

Short Communication

Column liquid chromatographic determination of flavanone glycosides in Citrus

Application to grapefruit and sour orange juice adulterations

Pierre Mouly*

Centre de Recherche et de Valorisation des Produits de Consommation, Parc Club des Ayalades, Bat. A7, 31 Bd. Frederic Sauvage, 13333 Marseille Cédex 14 (France)

Emile M. Gaydou

Laboratoire de Phytochimie de Marseille, Faculté des Sciences et Techniques de Saint Jerome, Avenue Escadrille Normandie Niemen, 13397 Marseille Cédex 13 (France)

Jacques Estienne

Institut de Chimie Analytique et du Contrôle de la Qualité, Université de Droit d'Economie et des Sciences d'Aix-Marseille, Avenue R. Schuman, 13380 Aix en Provence (France)

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ABSTRACT

Six flavone glycosides were separated by HPLC using RP-18 with a quaternary mobile phase mixture [water-acetonitrile-tetrahydrofuran-acetic acid (80: 16:3: 1, v/v)]. This procedure was applied to the separation and determination of the most important flavanone glycosides contained in pure grapefruit and sour orange juices and mixtures. This method can be used for quality control of industrial concentrates and juices.

INTRODUCTION

Flavonoid compounds are widespread in the plant kingdom. Whereas polymethoxylated flavones (PMFs) are generally found in large amounts in the peel of some *Citrus* [1-3],

flavanone glycosides (FGs) are specific of *Citrus* juices [3-5]. Among FGs, naringin and neohesperidin (Fig. 1) are important with regard to quality control and bitterness of grapefruit juice. The resolution and determination of these two compounds have been achieved by reversed-phase HPLC [6,7]. Two other FGs, hesperidin and narirutin (Fig. 1), have been determined in common sweet orange [6,8]. Neoeriocitrin and

* Corresponding author.

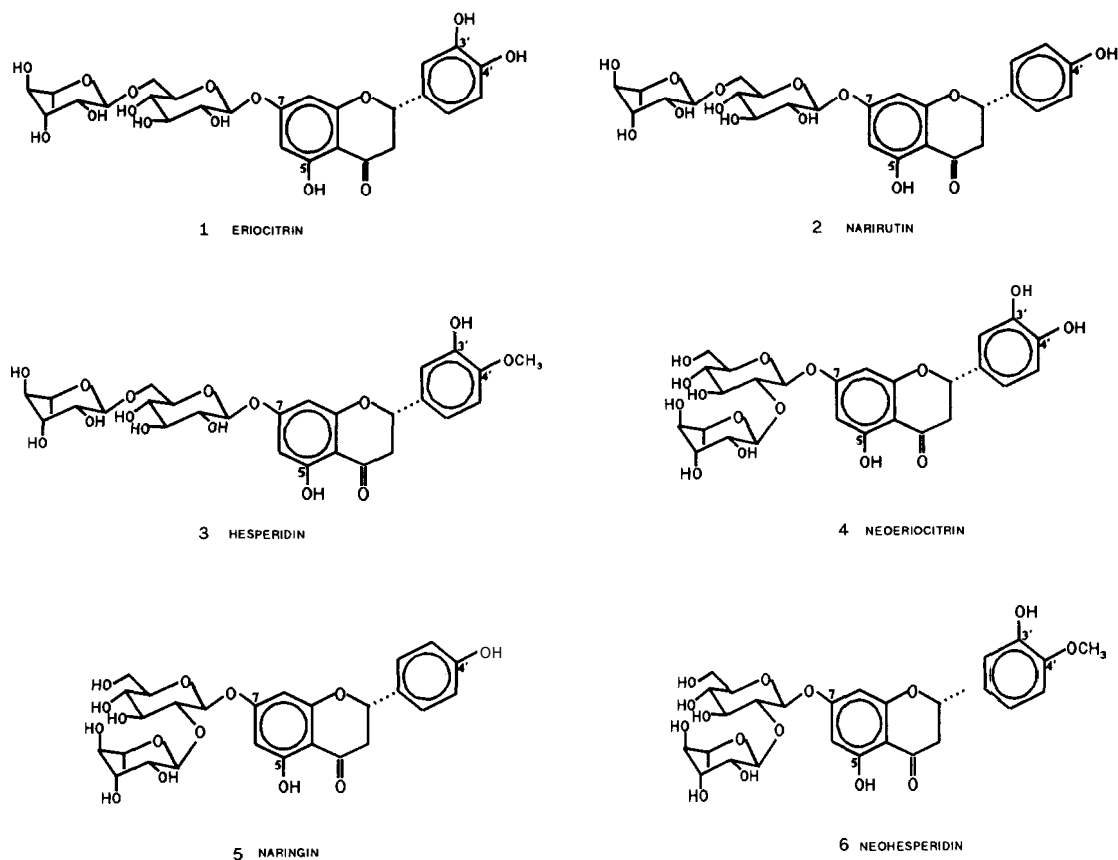


Fig. 1. Flavanone glycosides investigated.

eriocitrin are generally found in large amounts in sour orange [8] and lemon juices [4], respectively. As neeriocitrin was not completely resolved from the other unidentified peaks in HPLC, only naringin and neohesperidin contents and their ratio have been used for quality control of grapefruit juice [6].

The purpose of this paper is to report an adequate solution of the six FGs mentioned above and generally found in *Citrus* juices, using reversed-phase HPLC with a quaternary mobile phase mixture. It is shown that the detection of grapefruit juice adulteration by sour orange juice requires the determination of four FGs.

EXPERIMENTAL

Materials

Solvents were of HPLC grade. The six flavanone glycosides used as standards (Extra-

synthese, Lyon, France) were also of HPLC grade. Grapefruit samples (four of Florida origin and six of Israel origin) were purchased at a local market. Sour oranges were obtained from INRA San Giulano station, Corsica.

Chromatographic conditions

Separations were performed on a stainless-steel column (250 x 4.6 mm I.D.) packed with RP-18 UHS, 5 μm (Alltech, Paris, France), equipped with a precolumn (30 x 4.6 mm I.D.) filled with the same stationary phase. The mobile phase was water-acetonitrile-tetrahydrofuran (THF)-glacial acetic acid (80:16:3:1, v/v). A Shimadzu Model 10 A5 HPLC pump was used for analyses. Samples were introduced on to the column via a Rheodyne Model 7010 injector fitted with a 20- μl sample loop. A Shimadzu SPD 6 AV variable-wavelength UV-visible detector was set at 280 nm. The column was at

ambient temperature, the inlet pressure was 190 bar and the flow-rate was fixed at 1.5 ml min⁻¹.

Sample preparation

Hesperidin was diluted in dimethylformamide (DMF)-water (2: 1, v/v) at a 200 mg l⁻¹ concentration. All other reagents were diluted in the mobile phase. Working standard solutions were prepared freshly each week by dilution with the mobile phase. The final concentrations were 20 ppm for hesperidin and naringin and 10 ppm for the other FG.

Citrus juice and mixture preparations

Hand-squeezed *Citrus* juices (5 ml) were diluted in 10 ml of DMF and 10 ml of 0.05 M ammonium oxalate solution and then placed on a steam-bath for 10 min at 90°C. After cooling, the solutions were adjusted to 50 ml with a volumetric flask. All solutions were centrifuged at high speed (2500 g) for 10 min. The clarified sample juice solutions were filtered through Acrodisc filters (5 and 0.45 μm) (Gelman Science, Paris, France) and then injected in a 20-μl sample loop.

Determination of FG in Citrus juices

The flavanone glycosides contained in *Citrus* juices were identified by comparison of their retention times with those of standards. For each sample solution, concentrations were determined using response factors obtained with the single-point external calibration method.

RESULTS AND DISCUSSION

Optimization of flavanone glycoside separation

The formulae of the various FGs investigated are given in Fig. 1. These six compounds contain three aglycones having a flavanone skeleton (naringenin, hesperetin and eriodictyol) associated with rutosyl and neohesperidosyl moieties. Using a ternary mixture [water-acetonitrile-glacial acetic acid (79.5 : 20 : 0.5, v/v)] the separation of compounds 3 and 5 was incomplete [6]. Better results were obtained using a quaternary mixture [water-acetonitrile-THF-glacial acetic acid (80: 16: 3 : 1, v/v)], as shown in Fig. 2. The first compounds eluted are generally rutosides such as eriocitrin, narirutin and hes-

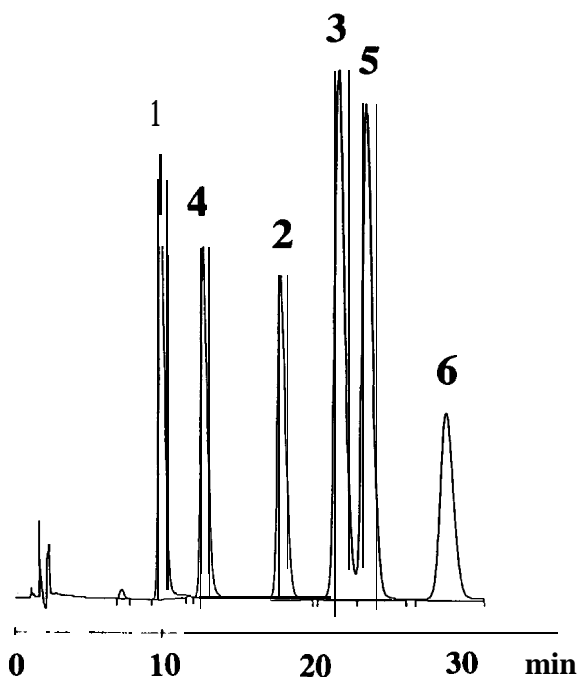


Fig. 2. Separation of standard flavanone glycosides. Column, 250 × 4.6 mm I.D.; stationary phase, RP-18 UHS; amount injected, 20 μl of a solution at 10 mg l⁻¹ for 1, 2, 4 and 6 and 20 mg l⁻¹ for 3 and 5; mobile phase, water-acetonitrile-THF-glacial acetic acid (80:16:3:1, v/v); inlet pressure, 190 bar; temperature, ambient; flow-rate, 1.5 ml min⁻¹; UV detection at 280 nm. For compound identification, see Table I and Fig. 1.

peridin, immediately followed by the corresponding neohesperidosides, neoeriocitrin, naringin and neohesperidin. For compounds 3 and 5, when THF is added to the mobile phase this elution order is reversed. In absence of THF, Rouseff [6] observed the same elution order for rutosides and neohesperidosides. Considering the substitution on the aglycone skeleton, the more hydroxylated derivative is eluted first. Retention times and capacity factors were determined for each FG. Capacity factors relative to hesperidin (3) and the corresponding response factors at 280 nm are reported in Table I.

Determination of flavanone glycosides in Citrus juices

Hand-squeezed *Citrus* juices of eleven samples of various origins (Israel, Florida and Corsica) were analysed for their FG contents. As shown

TABLE I
CAPACITY AND RESPONSE FACTORS OF FLAVANONE GLYCOSIDES

Column, 250 × 4.6 mm I.D.; stationary phase, RP-18 UHS; inlet pressure, 190 bar; temperature, ambient; mobile phase, water-acetonitrile-THF-glacial acetic acid (80:16:3:1, v/v); flow-rate, 1.5 ml min⁻¹.

Compound ^a	Name	<i>k'</i> ^b	<i>f'</i> ^c
1	Eriocitrin	0.445	1.159
2	Narirutin	0.846	1.100
3	Hesperidin	1.000	1.084
4	Neoeriocitrin	0.632	1.273
5	Naringin	1.090	1.139
6	Neohesperidin	1.330	1.173

^a See Fig. 1 for structural formulae.

^b Capacity factor relative to hesperidin.

^c Response factor × 10⁵.

in Fig. 3A, grapefruit juices were characterized by high contents of narirutin (2) and naringin (5). The content of 2 (Table II) for Israel juices (103-122 mg l⁻¹) is generally higher than that in Florida juices (59-73 mg l⁻¹). The same phenomenon was observed for compound 5 (33-675 vs. 138-176 mg l⁻¹, respectively). For Corsica sour orange samples (Fig. 3B), three compounds (4, 5 and 6) were found in higher amounts (320, 427 and 266 mg l⁻¹, respectively). In each sample eriocitrin (1) was found in smaller amounts.

As *Citrus* juices may be adulterated with one another, we prepared various juice mixtures and examined them for quality control using FG determination. For a sour orange juice blended with 10% of grapefruit juice (Fig. 4), the adulteration can be detected by the increasing amount of narirutin (2) (15-22 mg l⁻¹), as shown in Table II. For grapefruit juices blended with only 5% of sour orange (Fig. 5), whereas the neohesperidin content is slightly higher (18-36 mg l⁻¹ compared with 4-24 mg l⁻¹), the neoeriocitrin content may be used for adulteration determination (Table II).

The naringin-to-narirutin ratio has been proposed for quality control of grapefruit juices [6]. As shown in Table III, our ratio values are within the range of those already proposed by Rouseff [6] for sour oranges (1.6 compared with

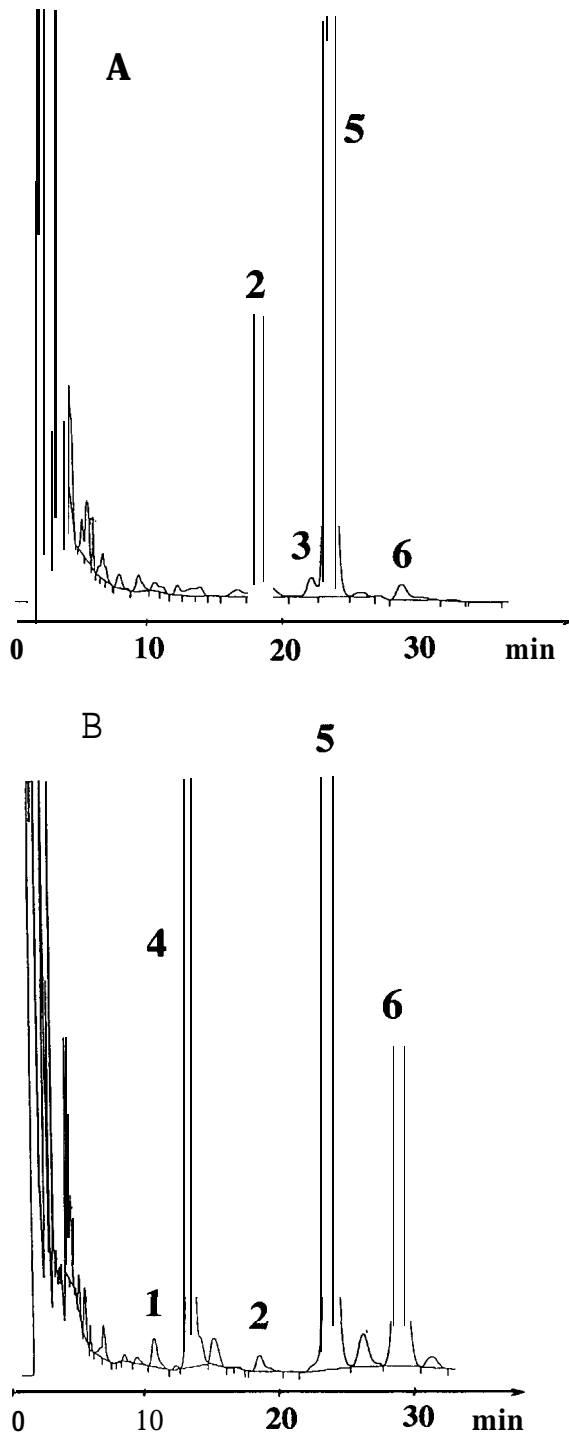


Fig. 3. Chromatogram of flavanone glycosides contained in Citrus juices. For sample preparation, see Experimental. For compound identification, see Fig. 1. (A) Grapefruit juice; (B) sour orange juice.

TABLE II
DETERMINATION OF FLAVANONE GLYCOSIDES IN PURE JUICES AND MIXTURES

Column, 250 X 4.6 mm I.D.; stationary phase, RP-18 UHS; temperature, ambient; flow-rate, 1.5 ml min⁻¹; mobile phase, water-acetonitrile-THF-glacial acetic acid (80:16:3:1, v/v). Results are concentrations in mg l⁻¹.

Compound ^a	Mixture ^c														
	Pure juice ^b			Sour orange 90%, grapefruit 10%			Sour orange 95%, sour orange 5%			Grapefruit 95%, sour orange 5%					
	Florida	Israel	Mean S.D.	Min.-max.	Mean S.D.	Min.-max.	Mean S.D.	Min.-max.	Mean S.D.	Min.-max.	Mean S.D.	Min.-max.			
1	13	<1	<1	11.4-12.1	11.8	0.27	11-12.3	11.6	0.50	<1	<1	18-20.1	19.0	1.00	
4	320	<1	<1	286-289	287	1.1	287-289	288.5	0.96	18.8-20.6	19.8	0.79	18-20.1	19.0	
2	9.9	70	6.6	103-122	116	9.3	18.3-22.2	20.6	0.91	58-74	67	5.9	w-12.6	111	
3	ND	7.5-11	9.8	6.9-16	9.9	3.44	<1	<1		7.2-10.4	9.3	1.24	6.6-13.0	9.0	
5	427	138-227	176	33	336-675	461	111.6	398-407	402	3.3	418-452	431	11.2	138-193	180
6	266	4.4-7.9	6.2	1.40	6.9-24.4	11.3	5.98	239-241	240	0.8	240-242	241	0.8	18.3-33.8	24.2
															25.9
															5.03

^a See Table I and Fig. 1 for names and structure formulae.
^b Hand-squeezed extraction obtained from Corsica variety (three-fold determination) for sour orange and four and six samples for Florida and Israel grapefruits, respectively.
^c Prepared from pure juices.

TABLE III
COMPARATIVE RESULTS OF GRAPEFRUIT ADULTERATION USING PUBLISHED RATIOS [6](NARINGIN/NEOHESPERIDIN) AND PRESENT DETERMINATIONS

Column, 250 X 4.6 mm I.D.; stationary phase, RP 18 UHS; temperature, ambient; flow-rate, 1.5 ml min⁻¹; mobile phase, water-acetonitrile-THF-glacial acetic acid (SO: 16:3:1, v/v). Ratios obtained from Table II values. Ranges are minimum and maximum values.

Compounds	Sour orange juices				Grapefruit juices			
	Pure juice	Mixture ^a	This work	90% so, 10% Florida GJ	Pure juice	Mixture ^a	This work	95% Florida GJ, 5% SO
Naringin/nehesperidin	1.3-2.5	1.61	1.67-1.69	1.74-1.87	14-83	23-31	28-69	16-20
Narirutin/nehesperidin	?	32.3	17.4-19.1	13-14.9	?	>60	>100	0.16-0.19

^a SO = sour orange juice; GJ = grapefruit juice.

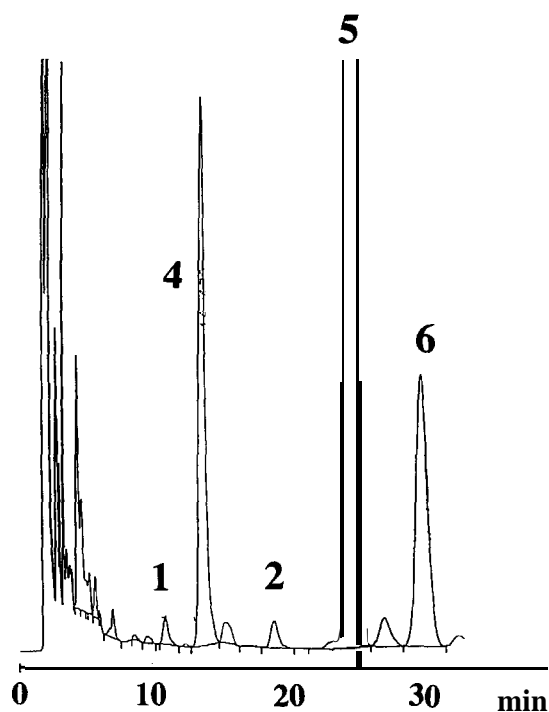


Fig. 4. Chromatogram of flavanone glycosides of sour orange juice blended with 10% of grapefruit juice. For sample preparation, see Experimental. For compound identification, see Fig. 1.

1.3-2.5) and grapefruit (23-31 for Florida and 28-69 for Israel samples compared with 14-83). Using this ratio alone, the adulteration of sour orange with 10% of grapefruit juice gave ratios (1.7-1.8) in agreement with the range ratio proposed by Rouseff [6] (1.3-2.5). For grapefruit juices adulterated with 5% of sour orange, whereas the ratios for Florida juices are low (4-9 compared with 14-83), the ratios obtained for Israel juices (16-20) are within Rouseff's range of values.

The narirutin-to-neoeriocitrin ratios may be useful for quality control of *Citrus* juices. As shown in Table III, this ratio is almost halved when sour orange is adulterated with 10% of grapefruit juice. This ratio is <1 for grapefruit juice adulteration with only 5% of sour orange juice.

CONCLUSIONS

The column liquid chromatographic separation of six flavanone glycosides was achieved, demonstrating the possibility of using this procedure for the determination of these compounds in natural

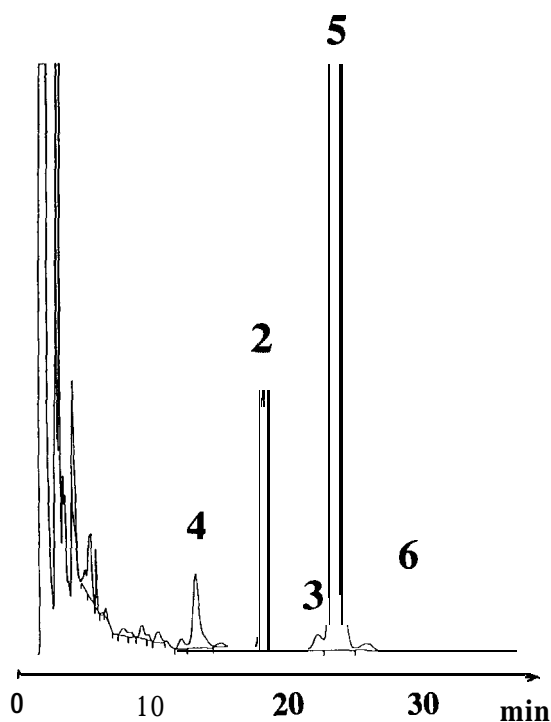


Fig. 5. Chromatogram of flavanone glycosides of grapefruit juice blended with 5% of sour orange juice. For sample preparation, see Experimental. For compound identification, see Fig. 1.

mixtures. This new HPLC procedure, applied to the separation and determination of the most important flavanone glycosides contained in *Citrus* juices, offers an alternative method for quality control of industrial concentrates and juices.

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