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Determination of phenolic constituents in *citrus* juices: Method of high performance liquid chromatography

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Abstract

Some flavonoid compounds, naringin, hesperidin, neohesperidin, and quercetin, generally present in fruit juices were separated and determined by high performance liquid chromatography using a C6-phenyl-phase column and diode array detector. The method was partly validated with good results. Flavanones naringin, hesperidin, and neohesperidin were identified as markers of 100% *Citrus* juices. Their contents in analysed juices were compared with published values established by extensive research of *Citrus* composition. Quercetin, as a constituent of grapefruit and some other fruit juices, was also studied.

Keywords: Flavonoids; 100% *citrus* juices; HPLC

1. Introduction

Flavonoids are widely distributed in fruits, vegetables, fruit juices, teas and wines. *Citrus* plants especially are rich flavonoid sources. They may have beneficial human health effects, such as antioxidant, antiallergic, and anticarcinogenic benefits and can protect against high blood pressure or cholesterol increase (Benavente-Garcia, Castillo, Marin, Ortuno, & Del Rio, 1997; Pszczola, 1998; Kawaii, Tomono, Katase, Ogawaa, & Yano, 1999). Flavonoid glycosides, were found in *Citrus* fruit. These compounds could be structural isomers, which contain rhamnose and glucose bound in either the 1–2 (neohesperidose) or 1–6 (rutinoside) positions (Rouseff, 1998). *Citrus* species should contain only one kind of flavanone and this fact is used for their differentiation in *Citrus* juices. Recent publications suggest advances in *Citrus* flavonoid determination, especially by HPLC (Kawaii et al., 1999; Mouly, Gaydou, & Auffray, 1998; Swatsitang, Tucker, Robards, & Jardine, 2000) in conjunction with diode array detection for their identification and characterization. Detection of these compounds can be also successfully achieved by UV (Da Queija, Queirós, & Rodrigues, 2001; Kawaii et al., 1999) and electrochemical detection (Achilli, Cellerino, & Gamache, 1993). To date the Davis method is still used for the measurement of naringin in grapefruit and the total flavanone glycosides in oranges (Ting & Rouseff, 1986). This is a non-specific test and estimates only the principal flavanone glycosides in *Citrus* juices. For complicated flavonoid mixtures, HPLC gradient elution was developed to improve flavonoid separation (Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999; Kawaii et al., 1999). For juice sample preparation, this is minimal and often requires only a simple filtration.

This paper, reports the separation and quantification of naringin, hesperidin, neohesperidin, and quercetin in some 100% *Citrus* juices and nectars in one run without relevant sample preparation. The determination was achieved using gradient C6-phenyl-phase chromatography and UV detection.

2. Materials and methods

2.1. Samples

The 100% commercial orange and grapefruit juices and nectars were taken from many brands available in

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the Slovak market network, as well as fresh fruits for recovering fresh-pressed *Citrus* juices. During testing, juices were maintained in a fridge at +4 °C and they were filtered through a paper filter before analysis. Undiluted samples were directly injected onto the column. To investigate an influence of sample preparation, some of the 100% orange juices were submitted to dilution with the mixture of methanol/water, 70/30 (v/v) as follows: 5 ml of unfiltered juice were mixed with 20 ml of methanol/water mixture and placed in a shaking apparatus for 15 min. The mixture was passed through a paper filter and pure filtrate was injected onto the column without further modification.

2.2. Standards and chemicals

Naringin (naringenin-7-rhamnosidoglucosidose), 97%; neohesperidin (S)-4′-methoxy 3′,5,7-trihydroxyflavanone-7-(2–O- (α-L-rhamnopyranosyl)-β-D-glucopyranoside), 98%, and quercetin dihydrate, 99%, were purchased from Fluka (Fluka Chemie, Buchs, Switzerland). Hesperidin (hesperetin-7-rutinoside), 97%, was obtained from Aldrich (Aldrich Chem. Co., Germany). Methanol (HPLC grade) was delivered from Merck (Darmstadt, Germany). Phosphoric acid (85%) was from Lachema (Brno, Czech Republic). Individual stock standard solutions of flavonoids, with concentration 0.1 mg/ml in methanol, were prepared. Working standard solutions were made by dilution of each component of the stock solutions with methanol in order to give required concentrations for calibration curve construction.

2.3. Equipment and HPLC analysis

The separation was performed on a liquid chromatograph PU (Pye Unicam Ltd., Cambridge, UK). The instrument consisted of programming unit and gradient pump PU 4003, multichannel detector UV–Vis PU 4021 at 285 nm in conjunction with a chromatographic data station CSW, version 1.7 (DataApex, Prague, Czech Republic), and injection valve Rheodyne, Model 7125 (Rheodyne, USA) fitted with 20 μl sample loop. The used column was Separon SGX C6-Phenyl 5 μm, 150 × 3 mm i.d. (Tessek, Prague, Czech Republic). Flavonoids were separated at ambient temperature using a gradient elution programme: 0–1 min 0% B, 2–40 min 0–100% B linear, 41–45 min 100–0% B linear, 45–55 min 0% B for column equilibration. Solvent A was 0.01 M phosphoric acid/methanol (80/20 v/v), solvent B was 100% methanol. The mobile phase flow rate was 0.8 ml/min.

3. Results and discussion

3.1. General

Initially the HPLC conditions were optimized for separation of four flavonoids. The order of flavonoid elution was confirmed by analyses of individual standard solutions and their mixtures. The separation of flavonoids took about 40 min under the described conditions including column equilibration.

3.2. Identification and determination

UV spectra of individual flavonoids were recorded in the range of 200–360 nm via the diode array detector. The wavelength of 285 nm was shown to be the absorption maximum of naringin, hesperidin, and neohesperidin. All three flavonoids had nearly identical spectra. The UV spectrum of quercetin was quite different with the absorption maximum at 260 nm. Quercetin showed about 70% absorption activity at 285 nm. Flavonoid identification was performed by characterizing the sample peaks in terms of retention times, compared with those in standard solutions. The purity of flavonoid examined could be checked by peak spectra scanning at the beginning and ending of the peak elution. Calculation of flavonoid concentration (expressed in mg/l) was carried out by an external standard method, using calibration curves.

3.3. Calibration

Six flavonoid standard solutions, in the concentration range 1–50 mg/l, were prepared and analysed. The calibration curves of the individual flavonoids were created by applying a statistical programme of the used chromatographic data station CSW. The peak area values (expressed in mV/s) were plotted as average values of duplicate injections. The results of calibration are sum-

Table 1
Calibration data of flavonoids

Flavonoid	LOD (mg/l)	LOQ (mg/l)	Linearity range (mg/l)	Correlation factor	Slope	Intercept
Naringin	1.25	2.50	2–50	0.99924	359.36	−60.35
Hesperidin	1.00	2.50	2–50	0.99459	391.60	−437.91
Neohesperidin	1.00	2.50	2–10	0.99449	456.86	−123.79
Quercetin	2.50	5.00	3–50	0.99970	134.03	22.46

marised in Table 1 and show good linearity ($r > 0.99$) for all the compounds in the range of concentration tested (1–50 mg/l) at 285 nm. Both the limit of detection (LOD) and limit of quantification (LOQ) were evaluated by statistical programme ADSTAT, version 1.25 (TriloByte™, Pardubice, Czech Republic).

3.4. Repeatability

Repeatability was determined by nine replicate injections of flavonoid mixture control standard solution (9.7 mg/l of naringin and hesperidin, 9.8 mg/l of neohesperidin and 9.9 mg/l of quercetin). The results, calculated as relative standard deviations, were 3.85% for naringin, 5.57% for hesperidin, 6.81% for neohesperidin, and 5.86% for quercetin.

3.5. Robustness

The flavonoid mixture control standard solution, mentioned above, was also used for the peak area stability monitoring for two months. During this time period the conditions of analyses were changed by new lots of solvents, C6-phenyl column and by slight changes in ambient temperature. The alterations also included an apparatus fluctuation. Results of analyses for $n = 16$ were evaluated as relative standard deviation of robustness and were 7.75% for naringin, 9.06% for hesperidin, 21.0% for neohesperidin, and 24.4% for quercetin.

3.6. Recovery

To evaluate the accuracy of used method, a recovery of flavonoids was performed. To two different sorts of 100% orange and grapefruit juices were added known amounts of mixed spiking solution (without quercetin). Samples were then passed through a paper filter and analysed. Each spiked sample was injected once and the results were averaged for each sort of juice. The recovery of quercetin was not studied. Recoveries are shown in Table 2. The higher values of relative standard deviations (12.7–17.4%) may be due to natural variability of juice composition and origin.

The recoveries were calculated using following formula: $\text{Recovery} = \text{concentration}_{\text{spiked sample}} - \text{concentration}_{\text{nonspiked sample}}/\text{concentration}_{\text{spiking solution}}$.

3.7. Yield of dilution

To find out whether the simple dilution had any influence on yield of flavonoids in orange juice, two procedures of pre-treatment of juices were compared. The first procedure included juice filtration (through a paper filter) and dilution with distilled water, the second one dilution with a mixture of methanol/water (70/30, v/v), followed by

Table 2
Recovery of flavonoids added to orange and grapefruit juice ($n = 2$)

Flavonoid	Concentration of nonspiked sample (mg/l)	Concentration of spiking solution (mg/l)	Concentration of spiked sample (mg/l)	Recovery
100% orange juice, $n = 2$				
Naringin	2.20	13.4	14.2 *12.7*	0.83
Hesperidin	47.0	40.6	76.8 *17.4*	0.74
Neohesperidin	12.9	13.5	25.1 *15.4*	0.91
100% grapefruit juice, $n = 2$				
Naringin	211	13.4	225 *16.4*	1.02

Relative standard deviation RSD in % (in italic).

Table 3
Influence of dilution on yield of flavonoids in orange juice

Stage of pre-treatment	Naringin (mg/l)	Hesperidin (mg/l)	Neohesperidin (mg/l)
Filtration through paper filter and dilution	2.43 *0.30*	56.1 *2.98*	11.5 *4.50*
Dilution with mixture of methanol/water 70/30, filtration through paper filter and dilution	2.13 *1.88*	38.5 *1.84*	5.00 *5.92*
Yield after dilution	0.88	0.69	0.44

Relative standard deviation RSD in % (in italic).

the filtration described above. One sort of orange juice was tested in four replicates. The results are given in Table 3. It can be seen that use of methanol mixture decreased the flavonoid content, mainly of neohesperidin. The losses of hesperidin were not significant (about 5%) against the typical recovery given in Table 2.

3.8. Measurement uncertainty

The measurement uncertainty was evaluated as combined uncertainty with the covering factor of 2 ($2U_C$) and a 95% confidence interval. In assessment, possible sources of uncertainty were taken into account such as standard purity, standard weighing, standard dissolving in exact volume, standard and sample dilution, and calibration curve linearity. Calculated uncertainties were 8.72% for naringin, 8.86% for hesperidin, 8.37% for neohesperidin, and 8.20% for quercetin.

3.9. Column performance criteria

From the five replicated analyses of the flavonoid mixture control standard solution, capacity and resolution factors were calculated to specify column performance. The capacity factor characterizes the retention of studied compounds and was calculated as $k' = (RT_i - T_0)/T_0$, where RT_i is component retention time, and T_0 is solvent front retention time. The resolution factor means separation between two compounds and was calculated by formula $R_i = (RT_i - RT_{i-1})/0.5(W_{i-1} + W_i)$, where W_i is the peak width given by tangent and baseline intersection (calculation was carried out by data station CSW). The resolution factor was considered to be higher than 1 or equal to 1 and should not be higher than 10 (a resolution factor of 1 means two peaks separation for about 98%). From the five results, an average value was calculated and relative standard deviation. The capacity factors were as follows: naringin 23.6, RSD 8.55%; hesperidin 25.2, RSD 7.09%; neohesperidin 25.7, RSD 6.62%; quercetin 27.2, RSD 5.85%, and the resolution factors were: naringin–hesperidin 2.60, RSD 13.9%, hesperidin-neohesperidin 0.71, RSD 14.1%, and neohesperidin-quercetin 2.27, RSD 6.61%. Hesperidin and neohesperidin were not separated from the baseline and this results in a capacity factor lower than 1 (0.71; Fig. 1).

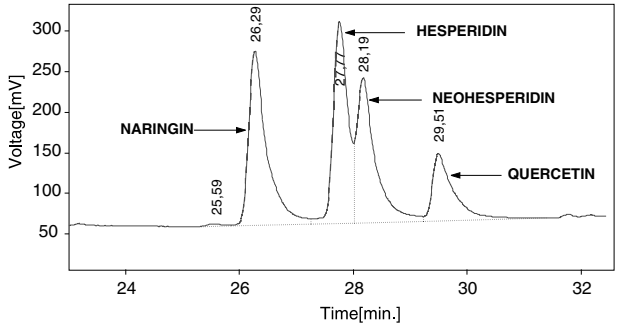

Fig. 1. Separation of flavonoids at 285 nm.

Table 4
The flavonoid content in filtered juices and nectars

Sort of juice	Naringin (mg/l)	Hesperidin (mg/l)	Neohesperidin (mg/l)	Quercetin (mg/l)
Fresh-pressed orange	2.0	92.7	7.0	23.0
	6.50	*6.57*	*5.03*	*4.72*
100% orange 1	2.4	56.1	11.5	6.9
	2.50	*2.97*	*6.35*	*5.31*
100% orange 2	3.4	44.5	11.0	7.1
	4.41	*2.11*	*4.85*	*3.28*
100% orange 3	7.0	47.8	5.3	5.3
	5.73	*1.27*	*6.56*	*3.25*
Reference values for sweet orange 11.8 Brix[a]	Less than 1 mg/kg	80–250 mg/kg	Less than 1 mg/kg	Not defined
Reference values for sour orange 11.8 Brix[a]	150–350 mg/kg	1–3 mg/kg	100–200 mg/kg	Not defined
Fresh-pressed grapefruit	211	15.4	10.8	6.8
	0.52	*1.56*	*1.29*	*4.68*
100% grapefruit	116	15.7	19.7	12.1
	4.33	*4.30*	*4.42*	*2.63*
Reference values for grapefruit 11.8 Brix[a]	100–800 mg/kg	Less than 20 mg/kg	4–10 mg/kg	Not defined
Fresh-pressed lemon	Not detected	Not detected	14.5	Not detected
			7.59	
50% orange	4.7	34.3	2.9	7.0
	2.13	*2.27*	*4.48*	*8.14*
12.5% orange	Not detected	7.8	1.6	Not detected
		4.49	*0.63*	
60% grapefruit	115	15.0	6.7	18.9
	3.28	*6.34*	*5.55*	*1.11*

[a] According to Dillon (1995); Relative standard deviation RSD in % for $n = 3$ (in italic).

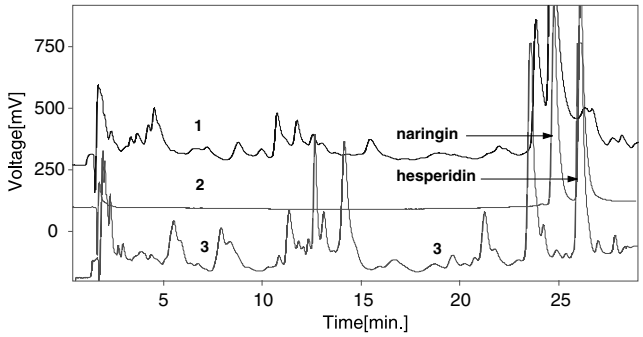

Fig. 2. Comparison of 100% juices chromatographic profiles. *Legend*: 1 – 100% grapefruit juice, 2 – naringin and hesperidin in standard solution (50 + 50 ng/ml), 3 – 100% orange juice.

3.10. Content of flavonoids in Citrus juices

The method described above has been applied to the study of the flavonoid glycoside compositions of different commercial *Citrus* juices, including orange, grapefruit, and lemon. Because it is known that the distributions of type, number, and amounts of flavonoids are not balanced among *Citrus* cultivars, their concentrations found in examined juices were compared with the published values (Dillon, 1995), which are based on evaluation of juice composition of various *Citrus* varieties. Some of the results are reported in Table 4. Significant amounts of hesperidin were found in orange juices; in contrast naringin was the major flavonoid of grapefruit juices. Also, a fresh-pressed lemon juice was analysed. The dominant flavanone glycoside of lemon juice is narirutin, which was not studied in this work, but there was some hesperidin in lemon juice (about 14.5 mg/l). In general, the highest concentrations of typical flavonoids were identified in fresh-pressed juices. The chromatographic profiles of 100% juices are in Fig. 2.

4. Conclusion

Naringin, hesperidin, and neohesperidin are the main flavonoid markers of *Citrus* juice. The chromatographic profile of these flavonoids is important information for originality and quality evaluation of the juices. The presented HPLC method has been targeted on simultaneous evaluation of these major flavonoids in commercial *Citrus* juices and nectars, including quercetin. The method allows identification and quantification of selected flavonoids in juices and is shown to be a good tool for their quality control. The results obtained by this method indicate sufficient correlation with some published references which represent valid values in the field of *Citrus* juices composition.

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