Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 419–422

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

# HPLC determination of hesperidin, diosmin and eriocitrin in Iranian lime juice using polyamide as an adsorbent for solid phase extraction

Iman Saeidi [a], Mohammad Reza Hadjmohammadi [b,*], Moazameh Peyrovi [b], Mehrdad Iranshahi [c], Behruz Barfi [b], Adel Beig Babaei [a], Abolghasem Mohammad Dust [d]

[a] Institute of Standard and Industrial Research of Iran, Mashhad, Iran
[b] Department of Chemistry, University of Mazandran, Niroo Havayii boulevard, 47416-95447 Babolsar, Iran
[c] Biotechnology Research Center and School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[d] School of Medical Science, Mashhad, Iran

ARTICLE INFO

*Article history:*
Received 13 January 2011
Received in revised form 17 April 2011
Accepted 15 May 2011
Available online 20 May 2011

*Keywords:*
Solid phase extraction
SPE
HPLC
Lime juice
Hesperidin
Diosmin
Eriocitrin

ABSTRACT

Solid phase extraction (SPE) and HPLC were used for simultaneous determination of hesperidin, diosmin and eriocitrin in Iranian lime juice samples. The method involved very simple efficient SPE with polyamide cartridge, the use of mixture of water/acetonitrile/acetic acid (78:19:3, v/v) as a mobile phase at a flow rate of 0.8 mL/min and UV detection at 280 nm. Optimum conditions for SPE were achieved using 8 mL water/methanol (85:15, v/v, pH = 3) as the washing solution and 4 mL methanol for elution. SPE parameters, such as maximum loading capacity and breakthrough volume, were also determined for each analyte. Good clean-up and high > 90% were observed for all analytes. Limits of detection, limits of quantification, linear range, recovery, repeatability of retention times, and peak areas for hesperidin, diosmin and eriocitrin were 0.0283–0.0512 μg/mL, 0.0857–0.155 μg/mL, 0.0283–105.0 μg/mL ($R^2$ > 0.99), 93.3–98.1%, 3.2–4.7% and 2.8–3.6%, respectively. The method was applied to analysis of lime juice samples obtained from different locations of Iran.



## 1. Introduction

Flavonoids are a group of polyphenolic compounds with health-related properties. They are potent antioxidants, free radical scavengers [1] and metal chelators; they inhibit lipid peroxidation [2] and exhibit various physiological activities [3,4], including anti-inflammatory, anti-allergic, anti-carcinogenic, antihypertensive and anti-arthritic activities [5]. Due to the importance of flavonoids as contributors of beneficial health effects of citrus fruit, determination of such compounds occurring in citrus fruits play an important role in many areas of science. Lime juice is characterized by the presence of significant amounts of the flavanones, hesperidin and eriocitrin. Lime juice is also quite rich in flavones: diosmin has been recognized as one of the main flavonoid components of this juice [6].

Several sample preparation techniques such as hydrolysis [7], filtration/dilution [8], liquid extraction [9], ultrasound-assisted extraction [10] and solid phase extraction using molecularly imprinted polymers [11] were developed to allow HPLC-based determination. Reverse-phase high-performance liquid chromatography combined with different detectors is the commonly used analytical method for separation and identification of flavonoids [12–18].

Although SPE is not a new technique, it has only recently been applied in flavonoid analysis. The SPE strategy comprises the isolation and preconcentration of the analyte from a complex matrix by adsorption onto an appropriate sorbent, removal of interfering impurities by washing with a suitable solvent system, and selective recovery of the retained analyte with a suitable solvent. Polyamide (PA) can be a good general-purpose phase suited to separation of flavonoids of varying polarity [19].

The objective of present study was to develop a SPE-HPLC method for the simultaneous determination of hesperidin, diosmin and eriocitrin in lime juice samples. This method was applied to determine of mentioned flavonoids in lime juice samples from different locations of Iran.

## 2. Experimental

### 2.1. Chemicals and stock solutions

Standards of hesperidin, diosmin and eriocitrin were purchased from Sigma (Steinheim, Germany). HPLC grade methanol and acetonitrile were from Fluka (Buches, Switzerland). Glacial acetic acid and HCl were from Merck (Darmstadt, Germany). Water used was double distilled deionized.

* Corresponding author. Tel.: +98 112 534 2350; Fax: +98 112 534 2350.
*E-mail address:* iman.analyst@gmail.com (M.R. Hadjmohammadi).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.05.015

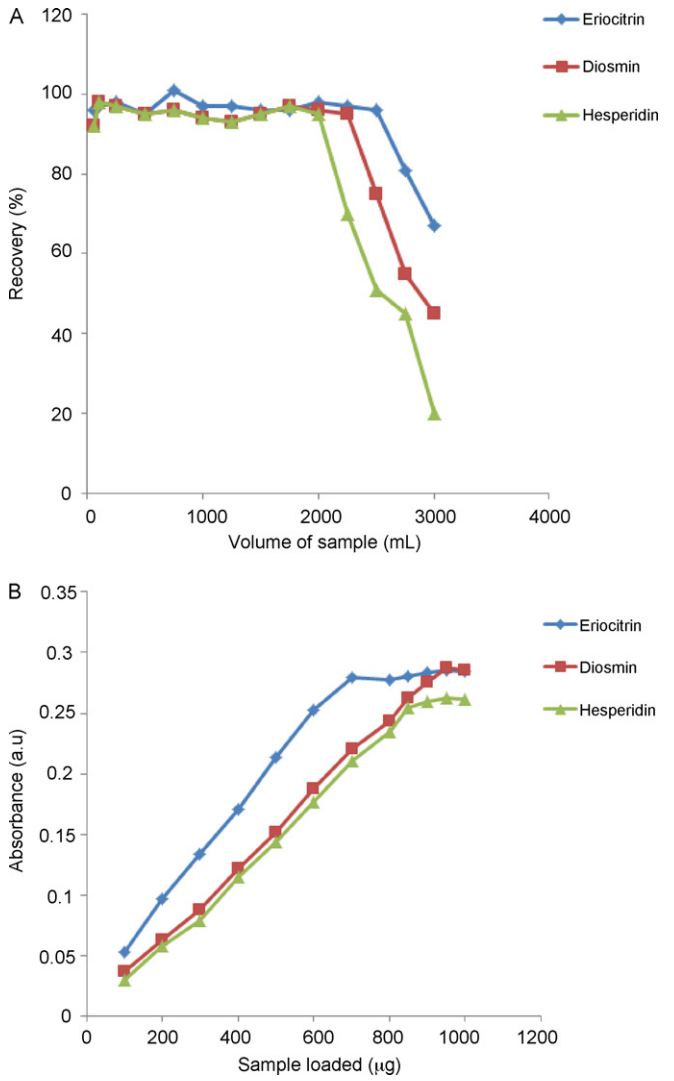


**Fig. 1.** Breakthrough volume of analytes using PA-cartridge (A) and maximum loading of PA-cartridge for adsorption of analytes (B). Conditions: sample loaded 50–3000 mL of standard solutions containing 5 μg of analytes (A) and loaded 10–100 mL of 10 ppm standards (B).

Stock solutions of hesperidin, diosmin and eriocitrin were prepared separately by dissolving appropriate amounts of the compounds in methanol/dimethyl sulfoxide (1:1) to achieve concentrations of 400 μg/mL for each compound. These solutions stored in the dark at 4 °C and were observed to be stable at least for 3 months. All solutions were filtered through 0.45 μm membrane filters (Millipore, Bedford, MA USA) prior to use. Lime juice samples were provided from different cities of Iran (Jahrom, Minab and Rudan).

### 2.2. Instrumentation

Chromatographic measurements were carried out using a Knauer HPLC system (Berlin, Germany) equipped with a K-1001 HPLC pump and a UV detector K-2800 was set at 280 nm. The other HPLC equipment included a Knauer K-1500 solvent organizer, Knauer K-500 degasser. Adjustments of pH of solutions were determined by a 3030 Jenway pH meter (Leeds, UK). Column used was C8 (25 × 4.6, 5 μm) from Capital (Broxburn, UK). The polyamide solid-phase extraction (SPE) cartridge (Chromabond PA, 3 mL/500 mg) was obtained from Macherey-Nagel (Düren, Germany). The system was equipped with Chromgate HPLC software, version 3.3.

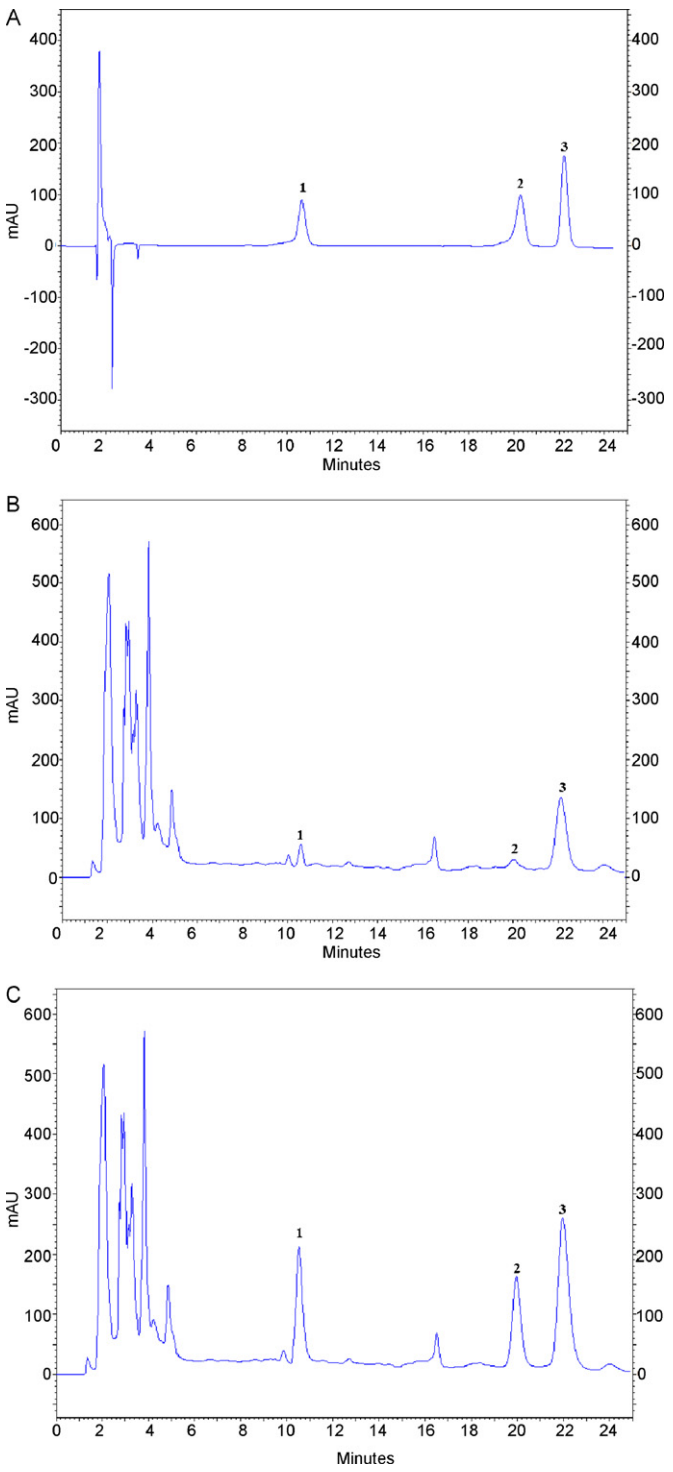


**Fig. 2.** Typical chromatograms of standards (5 μg/mL) (A), lime juice sample (B), spiked lime juice sample with 5 μg/mL (C), at optimum. 1: Eriocitrin, 2: Diosmin, 3:Hesperidin.

### 2.3. Sample preparation and solid phase extraction

Lime juice samples were directly collected from different areas of Iran without any commercial manipulation in order to avoid potential changes to occur during handling, storage and processing. Samples were stored at 4 °C in the dark prior to analysis.

Lime juice samples (5 mL) were centrifuged (4000 rpm for 10 min) and mixed with 25 mL of double distilled deionized water, adjusted at pH = 3 with concentrated HCl. The solution was filtered

**Table 1**
LOD, LOQ, linear range, squared correlation coefficient, repeatability of peak area and retention time and recovery of the method for the determination of hesperidin, diosmin and eriocitrin in lime juice.

| Analyte | LOD (μg/mL) | LOQ (μg/mL) | Linear range (μg/mL) | Squared Coefficient of determination ($R^2$) | Repeatability of peak area (RSD%) ($n$ = 8) | Repeatability of retention time (RSD%) ($n$ = 8) | % Recovery ± S.D. ($n$ = 3) |
|---|---|---|---|---|---|---|---|
| Hesperidin | 0.0512 | 0.155 | 0.0283–105.0 | 0.9928 | 2.8 | 3.2 | 94.2 ± 3.5–98.1 ± 4.2 |
| Diosmin | 0.0283 | 0.0857 | 0.0283–105.0 | 0.9987 | 3.6 | 4.7 | 93.3 ± 4.1–95.4 ± 3.9 |
| Eriocitrin | 0.0326 | 0.0987 | 0.0283–105.0 | 0.9935 | 3.3 | 3.9 | 94.8 ± 2.6–96.9 ± 5.1 |

Conditions: mobile phase water/acetonitrile/acetic acid (78:19:3, v/v); flow rate 0.8 mL/min; column C8 (25 × 4.6, 5 μm); λ 280 nm; room temperature.

**Table 2**
Content of analytes in lime juice samples under optimum condition.

| Lime juice sample | Concentration of flavonoid markers (μg/mL) ± S.D. | | | | | |
|---|---|---|---|---|---|---|
| | Instrumental | | | Hand-squeezed | | |
| | Hesperidin | Diosmin | Eriocitrin | Hesperidin | Diosmin | Eriocitrin Phosalone C Chlorpyrifos |
| Jahrom | 13.2 ± 1.6 | 96.5 ± 3.1 | 27.1 ± 2.7 | 10.4 ± 2.3 | 91.2 ± 4.6 | 22.4 ± 1.2 |
| Minab | 15.1 ± 2.4 | 87.2 ± 2.7 | 23.4 ± 2.5 | 11.8 ± 2.8 | 81.6 ± 6.1 | 18.7 ± 2.9 |
| Rudan | 19.8 ± 0.9 | 83.4 ± 4.2 | 21.6 ± 1.1 | 15.1 ± 1.5 | 77.7 ± 3.3 | 17.1 ± 2.1 |

Conditions: see Table 1.

**Table 3**
Comparison of the presented method with other methods which were used for determination of interested flavonoids.

| Method | Matrix | Analyte | LOD | LOQ | DLR | $r^2$ | Recovery | RSD% | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Centrifugation/ Dilution/HPLC-UV | Lemon | Hesperidin | – | 0.1 μg/mL | 0.25–20 μg/mL | 0.999 (r) | – | – | [12] |
| | | Diosmin | – | 0.1 μg/mL | 0.25–20 μg/mL | 0.999 (r) | – | – | |
| LLE/HPLC-DAD | *Citrus bergamia* juice | Hesperidin | 0.010 mg/mL | 0.016 mg/mL | 0.02–0.8 | 0.9999 | 93.4–98.97% | 3.13 | [16] |
| | | Eriocitrin | 0.014 mg/mL | 0.021 mg/mL | 0.02–0.8 | 0.9996 | 95.53–102.47% | 3.55 | |
| Centrifugation/ Dilution/RP-CEC | Lemon | Hesperidin | 2.5 μg/mL | 5 μg/mL | 5–200 μg/mL | 0.9989 | 71–112% | – | [17] |
| | | Eriocitrin | 2.5 μg/mL | 5 μg/mL | 5–200 μg/mL | 0.9982 | 71–112% | – | |
| SPE/HPLC-UV | Lime | Hesperidin | 0.0512 μg/mL | 0.1551 μg/mL | 0.0283–105.0 μg/mL | 0.9928 | 94.2–98.1% | 2.8 | this research |
| | | Diosmin | 0.0283 μg/mL | 0.0857 μg/mL | 0.0283–105.0 μg/mL | 0.9987 | 93.3–95.4% | 3.6 | |
| | | Eriocitrin | 0.0326 μg/mL | 0.0987 μg/mL | 0.0283–105.0 μg/mL | 0.9935 | 94.8–96.9% | 3.3 | |

through cotton to remove solid particles and then, filtered through 0.45 μm membranes. The SPE polyamide cartridge was sequentially conditioned with 5 mL of n-hexane, 5 mL of methanol and 10 mL of double distilled deionized water without allowing the cartridge to dry. The filtrate was passed through the cartridge, washed by 8 mL water/methanol (90:10 v/v, adjusted at pH = 3 with concentrated HCl) to remove interferences and eluted with 4 mL HPLC grade methanol. The eluate was dried by blowing $N_2$ stream and dissolved in 1 mL of acetonitrile, filtered through a 0.45 syringe filter and injected into the HPLC system.

### 2.4. Chromatographic conditions

HPLC separation of hesperidin, diosmin and eriocitrin was performed using a mobile phase consisting of water/acetonitrile/acetic acid (78:19:3, v/v) with a flow rate of 0.8 mL/min at room temperature. Prior to use, all mobile phases were passed through a 0.45 μm membrane filter and degassed under vacuum. The sample injection volume was 50 μL and analytes were monitored at 280 nm.

## 3. Results and discussion

### 3.1. Determination of breakthrough volume and maximum loading capacity of solid phase cartridge

Determination of breakthrough volume was performed according to the procedure presented by Hennion [20]. Standards of hesperidin, diosmin and eriocitrin (5 μg) were dissolved in 50–3000 mL of water (adjusted at pH = 3 with concentrated HCl) and passed through the cartridge.

Maximum loading capacity of analytes was determined by passing different volumes (10–100 mL) of 10 μg/mL standard (pH = 3 adjusted with concentrated HCl) through the cartridge. In both cases, the retained analytes were eluted with 4 mL methanol and dried by blowing $N_2$ stream.

Residue was dissolved in 1 mL of acetonitrile and injected into the HPLC system. Fig. 1A shows that the breakthrough volumes for hesperidin, diosmin and eriocitrin were 1850, 2250 and 2500 mL, respectively.

As illustrated in Fig. 1B, maximum loading capacities for hesperidin, diosmin and eriocitrin were 850, 950 and 700 μg, respectively.

### 3.2. Optimization of solid phase extraction

Effect of some parameters including percentage of methanol in washing solution, pH of washing solution, type and volume of elution solvent and flow rate of sample solution through the cartridge on extraction efficiency was investigated using PA-cartridge as the sorbent.

#### 3.2.1. Optimization of washing solution

For optimizing the washing solution, different percentages of methanol in water at different pH ranging from 2.5 to 7.5 were examined. According to the best recovery of analytes, the optimum

conditions for washing solution were: water/methanol (90:10 v/v), pH = 3 and 8 mL of washing solution.

*3.2.2. Selection of type and volume of elution solvent*

To obtain a suitable solvent for elution of analytes from the cartridge, different solvents such as methanol, acetonitrile, ethylacetate, diethylether, n-hexane and acetone were examined. The best elution solvent for the compounds was examined by using 5 mL of each solvent.

The best recovery of analytes was achieved by methanol. For determination of a suitable volume of the elution solvent, different volumes (2, 4, 6, 8 and 10 mL) of methanol were used for elution of retained analytes from the cartridge. The most suitable volume of elution, according to the best recovery for each analyte was 4 mL.

*3.2.3. Flow rate of sample solution through the cartridge*

Flow rate of aqueous sample solution always has a significant impact in the SPE procedure, because the sample flow rate affects both the recoveries of analytes and loading time in a SPE system. Results indicated that the best recovery of analytes in PA-cartridge was achieved at a flow rate of 1 mL/min.

### 3.3. Method Validation

Limit of detection (LOD), limit of quantification (LOQ), linear range, recovery, repeatability of retention times and peak areas for hesperidin, diosmin and eriocitrin were determined after 8 injections and are shown in Table 1. Results showed that the proposed method had good repeatability for determination of analytes in lime juice samples.

The optimum SPE conditions were evaluated in the analysis of real samples. The recovery percentages of the analytes in lime juice samples were measured by means of spiking at the same condition of extraction. The lime juice sample was spiked with 3 fortification levels of hesperidin, diosmin and eriocitrin, 0.0500, 5.00 and 50.0 μg/mL.

Method precision was determined by 5 replicate measurements for hesperidin, diosmin and eriocitrin in lime juice using a standard addition method ($n = 5$). Flavonoids were identified by spiking the sample with standards and by comparison with their retention times.

### 3.4. Determination of hesperidin, diosmin and eriocitrin in lime juice

Three different lime juice samples from different cities of Iran (Jahrom, Minab and Rudan) were prepared in October and November 2010. The concentration of hesperidin, diosmin and eriocitrin in lime juice samples were determined by 5 replicate measurements using standard addition method ($n = 5$). Table 2 presents the concentration of these compounds found in instrumental and hand-squeezed lime juice samples. Representative chromatograms after injection of standards, lime juice and spiked lime juice acquired at optimum mobile phase conditions are presented in Fig. 2.

## 4. Conclusion

In this work, determination of three flavonoids in lime juice was performed using SPE with a PA-cartridge and HPLC. SPE parameters (selection of a suitable solvent and its volume for elution of analytes, maximum loading capacity of PA-cartridge and breakthrough volume) and HPLC conditions (percentage of organic modifier, pH of mobile phase and temperature) were optimized. The use of PA-cartridge allowed for a fast sample treatment with low solvent consumption, good clean up and high recovery above 90% in lime juice samples. The method showed good sensitivity, linearity and repeatability. This method was found to be suitable for quality control and routine analysis of adulterated lime juice samples.

The method was compared with many techniques used for determination of interest analytes in different samples, and the results were shown in Table 3. One can see that comparable results were achieved for this method versus other conventional methods and without the use of advanced instruments such as LC–MS, LC–MS–MS or GC–MS.

## Acknowledgments

The authors are grateful to the financial support of institute of standard and industrial research of Iran (Khorasan-e-Razavi province). The authors also would like to special thank Mr. A. Gharah Baghi, M. Asgharian Rezaei, Institute of standard and industrial research of Khorasan-e-Razavi, Iran and Mr. M. Ferdowsi, IT department of institute of standard of khorasan-e-Razavi. This study was also financially supported by a grant (88398) from Mashhad university of medical science research council.

## References

[1] M. Sato, N. Ramarathnam, Y. Suzuki, T. Ohkubo, M. Takeuchi, H. Ochi, Varietal differences in the phenolic content and superoxide radical scavenging potential of wines from different sources, J. Agric. Food Chem. 44 (1996) 37–41.
[2] N.C. Cook, S. Samman, Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources, J. Nutr. Biochem. 7 (1996) 66–76.
[3] F.D. Dakora, Plant flavonoids: biological molecules for useful exploitation, J. Plant Physiol. 22 (1995) 87–99.
[4] A. Das, J.H. Wang, E.J. Lien, Carcinogenicity, mutagenicity and cancer preventing activities of flavonoids: a structure–system–activity relationship (SSAR) analysis, Prog. Drug Res. 42 (1994) 133–166.
[5] R. Ficarra, P. Ficarra, S. Tommasini, M.L. Calabro, S. Ragusa, R. Barbera, A. Rapisarda, Leaf extracts of some Cordia species: analgesic and anti-inflammatory activities as well as their chromatographic analysis, Farmaco 50 (1995) 245–256.
[6] G. Gattuso, D. Barreca, C. Gargiulli, U. Leuzzi, C. Caristi, Flavonoid Composition of Citrus Juices, Molecules 12 (2007) 1641–1673.
[7] A.M. El-Shafae, M.M. El-Domiaty, Improved LC methods for the determination of diosmin and/or hesperidin in plant extracts and pharmaceutical formulations, J. Pharm. Biomed. Anal. 26 (2001) 539–545.
[8] S. Carando, P.L. Teissedre, L. Pascual-Martinez, J.C. Cabanis, Levels of flavan-3-ols in French wines, J. Agric. Food Chem. 47 (1999) 4161–4166.
[9] Z.F. Katona, P. Sass, I. Molnar-Perl, Simultaneous determination of sugars, sugar alcohols, acids and amino acids in apricots by gas chromatography–mass spectrometry, J. Chromatogr. A 847 (1999) 91–102.
[10] J. Londoño-Londoño, V.R. Lima, O. Lara, A. Gil, T.B.C. Pasa, G.J. Arango, J.R.R. Pineda, Clean recovery of antioxidant flavonoids from citrus peel: optimizing an aqueous ultrasound-assisted extraction method, J. Food Chem. 119 (2010) 81–87.
[11] A. Molinelli, R. Weiss, B. Mizaikof, Advanced solid phase extraction using molecularly imprinted polymers for the determination of quercetin in red wine, J. Agric. Food Chem. 50 (2002) 1804–1808.
[12] F.I. Kanaze, C. Gabrieli, E. Kokkalou, M. Georgarakis, I. Niopas, Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations, J. Pharm. Biomed. Anal. 33 (2003) 243–249.
[13] C. Caristi, E. Bellocco, V. Panzera, G. Toscano, R. Vadala, U. Leuzzi, Flavonoids detection by HPLC-DAD-MS–MS in lemon juices from Sicilian cultivars, J. Agric. Food Chem. 51 (2003) 3528–3534.
[14] M.L.M. Bilbao, C. Andrés-Lacueva, O. Jáuregui, R.M. Lamuela-Raventós, Determination of flavonoids in a Citrus fruit extract by LC-DAD and LC–MS, J. Food Chem. 101 (2007) 1742–1747.
[15] S.A. Ross, D.S. Ziska, K. Zhao, M.A. ElSohly, Variance of common flavonoids by brand of grapefruit juice, Fitoterapia 71 (2000) 154–161.
[16] M.L. Calabrò, V. Galtieri, P. Cutroneo, S. Tommasini, P. Ficarra, R. Ficarra, Study of the extraction procedure by experimental design and validation of a LC method for determination of flavonoids in *Citrus bergamia* juice, J. Pharm. Biomed. Anal. 35 (2004) 349–363.
[17] C. Desiderio, A. De Rossi, M. Sinibaldi, Analysis of flavanone-7-*O*-glycosides in citrus juices by short-end capillary electrochromatography, J. Chromatogr. A 1081 (2005) 99–104.
[18] E. Rijke, P. Out, W.M.A. Niessen, F. Ariese, C. Gooijer, U.A.T. Brinkman, Analytical separation and detection methods for flavonoids, J. Chromatogr. A 1112 (2006) 31–36.
[19] A.J. Dick, P.R. Redden, A.C. DeMarco, P.D. Lidster, T.B. Grindley, Flavonoid glycosides of spartan apple peel, J. Agric. Food Chem. 35 (1987) 529–534.
[20] M.C. Hennion, Sample Handling and Trace Analysis of Pollutants Techniques, Application and Quality Assurance, Elsevier, Amsterdam, 2000, pp. 3–71.