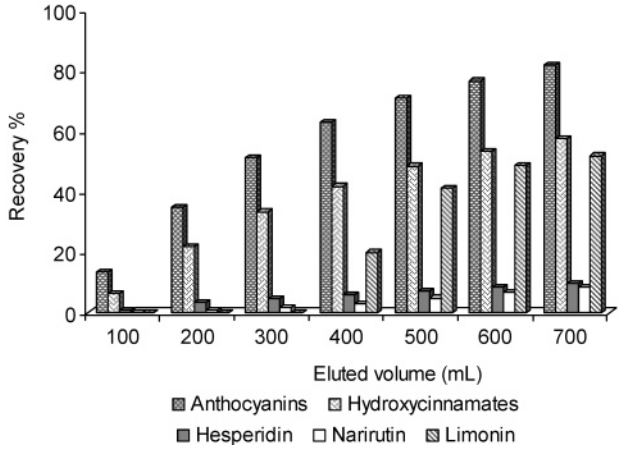

Figure 1. Cumulative percent of recovery of PW components from EXA-118 resin using 50% aqueous methanol as eluent.

volume. The contents of anthocyanins, hydroxycinnamates, hesperidin, narirutin, and limonin of each fraction are reported in Table 6 and both showed as cumulative recovery percentage (Figure 1) and cumulative distribution (Figure 2). The composition of the first three cumulative fractions is comparable with those of the previous experiment; in fact, the recovery and the selectivity of all detected compounds are almost superimposable. Prior to the 700 mL level, the volume collection showed an increase in pigments recovery from 50% up to 80%, which is

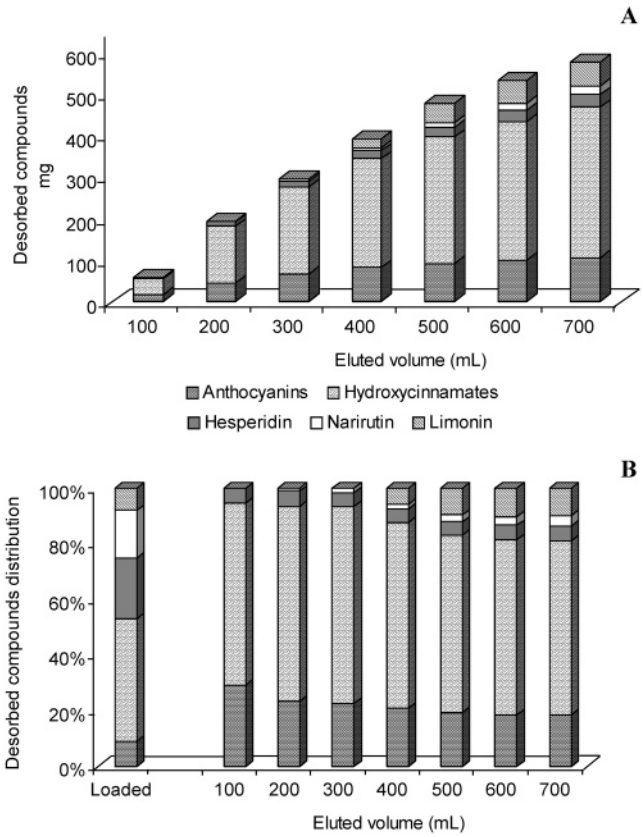

Figure 2. Cumulative desorption of PW components from EXA-118 resin using 50% aqueous methanol as eluent: (A) total amount; (B) percentage distribution.

associated with a corresponding increase of hydroxycinnamates from 34% to 58%. Anthocyanins and hydroxycinnamates showed a similar behavior during elution, being more hydrophilic than hesperidin, narirutin, and limonin, which were strongly retained by the hydrophobic resin. Analyzing every single fraction by HPLC, a maximum desorption of anthocyanins and hydroxycinnamates was noticeable, in particular in fractions 1 to 3 where the content of the two flavanone glycosides was similar and practically negligible. Considering the final volume of 700 mL, the hesperidin (31.2 mg) and the narirutin (20.8 mg) amounts remain more than 3 and 5 times lower than the amount of the pigments (106.4 mg) respectively, and over 10 times lower than hydroxycinnamates (361.6 mg). The limonin was absent in the first three fractions and has exhibited a massive outflow in the fourth and fifth fractions to slowly decrease in the latter. Its level in fractions 4, 5, and 6 was higher than those of the anthocyanins. In the total eluted volume, the limonin content (57.8 mg) was about half of that of anthocyanins, and its recovery (52%) was almost comparable with that of hydroxycinnamates (58%). In conclusion, the results indicate that the collection of smaller elution volumes (300 mL) is a reasonable compromise among a selective recovery of anthocyanins and hydroxycinnamates, the lowest amount of flavanones, and the total absence of limonin**.** The collection of larger eluate volumes (over 400 mL) results in a massive presence of the latter compound and in an increasing concentration of the flavanone glycosides.

Conclusions. This work has demonstrated that the PS-DVB EXA-118 copolymer, with a pore radius of 90 Å and a surface area of 1200 m^2/g, is the most suitable resin among selected commercial adsorbents for the recovery of anthocyanins from pigmented orange PW. This is due to the elevated uptake

capacity, the high cleanup capability, the good selectivity toward the pigments, and a great yield, with respect to the other tested resins under the same eluent conditions. The purity of the extracts primarily depended on the used eluents. One hundred percent methanol and 100% and 75% ethanol totally removed the adsorbed anthocyanins, but on the other hand, no affinity for the pigments was observed because of the substantial desorption of other phenolics together with limonin. Under the same eluted volume, it was also demonstrated that the ethanol/water mixtures proved a major cleanup capability even though no selectivity was shown for the anthocyanins with respect to the methanol/water mixtures. In contrast, the use of aqueous methanol decreases the yield of anthocyanins but increases their purity in the extracts. The best results, in terms of selectivity and percentage of recovery, were obtained using a methanol/water 50:50 (v/v) mixture on EXA-118 resin. The collection of a smaller volume of eluent instead of the larger one needed for the almost total recovery of the pigments could further increase the purity of the extract. Moreover, the extracts obtained could be further concentrated by alcohol distillation or spray−dry operation.

In conclusion, this study has evidenced the possibility to obtain highly concentrated alcoholic extracts of anthocyanins from citrus byproducts, completely free of sugars, acids, and other water-soluble compounds, with the application of a liquid chromatography purification step. The proposed procedure in fact utilizes commercial adsorbent resins and cheap eluents without requiring a highly expensive preparative-HPLC apparatus and time-consuming runs. To highlight products from the residues and to balance waste disposal costs, it can be applied in citrus processing plants without large investment costs. The stability of the anthocyanins extract is assured by the high quantities of hydroxycinnamates useful to stabilize anthocyanins (*32*−*35*) and by the absence of ascorbic acid responsible of pigments degradation (*33*, *36*−*38*). The resulting fractions could easily find application as a natural colorant, antioxidant ingredient for dietary supplements, functional foods, and/or as a raw material in cosmetic and pharmaceutical industry preparations.

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