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Journal of Chromatography A, 1081 (2005) 99–104
JOURNAL OF CHROMATOGRAPHY A
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Analysis of flavanone-7-*O*-glycosides in citrus juices by short-end capillary electrochromatography

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Available online 19 January 2005

Abstract

The separation of the major flavanone-7-*O*-glycoside constituents of *Citrus* was carried out by isocratic reversed phase capillary electrochromatography using a 75 μm i.d. silica fused column packed with 5 μm ODS silica gel. In comparison to HPLC mode, capillary electrochromatography resolution of flavanone glycosides was obtained with a high selectivity factor. Optimum separation conditions were found using a mixture of ammonium formate (pH 2.5)–acetonitrile (8:2, v/v) as the mobile phase by the short-end injection mode. Under these conditions all the investigated flavanones were baseline-resolved within short analysis time (i.e. between 5 and 10 min). A study, evaluating the intra- and inter-day repeatability as well as limit of detection and method linearity, was developed in accordance with the analytical procedures for method validation. The developed method was applied for the quantitative analysis of flavanone glycosides in commercial fruit juices (sweet orange, lemon and grapefruit).

Keywords: Flavanone-7-*O*-glycosides; Citrus fruit juices; Reversed phase capillary electrochromatography

1. Introduction

Flavanone-7-*O*-glycosides (FGs) are an important class of naturally occurring compounds specifically distributed in Citrus [1]. Due to their polyphenolic structure (Fig. 1), these compounds possess health-related properties, which are based on their antioxidant activity, including anticancer, antiviral and antiinflammatory activities, effects on capillary fragility and inhibition of human platelet aggregation [2–4].

Eriocitrin, hesperidin, neohesperidin, naringin and narirutin are the most abundant flavonoids in the edible part of many species of citrus fruits [5] and are known to greatly influence the quality of both fresh fruit and processed products, thus playing an important role in nutritional and pharmacological fields. The predominance of the nearly tasteless hesperidin or the bitter naringin, divides the citrus cultivars in two main groups characterized by a sweeter (mandarin, sweet orange) and a bitter taste (grapefruit, sour orange), respectively [6]. As reported in literature, naringin is the most representative FG in grapefruit [7], hesperidin and narirutin have been determined in common sweet orange [6], whereas eriocitrin and neoeriocitrin are typical in sour orange [8] and lemon juices [1]. Owing to this biodiversity, flavanone glycosides have been used as chemotaxonomic markers in quality control to identify adulterated processed foods [9–10].

Liquid chromatography (HPLC) using C_{18} or C_8 silica gel as the stationary phases and UV, coulometric and electrospray ionization (ESI) mass spectrometer detectors, is still the preferred method for the analysis and characterization of FGs in citrus [11–15], even though this technique requires a time consuming gradient elution.

It was recently demonstrated that FG standards can be fully separated by micellar electrokinetic capillary chromatography (MECC) and capillary zone electrophoresis (CZE) [16,17], but only in two cases the determination of FGs in citrus juices has been described, and such analyses concerned the resolution of some constituents as the

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doi:10.1016/j.chroma.2005.01.009

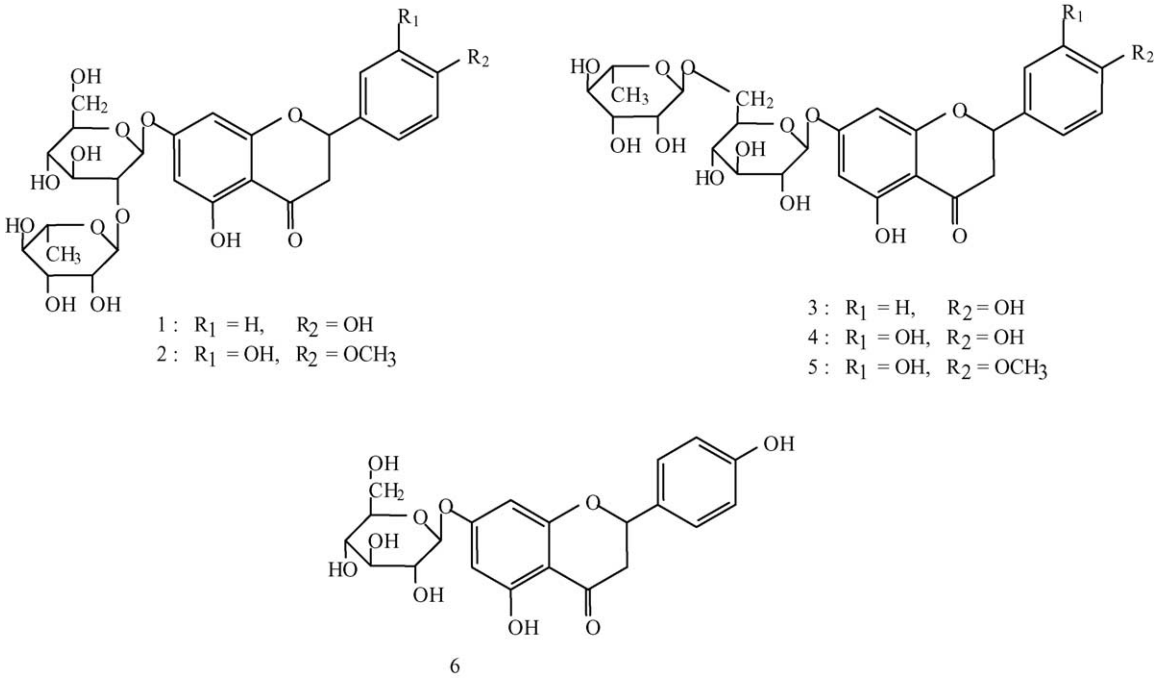

Fig. 1. Structures of the studied flavanone glycosides. (1) Naringin (naringenin-7-*O*-neohesperidoside); (2) neohesperidin (hesperitin-7-*O*-neohesperidoside); (3) narirutin (naringenin-7-*O*-rutinoside); (4) eriocitrin (eriodictyol-7-*O*-rutinoside); (5) hesperidin (hesperetin-7-*O*-rutinoside); (6) (naringenin-7-*O*-glucoside).

corresponding diastereomers [18,19]. To our knowledge, no separation of flavanone glycosides by capillary electrochromatography (CEC) has been so far reported and furthermore only few papers deal with CEC flavonoids analysis [20,21].

CEC is an hybrid analytical technique combining the separation principles and the best features of CE and HPLC because it is performed in capillaries packed with a stationary phase, and using the electroosmotic flow generated under an electric field as the mobile phase deliver. Owing to the high selectivity and efficiency in general exhibited, CEC has proved to be a very effective technique for the analysis of complex matrices allowing also to shorten and simplify the sample pretreatment procedures. Reversed phase CEC was initially developed and then extensively applied to the separation of non-polar neutral compounds [22–24]. At present, this technique is in rapid development especially for improving column fabrication technology and increasing its applicability in different fields of analysis.

Aim of the present paper was to investigate the applicability of reversed phase CEC technique to the analysis of FGs compounds particularly interesting either for their biological functions either for their chemical properties, namely the absence of charge at neutral and acidic pHs and the low differences in their strong polarity. The method was developed with the purpose of optimizing a rapid and high efficiency CEC method for the determination of FGs in different citrus juices (sweet orange, lemon and grapefruit) demonstrating the applicability of this powerful technique in the fields of food analysis and quality assessment.

2. Experimental

2.1. Chemicals

Ammonia solution (30%), formic acid (99%), acetonitrile and methanol were purchased from Carlo Erba (Milan, Italy). Double distilled water (Milli-Q, Millipore, Waters Milford, MA, USA) was used for preparation of solutions and for CEC experiments. Hesperidin (hesperetin-7-*O*-rutinoside) and naringin (naringenin-7-*O*-neohesperidoside) were purchased from Aldrich (Steinheim, Germany). Neohesperidin (hesperitin-7-*O*-neohesperidoside) was from Sigma (St. Louis, MO, USA). Eriocitrin (eriodictyol-7-*O*-rutinoside) and narirutin (naringenin-7-*O*-rutinoside) were from Extrasynthese (Lyon, France). Naringenin-7-*O*-glucoside (Extrasynthese) was used as the internal standard (I.S.). Concentrated analytes solutions (1 mg/mL) were prepared in methanol and stored at −18 °C. Hesperedin and neohesperidin were dissolved in warm methanol. Further dilutions were daily made with water in order to obtain a final methanol concentration in the solution of 20%. According to the citrus juice extraction procedure, the FGs diluted standard solutions were always containing the 20% of methanol in water.

2.2. Apparatus

Experiments were performed on an Agilent Technologies Capillary Electrophoresis automated apparatus (Waldbronn, Germany) equipped with diode array UV detector and external nitrogen pressure (up to 12 bar). The separations were performed on silica fused capillaries, 75 μm i.d.,

375 μm o.d. (Composite Metal Services, Hallow, Worcs, UK) packed with Lichrospher 100 RP_{18} (5 μm particles) (Merck, Darmstadt, Germany), following the procedure previously described [25]. The capillary was packed for the full total length (32 cm), that allowed the use of 24.0 or 8.0 cm length segments of the capillary as the effective column, depending whether the long or the short-end injection method was used. During the run the capillary was pressurised at both ends by applying 10 bar from the external pressure and air termostated at 25 °C. The separation was performed in long-end injection mode in positive polarity mode at 30 kV of applied voltage by injecting the sample at the anode and using 195 nm as the output UV wavelength. In short-end injection mode both the electric polarity and the capillary sample injection end were simply reversed taking constant other experimental conditions. Samples were injected using the high pressure at 12 bar × 3 min followed by mobile phase injection at 12 bar × 0.2 min. Between runs the capillary was rinsed with a mixture consisting of buffer-acetonitrile (3:7, v/v) at 12 bar × 1 min followed by filling with the running mobile phase at 12 bar × 2 min. The mobile phase was daily prepared by adjusting the pH value of a 50 mM formic acid aqueous solution at 2.5 with aqueous diluted ammonia, and diluting the resulted solution with water and acetonitrile up to the final buffer concentration and organic solvent content.

2.3. Sample extraction procedure

Citrus juices (400 μL) from the market were vortex mixed for 5 min and extracted with 400 μL of methanol for 15 min in ultrasonic bath at 60 °C. The warm extract was then vortex mixed and centrifuged (2000 × *g*) and the clear solution diluted with water (1:2.5) before analysis.

3. Results and discussion

The analysis of highly polar compounds on sorbents for reversed phase liquid chromatography requires the use of eluents with a low amount of the organic modifier in order to maximize the affinity of the analytes towards the stationary phase. Such eluent conditions can generate in CEC some troublesomeness as for instance noisy baseline due to the current increase and bubbles formation, low peak efficiency due to the long analysis time as well as low analyte detection. Current increase and bubbles formation can be prevented by lowering the buffer concentration in the mobile phase, however, the use of a proper buffering system is highly requested for ensuring column stability and injection repeatability.

The use of capillaries fully packed with the stationary phase offers some important advantages as high column stability and robustness, and fast method optimization due to the possibility to perform separations on a long or a short path inside the same capillary, by simply reversing the electric polarity and the sample injection end. As previously demonstrated [25,26], the short-end injection mode in CEC (8 cm as effective column length) advantageously shorten the analysis time, especially when mobile phases with low organic solvent content were used, and provided quantitative reproducible results.

3.1. Optimization of flavanone-7-O-glycosides separation

In order to optimize the CEC separation of FGs, preliminary experiments were performed using a mixture consisting of 2.5 mM ammonium acetate (pH 6.0)–acetonitrile (1:1, v/v) as the mobile phase. Under these conditions all the examined FGs eluted with similar retention times indicating a very weak affinity of the analytes to the stationary phase. By lowering the acetonitrile content to 30%, a partial separation was observed, but problems concerning column stability and injection repeatability impaired the use of this mobile phase.

Better results were obtained with an eluent containing an acidic buffer, namely ammonium formate at pH 2.5 and studying the effect of the organic solvent concentration in the range 50–20% (v/v). As it can be observed in Fig. 2, to the decrease of the amount of acetonitrile in the mobile phase corresponded an increase of the analyte retention times and selectivity. In fact, whether at 50% acetonitrile all FGs coeluted, full separation of the five examined compounds was obtained at 20%.

It was also observed that the logarithmic functions of the retention factor (*k*) for the analytes were not linearly dependent on the acetonitrile content (data not reported), and this means that, in addition to hydrophobic partition, other types of interactions between the analytes and the stationary phase concur to the separation process. Nevertheless, with decreasing acetonitrile concentration the retention order of the analytes remained the same, i.d. eriocitrin was more retained than narirutin > naringin > hesperidin > neohesperidin.

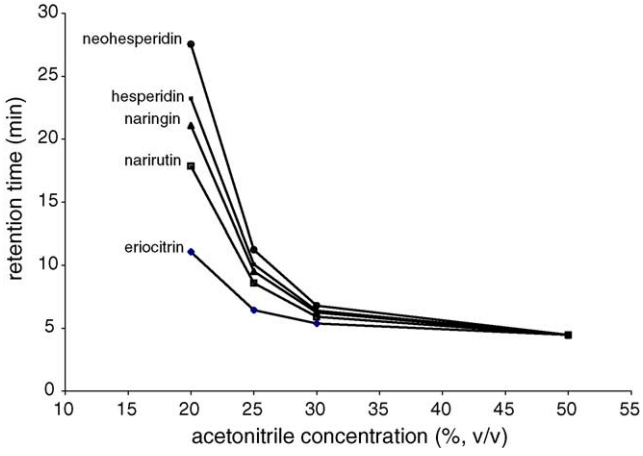

Fig. 2. Dependence of the retention time (t_R) on the acetonitrile content in the mobile phase (×100, v/v). Mobile phase: 2.5 mM ammonium formate (pH 2.5–acetonitrile (20–50%, v/v). Long-end injection mode (for other CEC conditions, see Section 2).

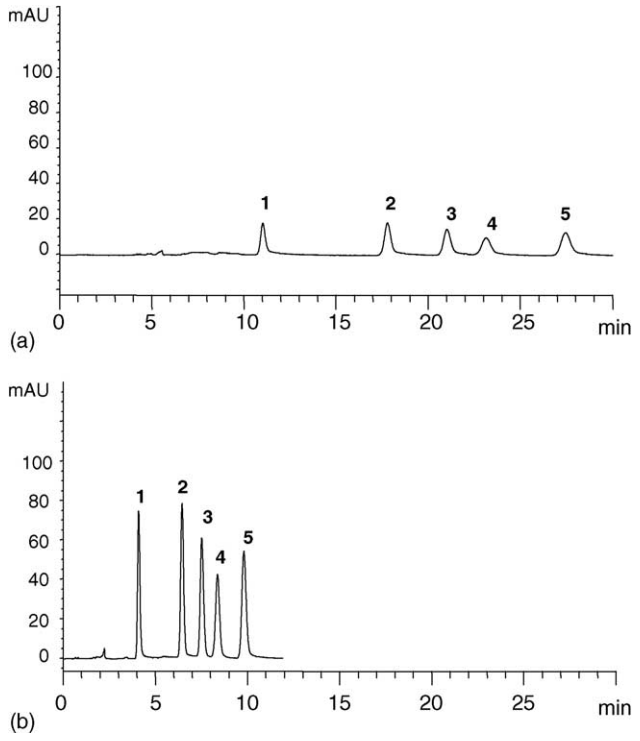

Fig. 3. Comparison of the reversed phase CEC separations of flavanone glycosides obtained under the same experimental conditions in (a) long-end and (b) short-end injection modes. Compounds: (1) eriocitrin; (2) narirutin; (3) naringin; (4) hesperidin; (5) neohesperdin. Mobile phase: 2.5 mM ammonium formate (pH 2.5)–acetonitrile (80:20, v/v) (for other CEC conditions, see Section 2).

However, with this eluent composition, too long retention times and low analytes signal were obtained.

In order to improve peak efficiency and to speed the analysis, similar operating conditions were employed, but the separation was carried out by the short-end injection mode, using as effective length a segment of 8 cm of the column, by simply reversing the electric polarity and the sample injection end. Fig. 3 shows the separation of the FG standard mixture by long-end (panel a) and short-end (panel b) injection modes, respectively, using 2.5 ammonium formate (pH 2.5) containing 20% acetonitrile as the mobile phase. It is interesting to note that in the latter operating mode all the analytes were still well separated even if in a shorter analysis time, showing a peak efficiency almost twice as higher (within the range between 38,541 and 56,817, and 63,637 and 103,800 N/m, in long- and short-end, respectively).

3.2. Method validation

A small variation of the acetonitrile mobile phase content (23%, v/v) was further necessary to allow the separation of naringenin-7-*O*-glucoside, the internal standard (I.S.), from the other examined FGs. With this mobile phase higher peak efficiencies (87,875–126,975 N/m) were achieved, with an improvement also of the detection (LOD, signal/noise = 3) and quantification (LOQ, signal/noise = 5) limits of the method, corresponding to 2.5 and 5.0 μg/mL for each compound, respectively. The optimized method was tested for quantitative estimation of repeatability and linearity tests using the internal standard method. In Table 1, the intra- and inter-day injection repeatability data (RSD), the regression equation as well as the relative squared correlation coefficients for each flavanone glycoside are reported. Intra-day repeatability was determined for each FGs by assaying the same standard mixture at concentration of 50 μg/mL (each compound) for ten consecutively runs. Inter-day repeatability was determined by assaying three times the same standard (50 μg/mL concentration, each compound) mixture consecutively for 3 days and measuring the main values per each day. Considering the wide range of concentrations of FGs in citrus, the method linearity was estimated in the range of concentration between 5 and 200 μg/mL on 10 calibration points and by measuring the average values of two consecutively runs per point. The final concentration of the internal standard in the FGs standard mixture and fruit juice extracts was 100 μg/mL.

3.3. Analysis of fruit juices

The method above described was applied to the analysis of FGs in different commercial fruit juices (lemon, orange and grapefruit). Before CEC analysis, the fruit juices were processed following the extraction method reported in Section 2. The extraction procedure was developed with the aim of minimizing the involved amounts of both sample and solvents in each step of sample preparation, thus reducing the analysis time as well as the cost and environmental impact effects. In fact, a volume as low quantity as 400 μL of fruit juice was submitted to the pretreatment procedure consisting of extraction with methanol at 60 °C in ultrasonic bath, centrifugation and dilution of the corresponding solution before injection. The procedure was tested for the recovery value by spiking in duplicate the commercially available lemon juice with all the studied FGs at a final concentration of 25 μg/mL and measuring the average value of three repeated runs. The same recovery method used for orange juice was also extended to the other fruits, namely orange and the grapefruit juices, by calculating the average values for triplicate runs. In all the experiments, the recoveries data of the methods were in the range 71–112%.

Furthermore, to evaluate the recovery from small juice volume (400 μL), it was compared with that obtained using higher volume (10 mL). Only negligible differences between the two measurements were observed (data not shown).

When analysing the extracted citrus juice samples an adjustment of the CEC pre-run conditioning procedure was necessary in order to provide reproducible results. In fact, the pre-run washing of the column with the mobile phase containing 23% of acetonitrile was not sufficient to replace the initial capillary conditions, probably due to the adsorption of

Table 1
RSD% data of intra- and inter-day injection repetability and method linearity data

Analyte	Intra-day repeatability ($n = 10$ runs)				Inter-day repeatability ($n = 3$ days)				Linearity data (concentration range 5–200 μg/mL)	
	R_t	Peak area	Peak area/R_t	Analyte/IS peak area ratio	R_t	Peak area	Peak area/R_t	Analyte/IS peak area ratio	Regression equation	r^2
Eriocitrin	0.90	1.97	1.90	2.11	5.20	4.48	2.98	1.68	$y = 6.0645\,x - 0.0103$	0.9982
Narirutin	1.22	0.85	1.53	1.82	5.07	2.73	3.98	0.61	$y = 6.8752\,x - 0.0086$	0.9986
Naringin	1.25	1.83	1.70	1.79	4.96	5.24	3.87	2.28	$y = 5.7319\,x - 0.0067$	0.9991
IS	1.04	1.47	0.80	–	4.64	3.27	3.97	–	–	–
Hesperidin	1.41	1.18	1.73	1.67	5.15	4.85	2.34	2.79	$y = 5.7982\,x - 0.0067$	0.9989
Neohesperidin	1.50	2.34	1.55	1.55	5.14	1.54	5.67	2.41	$y = 6.6363\,x - 0.0212$	0.9971

interfering material deriving from the sample matrix on the stationary phase. Between runs an additional preconditioning step with 70% acetonitrile mobile phase for 2 min at 12 bar was successfully used to ensure proper sample analysis repeatability and clean electrochromatograms profiles.

Fig. 4 shows the electrochromatograms related to the separation of FGs in (a) a standard mixture, (b) the extract from a commercial orange juice, and (c) the extract from an orange juice spiked with the standard compounds (before extraction). CEC analyses showed that FGs and the I.S. eluted free from interfering compounds.

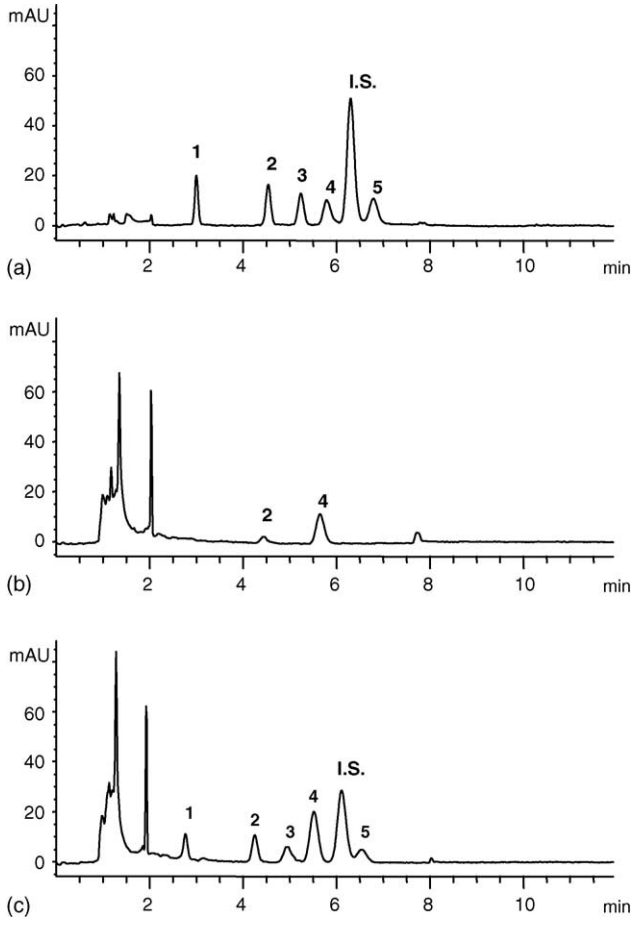

Fig. 4. Short-end CEC analysis of the extracts from orange juices under the optimum experimental conditions and sample pretreatment procedure; (a) flavanones glycosides standard mixture containing the internal standard (I.S.), (b) extracted orange juice; (c) extracted spiked orange juice (spiked concentration 50 μg/mL, each compound, before extraction). Mobile phase: 2.5 mM ammonium formate pH 2.5 containing 23% of acetonitrile. Other experimental conditions as in Fig. 3.

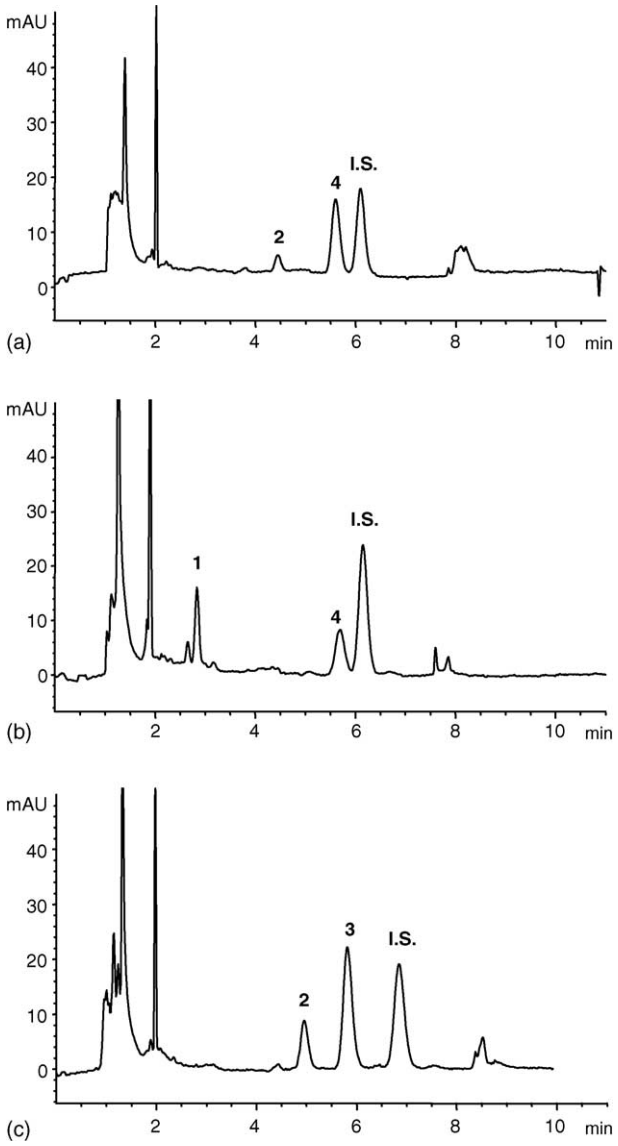

Fig. 5. Comparison of the electrochromatograms of fruit juice extracts from different citrus species: (a) orange, (b) lemon, (c) grapefruit (for other experimental conditions, see Figs. 3 and 4).

Table 2
Quantitative analysis of flavanone-7-*O*-glycosides in citrus juices

Fruit juice	Eriocitrin	Narirutin	Naringin	Hesperidin	Neohesperidin
Lemon[a]	16.02 ± 0.58			23.08 ± 0.08	
Orange[b]		3.40 ± 0.17		29.55 ± 0.78	
Grapefruit[b]		18.82 ± 0.27	62.62 ± 0.91		

[a] mg × 100 mL^{-1}, mean value of duplicate analysis of two determinations, not corrected for the recovery value.
[b] mg × 100 mL^{-1}, mean value of duplicate analysis, not corrected for the recovery value.

Fig. 5a–c compared the analysis of extracts from commercial orange, lemon and grapefruit juices, respectively, showing the typically different FG composition in the diverse citrus species. In particular, lemon juice is characterized by the presence of eriocitrin and hesperidin, orange juice by narirutin and hesperidin, and grapefruit juice by narirutin and naringin. Table 2 summarizes the quantitative data (mg × 100 mL^{-1}) obtained by analyzing commercially available fruit juice extracts from different citrus species.

The presence or absence of single FGs can be used as a sort of fingerprint for taxonomic purposes and identification of adulteration in citrus processed juices. Authentic orange juice should not contain naringin and the narirutin/hesperidin ratio can be used against the adulteration of processed food [6].

4. Conclusions

The present paper showed the first application of reversed phase CEC for the analysis of flavanone-7-*O*-glycosides using short-end injection mode for obtaining high efficiency and fast separation of strong polar compounds. The obtained results demonstrated that reversed-phase CEC can be successfully employed for the separation of flavanone glycosides providing their quantitative estimation in citrus fruit juices with high repeatability and linearity of assay and showed the powerful applicability of this technique in the field of food biomarker analysis. The optimized method provided the complete separation of flavanone-7-*O*-glycosides in short time by isocratic elution mode avoiding the time consuming gradient elution that is necessary in the FG analysis by liquid chromatography. Furthermore, the method developed a rapid and simple sample pretreatment procedure optimized on 400 μL of fruit juice contributing to further reduce the use of organic solvent and the environmental pollution impact and to speed the total analysis time.

Acknowledgment

The authors are indebted to Mr. Antonio Berti for technical assistance.

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