Flavonoids in Pigmented Orange Juice and Second-Pressure Extracts

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Flavanone glycosides (FGs) and polymethoxylated flavones (PMFs) have been studied in pigmented orange (*Citrus sinensis*) juices and second-pressure extracts (SPEs) by high-performance liquid chromatography and diode array detector. Detection was performed simultaneously at two different wavelengths: 278 nm (for determination of FGs) and 325 nm (for determination of PMFs). Qualitative distribution patterns of FGs and PMFs in juices and SPEs were similar, although the quantitative results are quite different. An increased narirutin/hesperidin ratio after centrifugation and the presence of high amounts of PMFs in SPEs, which remain unchanged after centrifugation, were observed. Therefore, a simple and affordable procedure to distinguish an orange juice from SPEs was proposed.

Keywords: *Orange juice; second-pressure extracts; flavonoids; flavanone glycosides; polymethoxylated flavones; HPLC*

INTRODUCTION

Citrus-processing industries are commercially interested in recovering residual amounts of soluble solids after juice extraction. The resulting liquids may have chemical−physical and organoleptic characteristics similar to those of juices, depending on the employed extraction technology.

In the United States, where pulp-wash is a widespread technique, the use of water-extracted soluble orange solids (WESOS) in addition (5−7%) to the juice for frozen concentrated orange juice (FCOJ) preparation is regulated by the Food and Drug Administration (FDA). Regulations in Brazil are similar to those in the United States.

In a few countries of the Mediterranean area, and in particular in Italy where *Polycitrus* extractors are common, the peel, still holding some juice, is pressed by a screw-press or extracted by a water extraction/diffusion process (Di Giacomo et al., 1991). These second-pressure extracts (SPEs) can be used only in the production of soft drinks and beverages.

Because, at present, in the European Community any addition to the juice which "shall come from the endocarp" is forbidden (EC, 1993), an analytical differentiation between juice and extracts of the remaining parts of fruits plays an important role in determining the genuineness of commercial products. Moreover, it could assume economical significance in consideration of the next likely adaptation of European Community law with regard to the use of SPEs in juices.

Several methods, essentially based on the difference in concentration of some minor components, including flavonoids, in pulp/peel with respect to juice, have been proposed (Hammond, 1996).

Flavonoids have been extensively studied for antioxidative, anticancer, antiviral, and anti-inflammatory activities, effects on capillary fragility, and an observed inhibition of human platelet aggregation (Benavente-Garcia et al., 1997; Miyake et al., 1997; Chen et al., 1997; Bocco et al., 1998). Moreover, flavonoids have relevant interest in the industrial, ecological, and chemotaxonomic fields (Robards and Antolovich, 1997).

Flavanone glycosides (FGs) present a typical pattern in different species or parts of fruits (Ooghe and Detavernier, 1997; Robards et al., 1997; Mouly et al., 1997; Sendra et al., 1988; Pupin et al., 1998a).

A method to determine non-*Citrus sinensis* additions to orange juice has recently been developed (Ooghe and Detavenier, 1999). Furthermore, the flavanone glycoside fingerprint looks more complex when the second extraction is compared to the first extraction of the same juice (Ooghe et al., 1994a; Postorino et al., 1998).

Citrus fruits contain a number of polymethoxylated flavones (PMFs) as minor flavonoids. PMFs can be observed in all fruit parts: flavedo, albedo, membranes, juice, and seed, the peel being richer by far in PMFs than the juice (Ting et al., 1979; Gaydou et al., 1987; Sendra et al., 1988; Ooghe et al., 1994b).

Some citrus species show characteristic PMF concentration patterns. Thus, their relative concentrations can be used to detect the presence of one species in the juice of another, whereas the variability in the amount and distribution of PMFs in juice, pulp-wash, and peel can be used to detect adulteration. Visual differences in the PMF chromatograms of hand-pressed orange juice in comparison with commercially diluted juice from concentrate and pulp-wash were reported, but no quantitative data were given (Heimhuber et al., 1988).

Pupin et al. (1998b) used the technique of HPLC to analyze PMFs in Brazilian orange juice. This technique

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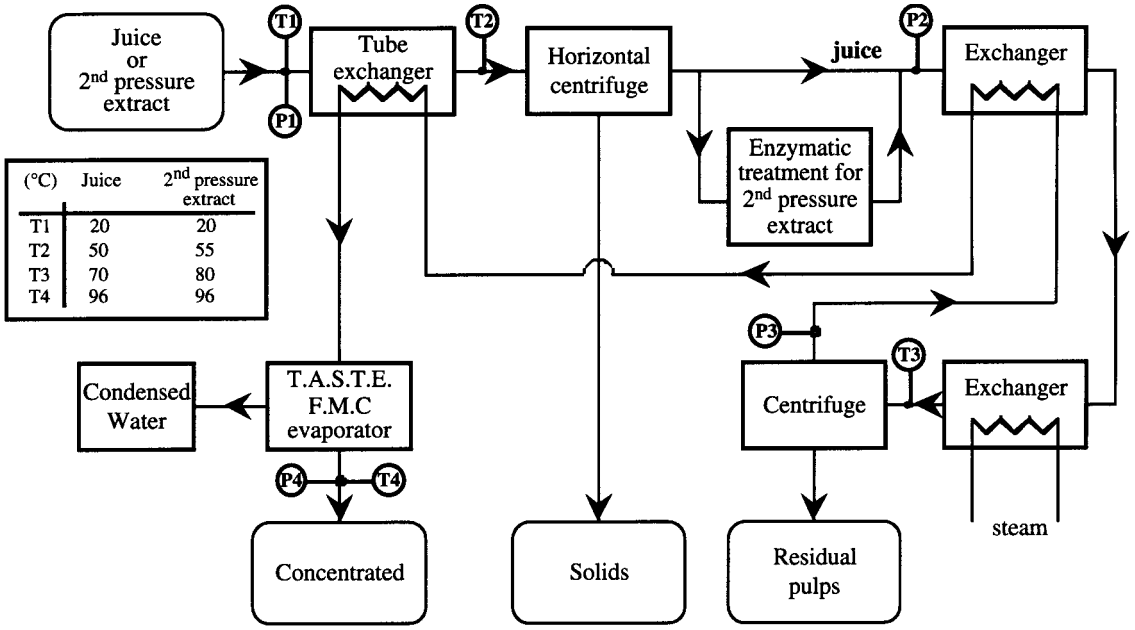

Figure 1. Production line.

is useful to distinguish the hand-squeezed juices from the commercial samples of FCOJ and frozen concentrated pulp-wash and from retail samples of FCOJ and freshly squeezed orange juice. Studies on the differentiation between juice and SPEs are very lacking.

In a previous paper (Leuzzi and Licandro, 1997), an analytical methodology based on ionic chromatography to distinguish orange juices from second-pressure extracts was described. In this paper bioflavonoid profiles in juices and SPEs were studied depending on the influences of the employed industrial processes. Simultaneous analysis by HPLC with diode array detection was applied according to the methodology proposed by Mouly et al. (1998). FGs and PMFs are both present in the orange juice, and SPEs and FGs are predominant. A different behavior of FGs and PMFs in the orange juice compared to the second-pressure extracts was observed when they were centrifuged.

Thus, a very simple and reliable procedure to distinguish an orange juice (single strength or concentrated) from a second-pressure extract was proposed. In addition, the procedure is also useful for identifying their mixtures and to determine the pigmented orange juice authenticity.

MATERIALS AND METHODS

The juices and SPEs were industrially processed (January−April 1999) from fruits (*Citrus sinensis*) deriving from various sites of Sicily (Italy). The selected cultivars Moro, Tarocco, and Sanguinello represent the major production of Italy.

Materials. Investigation has been carried out on 40 samples of industrially produced orange single-strength juices and related SPEs, 10 samples of concentrated orange juices, and 10 samples of orange second-pressure concentrates.

Industrial Juice Extraction Procedure. The juice was produced using FMC extractors and an FMC finisher. An FASO continuous press-machine processed the resulting peels and pulps to obtain the SPEs (60−90 g/L of soluble solids and 15−25% of pulp). Juices and SPEs, after centrifugation, were pasteurized, concentrated, and cooled below 0 °C.

The only SPE was treated, at fixed temperature and reaction time, with a suitable pectinolytic enzyme, Citrozym-LS Novonordisk Ferment. Figure 1 shows a flowchart of the above-described production line; sampling points (P) and temperature checks (T) are indicated.

Reagents and Standard Solutions. HPLC grade acetonitrile and acetic acid were supplied by Sigma-Aldrich, dimethylformamide (DMF) was supplied by Carlo Erba, and hesperidin, sinensetin, narirutin, and didymin standards were provided by Extrasynthèse (Genay, France). The standards were diluted in DMF, and calibration lines were obtained using solutions of known concentration (10−40 mg/L for narirutin and didymin; 1−40 mg/L for sinensetin; 40−200 mg/L for hesperidin).

Preparation of Samples. The concentrates were diluted with Millipore water (HPLC grade), to 11.2° Brix, whereas the single-strength juice was used as such. The samples (1.0 mL) were diluted in DMF (10 mL). The solutions were centrifuged (1000*g*) for 5 min and filtered through an ISO-DISC P-34 3 mm diameter PTFE membrane, 0.45 μm pore size. The samples were reanalyzed, using the same procedure, after centrifugation at 1000*g* for 5 min.

Analytical Determination. HPLC with gradient elution has been used. The analyses were carried out with an HPLC instrument, Shimadzu model LC 10 AD, equipped with a photodiode array. Detection by diode array was performed simultaneously at two different wavelengths: 278 nm (for determination of FGs) and 325 nm (for PMFs). The UV spectra were recorded between 250 and 350 nm. The analytical column used was a Discovery C18 Supelco 250 × 4.6 mm (i.d.) equipped with a guard column, Discovery C18 Supelco 20 × 4.0 mm. The column temperature was 30 °C, the injection loop was 20 μL, and the flow rate was 1.0 mL/min. The mobile phase consisted of eluant A (acetic acid 0.2%) and eluant B (acetonitrile). These were mixed to form the following linear gradient:

time (min)	% A	% B	time (min)	% A	% B
0	95	5	35	0	100
10	75	25	40	0	100
20	60	40	50	95	5
30	50	50	60	95	5

Each sample was determined three times, and peaks were superimposable. Peak identification was based on the elution order cited by literature and by comparison with UV spectra of available FG and PMF standards. The above-described experimental procedure allows the detection of 1.1 mg/L as lower content of sinensetin in the matrix. Recovery of sinensetin was always >99%.

Table 1. Average Values ($\bar{x}$), Standard Deviations (s), and Ranges of FGs and PMFs in the Single-Strength Juice and Second-Pressure Extract, Based on 11.2 °Brix

	single-strength juice				single-strength second pressure			
	as such		centrifuged		as such		centrifuged	
	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$xin \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)
narirutin	64.6 ± 8.2	50.0−80.0	44.2 ± 7.8	36.0−58.0	300 ± 41	248−378	176 ± 29	129−222
hesperidin	665 ± 65	555−761	114 ± 22	82−156	2739 ± 615	2053−3794	186 ± 49	134−309
didymin	35.3 ± 5.3	26.0−41.0	10.4 ± 2.3	7.0−15.0	147 ± 36	105−227	22.1 ± 4.1	17.0−29.0
narirutin/hesperidin	0.10 ± 0.01	0.08−0.11	0.39 ± 0.03	0.36−0.44	0.11 ± 0.01	0.09−0.13	0.65−1.42	0.98 ± 0.23
sinensetin	2.65 ± 0.72	1.7−4.5	2.40 ± 0.63	1.6−4.0	49.1 ± 7.3	38.5−62.1	41.2 ± 5.1	32.4−49.2
quercetogetin[a]	0.47 ± 0.08	0.4−0.6	0.42 ± 0.08	0.3−0.5	12.9 ± 1.9	9.2−15.6	10.0 ± 1.5	7.5−12.5
nobiletin[a]	2.94 ± 0.74	2.1−4.9	2.57 ± 0.57	2.0−4.1	45.8 ± 6.0	36.8−56.8	38.7 ± 5.2	30.6−46.8
isoscutellarein[a]	0.79 ± 0.08	0.7−0.9	0.73 ± 0.08	0.6−0.9	13.4 ± 3.0	8.9−19.3	9.62 ± 1.52	7.6−11.8
heptamethoxyflavone[a]	0.7 ± 0.11	0.5−0.8	0.63 ± 0.11	0.4−0.7	14.7 ± 3.4	8.0−20.2	11.9 ± 3.3	7.0−17.4
tangeretin[a]	0.41 ± 0.08	0.3−0.5	0.34 ± 0.10	0.2−0.5	4.33 ± 1.05	2.2−5.7	3.74 ± 0.93	2.0−4.9

[a] Expressed as sinensetin.

Table 2. Average Values ($\bar{x}$), Standard Deviations (s) and Ranges of FGs and PMFs in the Concentrate Juice and Second-Pressure Extract, Based on 11.2 °Brix

	juice				second pressure			
	as such		centrifuged		as such		centrifuged	
	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)	$\bar{x} \pm s$ (mg/L)	range (mg/L)
narirutin	73.0 ± 15.9	51−86	46.0 ± 12.7	28−57	205 ± 17	185−222	149 ± 23	132−181
hesperidin	513 ± 68	470−614	129 ± 40	83−167	1382 ± 168	1154−1521	278 ± 37	232−318
didymin	23.3 ± 2.8	20−26	8.75 ± 2.63	5.0−11.0	86.8 ± 18.7	61−102	28.0 ± 8.2	18.0−38.0
narirutin/hesperidin	0.15 ± 0.04	0.11−0.18	0.37 ± 0.08	0.28−0.48	0.15 ± 0.01	0.14−0.16	0.54 ± 0.11	0.45−0.68
sinensetin	3.58 ± 0.51	2.9−4.1	3.00 ± 0.42	2.6−3.5	61.7 ± 4.4	57.1−67.5	54.1 ± 4.5	49.7−60.1
quercetogetin[a]	0.28 ± 0.05	0.2−0.3	0.20 ± 0.0	0.2−0.2	13.9 ± 0.9	12.8−14.8	12.3 ± 0.4	11.7−12.7
nobiletin[a]	3.40 ± 0.22	3.1−3.6	3.03 ± 0.25	2.7−3.3	59.5 ± 4.0	54.0−63.4	50.8 ± 3.3	45.9−52.9
isocutellarein[a]	0.20 ± 0.08	0.1−0.3	0.18 ± 0.05	0.1−0.2	17.6 ± 1.8	15.4−19.7	12.7 ± 0.6	12.2−13.5
heptamethoxyflavone[a]	0.28 ± 0.10	0.2−0.4	0.23 ± 0.05	0.2−0.3	18.4 ± 1.9	16.2−20.5	15.3 ± 1.6	13.2−17.2
tangeretin[a]	0.63 ± 0.10	0.5−0.7	0.48 ± 0.10	0.4−0.6	4.95 ± 0.62	4.20−5.70	3.55 ± 0.47	2.9−4.0

[a] Expressed as sinensetin.

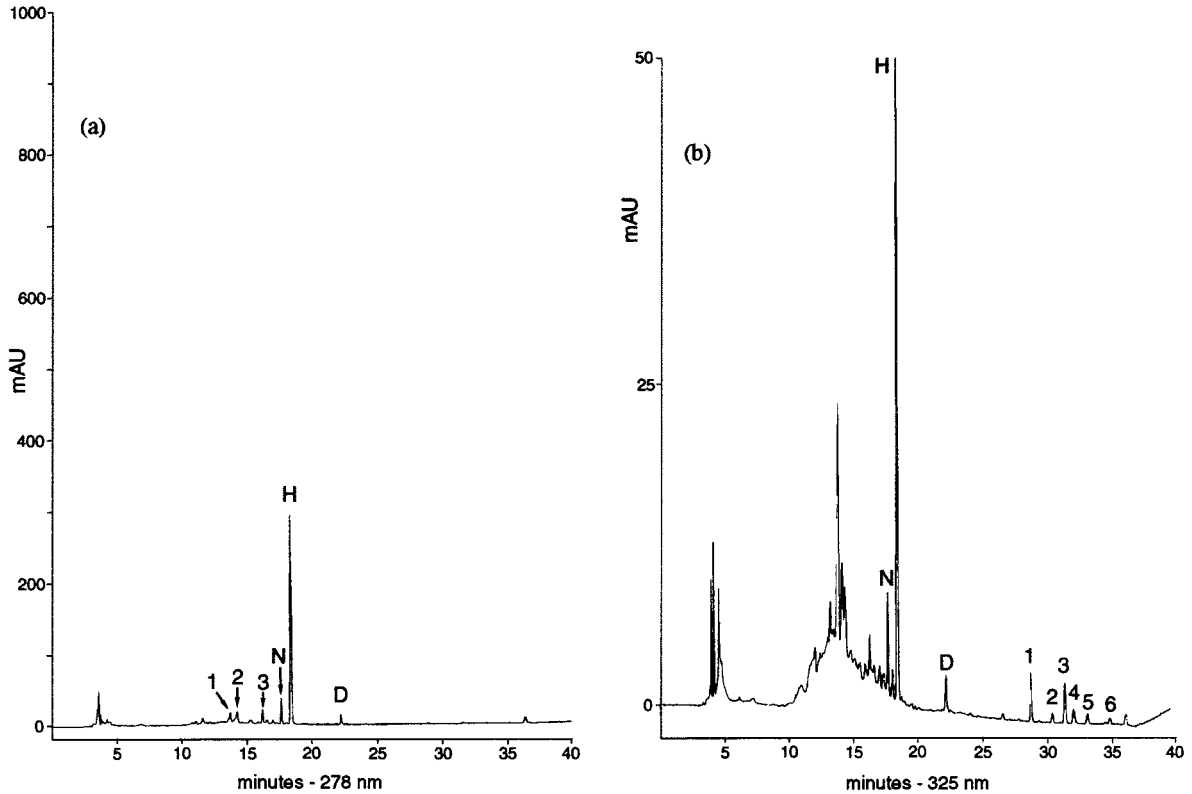

Figure 2. Typical chromatograms of a juice: (a) 278 nm [1, not identified (N.i.); 2, N.i.; 3, N.i.; N, narirutin; H, hesperidin; D, didymin]; (b) 325 nm [1, sinensetin; 2, quercetogetin; 3, nobiletin; 4, tetra-*O*-methylscutellarein; 5, heptamethoxyflavone; 6, tangeretin].

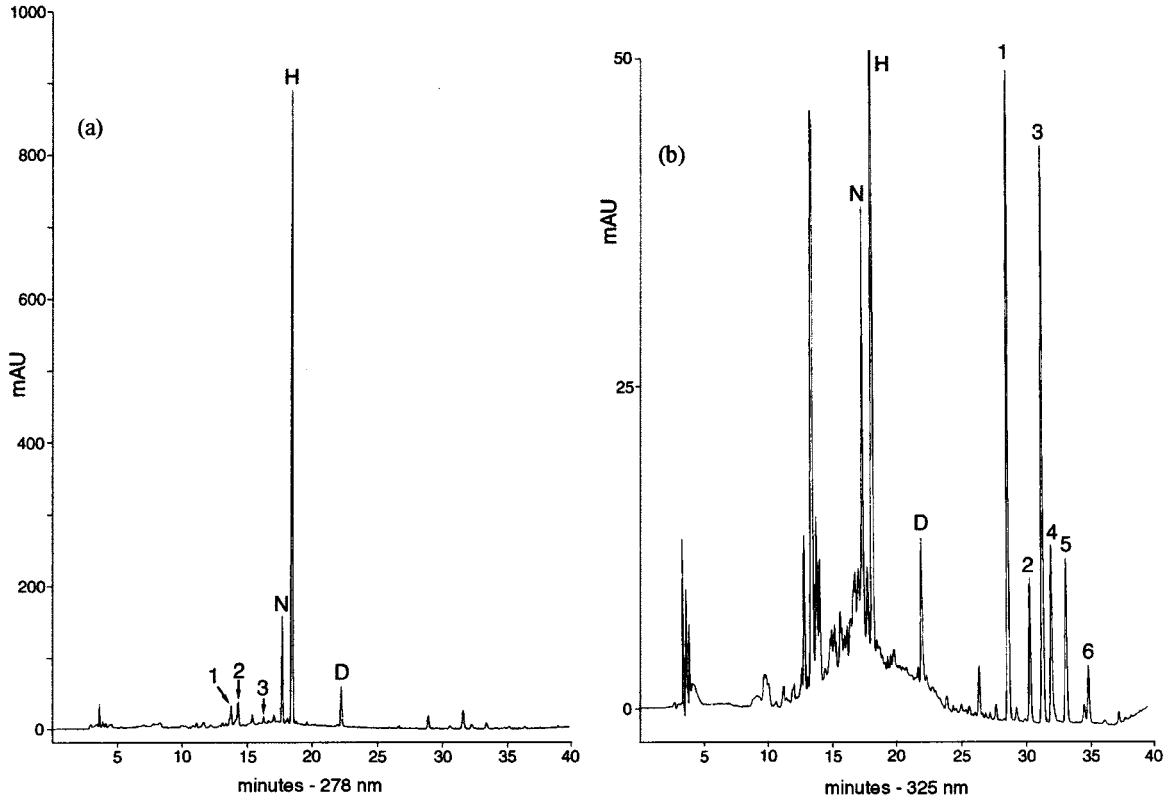

Figure 3. Typical chromatograms of a second pressure extract: (a) 278 nm [1, N.i.; 2, N.i.; 3, N.i.; N, narirutin; H, hesperidin; D, didymin]; (b) 325 nm [1, sinensetin; 2, quercetogetin; 3, nobiletin; 4, tetra-*O*-methylscutellarein; 5, heptamethoxyflavone; 6, tangeretin].

RESULTS AND DISCUSSION

FG and PMF contents, observed in pre- and postcentrifugation single-strength juices and related SPEs, are reported in Table 1. Determination was made simultaneously, and values were detected at 278 nm for FGs and at 325 nm for PMFs. Table 2 shows FG and PMF contents for concentrates. All values have been based on 11.2 °Brix. PMF value contents are expressed as sinensetin.

Flavanone Glycosides. Juices and SPEs showed similar FG distribution patterns but different contents of these components. Hesperidin was the principal component with a 555−761 mg/L concentration range for the single-strength juice and a 470−614 mg/L concentration range for the concentrate juices. In single-strength SPEs the values resulted about 4−5 times higher, whereas in the concentrated SPEs hesperidin showed a 1154−1521 mg/L range.

Similarly, narirutin and didymin showed concentration values for the single-strength and concentrate juices in the ranges of 50−86 and 20−41 mg/L, respectively, whereas for SPEs the values resulted significantly higher. In both single-strength and concentrated juices and SPEs, the narirutin/hesperidin (N/H) ratio was in the 0.1−0.2 range.

Typical chromatograms at 278 and 325 nm of a juice and an SPE are reported in Figures 2 and 3, whereas FG patterns are shown in Figure 4. Several peaks were also present at 13.773, 14.267, and 16.200 retention times (RT). Although concentration values of all components were lower in juice, unexpectedly the last peak was lower in SPE. The flavone nature of the unidentified compound, which originates at the peak at 13.773 RT, was claimed from the observed UV spectrum. Both RT and diode array spectrum suggest that the peak at

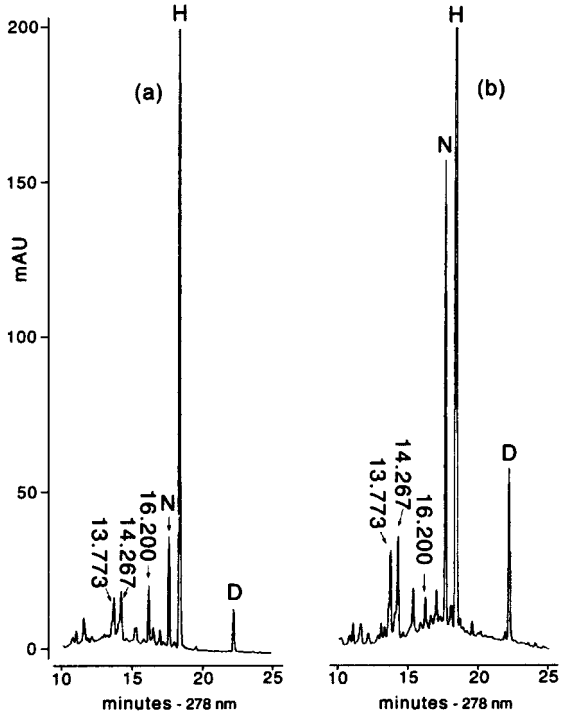

Figure 4. FG pattern of (a) juice and (b) second-pressure extract. N, narirutin; H, hesperidin; D, didymin.

14.267 RT was ascribed to narirutin 4′-glucoside according to the literature (Robards et al., 1997). HPLC-MS investigations are in progress to identify unambiguously all above components.

As previously reported for lemon juice (Grandi et al., 1994), the centrifugation plays an important role for FG contents in juices and SPEs. In centrifuged samples

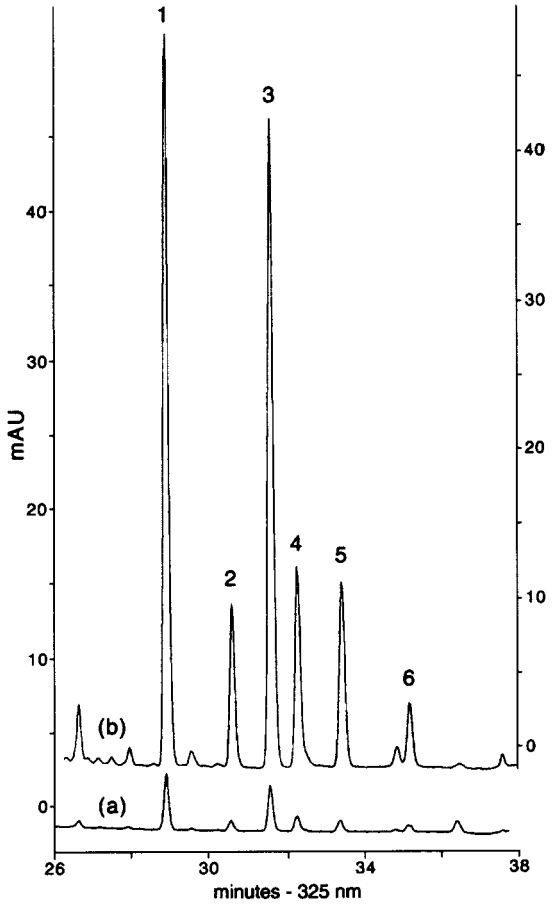

Figure 5. PMF pattern of (a) juice and (b) second-pressure extract. 1, sinensetin; 2, quercetogetin; 3, nobiletin; 4, tetra-*O*-methylscutellarein; 5, heptamethoxyflavone; 6, tangeretin.

(1000*g* for 5 min) hesperidin and didymin were reduced to ~20% of starting contents, whereas narirutin content reduction was quite limited. Thus, in single-strength juices the N/H ratio ranged from 0.36 to 0.44 and in concentrates from 0.28 to 0.48. In second-pressure single-strength extracts the N/H ratio ranged from 0.65 to 1.42 and in concentrates from 0.45 to 0.68.

The industrial production line of juices, SPEs, and their concentrates (Figure 1) was investigated by sampling in several points (P1−P4) to determine the eventual influence of the applied technology and temperature (T1−T4) in the FG distribution. In all cases an initial reduction of hesperidin content was observed during depulping in the horizontal centrifuge step, according to its low solubility. No other relevant changes were observed (Gherardi et al., 1980).

Polymethoxylated Flavones. Typical chromatograms at 278 and 325 nm of a juice and an SPE are reported in Figures 2 and 3, whereas PMF patterns are shown in Figure 5. Identification of PMF sinensetin is based on RT and UV spectrum between 250 and 350 nm. Quercetogetin, nobiletin, tetra-*O*-methylisoscutellarein, heptamethoxyflavone, and tangeretin were detected by comparison of their elution order and UV spectra with literature data (Sendra et al., 1988; Chen et al., 1997; Robards et al., 1997; Heimhuber et al., 1998; Pupin et al., 1998b). PMF amounts were found to be considerably higher in SPEs than in juices. Sinensetin and nobiletin were the major components for both juices and SPEs (Heimhuber et al., 1988; Robards and Antolovich, 1997; Mouly et al., 1998). Sampling along the industrial line (P1−P4) at different temperatures (T1−T4) showed no influence of the applied technology on PMF contents.

PMF traces were found in a hand-squeezed juice, but they were never detected in the endocarp itself when the latter was accurately separated.

Figure 6a shows the PMF chromatogram of benzene-extracted peel. Despite the few differences, the pattern is similar to that of an SPE. No PMFs could be detected in albedo extracted by either water or benzene.

An oil−water emulsion was obtained by hand squeezing thin peel strips; Figures 6b and 5c report PMF chromatograms of the two phases separated by centrifugation. The pattern of the water phase is similar

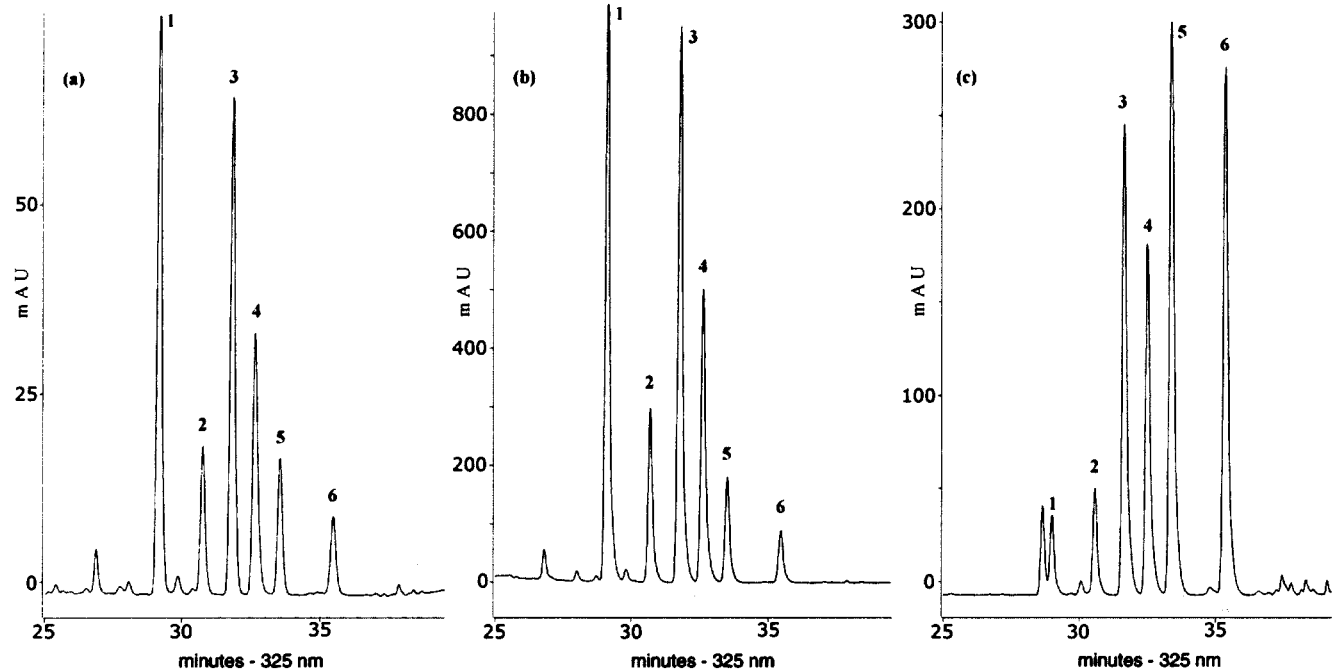

Figure 6. PMF pattern of (a) peels extract with benzene, (b) water phase of flavedo, and (c) oily phase of flavedo. 1, sinensetin; 2, quercetogetin; 3, nobiletin; 4, tetra-*O*- methylisoscutellarein; 5, heptamethoxyflavone; 6, tangeretin.

to that of an SPE, whereas the oily phase is typical of sweet orange oil (Dugo et al., 1997). In fact, total PMF amounts are lower and sinensetin, in particular, has reduced to ~10% of its water phase content. Heptamethoxyflavone and tangeretin contents, on the contrary, are considerably higher. The above results suggest that PMFs recovered in the SPE would be essentially derived from the flavedo water phase and confirm that the amounts of PMFs found in industrial juices are a consequence of the employed pressing process. Further investigations are in progress to better define a reasonable pathway of PMFs from the fruits to the juice or extract.

As reported above, due to their low solubility, FGs are very sensitive to centrifugation; this concerns especially SPEs. PMFs's behavior is quite different, and centrifugation produces a very low reduction of their contents (Tables 1 and 2). This typical behavior suggests a very simple and affordable procedure to test the genuineness of pigmented orange juice mainly based on the checking of PMF contents. In fact, whereas the FG content may be reduced by centrifugation and the N/H ratio value optimized to cover up adulteration, the amounts of PMFs remain unchanged.

Laboratory mixtures of juices and SPEs proved that >10% additions can be easily detected. The described methodology—direct injection of DMF diluted samples in the HPLC—is particularly useful for industrial product quality control given the relevance of adulterant additions.

In conclusion, the results show that FG and PMF distribution in juices and in SPEs is very closed. Therefore, SPEs present a content in hesperidin 4−5 times higher in comparison to the juice and sinensetin is ~20 times over. Thus, a high content of FGs and PMFs in a juice would suggest a probable mixture of juices and SPEs. By centrifugation SPEs reduce their FG contents to values similar to those of a juice, but the increase of the N/H ratio and, especially, unchanged amounts of PMFs, after centrifugation, indicate the adulteration.

ABBREVIATIONS USED

FGs, flavanone glycosides; PMFs, polymethoxylated flavones; N, narirutin; H, hesperidin; D, didymin; WESOS, water-extracted soluble orange juice; FCOJ, frozen concentrated orange juice; FMC, Food Machinery Co.; DMF, dimethylformamide; PTFE, poly(tetrafluoroethylene); SPE, second-pressure extract.

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