Characterization of Orange Juice (*Citrus sinensis*) by Flavanone Glycosides

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A fast and simple method has been developed to detect orange juice (*Citrus sinensis*) falsifications on the basis of the flavanone glycoside pattern. After a short sample preparation procedure, gradient HPLC is used, followed by photodiode array detection. Separation is done by means of a 15 cm Waters Novapak RP 18 column and a mobile phase gradient, partly replacing an aqueous phosphate buffer solution by acetonitrile. Peak identification is based on relative retention times as well as on UV-vis spectra. On the basis of a minimum hesperidin/narirutin ratio of 3, some doubtful technological processes, as further extraction of oranges, may be traced out, as well as the addition of *Citrus reticulata* and some orange hybrids. Also, the addition to *Citrus sinensis* of small amounts of *Citrus paradisi*, *Citrus aurantium*, and/or *Citrus bergamia* may be detected by the presence of naringin or some other flavonoids not present in *Citrus sinensis*.

Keywords: *Adulteration; orange juice; flavanone glycosides*

INTRODUCTION

Consumers consider orange juice to be a natural product containing many nutritive food components. However, this is not so evident because orange juice products may be adulterated very easily by adding water or other (cheaper) juices or some components, such as acids and sugars, in optimal proportions. In this way, fruit juice control has to be based on relevant parameters that, on the basis of their number and diversity, give an optimal guarantee for an honest product.

In the past many conventional parameters have been determined, which, however, could be adapted easily by unfair producers or manufacturers (Attaway et al., 1988; Ooghe, 1990). For this reason L-amino acids in the past were very important parameters, because they were very difficult to manipulate due to their cost and ratios (Ooghe and De Waele, 1982). The biotechnological development of recent years, however, has caused a decrease in price of these L-amino acids, so there is a need for new specific techniques to discourage potential falsifiers.

Recently, liquid chromatography techniques have been developed to determine natural plant pigments, as, for instance, flavonoids as flavanone glycosides and polymethoxylated flavones (Kirksey et al., 1988; Schnüll 1990; Wade, 1992; Rouseff and Ting, 1979; Sendra et al., 1988). These seem to be very promising as evaluation criteria for citrus juices.

The aim of this work is fourfold: proposition of an HPLC method for flavanone glycosides to be used as a routine procedure for the analysis of authentic orange juice; proposition of some analytical standards for the flavanone glycosides to guarantee the authenticity of *Citrus sinensis*; determination of a possible influence of fruit juice extraction and concentration technology on the flavonoid content; determination to what extent addition of some other citrus juices may be detected.

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FLAVANONE GLYCOSIDES

A. Flavonoids (Harborne et al., 1975). Flavonoids are yellow plant pigments that are widespread in nature. They belong to the benzopyrane derivatives and are the most important natural pigments, together with the carotenoids and the tetrapyrrole derivatives.

Flavonoids have a typical chemical structure consisting of two benzene rings enclosing a heterocyclic six-membered ring containing an oxygen atom (Figure 1).

Important also is the presence of some phenolic hydroxyl groups, which are free, methylated, or bound to sugars. The main flavonoids may be distinguished by means of differences in the heterocyclic ring and added groups. In citrus juices only three types of flavonoids occur: the flavanones, the flavones, and the flavonols (Park et al., 1983).

The flavanones predominate among the citrus flavonoids. The citrus flavonoids usually occur as mono- and diglycosides. The methoxylated flavones, however, occur as free aglycons.

Flavonoids are very promising for the determination of the authenticity of fruit juices as a result of the following reasons (Schnüll, 1990; Wade, 1992). Flavonoids are ubiquitous: they are found in nearly all plants; in fruit juices they appear in easily measurable quantities (10–1000 ppm). Flavonoids are very specific: their biosynthesis is under tight genetic control; their distribution is so characteristic for the various fruits that they may be used for taxonomic purposes. Flavonoids are multiple and diverse: a particular fruit may contain dozens of mostly not yet identified flavonoids; they look like a "fingerprint" for each fruit or juice. Even if identified, most of these compounds are not commercially available and cannot be synthesized inexpensively due to their structural complexity.

In this way it is not necessary to identify all compounds present: the total image is important as are the presence, absence, or ratios of some specific peaks.

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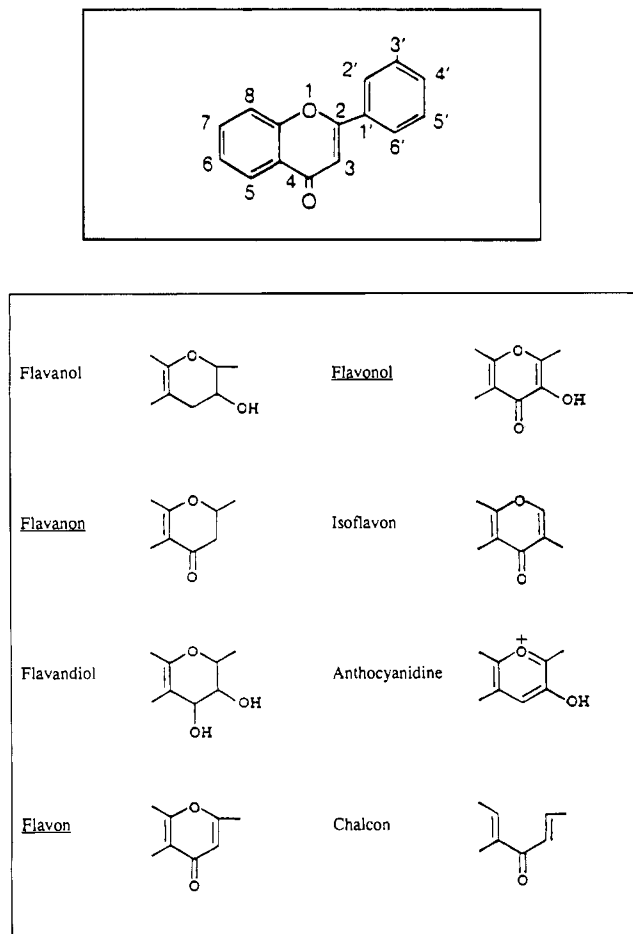


Figure 1. Typical chemical structure of the flavonoids and classification based on their heterocyclic ring.

B. Flavanone Glycosides. Kefford (1959) suggested a division of the citrus cultivars into two groups: those containing the nearly tasteless hesperidin as the most important flavanone glycoside (sweet orange, mandarin, lemon, ...) and those in which the bitter naringin is the most principal one (grapefruit, pummelo, sour orange, ...). As the structural composition became better understood, it seemed that with rhamnose 1−2 attached to glucose, the bitter flavanone neohesperidoside is formed. When, however, both sugars are 1−6 attached, the result is the flavanone rutinoside (Horowitz, 1961).

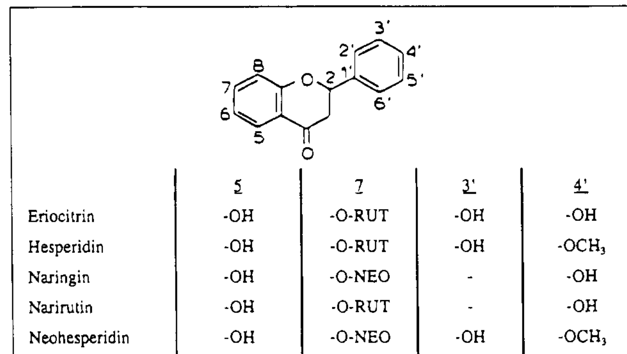
True citrus species only contain one of these structural isomers (Albach and Redman, 1969). The distribution of the number, kind, and amounts of the flavanone glycosides depends on the citrus cultivar (Rouseff et al., 1987; Rouseff, 1988). Cultivars containing, however, both isomers may be considered hybrids (tangor, tangelo, ...).

In Figure 2 the names and chemical structures of some important flavanone glycosides have been summarized: O-RUT means a rutinoside and O-NEO a neohesperidoside (Horowitz and Gentilli, 1977).

EXPERIMENTAL PROCEDURES

A. Description of the Samples. Five different groups of citrus juice products have been considered.

Group 1 comprised 100 authentic orange juice samples freshly squeezed by Solasun, Melle (B), on an industrial basis; these "SOL..." samples, characterized by their origin, variety, and/or squeezing date, have been reduced to 22 samples by



	5	7	3'	4'
Eriocitrin	-OH	-O-RUT	-OH	-OH
Hesperidin	-OH	-O-RUT	-OH	$-OCH_3$
Naringin	-OH	-O-NEO	-	-OH
Narirutin	-OH	-O-RUT	-	-OH
Neohesperidin	-OH	-O-NEO	-OH	$-OCH_3$

Figure 2. Names and chemical structures of some important flavanone glycosides (O-RUT, rutinoside; O-NEO, neohesperidoside).

preparing blended samples from orange juices from the same origin/variety and pressing period.

Group 2 consisted of 32 authentic orange juice samples obtained from the Schutzgemeinschaft der Fruchtsaftindustrie (SGF), Zornheim (D), and coded "SGF.../0" or "SGF.../1".

Group 3 was made up of 20 samples from SGF, either obtained by a different extraction technology or blended with other citrus juices ("SGF.../2 up to SGF.../7").

Group 4 comprised 17 authentic citrus juices or concentrates not belonging to the *Citrus sinensis* variety (code name ending on the name of the juice).

Group 5 consisted of 16 self-prepared "falsified" orange juices containing 10, 20, or 30% (m/m) of another citrus juice, coded "SIN + x%...".

The juices of groups 1 and 2 may be considered authentic orange juices. All juice samples were kept at −18 °C in a deep freezer until analysis.

B. Sample Preparation. The Brix value (expressed as grams of sucrose/100 g of juice) of all juices has been determined at 20 °C by means of an Abbe-Zeiss refractometer. Concentrates have been diluted either to the original juice strength, if known, or to 11.2° Brix, i.e., the value generally recognized as the minimum Brix value for industrially prepared orange juices.

Concerning sample preparation, however, we had a dilemna. Either the method of Schnüll (1990) and Wade (1992) has to be used, using water as a solvent and resulting in a fingerprint, or the method of Greiner and Wallrauch (1984), using dimethylformamide. Although the latter should result in a better solubility of some flavanone glycosides, this method has some disadvantages such as a further dilution resulting in a lower sensitivity and the lack of some fingerprint peaks at the beginning of the chromatogram (up to about 10 min).

We preferred the fingerprint method of Schüll (1990) and Wade (1992), slightly adapted by heating the sample to obtain a better and more reproducible solubility. The sample preparation of the juices and diluted concentrates for the flavanone glycosides may be summarized as follows. About 15 mL of sample is brought into a narrow glass tube of 35 mL with a stopper. To enhance the solubility of the flavanone glycosides and especially hesperidin, the tube is placed for 5 min in a boiling water bath. After cooling to room temperature, the tube content is centrifugated (Hettich Mikro Rapid/K centrifuge, type 1306) during 15 min at 4 °C and 12000 rpm. The ultracentrifugate is filtered through a 0.2 μm membrane filter (Macherey-Nagel Chromafil typ A20/25) and analyzed as soon as possible.

C. Chromatography. The chromatographic HPLC gradient system described by Schnüll (1990) and Wade (1992) has been adapted to obtain an optimal separation using a 15 cm Waters RP18 column (Novapak 4 μm; 3.9 × 150 mm). The resolution of this shorter Novapak column was about comparable to that of a 25 cm column. However, a shorter column has many advantages, such as a reduced analysis time, which results in a lower cost, and a lower column pressure, enhancing column lifetime.

Table 1. Waters HPLC Gradient Program for the Determination of Flavanone Glycosides (Flow Rate 1.2 mL/min)

time (min)	% eluent A	% eluent B	curve
0	100	0	*
3	100	0	1
38	58	42	6
40	0	100	6
43	0	100	6
46	100	0	3
58	100	0	3

The HPLC system used consists of a Waters 600 MS quaternary gradient pump and a Waters U6K manual injection system.

A photodiode array detector (UV−vis) is used to produce at the same time chromatographic (absorbance as a function of time) and spectral data (absorbance as a function of wavelength). In that way an unknown peak may be identified on the basis of its (relative) retention time and its spectrum. The purity of each peak is established as follows: (a) by means of the "spectrum index plot" (three spectra of each peak are registered, i.e., before, on, and after the top; if these spectra are comparable, the peak may be considered pure); (b) by means of "peak purity" (the PDA software compares the spectrum at the top of a peak to all spectra at both sides of the peak and indicates that part of the peak corresponding to a purity of at least 95%).

1. Preparation of the Standard Solution. Ten milligrams of eriocitrin (Extrasynthèse 1110), 10.0 mg of narirutin (Extrasynthèse 1130), 10.0 mg of naringin (Sigma 1376), 15.0 mg of hesperidin (Fluka 52040), and 10.0 mg of neohesperidin (Sigma 1887) are dissolved in dimethylformamide using a volumetric flask of 50 mL. The solution is ultrafiltrated through a membrane filter of 0.2 μm (Macherey-Nagel Chromafil type A-20/25). Using Eppendorf micropipets, this standard solution is diluted 1.33, 2.00, and 4.00 times to obtain four standard solutions for each component.

2. Working Conditions: column, Waters Novapak 4 μm (3.9 × 150 mm); column temperature, 35 °C; eluent flow rate, 1.2 mL/ min; injection volume, 20 μL; HPLC gradient, see Table 1; eluent A, 80.0 mL of 0.25 M KH_2PO_4 solution and 400 μL of 85% H_3PO_4 (Merck 573) pipetted into a volumetric flask of 2 L. [HPLC water (Janssen 26.830.58) is added up to the mark and, after mixing, the solution is ultrafiltrated through a 0.45 μm membrane filter (Gelman Supor 450) using a vacuum pump and an ultrasonic bath. The solution immediately is kept under a helium atmosphere.]; eluent B, about 200 mL of HPLC water (Janssen 26.830.58), 40.0 mL of 0.25 M KH_2PO_4 solution, 200 μL of 85% H_3PO_4 (Merck 573), and 507.65 g of HPLC acetonitrile far-UV (Janssen 25.826.54) brought together into a volumetric flask of 1 L [After mixing, HPLC water is added up to the mark and mixed again. The solution is ultrafiltrated through a membrane filter of 0.45 μm (Gelman Nylaflo) using a vacuum pump and an ultrasonic bath.]; wavelength range PDA, 210−400 nm; chromatogram wavelength, 280 nm; analysis time, 38 min; global time, 58 min.

3. Chromatography. Consecutively 20 μL of the four standard solutions and 20 μL of each of the samples are injected into the HPLC system. The spectra are registered every 2.4 s in the wavelength range from 210 to 400 nm. The chromatograms are presented at 280 nm, i.e., the wavelength giving a maximum of information.

The standard components elute in the following order: eriocitrin (25 min), narirutin (28 min), naringin (29 min), hesperidin (30 min), and neohesperidin (31 min). Unknown peaks are identified by comparing retention times and spectra. Hesperidin is used as an internal standard to calculate the relative retention times. Using the software package Maxima 820, version 3.3 (Waters Chromatography Workstation), for each of the five standard components, a calibration graph, forced through the origin, is drawn and the concentration of each of these components in the unknown sample is calculated. If this concentration is out of the calibration graph range, the unknown sample is diluted two or more times using HPLC water before it is injected again.

RESULTS AND DISCUSSION

Using the software package Maxima, a report is obtained containing the chromatogram at 280 nm. For each peak established the retention time, relative retention time (hesperidin as an I.S.), peak area, relative peak area, and concentration of the different components, identified only on the basis of the (relative) retention times, are reported (Figure 3).

In almost all orange juice chromatograms at 280 nm a little peak is established between narirutin and hesperidin, which, on the basis of only its relative retention time, was identified as naringin. However, this identification is not confirmed by comparison of its UV−vis spectrum to the spectrum of naringin in the standard solutions, as may be seen in Figure 4. This confirms that naringin is not present in an authentic orange juice (Rouseff et al., 1987; Galensa and Herrmann, 1980; Greiner and Wallrauch, 1984; Scholten, 1984; Galensa et al., 1986).

As eriocitrin and neohesperidin could not be detected in the orange juice chromatogram, only narirutin and hesperidin concentrations are considered in the authentic orange juices, as well as the ratio hesperidin/narirutin. In other citrus samples the eriocitrin, naringin, and/or neohesperidin concentrations were calculated, if present.

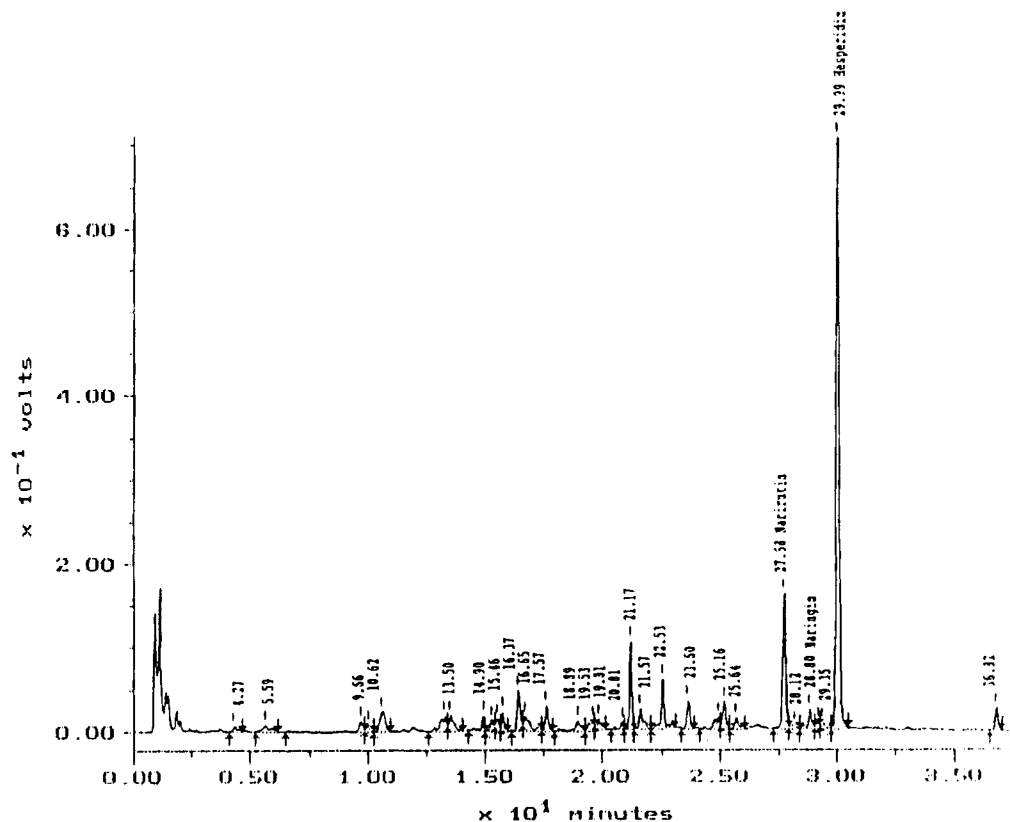
The results obtained for the authentic orange juices (groups 1 and 2) are summarized in Table 2.

The narirutin (N) and hesperidin (H) values and the hesperidin/narirutin ratios obtained for group 1 are compared to the values published by Rouseff (1988). For group 2 the narirutin value is significantly higher ($\alpha < 0.01$), resulting in a significantly lower ($\alpha < 0.01$) H/N ratio. Because both groups of authentic orange juices have been kept at −18 °C and because a paired t test of Student indicates that juice concentration does not have a significant influence ($\alpha > 0.40$) on the hesperidin and narirutin values and their ratio, the differences observed may only be explained by differences in the methods of juice extraction.

In almost all cases the calculated ratios are in accordance with the lower limit of 3, as indirectly proposed by Rouseff: i.e., the narirutin/hesperidin ratio has to be <0.399 for authentic orange juices (Schnüll, 1990; Rouseff, 1988). For the juices examined with a H/N ratio lower than 3, the explanation may be one of the following reasons: in some Italian so-called "polycitrus" juices of group 2, different cultivars, including hybrids, have been used, which results in ratios ranging from 1.65 to 4.33; juices containing some pulp do contain more narirutin, resulting in a lower H/N ratio (Schnüll, 1990; Wade, 1992; Rouseff, 1988); during authenticity tests based on the PMF pattern, three samples have been eliminated by an F test as outliers; navel orange juices seem to be characterized by a lower H/N ratio.

Otherwise, for some authentic orange juices, especially for some freshly squeezed juices, the H/N ratio is rather high, even exceeding the value of 10.

For the juices of group 3, however, rather lower H/N ratios are established, especially for juices treated by a different extraction technology. Although the presence of cells, as well as the addition of Lima and tangerine juice, mostly do not result in a H/N ratio lower than 3,



DETECTOR: 280.0 nm

PK#	Component Name	Retention Time (minutes)	Relative Time	Peak Area	Area Percent	Solution Conc
1		4.273	0.142	72194	0.50	
2		5.591	0.186	175126	1.21	
3		9.665	0.322	88171	0.61	
4		9.984	0.333	78079	0.54	
5		10.623	0.354	371436	2.57	
6		13.259	0.442	309674	2.14	
7		13.499	0.450	239696	1.66	
8		14.897	0.497	133594	0.92	
9		15.216	0.507	118383	0.82	
10		15.456	0.515	133103	0.92	
11		15.696	0.523	149357	1.03	
12		16.375	0.546	454783	3.14	
13		16.654	0.555	298099	2.06	
14		17.573	0.586	239168	1.65	
15		18.691	0.630	177103	1.22	
16		19.530	0.651	155121	1.07	
17		19.809	0.660	140193	0.97	
18		20.808	0.694	63913	0.44	
19		21.167	0.706	710444	4.91	
20		21.566	0.719	266559	1.84	
21		22.525	0.751	622916	4.30	
22		23.603	0.767	298542	2.06	
23		24.921	0.831	207054	1.43	
24		25.161	0.839	299899	2.07	
25		25.640	0.855	147834	1.02	
26	Narirutin	27.677	0.923	1537677	10.62	63.01
27		28.116	0.937	57820	0.40	
28	Naringin	28.795	0.960	224073	1.55	10.46
29		29.155	0.972	74554	0.52	
30		29.354	0.979	57749	0.40	
31	Hesperidin	29.993	1.000	6309555	43.59	260.17
32		36.823	1.228	262552	1.81	
TOTAL				14474819		

Figure 3. Flavanone glycoside chromatogram at 280 nm of *C. sinensis* and corresponding MAXIMA report.

further extractions of Italian juices seem to have a clear influence on that ratio, ranging from 0.95 to 1.67, mainly as a result of the strongly increased narirutin value. Further, as indicated also in Figure 5, the flavanone glycoside fingerprint looks more complex when the second extraction (B) is compared to the first extraction (A) of the same Italian polycitrus juice.

Finally, it may be remarked that for pulp wash or

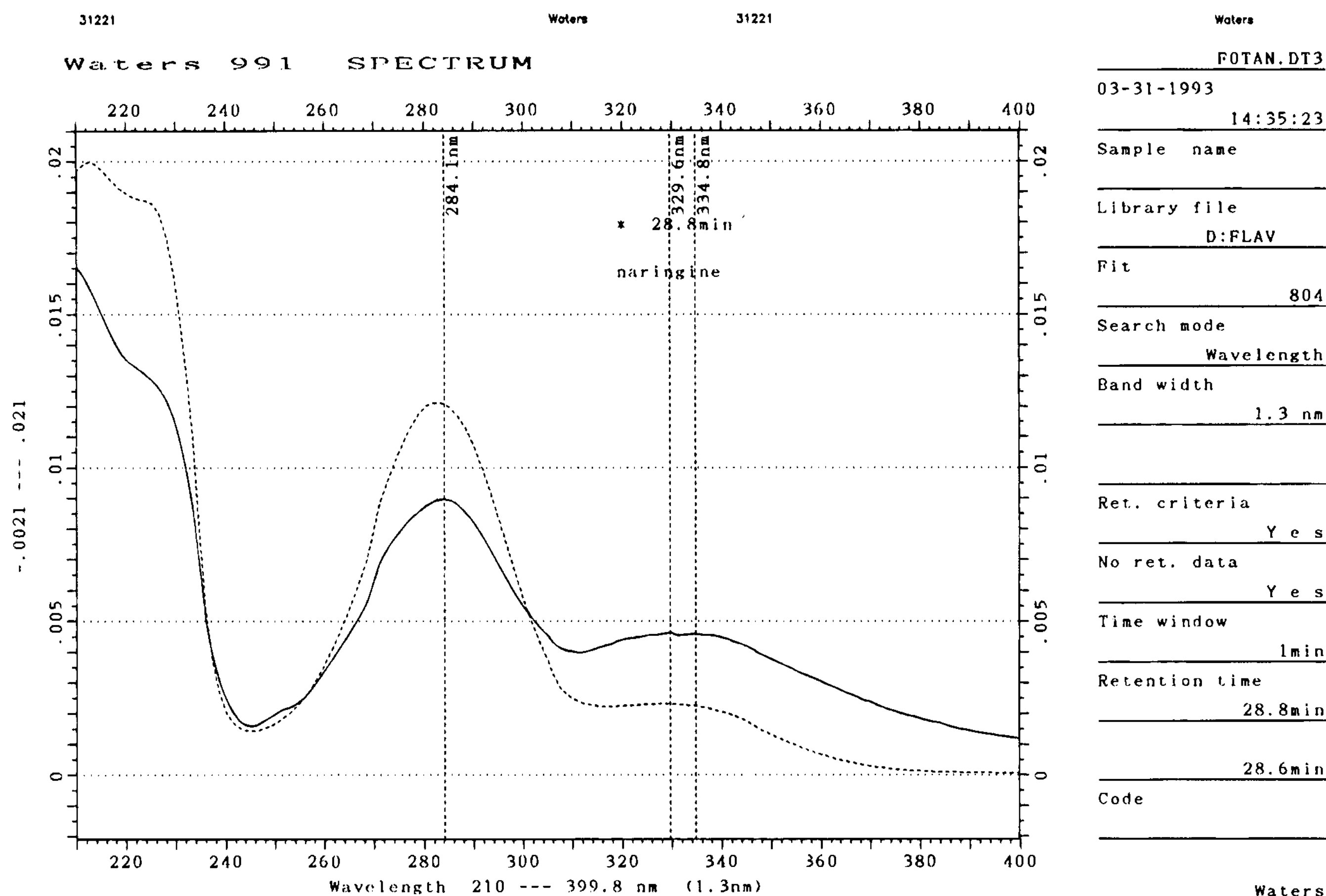


Figure 4. Comparison of the naringin spectrum (210–400 nm) of an orange juice (full line) to the spectrum of the naringin standard (dotted line).

Table 2. Average Values, Standard Deviations, and Ranges of the Flavanone Glycosides in 54 Authentic Orange Juices (Groups 1 and 2)

	group 1	*t* value	group 2	group 1 + 2	range
narirutin (N)	30.9 ± 16.4	4.745[a]	61.5 ± 27.0	49.0 ± 27.6	18.5–120.7
hesperidin (H)	222.7 ± 33.1	1.223	237.0 ± 47.2	231.2 ± 42.3	129.5–364.9
ratio H/N	8.15 ± 2.31	6.328[a]	4.52 ± 1.90	6.00 ± 2.73	1.65–12.00

[a] Significantly different ($\alpha < 0.01$).

addition of pulp wash the H/N ratio is rather low, ranging from 2.52 to 3.93.

Considering citrus juices and concentrates (group 4), Murcott juices show very low H/N ratios (range 0.33–0.48), as a result of the very high narirutin values (range 179–233 mg/L). Tangerine juice, which is the parent of the Murcott tangor, shows a H/N ratio of 2 up to 3, as a result of the rather low hesperidin value (range 73–130 mg/L).

As already mentioned for Lima juice, Baia juice also possesses a rather low H/N ratio, although both are considered *C. sinensis* juices.

In lemon juices the H/N ratio is very high (7 or more) as a result of a rather low narirutin value. A more important marker for lemon juice, however, is the presence of eriocitrin as the most important flavanone rutinoside peak.

For grapefruit juice, as well as for the less important bergamot and pommerans juices, a totally different flavanone glycoside pattern is established. In contrast to the flavanone rutinosides (hesperidin, narirutin, eriocitrin) present in orange, tangor, and lemon juices, grapefruit juice mainly contains flavanone neohesperidosides (naringin, neohesperidin). In grapefruit juice naringin is the most important flavanone glycoside, comprising at least 75% of total glycosides. While the H/N ratio is very low (≤0.10), the naringin (NG)/neohesperidin (nH) ratio in our samples is situated in the range 30–47, which is within the range (14–83) published by Rouseff (1988).

Pommerans juice has a H/N ratio comparable to that of orange juice but contains also eriocitrin, naringin, and neohesperidin. It may be differentiated from grapefruit juice not only by a lower NG/nH ratio of about 1 but also by the presence of an important peak (RRT 0.87) between eriocitrin and narirutin, which is identified as neoeriocitrin (nE). Furthermore, two important peaks, as yet unidentified, with relative retention times of 1.12 and 1.18, respectively, are present after neohesperidin (Figure 6).

Bergamot juice is characterized by very high concentrations of naringin and neohesperidin with a NG/nH ratio of about 1.4. Also present are eriocitrin, neoeriocitrin, and both peaks after neohesperidin, as mentioned above.

After the addition of 10, 20, or 30% of another type of citrus juice to orange juice (group 5), the following conclusions may be drawn from these self-prepared falsified orange juices: for increasing amounts of Murcott juice, grapefruit juice, or pulp wash, narirutin

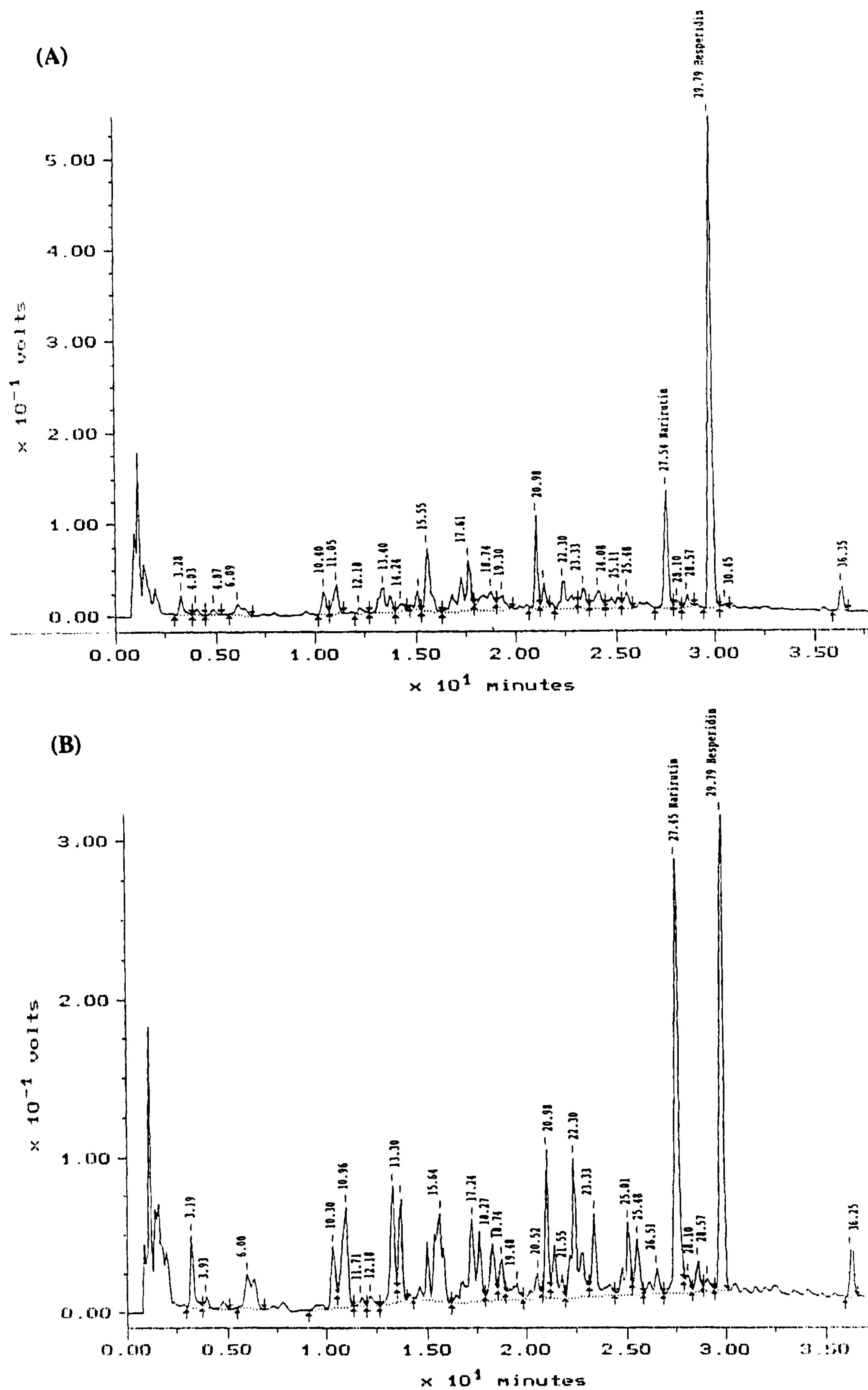


Figure 5. Comparison of the flavanone glycoside fingerprint at 280 nm after the first extraction (A) to that of the second extraction (B) of an Italian polycitrus juice.

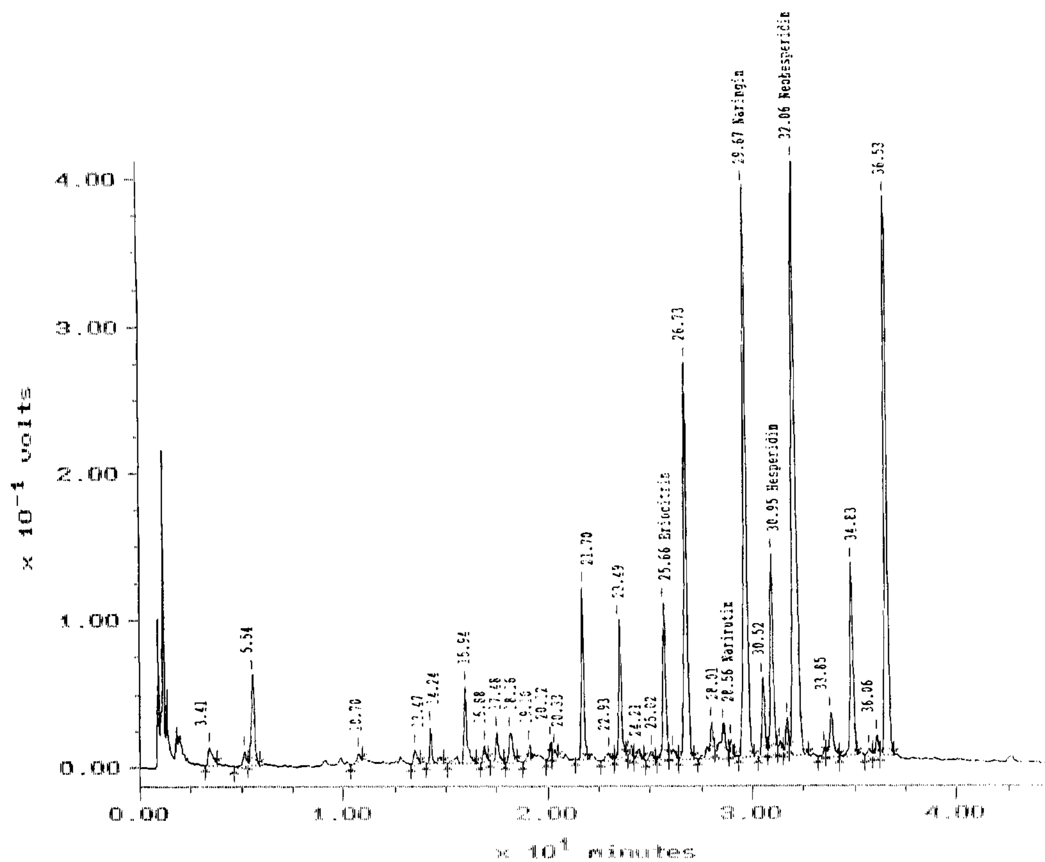


Figure 6. Flavanone glycoside chromatogram at 280 nm of a pommerans juice.

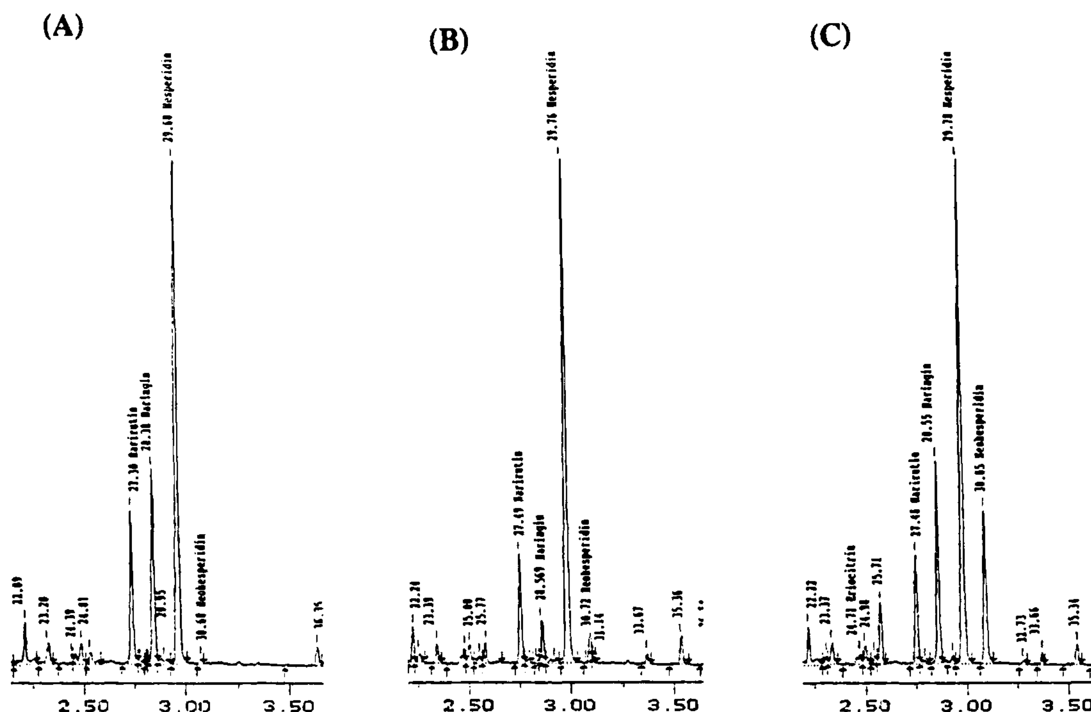


Figure 7. Comparison of some flavanone glycoside chromatograms of *C. sinensis* at 280 nm falsified with 10% grapefruit juice (A), 10% pommerans juice (B), and 10% bergamot juice (C).

increases, resulting in a lower H/N ratio; for increasing amounts of pommerans or bergamot juice, only a (slight) decrease of narirutin is established; blending with grapefruit, pommerans, or bergamot juice, however, may clearly be proved by the presence of naringin and neohesperidin as shown in Figure 7. On the basis of the naringin concentration, it even may be possible to trace out the addition of very low percentages (<2%) of these juices in orange juice.

CONCLUSION

It may be concluded that the aim of this work, as explained in the Introduction, has been attained.

First, a fast and simple routine procedure has been

developed or adapted from the literature. After a short sample preparation, gradient HPLC is used followed by photodiode array detection. An RP-18 Novapak column (3.9 × 150 mm) is used, and a gradient is made by increasing the acetonitrile concentration. The PDA detector is necessary to identify the flavanone glycosides, on the basis of their (relative) retention times and their spectra. The heat treatment described enhances the solubility of the flavanone glycosides, especially for diluted deep frozen concentrates. In some cases, however, the solubility remains limited to some extent and may be enhanced by using dimethylformamide.

Second, authenticity criteria have been proposed to evaluate *C. sinensis* juices. For the flavanone glycosides the hesperidin/narirutin ratio has to be 3 at least.

Third, the influence of the extraction and concentration technology on the flavanone glycosides and the influence of the addition of other citrus juices have been established. In contrast to the addition of cells to or concentration of an orange juice, a further extraction of oranges may be traced out on the basis of a decreased hesperidin/narirutin ratio and a more complex fingerprint.

Finally, the addition of low concentrations of *Citrus paradisi*, *Citrus aurantium*, and/or *Citrus bergamia* to *Citrus sinensis* may be detected by the presence of naringin and some other specific flavonoids not present in *Citrus sinensis*. The addition of tangerine (*Citrus reticulata*) and Murcott juice (a tangor or hybrid of orange and tangerin) may be established by a (strongly) decreased hesperidin/narirutin ratio.

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