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Recovery of Hesperidin from Orange Peel by Concentration of Extracts on Styrene–Divinylbenzene Resin

Alfio Di Mauro,[†] Biagio Fallico,[†] Amedeo Passerini,[‡] Paolo Rapisarda,[§] and
Emanuele Maccarone*,[†]

Istituto di Industrie Agrarie, Università di Catania, Via S. Sofia 98, 95123 Catania, Italy, Dipartimento di
Chimica Organica e Biologica, Università di Messina, Salita Sperone 31, 98166 Messina, Italy, and Istituto
Sperimentale per l'Agrumicoltura, Corso Savoia 190, 95024 Acireale, Italy

This paper describes a new procedure for obtaining hesperidin from the waste orange peel of the citrus industry. It is based on the adsorption of dilute extracts of hesperidin on a styrene–divinylbenzene (SDVB) resin and the desorption in much more reduced volumes by means of alkaline eluents. Hesperidin immediately precipitates with good yield and high purity after acidification of the concentrated solutions, thus overcoming disadvantages due to the high dilution. Different experiments were carried out to examine operating conditions in each phase of the process. Hesperidin was extracted from peel with an aqueous saturated $Ca(OH)_2$ solution, allowing precipitation of calcium pectates from colloidal pectins that can interfere in the subsequent phases of adsorption and separation of hesperidin. The clear extracts were neutralized to optimize adsorption on resin. The most effective eluent was 0.5 N NaOH solution containing 10% ethanol. Recycling of the crystallization liquor improved the yield and purity of the product and reduced the acid amount required for neutralizing fresh alkaline extracts. Resin must be washed after each adsorption–desorption cycle and regenerated after five cycles. Results can constitute a useful starting point for an industrial application. A flow scheme of the process is also reported.

Keywords: *Adsorption on resin; Citrus sinensis; hesperidin; orange peel; styrene–divinylbenzene resin*

INTRODUCTION

Flavonoids constitute a widespread class of plant phenolics often responsible for the coloring of leaves, flowers, and fruits. Their natural occurrence and biosynthesis, the methods of isolation and structural elucidation, and their applications in food and pharmaceutical industries have been extensively reviewed (Harborne, 1967, 1988; Harborne et al., 1975; Harborne and Mabry, 1982; Benavente-Garcia et al., 1997). Sweet oranges (*Citrus sinensis* L. var. Osbeck) are a rich source of flavonoids, particularly of flavanone glycosides (Rouseff et al., 1987; Bronner and Beecher, 1995), polymethoxylated flavone aglycons (Manthey and Grohmann, 1996), and anthocyanins, the latter occurring in the juice of blood orange cultivars (Chandler, 1958; Maccarone et al., 1983, 1985, 1998a). The predominant flavanone glycoside in sweet oranges is hesperidin, namely, 2-*S*-hesperetin-7-rutinoside (Figure 1). It is present in the blossoms, in the small unripe fruits, and in peel of the mature fruits (Hendrickson and Kesterson, 1964). It is very abundant in the first stages of fruit development, but it decreases with increasing maturity (El Nawawi, 1995). The hesperidin content in Italian blood oranges varies from ~35% of the dry matter in immature fruits to ~4% in the mature ones (Fuster Soler, 1997). Quantitative analysis in citrus juices has
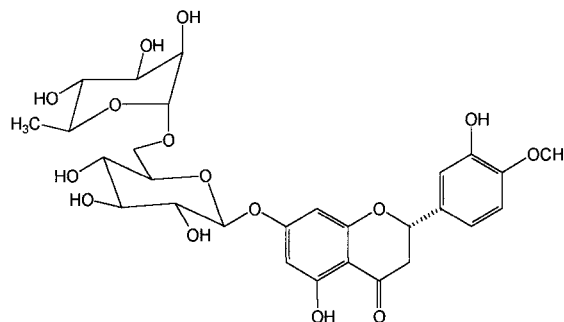

Figure 1. Structure of hesperidin.

been performed by reversed phase HPLC (Fisher, 1978; Trifirò et al., 1980; Widmer and Martin, 1992; Bronner and Beecher, 1995). Scarcely soluble in water, hesperidin has been considered as one of the factors of orange juice cloudiness (Kimball, 1991). It has been also characterized by UV, ^{1}H NMR (Mabry et al., 1970), ^{13}C NMR (Markham and Chary, 1982), and mass spectra (Mabry and Markham, 1975; Hedin and Phillips, 1992). Unlike other flavanone glycosides that are bitter in taste, hesperidin is tasteless, although its dihydrochalcone glucoside derivative is a strong sweetener (Horowitz and Gentili, 1963, 1971). Like other flavonoids it has antioxidant capacity (Foti et al., 1994; Jovanovic et al., 1996; Deng et al., 1997) and therapeutic importance to many diseased capillary conditions (Struckman and Nicolaides, 1994; Emin et al., 1994; Galati et al., 1994, 1996; Suarez et al., 1996; Kawaguchi et al., 1997). Recently, hesperidin has been successfully tested as a chemopreventive of variously induced tumors in rats

* Author to whom correspondence should be addressed (fax +39 95 714 1960).
† Istituto di Industrie Agrarie.
‡ Dipartimento di Chimica Organica e Biologica.
§ Istituto Sperimentale per l'Agrumicoltura.

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and mice (Tanaka et al., 1994, 1997a−c; Yang et al., 1997; Berkarda et al., 1998), and its metabolic pathway in humans has been also studied (Ameer et al., 1996).

The food and pharmaceutical industries performed research work and obtained patents concerning extraction and purification of hesperidin (Higby, 1944, 1946, 1947; Baier, 1948; Hendrickson and Kesterson, 1955). These methods are based on the alkaline treatment of citrus peel and on the direct precipitation of hesperidin from the acidified solutions, often followed by a crystallization to increase the purity of the commercial product. Lopez Sanchez (1986) extracted hesperidin from the peel of Satsuma mandarins using a mixture of quicklime and slaked lime to avoid gel formation and to favor filtration. Lo Curto et al. (1992) performed an acidic treatment of orange peel to hydrolyze pectins before alkaline extraction. Inaba et al. (1993a,b) degraded pectins in the fresh peel of Satsuma mandarins using a commercial *Aspergillus niger* enzyme preparation. El Nawawi (1995) increased hesperidin yield by recycling the extracting liquor. Resins have been also tested for adsorption of flavonoids and other valuable compounds from products and byproducts of the citrus industry (Doner et al., 1993; Calvarano et al., 1996). In particular, styrene−divinylbenzene resins (SDVB) have been utilized to remove naringin and limonin from citrus juices (Mattews et al., 1990) and to recover cold-pressed grapefruit oil from waste waters (Ericson et al., 1990).

In the past few years our research group studied the effects of thermal damage on Italian blood orange juice (Maccarone et al., 1996; Fallico et al., 1996) and contributed to the characterization of its constituents, such as cyanidin-3-(6″-malonyl)-β-glucoside (Maccarone et al., 1998a), *trans*-4-hydroxycinnamic acids (Rapisarda et al., 1998; Arena et al., 1998a), aroma components (Maccarone et al., 1998b), and fatty acids (Arena et al., 1998b). Because the citrus industry produces every year in Italy several hundred thousand tons of peel and more residues that contain flavonoids and other valuable products, we have recently undertaken studies about such waste materials. The aim of this work is to describe a new methodology to recover hesperidin from orange peel. The extraction was basically the same as in the past, that is, caustic extraction from peel and acidification; what is new is work to optimize the process of adsorption of dilute alkaline extracts on SDVB resin and the subsequent desorption in much more concentrated solutions to favor a rapid crystallization in high yield and purity for potential commercial scale-up.

MATERIALS AND METHODS

Samples of peel of Italian blood oranges were drawn from FMC extractors in line (Ruby International, Catania, Italy) during the citrus season from January to April 1998. Standard hesperidin was purchased from Sigma-Aldrich (Milan, Italy). The solvents used were of HPLC purity grade. Adsorbing material was a Kastell S 112 resin (Dow Italia, Milan, Italy), a nonpolar SDVB copolymer having a high degree of cross-linkage and the following physical properties: specific surface area, 450−600 m²/g, dry; mean pore diameter, 40−60 Å; density, 1.10 g/cm³; porosity, 40−70%. Before use, resin was treated with 35% ethanol/water to remove soluble compounds and then washed with distilled water (pH 6).

The dry matter content of wet peel was previously determined on different samples according to standard methods, and an average was 17.7 ± 2.0 g for 100 g of dry matter. A typical procedure of extraction, concentration by resin, and isolation of hesperidin was as follows. Wet peel (500 g) was
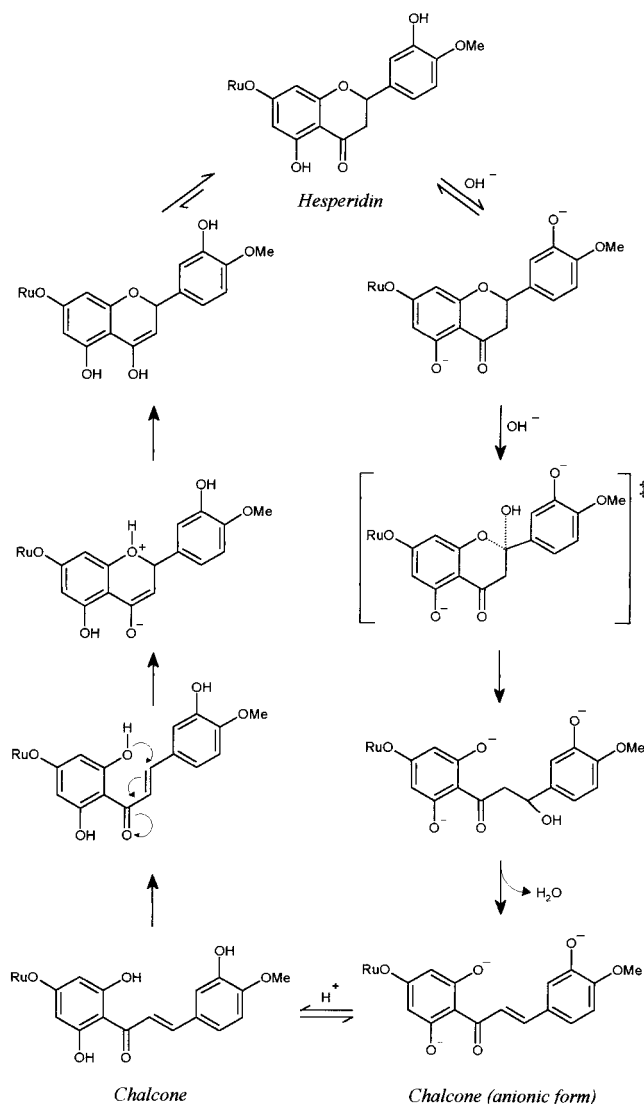

Figure 2. Structural transformations of hesperidin in aqueous solution.

triturated in an electric mixer, and particles <3 mm size (7 mesh) were used for the extraction of hesperidin. It was treated with aqueous saturated solution of Ca(OH)₂ (∼10 g/L) until pH 12 was reached and then heated at 60 °C for 1 h under stirring. The solid residue was filtered and extracted once more according to the same procedure. The clear solutions were mixed and made up to a known volume to determine the concentration of hesperidin. This solution was neutralized by HCl until pH 6 and loaded on a glass column (length, 30 cm; i.d., 2 cm) filled with 50 mL of resin and equipped with a peristaltic pump. Outflow (15 mL/min) was continuously monitored with photometer at a fixed wavelength (254 nm) to check saturation of resin. Hesperidin was then desorbed from the column using NaOH solutions at different concentration, both in the absence or in the presence of ethanol. Different fractions were collected. The eluted fractions were analyzed by HPLC to measure the concentration of hesperidin, and those having the highest concentration were acidified to pH 3.5 and allowed to separate hesperidin. Flavanone was filtered, dried, and analyzed by HPLC to determine purity. After the adsorption and desorption steps, resin was washed with 2−3 volumes of hot water and with 2−3 volumes of distilled water (pH 6) to regenerate it for another adsorption−desorption cycle. After five cycles, the resin must be regenerated with 5 volumes of a mixture of 2.0 N NaOH and 95% ethanol (50:50, v/v) and then with 5 volumes of distilled water.

The samples for HPLC analyses were prepared by neutralizing a known volume of alkaline extracts or eluted fractions

Table 1. Effect of Ca(OH)₂ on the Extraction of Hesperidin

	expt				
	1	2	3	4	5
peel, g	50	50	50	50	200
Ca(OH)₂, g	2.5	5	7.5	10	50
water, g	250	250	250	250	1000
peel/Ca(OH)₂	20	10	6.7	5	4
water/Ca(OH)₂	100	50	33.3	25	20
hesperidin,[a] mg	122	115	136	167	671
hesperidin, % dried peel	1.35	1.28	1.51	1.81	1.86

[a] Each extract was analyzed in triplicate. Standard error of estimate did not exceed ±6%.

with 2 N HCl and diluting them to a final known volume with an acetic acid/acetate buffer solution (pH 4). Purity was determined by dissolving a weighed quantity of hesperidin in 0.3 mL of *N*,*N*-dimethylformamide and dilution with the HPLC mobile phase as described later. Analyses were performed using a Varian 5000 liquid chromatograph equipped with a UV−vis detector LC 100 and a 25 cm Lichrospher 100 RP-18 (5 μm) column (Merck). The mobile phase was isocratic H₂O/CH₃CN/CH₃CO₂H (78:20:2, v/v/v) at a rate flow of 0.7 mL/min. Samples of 10 μL were injected and monitored at 287 nm. Each analysis was carried out in triplicate within 1 h from the sample preparation. Standard errors of the replicated HPLC measurements did not exceed ±6%, and the quantitation of hesperidin was performed according to the external standard method.

RESULTS AND DISCUSSION

Extraction of hesperidin from orange peel with aqueous alkaline solutions is due to the formation of water soluble polyphenolate anion. In such medium the open-

ing of the 2,3-dihydropyranone ring also occurs, affording the corresponding chalcone (Horowitz and Gentili, 1963, 1971). After separation of exhausted peel, acidification of the clear solution yields undissociated phenolic groups and ring closure, thus allowing separation of hesperidin. The chemistry of the process is illustrated in Figure 2. Several experiments were carried out to investigate operating conditions in each of the following phases: (i) treatment of peel with a Ca(OH)₂ solution until pH 12 is reached; (ii) separation of solid waste from solution; (iii) mild acidification of solution to pH 6; (iv) adsorption on SDVB resin; (v) desorption of hesperidin with NaOH solutions without or with ethanol; (vi) acidification of eluted fraction at pH 3.5; (vii) filtration of hesperidin; (viii) washing and regeneration of resin.

Preliminary trials confirmed that 7-*O*-glycoside linkage in hesperidin is very stable in the alkaline conditions, as Horowitz and Gentili (1963) pointed out. No traces of hesperetin aglycon were observed in RP-HPLC chromatograms (retention time of standard compound occurs at ~40 min).

The relative ratio between peel and Ca(OH)₂ was investigated to determine the maximum amount of extractable hesperidin (Table 1). For this purpose aliquots of the same sample of orange peel were used at a constant water/peel ratio (w/w). The extracted hesperidin increased, from ~1.3 to ~1.8 wt % of dried peel, with increasing loading of Ca(OH)₂ from 5 to 25% of the wet peel weight. However, a great excess of Ca(OH)₂ can be avoided to reduce the economic and ecological cost of the alkaline treatment, without a remarkable lowering of yield. Table 2 reports the results of extraction in nine different samples of peel, drawn from FMC extractors at different times, using a saturated solution of Ca(OH)₂

Table 2. Extraction of Hesperidin from Different Samples of Orange Peel Using a Saturated Solution of Ca(OH)₂[a]

	sample								
	1	2	3	4	5	6	7	8	9
peel, g	100	200	200	200	200	500	500	500	500
Ca(OH)₂, L	0.5	1	1	1	1	2	2	2	2
peel/Ca(OH)₂	20	20	20	20	20	25	25	25	25
water/peel	5	5	5	5	5	4	4	4	4
hesperidin,[b] g	0.48	1.12	1.06	0.77	0.57	1.71	1.26	1.23	1.06
hesperidin, % dried peel	2.7	3.1	2.9	2.1	1.6	1.9	1.4	1.4	1.2

[a] 10 g/L. [b] Each extract was analyzed in triplicate. Standard error of estimate did not exceed ±6%.

Table 3. Adsorption, Desorption, and Isolation of Hesperidin from Orange Peel

	expt							
	1	2	3			4		5	6
adsorption									
vol, L	2.1	3.4	4.8			1.9		4.95	1.7
hesperidin, g	2.47	2.66	2.89			2.17		3.36	3.21
hesperidin,[a] g/L	1.18	0.78	0.60			1.14		0.68	1.89
desorption									
eluent (NaOH), N	1.0	1.0	1.0			2.0		2.0	2.0
fraction	1	1	1	2	(1 + 2)	1	2	(1 + 2)	1	1
fraction vol, L	0.15	0.15	0.075	0.125	0.20	0.10	0.10	0.20	0.15	0.20
hesperidin, g	1.49	1.60	1.47	0.53	2.00	0.90	0.47	1.37	2.25	2.63
hesperidin, yield %	60.3	60.2	50.9	18.3	69.2	41.5	21.7	63.1	67.0	81.9
hesperidin,[a] g/L	9.9	10.7	19.6	4.2	10.0	9.0	4.7	6.85	15.0	13.2
concentration factor	8.4	13.7	32.7	7.0	16.7	7.9	4.1	6.0	22.1	7.0
isolation									
hesperidin, g	0.93	0.83	0.75	0.16	0.91	0.70	0.36	1.06	1.23	1.61
hesperidin, yield %[b]	38	31	26	6	32	32	17	49	37	50
hesperidin, yield %[c]	62	52	51	30	46	78	77	77	55	61
hesperidin,[a] purity	89	91	97	91	96	86	98	90	95	94

[a] Each analysis was carried out in triplicate. Standard error of estimate did not exceed ±6%. [b] On the adsorbed hesperidin. [c] On the desorbed hesperidin.

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Table 4. Adsorption of 1 g of Hesperidin and Desorption with Ethanol Aqueous NaOH Solutions

	expt												
	1		2		3		4		5		6		7
adsorption													
vol, L	0.47		1.21		0.78		1.40		1.78		1.08		1.56
hesperidin,[a] g/L	2.13		0.83		1.28		0.71		0.56		0.93		0.64
desorption													
eluent													
NaOH, N	0.1		0.25		0.5		1.0		1.0		2.0		2.0
C_2H_5OH, %	10		10		10		5		10		5		10
fraction[b]	1	2	1	2	1	2	1	2	1	2	1	2	1	2
hesperidin, g	0.22	0.33	0.52	0.14	0.80	0.09	0.44	0.08	0.71	0.05	0.20	0.03	0.26	0.05
hesperidin, yield %	21.9	32.2	52.5	14.2	80.4	8.5	43.7	7.8	71.0	5.3	19.6	2.9	26.5	4.6
hesperidin,[a] g/L	4.42	6.51	10.50	2.85	16.08	1.70	8.73	1.56	14.20	1.05	3.93	0.59	5.29	0.91
concentration factor	2.1	3.1	12.7	3.4	12.6	1.3	12.3	2.2	25.4	1.9	4.2	0.6	8.3	1.4

[a] Each extract was analyzed in triplicate. Standard error of estimate did not exceed ±6%. [b] Each fraction was 50 mL in volume.

(10 g/L): it was sufficient for keeping the pH at 12 and ensuring both a fair extraction yield and an almost complete precipitation of insoluble calcium pectates from the colloidal pectins, which can interfere in the subsequent phases of adsorption and separation of hesperidin. The average of extracted hesperidin was ~2% of the dried peel, with a minimum of 1.2% and a maximum of 3.1%. The best results were obtained when 100 and 200 g of peel were extracted at a $Ca(OH)_2$ saturated water solution/peel ratio of 5.

Before loading of the adsorption column, the alkaline extracts were acidified to the same pH value as the resin (pH 6). This pH value was suitable to give rise to complete protonation of the chalcone anion and cyclization (Figure 2), avoiding massive precipitation of hesperidin on resin. Table 3 reports the hesperidin content in the eluted fractions and the yield and purity of the isolated hesperidin. The amount of adsorbed hesperidin changed from 2.17 to 3.36 g, while its concentration in the extracts changed from 0.60 to 1.89 g/L. Desorption of hesperidin was performed using 1.0 or 2.0 N NaOH solutions as eluents. Different fractions from each desorption experiment were obtained. The first fraction was always the most concentrated, and from it hesperidin was isolated by acidification. In experiments 3 and 4 two fractions (1 and 2) were used to isolate the product. A greater hesperidin concentration in the loaded extract afforded a higher yield of desorbed product independent of the eluent type; however, 2.0 N NaOH as eluent must be avoided because it causes swelling and floating of the resin. After acidification of the first fractions to pH 3.5, most of the hesperidin rapidly crystallized. The highest purity was obtained in those experiments carried out with the less concentrated extracts, apart from experiment in which 94% pure hesperidin was obtained notwithstanding the highest concentration of extract; however, in this case the crystallization mother liquors from the first fraction of experiment 5 (containing 1.02 g of hesperidin) was recycled and loaded together with a fresh extract. Therefore, the loading of crystallization liquor caused an increase in yield and purity, the latter due to the recycling of a solution just purified from byproducts. Purity of the isolated hesperidin was always >86%, with a maximum of 98% (mean of 92.7%). Figure 3 shows the HPLC chromatogram of a sample of hesperidin: purity is >96% and the peak at ~8 min is due to narirutin, namely, naringenin 7-rutinoside (2.5% of HPLC total area), assigned by comparison of retention time, HPLC in mixture, and UV−vis spectra (diode array detector) with a standard sample.
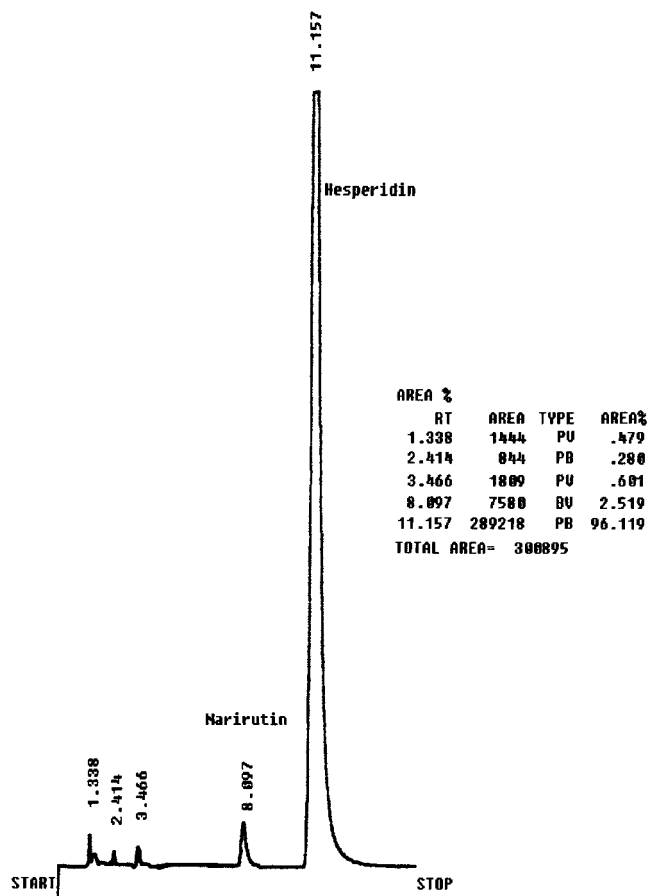

Figure 3. HPLC chromatogram of isolated hesperidin from orange peel.

Preliminary trials pointed out a more effective desorption of hesperidin from resin using ethanol-containing eluents. Additional experiments were then carried out to investigate the effectiveness of such eluents. NaOH concentration was varied from 0.1 to 2.0 N in the presence of added 2.5, 5, and 10% ethanol (v/v), whereas the amount of loaded hesperidin was 1 g in each experiment, regardless of the concentration in extracts. The 2.5% ethanol-containing eluent did not significantly improve the corresponding result obtained in the absence of ethanol. Table 4 reports the results with 5 and 10% ethanol-containing eluents. Desorption by 2.0 N NaOH solution with 5% ethanol was much less effective than desorption with the corresponding 1.0 N NaOH solution, owing to remarkable swelling and floating effects. The resin hindered contact with eluent.
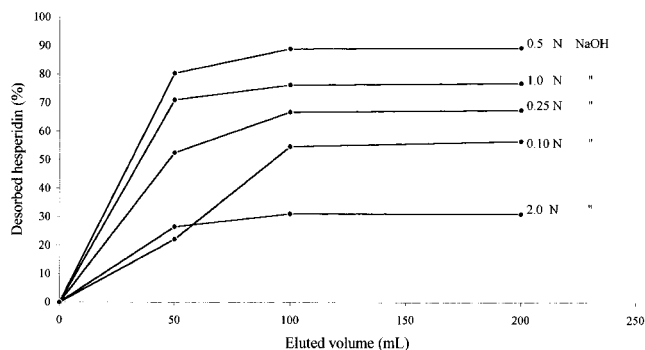
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Figure 4. Desorption of hesperidin by 10% ethanol containing aqueous NaOH eluents.
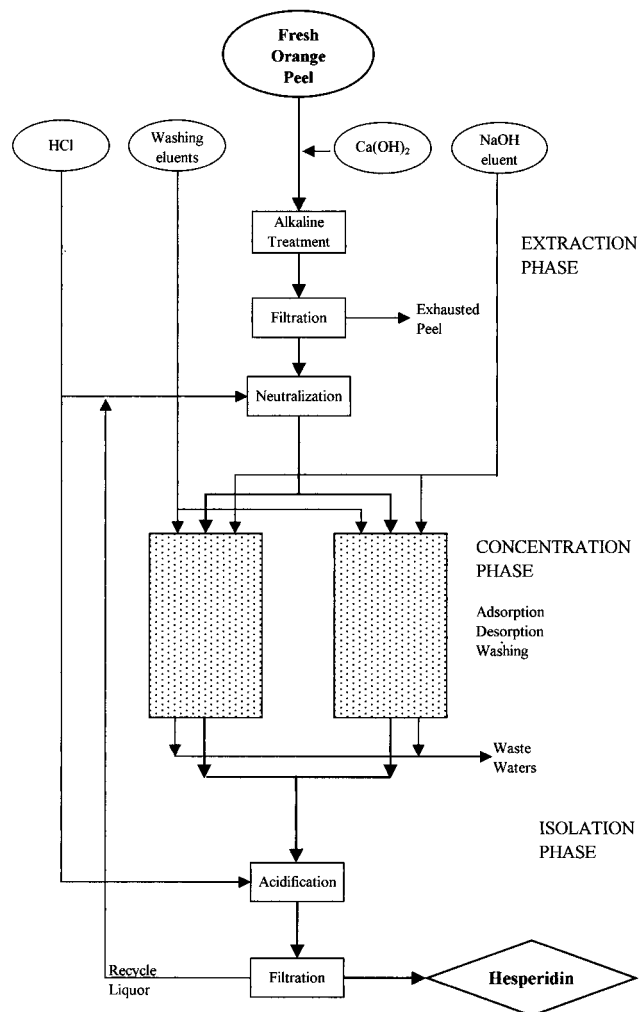

Figure 5. Flow scheme for the recovery of hesperidin from orange peel.

This trend was confirmed using 2.0 N NaOH with 10% ethanol as eluent, where the worst result was obtained notwithstanding the highest NaOH concentration. Figure 4 displays cumulative percentages of desorbed hesperidin in the experiments carried out with 4 resin bed volumes using 10% ethanol as eluent at different NaOH concentrations. About 70% yield was obtained with 0.25 and 1.0 N NaOH. The presence of 10% of alcohol in the eluent mixture performs a more effective elution of hesperidin in the first bed volume of resin, with the exception of the desorption with 0.1 N NaOH. The most effective desorption was achieved with 0.5 N NaOH. In such an experiment ~90% of the loaded

hesperidin was desorbed in the first two eluted fractions (50 mL each), and the former was much more concentrated. It gave 0.323 g of hesperidin, 40% yield, and 93% HPLC purity grade.

CONCLUSION

The procedure of adsorption and desorption on SDVB resin significantly increases the concentration of hesperidin in eluted fractions, favoring its precipitation in good yield and purity from solutions from which colloidal pectins have been removed. Recycling of the acidic liquor of crystallization improves the yield and purity of the product and reduces the amount of acid required for neutralization of the fresh alkaline extract. The results of laboratory experimentation appear to be encouraging and represent a useful starting point design for an industrial pilot plant. Figure 5 reports the flow scheme of the process including phases of extraction and isolation of hesperidin. The concentration phase involves two parallel columns that alternately operate in both the adsorption and desorption–washing steps. The working times of each step indicate that concentration phase can operate in a continuous flow.

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