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Recovery of Anthocyanins from Pulp Wash of Pigmented Oranges by Concentration on Resins

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A pulp wash (PW) coming from a plant for citrus processing of pigmented oranges was utilized as a starting material to recover anthocyanins, using the procedure of concentration on resin. Six commercial food-grade resins were tested to find the more suitable ones for adsorbing anthocyanins, and 96% ethanol was used as eluent for desorbing them. An automated experimental apparatus was developed to perform the adsorption−desorption procedure on column. The kinetics results in a batch system and experiments on column showed that the more efficient resins are those made of styrene−divinylbenzene having a pore radius ranging from 70 to 150 Å and a surface area from 600 to 800 m^2/g, namely, Sepabeads SP 70 and Relite EXA 90. The richest fractions collected from these resins contained about 95% of the anthocyanins in a volume of about 2% of the loaded PW. The HPLC profile of the desorbed anthocyanins is the same as that in PW. These fractions contain other phenol compounds, such as hesperidin and derivatives of hydroxycinnamic acids, in remarkable amounts. Ethanol can be easily removed from the solution and recycled, thus affording a much more concentrated product which can find application as a food colorant or antioxidant ingredient for a nutritional integrator.

KEYWORDS: Anthocyanins; citrus byproducts; oranges; pulp wash; styrene−divinylbenzene resins

INTRODUCTION

The Italian citrus industry prevalently processes pigmented oranges, producing both natural and concentrated red juices, together with essential oil. Moreover, it discharges every year several hundred thousand tons of peel, wet pulp, and wastewater containing high quantities of organic compounds. Disposal and purification of wastes require technological and economic efforts, so that the recovery and valorization of products from residues has become a necessity, to balance costs and increase competitiveness by diversification of products. Among the valuable compounds are hesperidin and anthocyanins that have found application in the food and pharmaceutical industries (*1*, *2*). The first product can be recovered from peel or from the wastewater flowing from centrifuges of essential oil separation, namely, yellow water, while anthocyanins can be obtained from the pulp still rich in red juice, outflowing from finishers (**Figure 1**).

Previously, we studied the recovery of hesperidin from the aforementioned sources (*3*, *4*), utilizing an apolar styrene−divinylbenzene resin (SDVB) to adsorb the flavanone from diluted extracts, and eluting in an aqueous alkaline in a very reduced volume, yielding pure hesperidin after acidification. The present paper concerns the recovery of anthocyanins from pulp wash (PW) using the same procedure of concentration on resin previously applied for hesperidin. PW, defined also as "water-extractable soluble orange solids" (WESOS), is obtained by washing and refinishing discharged pulp several times in a countercurrent system (*5*), representing about 5% of processed oranges. PW contains a significant amount of sugars, organic acids, flavonoids, phenol compounds, pectins (*6*), and anthocyanins. Six resins, having different chemical and physical properties, were investigated to find the best one for adsorbing anthocyanins.

Resins are currently used for adsorption of flavonoids and other components from products and byproducts of the citrus industry (*7*, *8*). In particular, SDVB resins have been used to remove naringin and limonin from citrus juices (*9*) and to recover cold pressed grapefruit oil from wastewater (*10*). Amberlite XAD 16 and other nonionic and macroporous resins have been used recently to remove limonoids and phenol compounds from citrus peel juice and molasses (*11*).

Anthocyanins have been extracted from various plant sources and residual materials to produce authorized food colorants (European Code, E 163), nutraceuticals, and drugs. Enocianina (*12*) and Myrtocyan (Indena, Milan, Italy) are two typical anthocyanin-based products obtained from the dregs of red grapes and bilberry fruits, respectively. Resins for recovering

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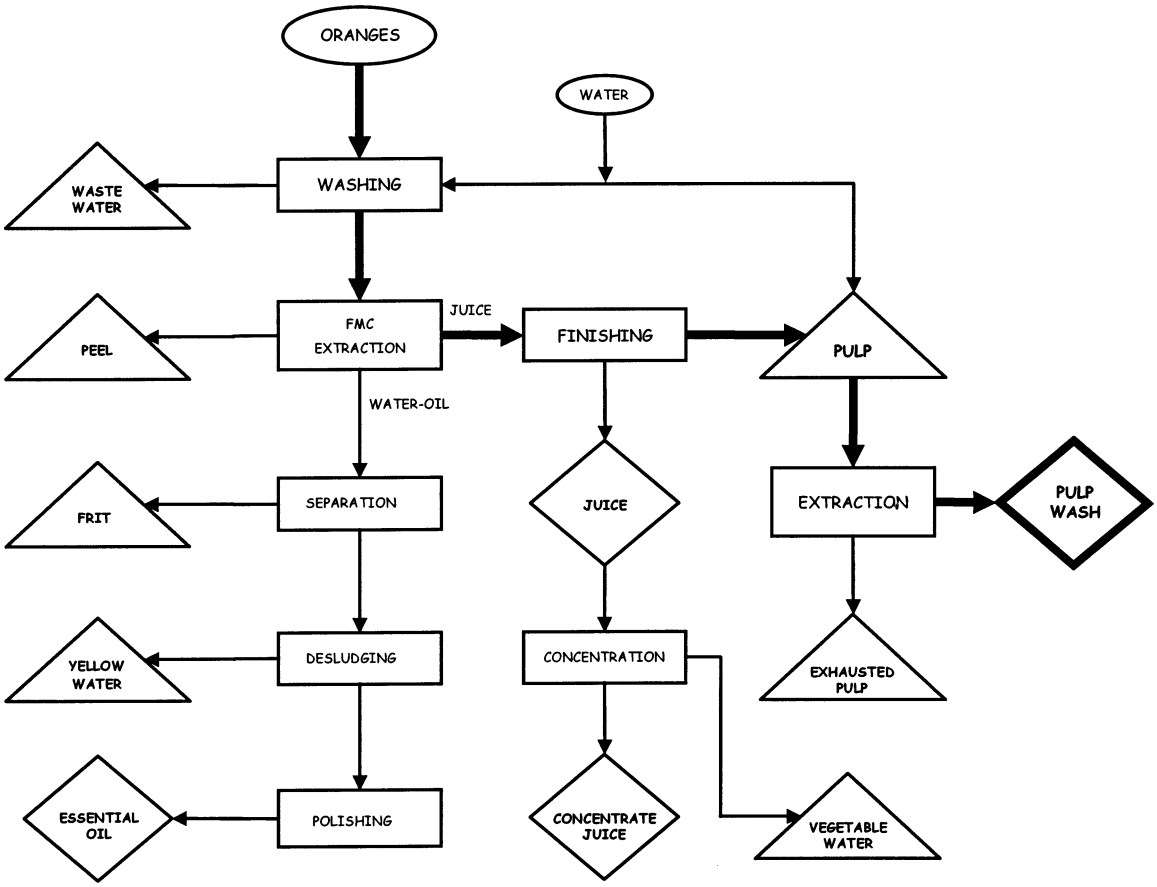

Figure 1. Flow scheme of a plant for production of orange juice and essential oil. The bold lines indicate the unit operations to obtain pulp wash.

Table 1. Chemical and Physical Properties of Resins

trade name	structure	pore radius (Å)	surface area (m^2/g)	particle diameter (mm)	code of regulations
SP 70	SDVB	~70	800	0.45	FDA CFR 173.25
EXA 31	methacrylic	150–200	470	0.3–0.8	EC R AP (97) 1
EXA 32	SDVB	200–300	600	0.3–0.8	EC R AP (97) 1
EXA 45	SDVB	50–60	1000	0.3–0.8	EC R AP (97) 1
EXA 90	SDVB	100–150	600	0.3–0.8	EC R AP (97) 1
XAD 16	SDVB	50	800	0.3–0.7	FDA CFR-173.65

Table 2. Chemical Composition of the Centrifuged PW[a]

Brix	5.4(0.1)
pH	3.9(0.1)
acidity (g/L)[b]	0.32(0.17)
glucose (g/L)	12.8(0.5)
fructose (g/L)	13.9(0.6)
sucrose (g/L)	14.9(0.6)
hesperidin (mg/L)	285(32)
hydroxycinnamic acids (mg/L)[c]	120(9)
anthocyanins (mg/L)[d]	17.1(0.4)

[a] Mean values from four determinations of the same sample (standard deviation in parentheses). [b] As anhydrous citric acid. [c] Sum of sinapic, caffeic, ferulic, and *p*-coumaric acids. [d] As cyanidin-3-glucoside chloride.

anthocyanins have been using for many years (*13*); in particular, Amberlite XAD 16 was used to obtain anthocyanins from pigmented oranges (*14*). These fruits (Cv. Tarocco, Moro, and Sanguinello) contain pigments prevalently derived from cyanidin aglycon (*15*, *16*); among them, cyanidin-3-β-*O*-glucoside and cyanidin-3-(6″-malonyl)-β-*O*-glucoside predominate (*17*). Cyanidin derivatives behave as very active scavengers of radical species (*18*, *19*), thus contributing to increase the antioxidant capacity of pigmented orange juices (*20*, *21*). Therefore, obtaining concentrated extracts of these pigments is of interest in the food and pharmaceutical industries.

MATERIALS AND METHODS

Pulp Wash, Standards, Solvents, and Resins. A sample of PW was drawn from the plant of Ruby Co. (Catania, Italy) during the 2001 season, distributed in 5 L plastic containers, and stored at −15 °C to avoid fermentation. Before use, PW was centrifuged at 13000*g* for 20 min at 4 °C (Braun Biotech GMBH DR 15, Melsungen, Germany), and then the supernatant was filtered through Whatman No. 1 filter paper by vacuum suction using a Buchner funnel. PW was characterized by measurements of pH, Brix, and acidity. Glucose, fructose, and sucrose were quantified individually by an enzymatic−spectrophotometric procedure using a kit purchased from Boheringer (Mannheim, Germany). Anthocyanins were determined by spectrophotometry and by HPLC (*22*, *23*). Hesperidin and hydroxycinnamic acids were quantified by HPLC following Di Mauro et al. (*3*, *4*) and Rapisarda et al. (*24*), respectively.

Standard compounds for spectrophotometry and HPLC were used without further purification: cyanidin-3-glucoside chloride (Extrasynthèse, Genay, France), hesperidin, sinapic acid, ferulic acid, and *p*-coumaric acid (Sigma Aldrich, Milan, Italy). HPLC grade solvents

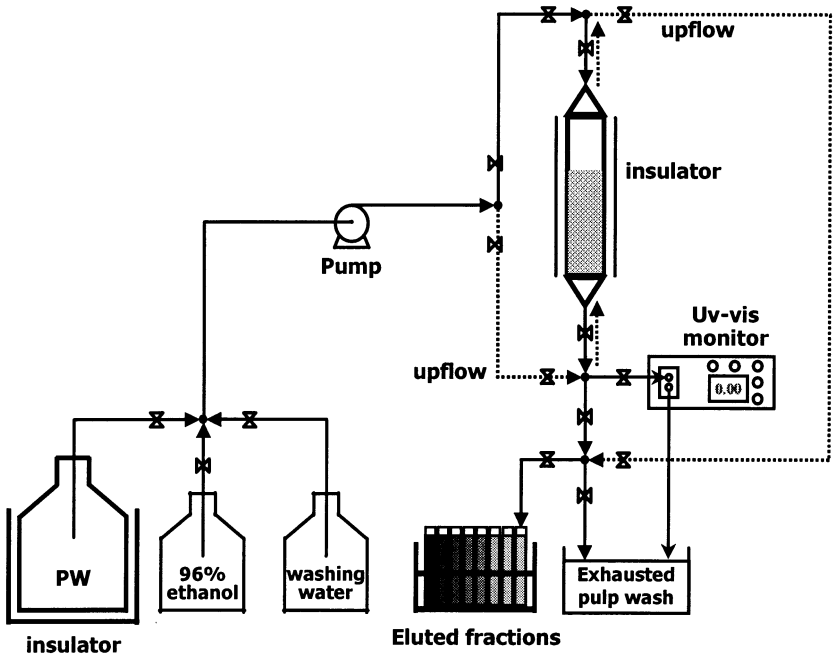

Figure 2. Flow scheme of the apparatus for recovery of anthocyanins from pulp wash.

(methanol, tetrahydrofuran, acetonitrile, and water) were from Merck (Milan, Italy), and 96% ethanol as eluent was from Panreac Quimica (Barcelona, Spain).

The tested resins were Sepabeads SP 70, Relite EXA 31, Relite EXA 32, Relite EXA 45, Relite EXA 90 (Resindion, Mitsubishi Chemical Co., Milan, Italy), and Amberlite XAD 16 (Sigma Aldrich, Milan, Italy). Apart from Relite EXA 31 (methacrylic polymer), all resins are styrene−divinylbenzene (SDVB) copolymers. These polymers may be used for the removal of organic substances from aqueous food under prescribed preparation conditions. The resins were pretreated with water according to the manufacturer's recommendation to guarantee food-grade purity at the time of use, in accordance with current good manufacturing practice. Chemical and physical properties of resins, together with the European and FDA codes of regulations, are shown in **Table 1**.

Resin Activation and Kinetics of Adsorption in Batch System. Activation of resins was performed by overnight treatment with 2 bed volumes (BV) of 96% ethanol, followed by rinsing with 2 BV of distilled water. After being stirred for 10 min, the resin was filtered, and 150 mL was introduced onto a glass column and washed with 5 BV of distilled water. The volume of packed resin did not exceed 60% of the total volume of the column to allow washing of the resin by an upflow fluidized-bed column system.

Some adsorption experiments were carried out in a standard batch system at 20 °C, under stirring: 10 mL of activated resin was mixed with 700 mL of centrifuged PW. The initial concentration of anthocyanins was 17.7 mg/L in all experiments. Aliquots of 1 mL were drawn from the solution at regular intervals up to 200 min to determine the residual anthocyanin content (*22*). Kinetics were conducted in duplicate runs, and the results did not differ by more than 8% for each resin. The amount of pigments adsorbed into the resin was calculated by difference.

Recovery of Anthocyanins. An automated apparatus was developed to perform the concentration of anthocyanins (**Figure 2**). It consists of a glass column having two flanges and two removable septa (distance between septa, 46.5 cm; 2.7 cm i.d.; volume, 266 mL), a membrane pump having variable and controlled flow rate, equipped with an autoblocking valve (Laboport FM 30, TT 18, KNF Italia, Milan, Italy), and a UV detector to monitor on-line the absorbance of effluent (Knauer, Germany). The apparatus includes a thermally isolated reservoir for the refrigerated PW (4 °C) and two other reservoirs for 96% ethanol and washing water, and many faucets of Teflon and independent lines of Rilsan tube for loading PW, ethanol, and washing water and for collecting eluted fractions and exhausted PW. The column was insulated to minimize heating of the refrigerated PW and degradation of anthocyanins. PW was pumped down through the column at 30 mL/min (12 BV/h). Feeding was stopped when the effluent achieved an absorbance value of about 50% of the loaded PW, as indicated by the UV monitor. The saturated resin was washed with distilled water (5 BV) to remove the sugars and other water-soluble compounds, and anthocyanins were desorbed using 96% ethanol (3

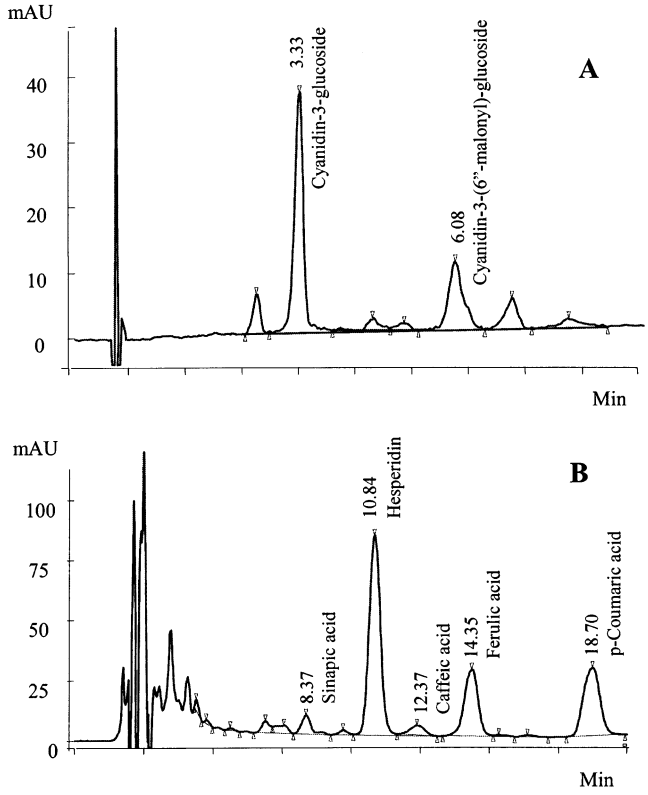

Figure 3. HPLC profiles of phenol compounds in pulp wash: anthocyanins monitored at 520 nm (A), hesperidin and hydroxycinnamic acids at 283 nm (B).

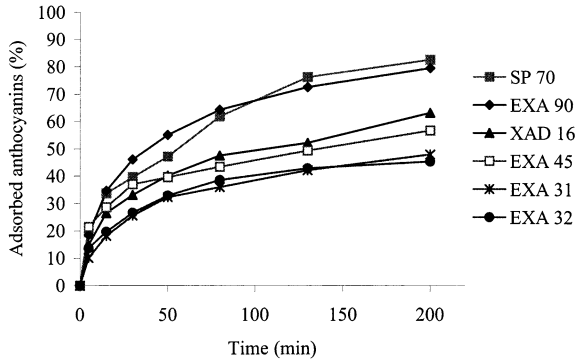

Figure 4. Kinetics of adsorption in batch system of the PW anthocyanins on different resins at 20 °C.

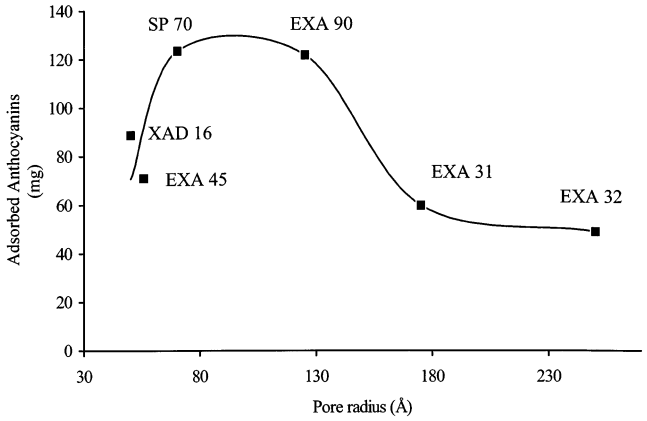

Figure 5. Adsorption of anthocyanins vs the radius pore of resins.

BV/h). The ethanol fractions (50 mL each) were collected in graduated cylinders until the anthocyanins in the column were exhausted.

The resin regeneration procedure includes the following steps: (i) leaving the resin overnight in 96% ethanol, (ii) upflow rinsing with 3 BV of 50% aqueous ethanol to remove soluble impurities, and (iii) upflow washing with 5 BV of distilled water to eliminate traces of ethanol. The upflow system was useful for removing gas bubbles and the fine particles accumulated on the top of the resin, favoring the regular sedimentation of resin.

The amount of sugars in the effluent was determined, as previously indicated. The concentration of anthocyanins was determined in the effluent and in each eluted fractions, while the HPLC profile of anthocyanins was determined in the richest fractions collected from each resin. Hesperidin and hydroxycinnamic acids were quantified in the first four fractions collected from SP 70 and EXA 90, using the same procedures described for PW analysis.

RESULTS AND DISCUSSION

Table 2 reports the mean composition of the centrifuged PW used for all experiments. It is a slightly acidic solution containing about 4% sugars, but the relative ratios of glucose, fructose, and sucrose are different from those found in orange juices, probably due to the partial inversion of sucrose during storage. It also contains several phenol compounds, such as anthocyanins, hesperidin, and derivatives of hydroxycinnamic acids. HPLC profiles of these compounds (**Figure 3**) are almost identical to those of the pigmented orange juices (*3*, *4*, *16*, *17*, *22*, *24*).

The kinetics of PW adsorption in a batch system demonstrated the different capacity of resins for adsorbing anthocyanins (**Figure 4**). After 200 min at 20 °C, no significant change occurs, indicating that the rates of adsorption and desorption of anthocyanins achieved equilibrium. The six resins can be divided into three different couples. The most effective are SP 70 and

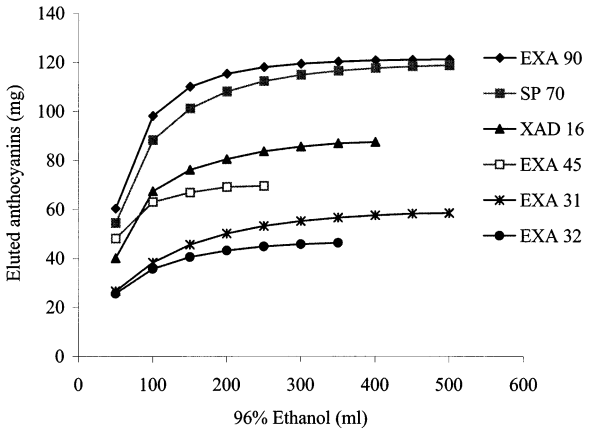

Figure 6. Elution of anthocyanins vs total volume of the fractions for different resins.

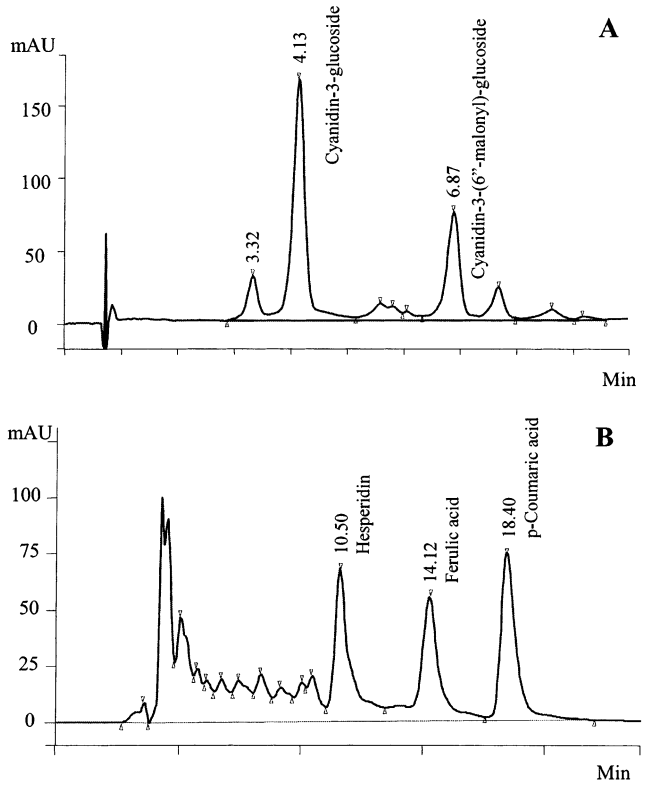

Figure 7. HPLC profiles of phenol compounds in the eluted fractions from EXA 90 resin: anthocyanins monitored at 520 nm (A), hesperidin and hydroxycinnamic acids at 283 nm (B).

EXA 90, which adsorb about 80% of the anthocyanins present in solution; XAD 16 and EXA 45 show an intermediate capacity, adsorbing about 60% of the pigments; and the worst are EXA 31 and EXA 32, which adsorb less than 50% at equilibrium. Concerning the methacrylic resin EXA 31, which is less hydrophobic than SDVB resins, it was expected to show a higher adsorption for the polar species such as flavylium salts, but these pigments are probably adsorbed into the resin through the neutral and hydrophobic species present at equilibrium in aqueous solution (*25*).

Table 3 reports the results of the experiments carried out on column for each resin. The reproducibility of the data is very good if the resin is regenerated after every cycle. For instance, the mean adsorption of anthocyanins by EXA 45 (four cycles) is 71.2 ± 6.6 mg, while the corresponding desorption is 70.1 ± 6.5 mg, with a standard deviation of less than 10% in both

Table 3. Adsorption of PW Anthocyanins on Different Resins and Desorption by 96% Ethanol[a]

adsorption	desorption	anthocyanins			
resin	fraction no.	mg/L	mg	yield (%)[f]	concentration factor
SP 70[b]					
PW: loaded volume 8 L	1	1090(131)	55(7)	44.1(4.2)	58(7)
anthocyanins:	2	661(44)	40(2)	27.4(0.8)	36(2)
18.7(0.5) mg/L	3	254(23)	13(1)	10.4(0.6)	14(1)
149.9(4.0) mg	4	145(33)	6.9(1.6)	5.6(1.2)	7.4(1.6)
hesperidin: 2.28 g	5	87(22)	4.2(1.1)	3.4(0.8)	4.4(1.1)
adsorbed anthocyanins:[c]	6	56(17)	2.6(0.9)	2.1(0.7)	2.8(0.9)
123.5(4.2) mg	7	38(16)	1.7(0.8)	1.3(0.6)	1.8(0.8)
yield: 82.4(31.5)%	8	24(12)	1.0(0.6)	0.8(0.5)	1.1(0.6)
	total		117.6(1.0)	95.1(5.7)	
EXA 31[d]					
PW: loaded volume 4 L	1	534(11)	27(1)	44.6(0.3)	31.5(0.5)
anthocyanins:	2	234(14)	12(1)	19.6(1.5)	13.8(0.9)
17.0(0.1) mg/L	3	146(6)	7.3(0.3)	12.2(0.3)	8.6(0.3)
67.8(0.3) mg	4	90(9)	4.5(0.4)	7.5(0.6)	5.3(0.5)
hesperidin: 1.14 g	5	61(1)	3.1(0.1)	5.1(0.2)	3.6(0.1)
adsorbed anthocyanins:[c]	6	40(3)	2.0(0.1)	3.3(0.3)	2.4(0.2)
59.8(0.8) mg	7	28(3)	1.4(0.1)	2.3(0.2)	1.7(0.2)
yield: 88.2(0.9)%	8	20(3)	1.0(0.1)	1.7(0.2)	1.2(0.2)
	total		57.7(0.4)	96.4(0.8)	
EXA 32[b]					
PW: loaded volume 6.3 L	1	514(71)	26(4)	52.7(5.0)	40.5(6.6)
anthocyanins:	2	202(91)	10(5)	20.0(6.5)	16.1(7.9)
12.7(0.8) mg/L	3	97(32)	4.8(1.6)	9.7(1.7)	7.7(3.1)
80.1(15.6) mg	4	53(20)	2.7(1.0)	5.4(1.2)	4.3(1.9)
hesperidin: 1.80 g	5	32(16)	1.6(0.8)	3.2(1.0)	2.6(1.4)
adsorbed anthocyanins:[c]	6	19(8)	0.9(0.4)	1.9(0.5)	1.5(0.7)
49.0(7.9) mg	7	11(4)	0.6(0.2)	1.1(0.3)	0.9(0.4)
yield: 61.5(3.7)%	total		46.4(11.0)	93.9(8.4)	
EXA 45[e]					
PW: loaded volume 4 L	1	964(173)	48(7)	67.7(9.1)	47(8)
anthocyanins:	2	297(72)	15(4)	21.1(6.3)	15(4)
20.4(0.7) mg/L	3	80(19)	4.0(0.9)	5.6(1.7)	3.9(1.1)
81.7(6.6) mg	4	44(18)	2.2(0.6)	3.1(1.5)	2.2(1.0)
hesperidin: 1.14 g	5	17(5)	0.8(0.2)	1.2(0.6)	0.8(0.3)
adsorbed anthocyanins:[c]	total		70.1(6.5)	98.4(0.1)	
71.2(6.6) mg					
yield: 87.1(5.7)%					
EXA 90[b]					
PW: loaded volume 9 L	1	1209(55)	60(3)	49.7(1.8)	73(6)
anthocyanins:	2	755(64)	38(3)	31.0(1.1)	46(5)
16.6(1.4) mg/L	3	239(42)	12(2)	9.8(1.0)	14(2)
148.5(4.6) mg	4	105(17)	5.2(0.8)	4.3(0.4)	6.3(0.7)
hesperidin: 2.57 g	5	53(16)	2.7(0.8)	2.2(0.6)	3.2(0.7)
adsorbed anthocyanins:[c]	6	28(16)	1.4(0.8)	1.2(0.6)	1.6(0.8)
121.7(9.2) mg	7	18(6)	0.9(0.3)	0.7(0.2)	1.1(0.3)
yield: 82.0(6.4)%	8	11(5)	0.5(0.2)	0.4(0.2)	0.6(0.2)
	total		120.9(9)	99.3(0.2)	
XAD 16[b]					
PW: loaded volume 6 L	1	804(16)	40(1)	45.3(1.2)	47(1)
anthocyanins:	2	546(33)	24(2)	30.7(2.3)	32(3)
17.0(0.7) mg/L	3	174(23)	8.7(1.1)	9.8(1.1)	10(1)
102.0(3.9) mg	4	88(14)	4.4(0.7)	5.0(0.7)	5.2(0.7)
hesperidin: 1.71 g	5	63(12)	3.1(0.6)	3.5(0.5)	3.7(0.7)
adsorbed anthocyanins:[c]	6	39(5)	1.9(0.3)	2.2(0.2)	2.3(0.3)
88.8(3.2) mg	7	26(4)	1.3(0.2)	1.5(0.2)	1.5(0.2)
yield: 87.1(3.7)%	8	17(4)	0.9(0.2)	1.1(0.2)	1.0(0.2)
	total		87.5(3.2)	98.9(0.2)	

[a] Standard deviation in parentheses. [b] Mean values from three runs. [c] Calculated from the difference between anthocyanins in the loaded PW and those present in the effluent. [d] Mean values from two runs. [e] Mean values from four runs. [f] Calculated on the adsorbed anthocyanins.

cases. The loaded volume of PW in each run ranges from 4 to 10 L, depending on specific adsorbing capacity of resin and on the content of anthocyanins in PW, which varies from 21 to 12 mg/L, due to partial degradation of the pigments during storage.

The capacity of resins to adsorb anthocyanins on column strictly follows that measured in the standard kinetics. A critical factor in determining differences in adsorption seems to be the pore radius (**Table 1**). The most efficient resins have a pore radius ranging from 70 to 150 Å and a surface area from 600 to 800 m^2/g. Resins having a pore radius smaller than 70 Å are less efficient, even if their surface area is higher than 800 m^2/g. Those having a pore radius larger 150 Å and a surface area less than 600 m^2/g are unsuitable for adsorption (**Figure 5**). In other words, too small pores do not allow penetration of

anthocyanins into the reticule, and too large ones are not able to retain them. The most efficient resins, EXA 90 and SP 70, adsorb 122 ± 9 and 124 ± 4 mg, respectively, i.e., 0.82 mg per milliliter of resin, whereas EXA 32, the worst resin, adsorbs only 49 ± 8 mg, i.e., 0.33 mg per milliliter of resin. The adsorption yield is about 85%, except for EXA 32, where it is 62%. After the adsorption process, the effluent contains almost all the sugars originally present in the starting material.

As acidic solutions have been used to elute anthocyanins from resins (*13*, *14*), different citric acid/ethanol solutions have been tested in preliminary experiments. These trials demonstrated that citric acid in 96% ethanol does not improve the elution of anthocyanins with respect to 96% ethanol. **Figure 6** reports the cumulative amount of anthocyanins eluted from the column. Using 96% ethanol led to almost quantitative elution of the adsorbed anthocyanins. Analysis of the eluted fractions shows a remarkable concentration of anthocyanins in the initial fractions. In particular, for the best resins, EXA 90 and SP 70, the first 200 mL of eluted ethanol are 30−35 more concentrated than the starting PW (540−580 mg/L), corresponding to about 2% of the loaded PW volume. The HPLC profile of these anthocyanins (**Figure 7A**) is very similar to that of PW (**Figure 3A**), indicating that concentration on resin did not change significantly the relative distribution of the individual pigments.

The choice of 96% ethanol as eluting solvent, rather than aqueous ethanol mixtures, allowed further concentration of the solution by azeotropic distillation, with the advantage of recovering a large part of solvent for recycle purposes. Nevertheless, 96% ethanol is not selective for the elution of anthocyanins; in fact, HPLC analysis of the anthocyanin-rich fractions from SP 70 and EXA 90 resins revealed the presence of remarkable quantities of hesperidin and hydroxycinnamic acids (**Figure 7B**), amounting to about 2 and 1 g/L, respectively. The content of these compounds depends on the volume of PW loaded on column, which in turn depends on the saturation of anthocyanins on the resin. Therefore, in all cases there is an excess of neutral phenol compounds with respect to anthocyanins. This result indicates that SDVB resins preferentially adsorb the hydrophobic species because they are sparingly soluble in water. In particular, the water solubility of hesperidin is about 20 mg/L (*26*), while that of cyanidin-3-glucoside increases upon increasing the acidity of the aqueous solution, due to shifting of the equilibrium toward the polar flavylium salt form (*25*, *27*). In fact, the rate of adsorption of cyanidin-3-glucoside in a model solution increases on increasing the pH value of the solution, thus indicating that anthocyanin is adsorbed into the apolar SDVB resin through the apolar forms (E. Maccarone et al., unpublished results). A comparison of the adsorbed amounts indicates thet 1 mL of SDVB resin is able to adsorb up to 40 mg of hesperidin (*3*, *4*), whereas it adsorbs less than 1 mg of anthocyanins. Therefore, anthocyanins compete with the other phenol compounds during the adsorption process. During elution, ethanol replaces water and weakens the hydrophobic bindings between the adsorbed species and the resin sites, thus favoring a rapid desorption of all solutes.

The product is then an ethanol solution rich in anthocyanins, hesperidin, and derivatives of hydroxycinnamic acids. After ethanol distillation or spray-dry processing, the final product can find application as a food colorant or nutritional integrator, simply for the presence of valuable antioxidant substances in high concentration. Moreover, the presence of phenol compounds, together with anthocyanins, provides a further technological advantage, because these substances are able to stabilize anthocyanins through the formation of intermolecular hydrophobic complexes, which are more resistant than the free anthocyanins toward the degradation factors (*27*−*30*).

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