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Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography

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Abstract

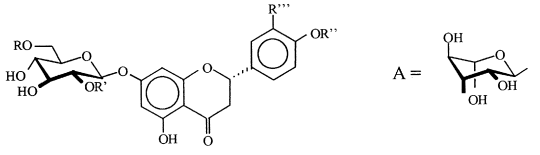
We present a simultaneous liquid chromatographic method for the separation of two flavonoid compound families, flavanone glycosides (FGs) and polymethoxylated flavones (PMFs), which are usually found in citrus fruit species and varieties. This technique permits the quantitation of six FGs (narirutin, naringin, hesperidin, neohesperidin, didymin, poncirin) and six PMFs (sinensetin, hexamethoxyflavone, nobiletin, scutellarein, heptamethoxyflavone and tangeretin). This technique, to be used to characterize a citrus juice by its polyphenolic profile, has been applied to the determination of flavonoid compounds in grapefruit- and orange juice. Differentiation of orange juice varieties and mixtures containing tangor juice using polyphenolic profiles and flavonoid content has been achieved. © 1998 Elsevier Science B.V.

Keywords: Fruit juices; Food analysis; Flavanone glycosides; Glycosides; Flavanones; Polyphenols; Flavonoids

1. Introduction

Flavanone glycosides (FGs, Table 1) are widely used for differentiation of species and varieties of *Citrus* [1–4]. Nevertheless, in some cases, for mandarins such as *C. reticulata* var. Ortanique or oranges such as *C. sinensis* var. Salustania, chromatographic differentiation based only on the content of FGs is difficult. Multidimensional statistical analyses are useful for the differentiation of these *Citrus* varieties [5,6]. The polymethoxylated flavones (PMFs, Table 2), which are located mainly in the

Table 1
Common flavanone glycosides (FGs) found in *Citrus* fruits



Name	Abbreviation	*R*	*R′*	*R″*	*R‴*
Narirutin	NAT	A	H	H	H
Naringin	NAR	H	A	H	H
Hesperidin	HES	A	H	CH_3	OH
Neohesperidin	NEH	H	A	CH_3	OH
Didymin	DID	A	H	CH_3	H
Poncirin	PON	H	A	CH_3	H

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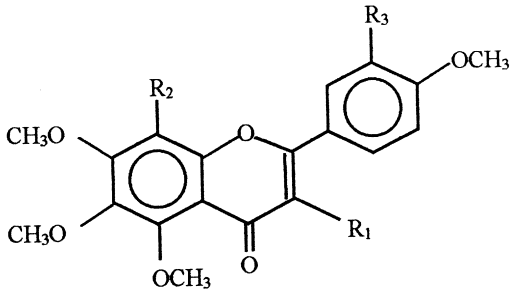
Table 2
Common polymethoxylated flavones (PMFs) found in *Citrus* fruits



Name	Abbreviation	R_1	R_2	R_3
Sinensetin	SIN	H	H	OCH_3
Hexamethoxyflavone	HEX	OCH_3	H	OCH_3
Nobiletin	NOB	H	OCH_3	OCH_3
Scutellarein	SCU	H	H	H
Heptamethoxyflavone	HEP	OCH_3	OCH_3	OCH_3
Tangeretin	TAN	H	OCH_3	H

flavedo part of the fruit, are widely used in quality control for the differentiation of orange (*C. sinensis*) and mandarin (*C. reticulata*) juices [7–9], since some essential oil is always found in citrus juice during the industrial production of juice. Taking into account, on the one hand, the differences between the concentrations of FGs and PMFs and, on the other hand, the structural differences between these two families of compounds [10,11], quantitative methods for the determination of flavonoids (FGs and PMFs) have been developed [12–14]. It is possible, with these two methods, to quantify low amounts of juice addition in some species and variety cases. Nevertheless, the disadvantage of these methods is that they are specific for a family of compounds.

For citrus juice mixtures, characterized by a similar composition of FGs and/or PMFs, it is necessary to employ multidimensional statistical techniques to differentiate these mixtures [12,15,16]. Polyphenolic profiles are now used by many laboratories for quality control, which represent a fingerprint of citrus juices and can be used to characterize varieties or added substances, such as pulpwash [17,18]. This technique can be used in liquid chromatography (LC) at 280 nm [19,20].

The purpose of this paper is to present a simultaneous global method for the separation and quantification of two flavonoid families using reversed-phase liquid chromatography with a binary gradient mobile-phase mixture. Firstly, we show the polyphenolic profile obtained on pure valencia orange juices coming from different countries. Secondly, the differentiation of various pure orange juice varieties from Spain was achieved and we were able to determine a mixture of Florida orange juice and mandarin juice (*C. reticulata* var. Ortanique) using the quantitative determination of PMFs.

2. Experimental

2.1. Materials

Solvents were of HPLC grade. Six commercial FGs [narirutin (NAT), naringin (NAR), hesperidin (HES), neohesperidin (NEH), didymin (DID) and poncirin (PON), Table 1] and three commercial PMFs [sinensetin (SIN), scutellarein (SCU) and tangeretin (TAN)] were also pure for analysis (Extrasynthese, Genay, France). The other PMFs were characterized as previously reported [8]. Ortanique citrus fruit samples (Israel) and pure Salustania orange juice from Spain were purchased at a local market. Pure Valencia orange juice (Spain) and pure Navel orange juice from Spain were given to us by the Couecou society (Biarritz, France) and a mixture of Early mid/Valencia orange juice from Florida was obtained from the Fruival society (Valence, France). For recovery and repeatability determinations, citrus juice samples (var. Pink Marsh and Ruby Red from Florida (grapefruit juices) and var. Valencia from Brazil (orange juice) were purchased at a local market.

2.2. Chromatographic conditions

Separations were performed on a stainless-steel column (250×4.6 mm I.D.) packed with C_{18} Alltima, 5 μm (Alltech, Paris, France), equipped with a precolumn (7.5×4.6 mm I.D.) that was filled with the same stationary phase. The gradient profile and the mobile phase are given in Table 3. A Waters 600 controller pump was used for analyses. Samples

Table 3
Gradient profile used in the liquid chromatographic separation of flavanone glycosides and polymethoxylated flavones contained in *Citrus* fruit juices

	Elution characteristics					
Time (min)	0	12	43	44	49	50[a]
% of A[b]	0	8	34	70	70	0
% of B[c]	100	92	66	30	30	100
Effect	Concave			Linear		Convex

[a] Equilibrating time, 10 min.
[b] Solvent A, acetonitrile.
[c] Solvent B, water–acetic acid (96:4, v/v).

were introduced onto the column via an automatic injector (Waters 717) that was equipped with a sample loop (20 μl). A waters 996 diode array detector was set at 280 nm for the quantitative determination of FGs, at 330 nm for PMFs and at between 260–350 nm for polyphenolic profiles with each sample. The chromatographic data were handled using a Millennium driven station; maxplot (Waters software) is a chromatographic channel where each data point is the absorbance maximum of the spectrum acquired at that point in time. Maxplot maximizes sensitivity for integration and quantitation. The column temperature was 35°C, the inlet pressure was 12 MPA and the flow-rate was fixed at 1.0 ml min^{-1}.

2.3. Sample preparation

2.3.1. Standards

The FG standards (NAT, NAR, NEH, DID and PON) were diluted with water–dimethylformamide (DMF) (70:30, v/v) (Labosi) to obtain a FG stock solution of 30 mg l^{-1} for NAT, 80 mg l^{-1} for NAR and 10 mg l^{-1} for NEH, DID and PON. The HES flavanone glycoside was diluted in water–DMF (30:70, v/v) to give a 200 mg l^{-1} standard solution. The PMF standards, SIN, SCU and TAN, were diluted in methanol to give a PMF stock solution of 25 mg l^{-1}. The standard solutions were prepared as follows: a 50-ml volume of the FG stock solution, 25 ml of the HES solution and 10 ml of the PMF stock solution were introduced into a 100-ml volumetric flask (see above), and the pH was adjusted with methanol. The standard solution contained 15 mg l^{-1} of NAT, 40 mg l^{-1} of NAR, 50 mg l^{-1} of HES, 5 mg l^{-1} of NEH, DID and PON and 2.5 mg l^{-1} of each PMF.

2.3.2. Citrus juice preparation

Hand-squeezed ortanique citrus fruit juice and industrial citrus fruit juices (25 ml) were diluted in DMF (20 ml) and placed in a water-bath for 10 min at 90°C. After cooling, the solutions were adjusted to 50 ml in a volumetric flask with water. All solutions were centrifuged (2500 *g*) for 10 min. The clarified solutions of sample juice were filtered through Acrodisc filters (5 and 0.45 μm) (Gelman Science, Paris, France) and then injected into the 20 μl sample loop.

2.3.3. Determination of FGs and PMFs in citrus juices

The FGs and PMFs contained in citrus juices were identified by comparing their retention times and UV spectra with those of standards. For each sample solution, concentrations were determined using response factors obtained from the single external calibration at 280 nm for FGs and using the mean response factors of SIN, SCU and TAN standards at 330 nm for PMFs. For the recovery study, four samples of citrus juices were used (two grapefruit juices and two orange juices). The flavonoids used for the recovery studies were commercially available flavonoids (NAT, NAR, HES, NEH, DID and PON for FGs, and SIN, SCU and TAN for PMFs). The standards were incorporated into the sample juices before the addition of DMF. The amounts of flavonoid standards added represented about 30% of the content of each FG and PMF in the samples.

3. Results and discussion

Fig. 1 shows the standard separation of commercially available FGs and PMFs using the chromatographic conditions given in Table 3. Better peak resolution was obtained using a concave gradient. Since the absorption maxima is near 280 nm for FGs

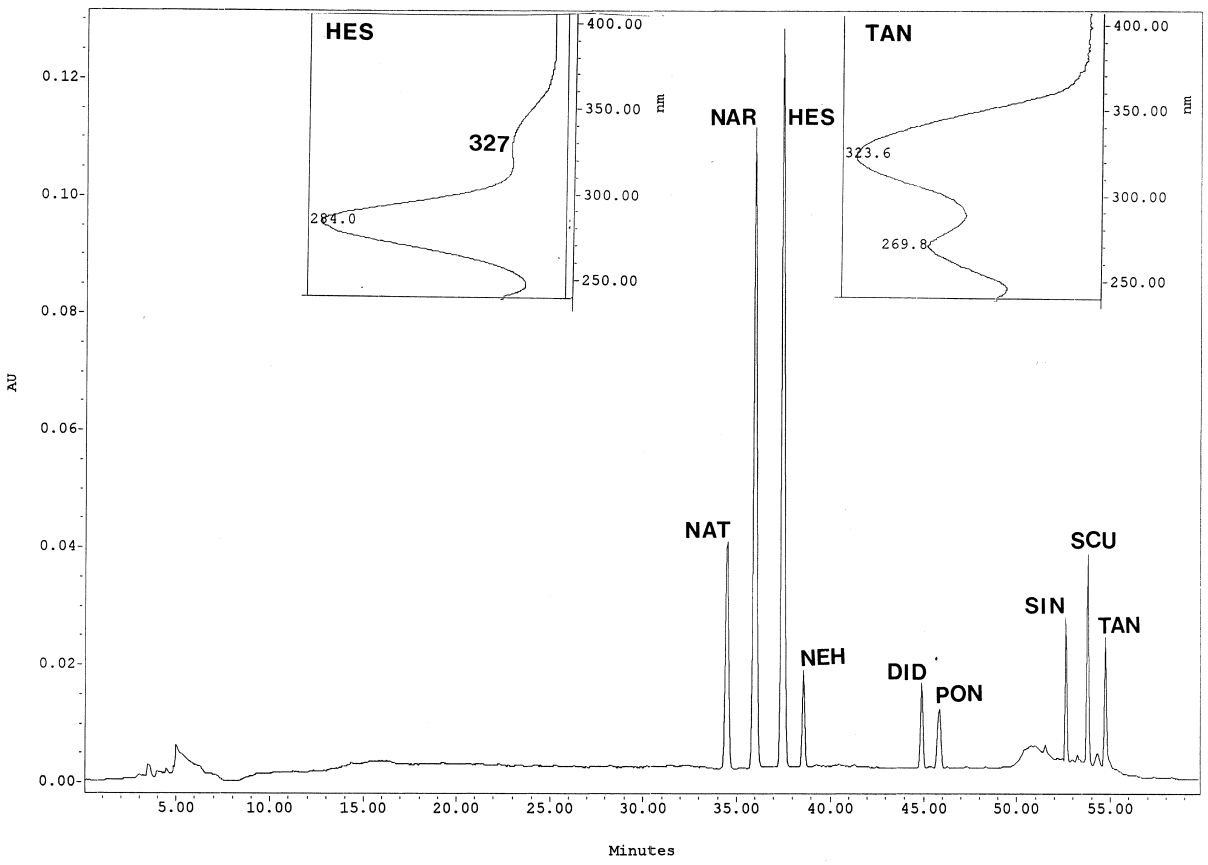


Fig. 1. Separation of flavanone glycoside and polymethoxylated flavone standards: Column, 250×4.6 mm I.D.; stationary phase, RP_{18} Alltima; amount injected, 20 μl of the solution at 15 mg l^{-1} for NAT; 40 mg l^{-1} for NAR; 50 mg l^{-1} for HES; 5 mg l^{-1} for NEH, DID and PON and 2.5 mg l^{-1} for SIN, SCU and TAN. See Tables 1 and 2 for compound identification; temperature, 35°C; flow-rate, 1 ml min^{-1}; UV detection, the chromatogram represents the absorbance maxima of each compound between 260 and 350 nm. See Table 3 for other chromatographic conditions.

and 330 nm for PMFs, we have done a maxplot between 260 to 350 nm and we obtained polyphenol profiles that absorbed at different wavelengths. For example, the UV spectra of HES (a FG) and TAN (a PMF) are given in Figs. 1 and 3. Table 4 shows the results for FGs (grapefruit and orange) and PMFs (orange) in the case of four commercial citrus juice samples. Good repeatabilities, as relative standard deviations for FGs and PMFs, were observed (2.4 and 2.8%, respectively; Table 4). The recovery for flavonoid was better in the case of compounds present in large amounts in citrus fruit juices (105%). Recoveries of between 102 and 130% were observed for compounds that were present in low amounts, PMFs in orange juices and HES, NEH and DID in grapefruit, indicating that the limit of quantitation (LOQ) of this method which is below 0.1 mg l^{-1}.

Fig. 2 shows three authentic samples of pure orange juice varieties from Spain (Valencia, Salustania and Navel). The navel variety is characterized by large amounts in NAT and DID compared to HES [6]. Although an unknown peak differentiates the Salustania variety, a qualitative operating method for polyphenolic profiles is not sufficient to allow the detection of a small amount of mandarin juice that had been added. Mixtures of Early–mid/Valencia orange juice (EM/V, 90:10 and 95:5, v/v) from Florida with mandarin juice (tangor, Ortanique variety) from Israel were prepared. These two citrus fruits mature at the same period [21]. There are two reasons for adding mixtures of tangor or tangerine

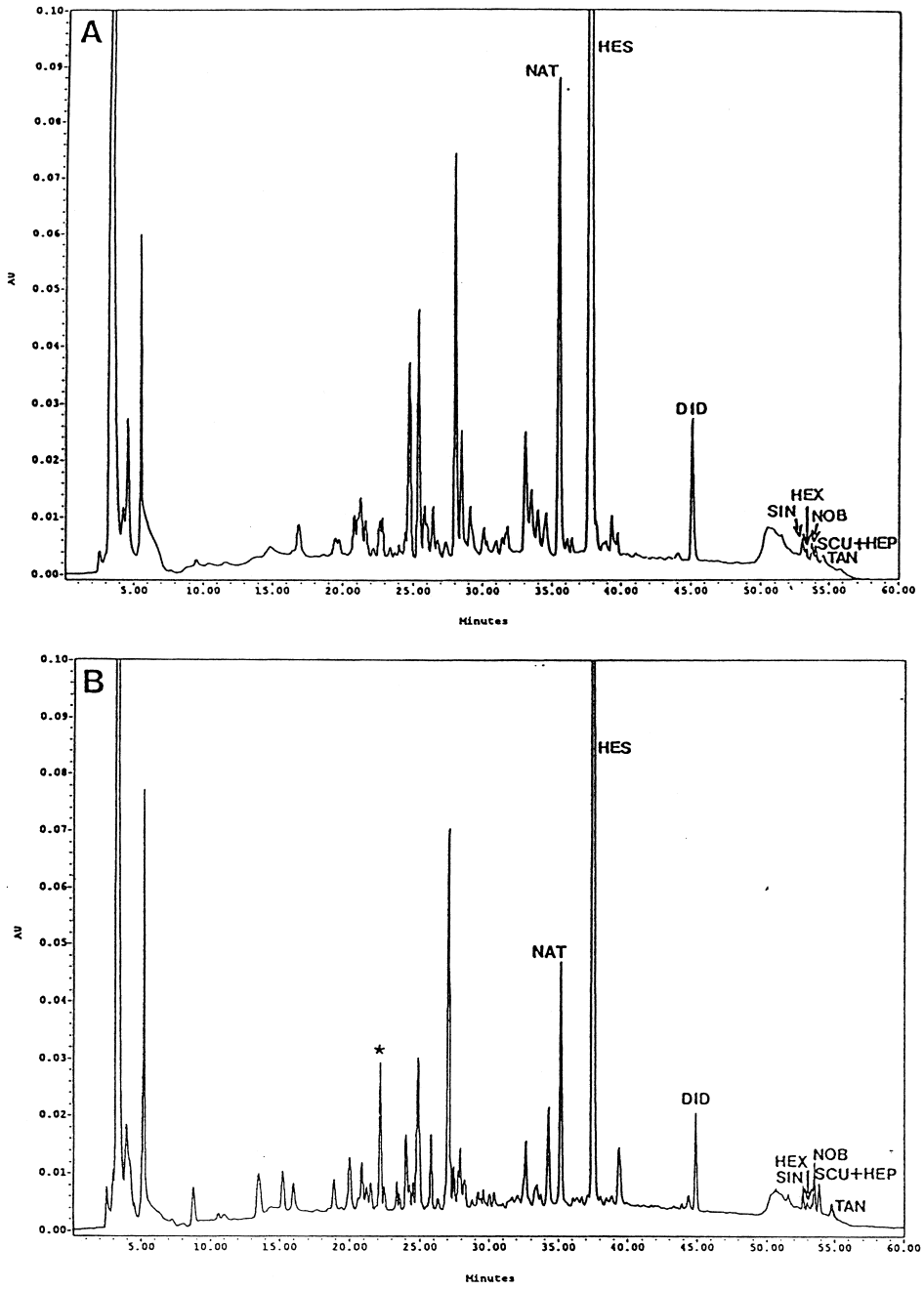


Fig. 2. Polyphenolic profiles of pure Spanish orange juices; (A) Valencia; (B) Salustania; (C, see next page) Navel. For sample preparation, see Section 2; for chromatographic conditions, see Fig. 1.

juice to Florida orange juice; (i) the low cost of the first and (ii) the colour due to carotenoids, since the Early–mid varieties that confer a typical Florida taste are colourless. The addition of tangor or tangerine juice in small proportions increased the colour. Fig. 3 shows different profiles obtained with an EM/V, Ortanique variety of juices and a mixture (95:5, v/v) of EM/V–ortanique. We can see that there is very

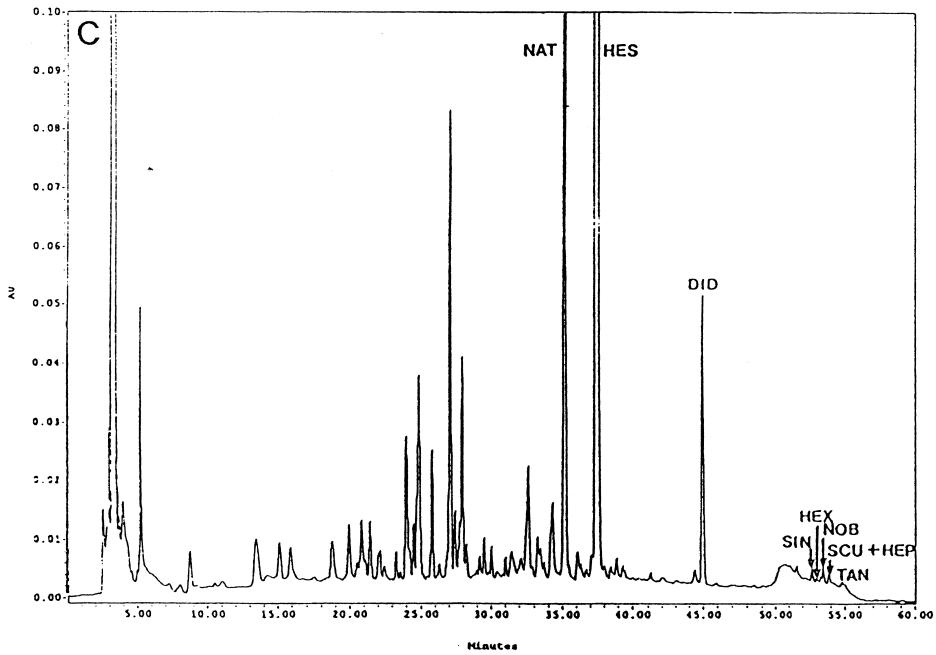


Fig. 2. (*continued*)

Table 4
Repeatability and recovery tests of *Citrus* juice polyphenolic profiles

Compound[d]	Grapefruit						Orange					
	Pure juice[a]			Concentrate[b]			Concentrate I[c]			Concentrate II[c]		
	Mean[e]	R.S.D.[f]	R^g	Mean[e]	R.S.D.[f]	R^g	Mean[e]	R.S.D.[f]	R^g	Mean[e]	R.S.D.[f]	R^g
NAT	159	0.6	102	129.6	1.4	109	52.2	2.2	106	71.2	0.3	107
NAR	428	0.8	105	420.1	1.4	102	–	–	–	–	–	–
HES	5.8	3.6	117	10.4	5.3	114	475	3.8	108	585	1.0	103
NEH	8.2	3.2	109	12.7	1.6	112	–	–	–	–	–	–
DID	8.3	1.8	114	6.2	3.2	102	17.4	3.9	102	29.2	0.7	106
PON	21.4	1.8	109	18.8	6.0	110	–	–	–	–	–	–
SIN	–	–	–	–	–	–	1.8	1.2	110	1.7	3.3	118
HEX	–	–	–	–	–	–	0.3	4.6	–	0.4	5.8	–
NOB	–	–	–	–	–	–	2.3	1.5	–	1.8	2.1	–
SCU+ HEP[f]	–	–	–	–	– – –	–	1.9	1.3	117	1.8	2.7	90
TAN	–	–	–	–	–	–	0.5	3.7	121	0.2	2.2	130

[a] *Citrus paradisi* var. Pink Marsh from Florida.
[b] *Citrus paradisi* var. Ruby Red from Florida; the juice was made from concentrate.
[c] *Citrus sinensis* var. Valencia from Brazil; the juice was made from concentrate.
[d] Determination of FGs was carried out at 280 nm and for PMFs at 330 nm.
[e] Mean of four samples, expressed in mg l^{-1}.
[f] Relative standard deviation (%).
[g] Recovery (%).
[h] Coeluted compounds.

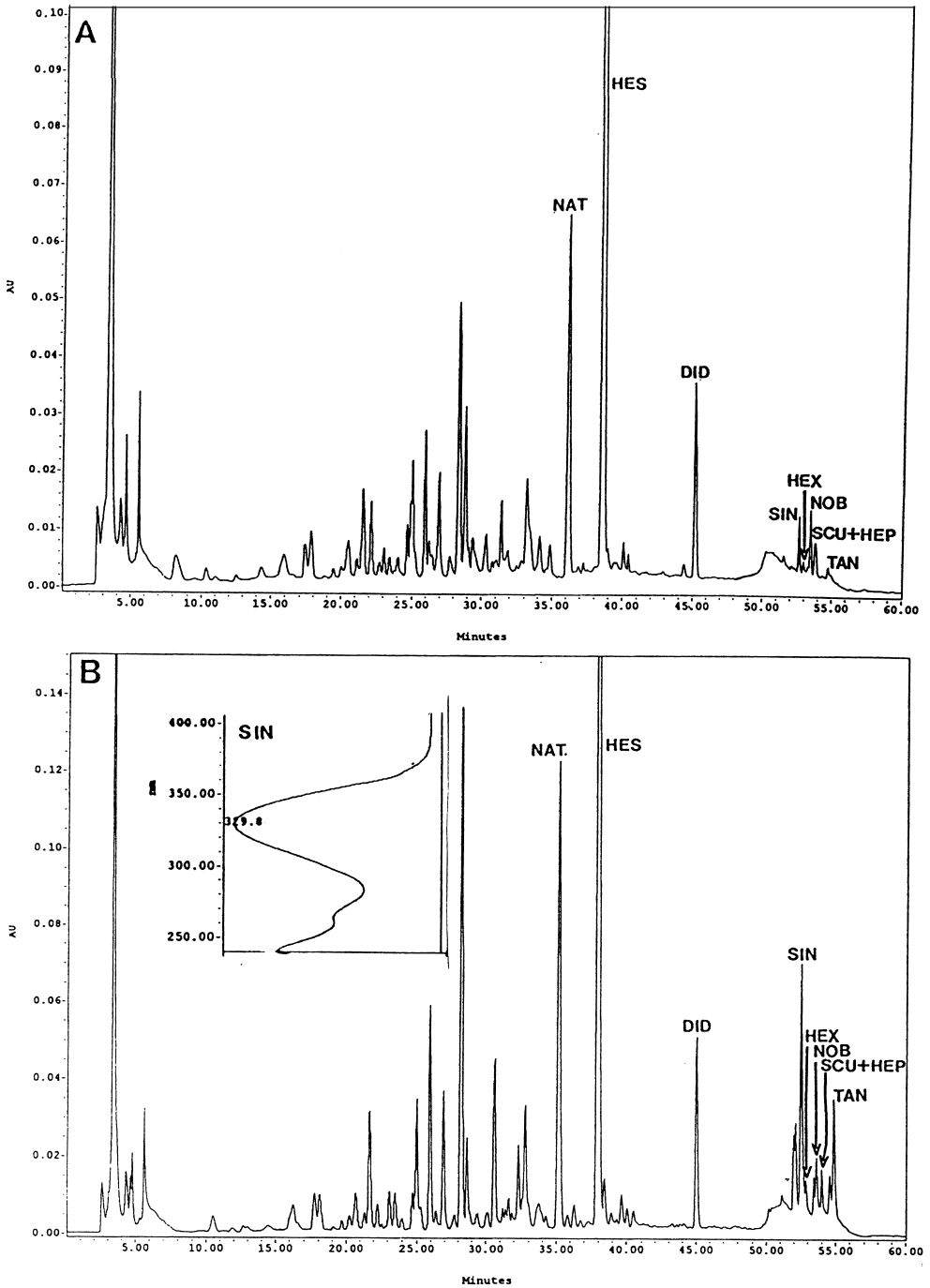


Fig. 3. Polyphenolic profiles of pure juice and mixtures; (A) Early–mid/Valencia from Florida; (B) Ortanique (tangor) from Israel; (C, see next page) a mixture of Early–mid/Valencia–Ortanique (95:5, v/v). For sample preparation, see Section 2; for chromatographic conditions, see Fig. 1.

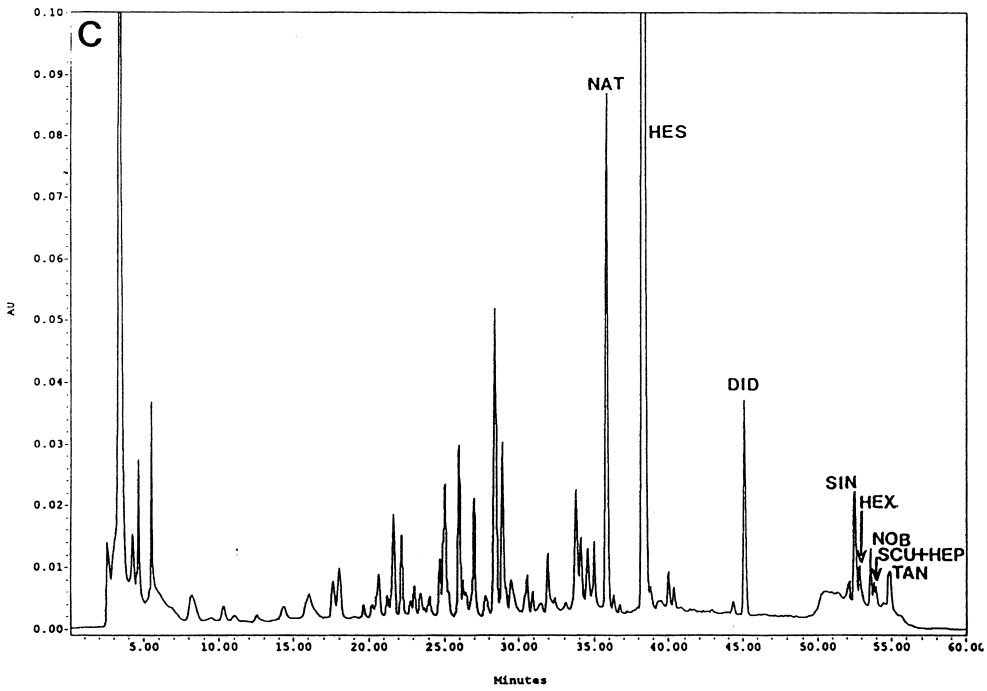


Fig. 3. (*continued*)

little difference between the polyphenolic profiles for these, except for the PMFs, which increased when tangor juice was added. The SIN spectra at 330 nm is shown in Fig. 3B.

Table 5 shows the contents of FGs and PMFs in EM/V, ortanique and juice mixtures. Compared to EM/V results, the addition of tangor juice cannot be detected using only the concentrations of FGs. The

Table 5
Flavanone glycoside and polymethoxylated flavone determinations in *Citrus* juices. Comparative results using various polymethoxylated flavone ratios

Compound (mg l^{-1})	Florida orange (Early–mid/Valencia)	Tangor Israel (Ortanique)	Mixture Orange–tangor (90:10, v/v)	Mixture Orange–tangor (95:5, v/v)
NAT[a]	45–47	89–91	49–51	47–49
HES[a]	335–351	273–287	320–336	320–336
DID[a]	21–23	30–32	22–24	22–24
SIN[b]	2.7–2.9	10.3–10.7	3.4–3.6	3.2–3.4
HEX[b]	0.48–0.52	0.58–0.62	0.48–0.52	0.48–0.52
NOB[b]	2.7-2.9	1.4–1.6	2.6–2.8	2.6–2.8
SCU+HEP[b]	2.3–2.5	1.2–1.4	2.2–2.4	2.2–2.4
TAN[b]	0.59–0.61	5.05–5.35	1.07–1.13	0.88–0.92
SIN/TAN	4.57–4.75	2.00–2.03	3.17–3.18	3.63–3.69
PMFs[c]/TAN	13.86–14.45	2.66–2.67	8.11–8.24	9.63–9.91

[a] For flavanone identification, see Table 1.
[b] For flavone identification, see Table 2.
[c] Sum of SIN, HEX, NOB, HEP and SCU.

content of PMFs clearly shows a difference between the EM/V juice and the mixture of juices. The tangor juice has lower SIN–TAN and PMF–TAN ratios than the EM/V juice (2.0 vs. 4.7 and 2.7 vs. 14.2, respectively). The orange–tangor mixture (95:5, v/v) can be clearly differentiated using these two ratios; SIN–TAN and PMF–TAN ratios decreased significantly (from 4.7 to 3.7 and from 14.2 to 9.8, respectively).

4. Conclusion

A simultaneous and rapid liquid chromatographic method can be applied for the quality control of citrus juices. With this method, the results obtained are equivalent to those found using three analyses for the characterization of commercial citrus juices (quantitative amounts of FGs, PMFs and polyphenolic juice profiles). Mixtures of orange juice with low amounts of tangor juice (5%) can be detected using PMF quantification. Moreover, this technique allows the characterization of various *C. sinensis* varieties as the polyphenolic profiles and the amounts of FGs and PMFs are known.

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