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Evaluation of matrix effect in determination of some bioflavonoids in food samples by LC–MS/MS method

Andrija Ćirić [a], Helena Prosen [b], Milena Jelikić-Stankov [c], Predrag Đurđević [a,*]

[a] University of Kragujevac, Faculty of Science, P.O. Box 60, 34000 Kragujevac, Serbia
[b] University of Ljubljana, Faculty of Chemistry and Chemical Technology, Aškerčeva 5, SI-1000 Ljubljana, Slovenia
[c] University of Belgrade, Faculty of Pharmacy, V. Stepe 450, 11000 Belgrade, Serbia

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ABSTRACT

In the present work the LC–MS/MS method with solid phase extraction for simultaneous determination of bioflavonoids rutin, quercetin, hesperidin, hesperetin and kaempferol in some food samples (red onion, orange peel and honey) was developed and the matrix effect accompanying this determination was quantified.

The matrix effect evaluated using a postextraction addition method was found to be negative in the range −44 to −0.5%, indicating ionization suppression and strongly depended on bioflavonoid concentration. The observed matrix effect was explained taking into account the co-elution of phenolic acids, in terms of their acid–base and hydrophilic properties. The efficacy of extraction expressed as the absolute recoveries of flavonoids were 88–96%, indicating very good efficiency of extraction.

The extracts of food samples obtained either by Soxhlet or ultrasonic extraction were analyzed for bioflavonoid content by the LC–MS/MS method in selected reaction monitoring mode using a triple quadrupole detector and standard addition method, which was found to be the most suitable calibration approach for these samples. The optimized separation was achieved on a Phenomenex Gemini C18 column with gradient elution and mobile phase composition A: 2% acetic acid in water and B: acetonitrile. R_s values were in the range from 1.3 to 3.1, indicating good selectivity of the method. The obtained results (mg/100 g fresh weight) for different bioflavonids were for rutin 0.16, for quercetin in the range 0.65–56, for hesperidin 0.016–24, for hesperetin 0.0068–36.4 and for kaempferol 0.14–1.63 and generally show good agreement with published data. Low detection limits (0.014–0.063 μg/mL) were obtained with acceptable recoveries (86–114%). Total time of analysis was less than 40 min, therefore the proposed method represents significant improvement over existing methods.

1. Introduction

Flavonoids are a large class of phenolic compounds which are subclassified as flavones, flavonols, isoflavones, flavanones and catechins, chalcones and anthocyanidins depending on phenyl substituent in the C_2 or C_3 position in benzo-γ-pyrone nucleus. Interest in the bioflavonoids is related to their diversity, biological significance as secondary plant metabolites and ecological role [1], use as chemotaxonomic markers [2], impact on fruit quality [3], physiological effects [4–6] and industrial applications [7].

The flavonoids are potent antioxidants, free radical scavengers [8] and metal chelators; they inhibit lipid peroxidation [9] and exhibit various physiological activities [10–15], including anti-inflammatory [16], anti-allergic, anti-carcinogenic, antihypertensive and anti-arthritic activities [17].

Various methods have been developed for the determination of bioflavonoids and reviewed: capillary electrophoresis [18], thin-layer chromatography [19], gas-chromatography [20], high-performance liquid chromatography with UV/visible, fluorescence detection [21,22], and electrochemical detection modes [23].

HPLC techniques are now the most widely used both for separation and quantitation of phenolic compounds [24]. LC–MS and in particular LC–MS/MS methods have been recognized [25] as the best tool to analyze samples of biological origin due to their selectivity, sensitivity and speed of analysis.

Red onion and honey are food samples which are rich with bioflavonoids and constitute common part of the everyday diet [26,27]. Orange peel is not used for food but it is recommended as a flavoring agent to improve taste because it is rich in bioflavonoids [28].

Bioflavonoids narirutin, hesperidin, didymin, diosmin, sinensetin, nobiletin, tangeretin, quercetin, kaempferol, myricetin, luteolin, apigenin quercetin 3-glucoside, quercetin 7,4'-diglucoside, quercetin 3,7,4'-triglucoside, isorhamnetin 4'-glucoside and isorhamnetin

* Corresponding author. Tel.: +381 34 300261; fax: +381 34 335040.
E-mail address: preki@kg.ac.rs (P. Đurđević).

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3,4'-diglucoside were analyzed in orange peel [29–32], red onion [33–44] and honey [45–50] employing HPLC–MS/MS techniques. The content of bioflavonoids (in mg/100 g fresh weight) in orange peel for hesperetin, hesperidin and quercetin were in the range 2.2–67 with LOD 0.03–0.4 μg/mL [28–32], for hesperetin, hesperidin, quercetin, rutin and kaempferol were in the range 0.001–192 with LOD 0.0004–5 μg/mL in red onion [26,33–44] and finally, for hesperetin, hesperidin, quercetin and kaempferol in honey were in the range 0.02–26 with LOD 0.01–3 μg/mL [27,45–50]. Main problems encountered in the analysis of these samples are ionization suppression or enhancement depending on "visible" and "invisible" matrix interferences. It was supposed that the matrix effect was eliminated by sample extraction and clean-up using SPE. The matrix effect can be a serious problem as it could severely compromise quantitative analysis of the compounds at trace levels as well as method reproducibility [51]. Little is known about the matrix effect due to co-eluting substances in red onion, orange peel and honey sample extracts. The matrix effect in determination of bioflavonids in food samples was not studied to the best of our knowledge. Knowing the source and level of the matrix effect possibly false results in determination of bioflavonoids content in food samples could be eliminated.

Matrix effects in LC–MS analysis occur when molecules co-eluting with the compound/s of interest (analytes) alter the ionization efficiency of the electrospray interface. A matrix effect is defined as a change in the analytical signal caused by anything else in the sample other than analyte. The influence of matrix effect on the reliability of LC–ESI–MS/MS method was investigated in terms of trueness and precision of determination and it has been shown that when ionization suppression occurs, the sensitivity and limit of quantification of the method may be adversely affected [52].

The most effective way to eliminate matrix effect affecting trueness and precision of the analytical method is to use the standard addition technique [53]. Standard addition is especially appropriate when the sample composition is unknown or complex and affects the analytical signal. If small volume of concentrated standard is added to the unknown the concentration of the matrix will not be significantly changed. The assumption in standard addition method is that the matrix has the same effect on added analyte as it has on the original analyte in the unknown.

The aim of this study was to develop an optimized HPLC method in terms of resolution ($R_s \geq 1.5$), analysis time and selectivity which could be used in food quality control laboratories and nutritional and pharmaceutical research. As a part of the method development, the matrix effect was studied to establish the dependence of MS/MS response on type of sample and solvent used for extraction. In addition the purpose of this work was to evaluate the degree of the signal suppression by co-eluting substances in food samples extracts to correct matrix effect by appropriate adjustment of LC–MS/MS parameters. The data presented in this paper clearly demonstrate that the study of matrix effect should constitute an integral and important part of quantitative determination of bioflavonoids in food samples.

First the matrix effect on LC–ESI–MS/MS determination of bioflavonoids (rutin, hesperidin hesperetin, quercetin and kaempferol) in some food samples (red onion, orange peel and honey) was recorded. Since the main source of matrix effect arises from extraction procedure we paid special attention to the extraction of analytes [54].

The extraction methodology for flavonoids generally includes extraction by solvents such as methanol, ethanol, acetone or mixture of solvent with water, cleaning-up and further fractionation by liquid–liquid extraction, column chromatography and solid-phase extraction. According to the literature [55–57], an ultrasonic bath at room temperature is a suitable extraction method for flavonoids. The ultrasonic and Soxhlet extraction were compared since it is widely accepted that Soxhlet extraction of flavonoids yields 100% recovery [55,56]. For evaluation of the extent of matrix effect the postextraction addition method was used. In an attempt to optimize a method for a simultaneous determination of five flavonoids, we developed a clean-up of analytes from food sample matrix prior to LC–MS/MS analysis. Bearing in mind the polar nature of the analytes a commercially available Supelco LC-18 end-capped SPE cartridge was used for clean-up of bioflavonoids from the food matrix. The use of reversed-phase cartridge effectively eliminated the interfering material with efficient extraction of flavonoids. Analytes from the extract were separated by HPLC using C18 column and gradient elution with ESI–MS/MS detection. Quantification was performed using the standard addition method.

Bearing in mind important beneficial effects of bioflavonoids on human health, the results obtained in the present study may be of interest not only to analytical chemists but also to food chemists and nutritionists.

2. Experimental

2.1. Materials and solutions

Rutin hydrate (>95%), hesperidin (>85%), hesperetin (>80%), quercetin (>99%), kaempferol (>95%) and caffeine (>99%) were from Sigma-Aldrich (Vienna, Austria), acetic acid, acetonitrile and methanol (HPLC grade purity) were from JT Baker (Deventer, Holland). Water was obtained from a Millipore Milli-Q system (Watford, UK).

2.2. Preparation of stock and sample solutions

1.00 mg/mL rutin, hesperidin, hesperetin, quercetin and kaempferol standards in methanol were prepared. Working calibrators (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0) μg/mL were prepared by appropriate dilution of these standard stock solutions with methanol. For standard addition method five solutions containing (10.0, 20.0, 40.0, 100.0 or 200.0) μg/mL of each flavonoid in methanol were prepared. Extracted samples were spiked with each of these solutions. Three food matrices were selected for the bioflavonoids analysis—red onion, orange peel and honey. Samples were purchased from an organic firm grocery. For sample treatment, the procedure of Vacek et al. [57] was followed. Samples were cut into small pieces and chopped. Water, methanol and acetonitrile extracts were prepared by extracting 10 g of the material with corresponding pure solvents. Honey was thoroughly mixed until a homogeneous sample was obtained. 10 g of sample was quantitatively transferred to beaker with water. Extraction with acetonitrile could not be performed due to formation of insoluble viscous material.

2.3. Soxhlet extraction

The ground powder of sample (5 g) and 50 mL of methanol were placed into the capsule. The extraction was performed for 120 min at 60 °C. After 120 min the extract was separated from the remaining material to which a new portion of 50 mL of methanol was added and extraction was continued for 60 min until completeness. The combined extract was evaporated in rotary vacuum evaporator to the volume of 5 mL and passed through a 0.45 μm microporus membrane filter; the filtrate was diluted with methanol in 10 mL normal flask to the mark and used for HPLC analysis. All extraction procedures were performed in triplicates for statistical analysis.

2.4. Ultrasonic extraction

The ground powder of sample (2.5 g) and 50 mL of methanol as solvent was first loaded into a 250 ml beaker and sealed by plastic film to avoid loss of solvent. The sample beakers were immersed into the ultrasonic cleaning bath for ultrasonication. After 30 min of extraction the extract was separated from the rest of the solid material on which was added a new portion of 50 mL of methanol and extraction was continued for 15 min. The combined extract was evaporated in rotary vacuum evaporator to the volume of 5 mL and passed through a 0.45 μm microporus membrane filter; the filtrate was diluted with methanol in 10 mL normal flask to the mark and used for HPLC analysis. All extraction procedures were performed in triplicates for statistical analysis.

The results shown in Fig. 1 indicate that no significant difference is found in extraction efficiency between Soxhlet and ultrasonic bath extraction method. Owing to its simplicity and rapidity, the ultrasonic bath extraction was chosen as the preferred method.

2.5. Development of the solid phase extraction procedure

The solid phase extraction was optimized in terms of cartridge and eluting solvent. To develop the most efficient SPE method five different cartridges (LC-C8, LC-SAX, LC-18, LC-NH_2 and DSC-NH_2) of different loading polarity, from the same manufacturer (Supelco, USA) were tested. The cartridges were pre-conditioned with 5 mL of tested solvent (methanol, acetonitrile or water) followed by 5 mL of deionized water. The standard solution of analyte mixture (0.1–0.3 mL) was diluted with water to 10 mL and this solution was forced through the cartridge at a flow rate of 1 mL/min. After loading, the SPE cartridge was washed with 5 mL of water and subsequently dried by vacuum drying at room temperature for 10 min. In this way excessive and residual water was removed from the cartridge. Finally, the analytes were eluted with 5 mL of tested solvents. The optimal extraction conditions in terms of efficacy and elution time was obtained with methanol as eluting solvent. The extracts obtained in this way were chromatographed and the efficiency of the method was calculated as ratio between peak areas of the standard solution before and after solid phase extraction multiplied by 100. The obtained recoveries ($\pm$standard deviation, SD) for extraction of flavonoids with different types of cartridges are presented in Table 1.

It may be seen from Table 1 that the best recoveries were obtained with Supelco LC-18 cartridge, so for further SPE we used this cartridge.

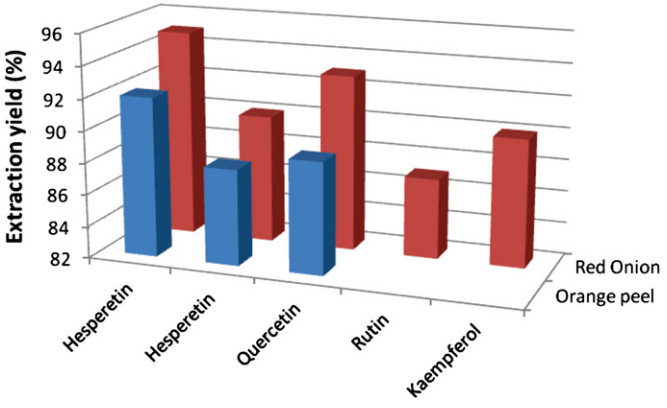

Fig. 1. Extraction yield of bioflavonoids from orange peel and red onion compared (normalized) to Soxhlet extraction.

Table 1
Recoveries $\pm$ SD (%) of flavonoids obtained with different SPE cartridges.

Bioflavonoids	Supelco cartridges				
	LC-18	ENVI-18	DSC-18	SepPak Plus C18	DSC-SAX
Quercetin	97 ± 2	77 ± 2	54 ± 1	70 ± 2	74 ± 1
Rutin	95 ± 1	66 ± 2	48 ± 2	72 ± 2	68 ± 2
Hesperetin	96 ± 2	86 ± 2	54 ± 2	64 ± 2	65 ± 1
Hesperidin	96 ± 1	95 ± 1	42 ± 1	61 ± 2	58 ± 2
Kaempferol	93 ± 4	95 ± 1	48 ± 2	67 ± 2	61 ± 2

2.6. Instruments

The separation was carried out using an HPLC system Perkin Elmer PE200 (Norwalk, CT, USA), composed of binary pump, autosampler and UV/VIS DAD detector. The mass spectrometer was 3200 QTRAP MS/MS (Applied Biosystems/MDS Sciex, USA) with electrospray ionization (ESI). The data were processed using an Analyst (PE Sciex) software. HPLC column was Phenomenex Gemini C18 (150 × 4.6 mm, 3 μm particle size), Phenomenex, Torrance, USA. Solid phase extraction (SPE) was carried out with a Supelco (Bellefonte, PA, USA) vacuum tank on Supelco LC-18 cartridges.

Ultrasonic extraction experiments were carried out in ultrasonic cleaning baths produced by Elma Hans Schmidbaner GmgH and Co. KG Singen, Germany, which can work at 20 kHz, 60 kHz, and 100 kHz frequencies with a variable power output, and have a digital timer to set up time and a temperature controller..

2.7. LC–MS/MS conditions

All MS and MS/MS data were collected in negative ion ESI mode. Both quadrupoles (Q1 and Q3) were operated at unit resolution. To establish the optimum ESI conditions infusion of individual rutin, quercetin, hesperidin, hesperetin and kaempferol standard solutions was performed. Potentials were chosen to obtain the maximum resolution and intensity of the signals, clean spectral area as well as minimal background emission. The measurements were made at a 500 °C source temperature, −4500 V ion spray voltage, 300 V focusing potential. The assay is based on monitoring the $[M–H]^-$ ions for the analytes in the first quadrupole and their corresponding product ions in the third quadrupole with a dwell time of 50 ms. Selected reaction monitoring data were collected using a Sciex Analyst software. Working parameters and other instrumental parameters were manually adjusted to get the best performance from the instrument. Identification of precursor and product ions and MS/MS optimization were established by direct infusion of 100 μg/mL solutions of single analyte in methanol. Infusion was made by a syringe pump. Typically, flow rate was 10 μL/min. The manual tuning of the instrument comprised the optimization of resolution, sensitivity and calibrating mass scale. Mass scale calibration was accomplished by 100 μg/mL of caffeine solution as calibration standard. Optimal experimental conditions are listed in Table 2.

For the LC separation the mobile phase composed of A: 2 wt% of acetic acid in water and B: acetonitrile was used. The solvents were mixed in a linear gradient: 0 min–85% A and 15% B, 5 min–85% A and 15% B, 25 min–10% A and 90% B, 30 min–10% A and 90% B, 35 min–85% A and 15% B, 40 min–85% A and 15% B; flow rate of the mobile phase was 0.7 mL/min, injected volume 20 μL.

3. Results and discussion

For quantitative LC-ESI-MS/MS the negative ionization mode was selected because of improved sensitivity due to the presence

Table 2
Optimal instrumental conditions for the analytes in ESI–MS/MS.

	Hesperetin	Hesperidin	Rutin	Quercetin	Kaempferol
GS1 (gas 1) the nebulizer gas (mL/min)			20		
GS2 (gas 2) the auxiliary gas (mL/min)			60		
TEM (temperature) (°C)			500		
CUR (curtain gas)			10		
Ion spray voltage (V)			−4500		
DP (declustering potential) (V)	−40	−57	−86	−21	−22
EP (entrance potential) (V)	−5	−5	−8	−8	−3
CE (collision energy) (eV)	−25	−34	−56	−31	−45
CXP (collision cell exit potential) (V)	−6	−9	−4	−12	−6

of hydroxyl groups which are easily deprotonated. Representative total ion chromatograms of methanol solution containing mixture of analytes and that of orange peel, red onion and honey extracts are shown in Fig. 2.

From Fig. 2a it can be seen that the investigated flavonoids are separated with resolutions $R_{s1,2}=1.3$ for the rutin and hesperidin, $R_{s2,3}=3.2$ for hesperidin and quercetin, $R_{s3,4}=2.1$ for quercetin and kaempferol and $R_{s4,5}=1.3$ for kaempferol and hesperetin. Peaks are resolved at a baseline so that conditions for good chromatography are fulfilled. The assignment of peaks in chromatograms of food samples was done on the basis of retention times and mass spectra corresponding to peak maxima by comparison with corresponding standards.

The matrix effect on simultaneous determination of quercetin, rutin, hesperetin, hesperidin, and kaempferol in food samples extracts (orange peel, red onion and honey) by the LC–ESI–MS/MS method was quantified. The efficient clean-up of analytes from the samples extracts was achieved by Supelco LC18 cartridges yielding the extracts free of particulate matter and endogenous interfering material.

3.1. Mass spectra and total ion chromatograms

In all analytes the precursor ion $[M–H]^-$ where M is the molecular mass of the respective analyte, is formed as a result of the loss of a proton to form a negatively charged molecular ion. The base peak and relative abundance in percents is for quercetin m/z 301 (100%), rutin m/z 609 (100%), hesperetin m/z 301 (100%), hesperidin m/z 609 (100%), and kaempferol m/z 285 (100%). Since the values of m/z are the same for rutin and hesperidin and for hesperetin and quercetin, further fragmentation (i.e. MS/MS mode) were employed for their identification and quantitation. The precursor and major product ions and relative abundance in percents of the analytes were monitored in selected reaction mode (SRM) as follows: quercetin 301→179 (63%), 301→151 (95%), 301→107 (31%), 301→97 (36%), rutin: 609→301 (12%), 609→273 (44%), 609→257 (24%), 609→179 (31%), hesperetin: 301→286 (37%), 301→244 (25%), 301→179 (34%), 301→151 (63%), hesperidin: 609→343 (45%), 609→325 (30%), 609→174 (37%), 609→151 (24%) and kaempferol: 285→256 (43%), 285→243 (29%), 285→228 (86%), 285→125 (61%). These MS/MS fragments were chosen because they the most intensive peaks in the product ion MS spectra. Possible fragmentation scheme for some analytes is given in Fig. 3.

3.2. Matrix effect evaluation

The matrix effect during the development of the analytical method may be examined by comparing MS/MS response (peak areas and heights) of an analyte in spiked sample extract with the MS/MS response of the same analyte present in the "neat" mobile phase, at several concentration levels. The relative matrix effect, ME%, is defined as the difference between the MS/MS response of an analyte present in the real sample extract and response from the same analyte present in the "neat" mobile phase or a solvent, but without the compounds extracted from a real sample

$$\mathrm{ME}(\%)=\frac{\text{peak area of post extraction}-\text{peak area of pure solution}}{\text{peak area of pure solution}}\times 100 \quad (1)$$

To investigate the influence of matrix effect on the determination of some bioflavonoids by post-extraction method, spiking of matrix with suitable concentrations (0.5 to 5.0 μg/mL) of analytes was employed (four concentration levels). First, water extract of food sample was prepared, then extracts were passed over the SPE cartridge, resulting in almost complete adsorption of bioflavonoids. Remaining isolated matrix was then spiked with known concentrations of bioflavonoids and resulting matrix effect, calculated using Eq. (1). The obtained results are given in Table 3.

The negative matrix effect represents a loss of the analytical signal (ion suppression) due to alterations in ionization efficiency. By inspecting the Table 3 it may be concluded that process efficiency is sufficiently high so that trueness and LOD may be obtained with satisfactory degree. The matrix effect decreases with increasing concentration of hesperetin and increases with increasing concentration of hesperidin. The matrix effect was generally much lower in honey than in other food samples. It is also less pronounced for the most intensive peaks in MS/MS spectra for all bioflavonoids. We tried to increase collision energy to optimize the formation of product ions. However, only a large number of fragments was obtained without improvement in the sensitivity of the assay.

To investigate the influence of solvents on the amount of co-eluting substances originating from the real sample extracts and on the MS responses, the standard solution of flavonoids was spiked into the extract of samples obtained with different solvents (methanol, acetonitrile and water). Matrix effect was calculated from Eq. (2)

$$\mathrm{ME}(\%)=\frac{\text{peak area from standard addition}-\text{peak area from calibration curve}}{\text{peak area from calibration curve}}. \quad (2)$$

The results are given in Table 4.

Increased ionization suppression was seen with methanol as compared to water for honey samples while in orange and onion the matrix effect was almost the same for different solvents. The ionization suppression was much less pronounced in the post-extraction addition than in the standard addition method (compare with Table 3). Postextraction addition as a calibration approach, however, is feasible only with closely matrix-matched extract without the analytes, which could be difficult to obtain. It was therefore decided to rely on standard addition procedure as a calibration method, which, although time-consuming, is

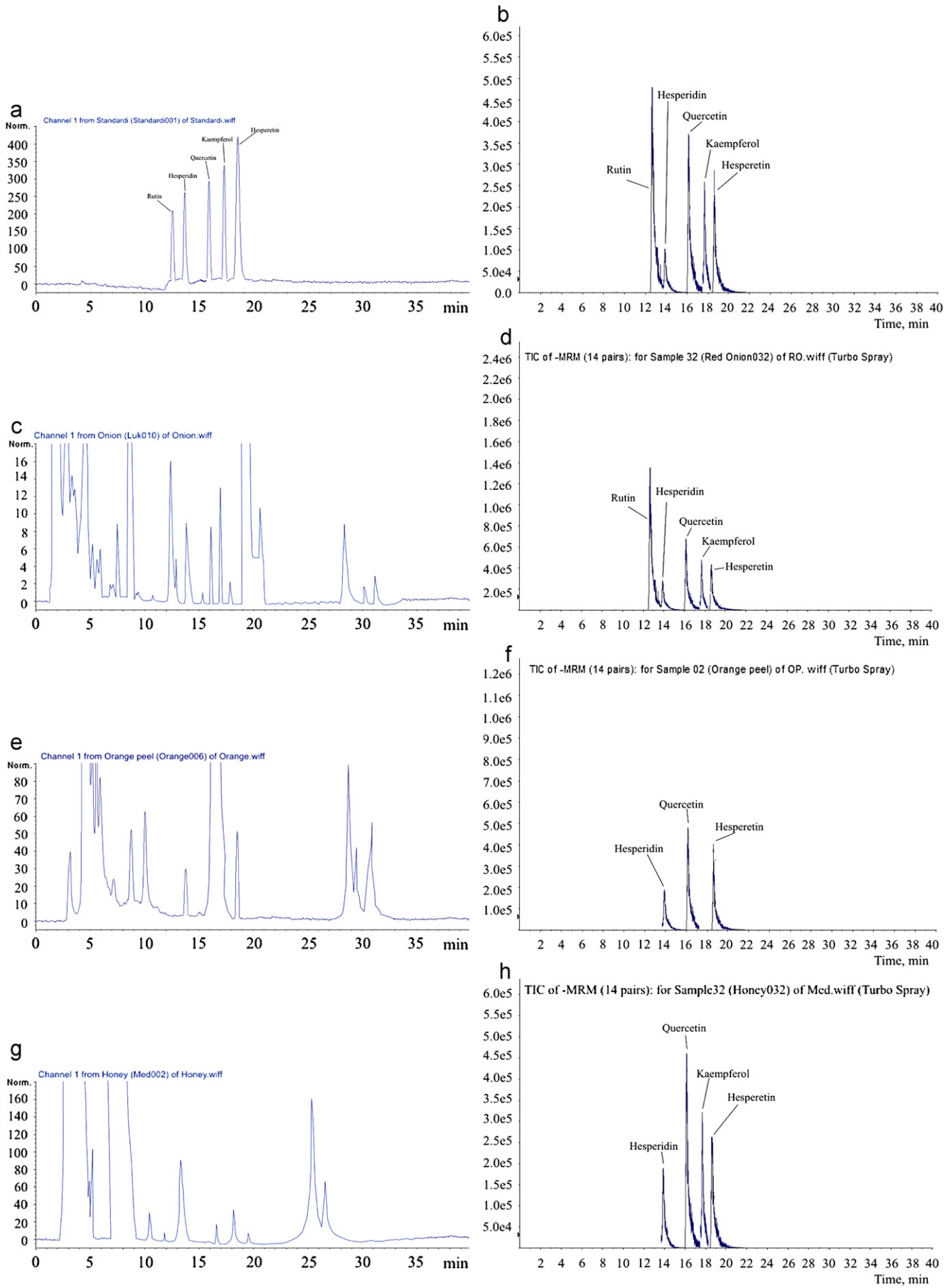

Fig. 2. (a) HPLC/UV chromatograms for bioflavonoid standards, (b) TIC (total ion chromatogram) of standards, (c) HPLC/UV chromatogram of red onion extract, (d) TIC red onion extract, (e) HPLC/UV chromatogram of orange peel extract, (f) TIC of orange peel extract and (g) HPLC/UV chromatogram of honey extract and (h) TIC of honey extract.

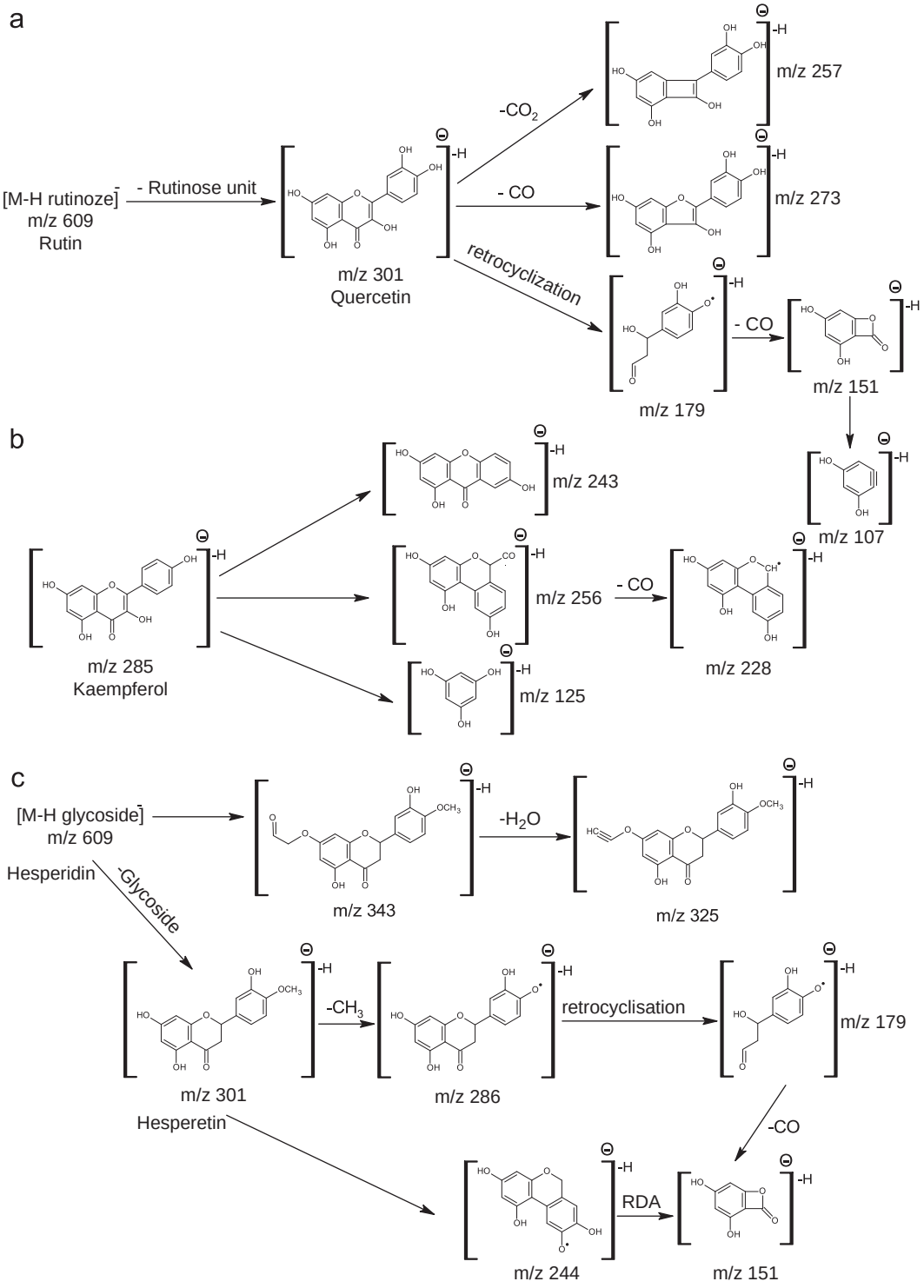

Fig. 3. Proposed fragmentation pathway for (a) rutin and quercetin (b) kaempferol and (c) hesperidin and hesperetin.

recommended as the most reliable in the analysis of samples with many interfering compounds [53].

Our method for preparation and clean-up of samples, did not result in a scrupulously clean extract. The method failed to sufficiently remove endogenous compounds such as polyphenolic acids and phospholipids from analytes. It is a result of the effort to achive acompromise between high recovery of the analytes and low co-extraction of endogenous substances. Coelution of these compounds with the compounds of interest is the main source of matrix effect.

Coelution is unavoidable bearing in mind that retention of organic analytes (polyphenolic acids, bioflavonoids and biomacromolecules) from samples onto the SP material is determined primarily by hydrophobic interactions between nonpolar parts of biomolecules contained in extracts and hydrocarbon C18 chain from silica surface. Thus, hydrophobicity of molecules present in extract will play dominant role in their retention onto the SP cartridge material. Since, silica surface of C18 sorbent also contains some remaining OH groups, hydrogen bonding between ionized hydroxyl groups of SP material and carboxyl groups

Table 3
Matrix effect on determination of bioflavonoids extracted in H_2O by postextraction method in food samples.

	Conc. (μg/mL)	SRM transition	0.5 (ME ± SD) (%)	1.0 (ME ± SD) (%)	2.5 (ME ± SD) (%)	5.0 (ME ± SD) (%)
Orange peel	Hesperetin	301/286	−33 ± 8	−30 ± 2	−28 ± 1	−24.45 ± 0.03
		301/244	−41 ± 2	−40 ± 2	−38 ± 1	−30.8 ± 0.7
		301/179	−41 ± 6	−39 ± 2	−39 ± 2	−30.2 ± 0.9
		301/151	−36 ± 3	−35 ± 3	−34 ± 1	−29 ± 2
	Hesperidin	609/343	−40.3 ± 0.2	−36.84 ± 0.06	−30.1 ± 0.5	−21.9 ± 0.4
		609/325	−43.4 ± 0.2	−36.00 ± 0.09	−32.8 ± 0.1	−23.9 ± 0.1
		609/174	−43 ± 1	−38.3 ± 0.7	−34 ± 1	−23.5 ± 0.5
		609/151	−39.4 ± 0.2	−35.4 ± 0.2	−29.9 ± 0.6	−23 ± 2
	Quercetin	301/179	−27 ± 2	−22 ± 5	−18 ± 3	−16 ± 2
		301/151	−26 ± 2	−23 ± 4	−17 ± 2	−14 ± 1
		301/107	−29 ± 3	−24 ± 3	−17 ± 2	−15 ± 2
		301/97	−29 ± 3	−25 ± 4	−18 ± 3	−15 ± 2
Honey	Hesperetin	301/286	−10 ± 1	−8 ± 1	−6 ± 2	−5 ± 2
		301/244	−13 2	−11 ± 3	−9 ± 1	−6 ± 4
		301/179	−12 ± 3	−7 ± 2	−6 ± 2	−6 ± 1
		301/151	−4 ± 3	−3 ± 2	−3 ± 2	−0.6 ± 0.3
	Hesperidin	609/343	−15 ± 5	−8 ± 3	−6 ± 2	−3 ± 1
		609/325	−23 ± 2	−16 ± 2	−8 ± 3	−8 ± 2
		609/174	−15 ± 2	−10 ± 5	−5 ± 2	−4 ± 2
		609/151	−25 ± 2	−20 ± 2	−9 ± 4	−5 ± 3
	Quercetin	301/179	−16 ± 4	−10 ± 2	−6 ± 3	−4 ± 1
		301/151	−16 ± 3	−11 ± 2	−7 ± 2	−4 ± 2
		301/107	−16 ± 2	−11 ± 2	−7 ± 3	−3 ± 1
		301/97	−16 ± 3	−10 ± 2	−7 ± 3	−4 ± 2
	Kaempferol	285/256	−11 ± 2	−8 ± 3	−6 ± 1	−3 ± 2
		285/243	−11 ± 2	−8 ± 3	−6 ± 1	−2 ± 2
		285/228	−13 ± 2	−8 ± 3	−6 ± 2	−3 ± 3
		285/125	−11 ± 2	−8 ± 4	−5 ± 1	−3 ± 3
Red onion	Hesperetin	301/286	−9.3 ± 0.4	−7.4 ± 0.7	−5 ± 2	−1 ± 5
		301/242	−11 ± 1	−10 ± 1	−8 ± 1	−3.0 ± 0.4
		301/174	−13 ± 4	−12.2 ± 0.6	−10 ± 1	−6 ± 6
		301/150	−6 ± 2	−3 ± 2	−2 ± 2	−1.0 ± 0.8
	Hesperidin	609/343	−14 ± 2	−8 ± 4	−5.66 ± 0.05	−5 ± 3
		609/325	−23 ± 7	−16.10 ± 0.03	−8.1 ± 0.9	−7.8 ± 0.2
		609/174	−48 ± 7	−10 ± 2	−9 ± 2	−5 ± 1
		609/151	−21 ± 5	−16 ± 7	−7 ± 3	−5 ± 1
	Quercetin	301/179	−22 ± 2	−17 ± 2	−11 ± 3	−5 ± 4
		301/151	−23 ± 5	−16 ± 5	−12 ± 4	−5 ± 3
		301/107	−22 ± 6	−15 ± 4	−12 ± 4	−5 ± 4
		301/97	−23 ± 4	−14 ± 4	−11 ± 2	−5 ± 2
	Rutin	609/300	−17.2 ± 0.6	−15 ± 4	−10 ± 3	−6.4 ± 0.3
		609/271	−15 ± 2	−12.6 ± 0.5	−6.8 ± 0.2	−5 ± 1
		609/255	−48 ± 1	−20 ± 7	−14 ± 1	−9 ± 5
		609/179	−14 ± 4	−12 ± 4	−9 ± 1	−3 ± 2
	Kaempferol	285/256	−20 ± 4	−16 ± 3	−12 ± 2	−4 ± 2
		285/243	−19 ± 5	−15 ± 7	−11 ± 4	−5 ± 2
		285/228	−19 ± 5	−14 ± 3	−10 ± 4	−5 ± 1
		285/125	−18 ± 3	−15 ± 4	−10 ± 3	−5 ± 2

present in phenolic acids and higher fatty acids or other acidic groups from biomacromolecules also contributes to retention. Thus, SP material will adsorb and retain not only analyte molecules but also other nonpolar molecules. Compound elution from SP material with polar solvent is similar to that of RP-LC i.e. more hydrophilic compounds (e.g. phenolic acid) elute first followed by those with increasing hydrophobicity. In our case elution order is phenolic acids before flavonoids.

Other biomacromolecules (lipids, fatty acids) are retained by SPE. From the above discussion it may be concluded that the main interferences for ES ionization and chromatographic separation of bioflavanoids would arise from polyphenolic acids, so the development of the LC method should involve separation of phenolic acids from analytes.

The result presented in Fig. 4 shows that co-eluting substances cause equal suppression because of the degradation of ionization efficiency. Degradation of ionization efficiency may be linked to the pronounced tendency of phenolic acids to release proton at lower energies than bioflavonoids leading to incomplete dissociation of phenolic OH group of bioflavonoids. Also, hydrogen bond formation between carboxyl group from phenolic acids and OH from flavonoids may contribute to the effect.

A second process is linked to the increased viscosity and surface tension of the droplets produced in electrospray interface due to hydrophilic nature of phenolic acids. This could reduce capability of the analytes to be emitted in the ionized form from droplets (ion evaporation model of small ion formation in ESI) and to enrich gas phase.

3.3. Determination of bioflavonoids in food samples

The method to improve accuracy of the quantitation methods and eliminate interferences should be considered in the quantitation of bioflavonoids in food matrices. Complete removal of co-eluting substances by sample clean-up could not be achieved since in the case of our samples, the matrices are complex and different in composition from sample to sample. Consequently, even if the same extraction procedure is used for each sample, the

Table 4
Matrix effect (%) on bioflavonoids in food samples by standard addition method in different solvents.

Food sample	Compound	SRM transition	MeOH Mean ME (%) ± SD (%)	H_2O Mean ME (%) ± SD (%)	ACN Mean ME (%) ± SD (%)
Orange peel	Hesperetin	301/286	−110 ± 1	−102 ± 1	−112 ± 1
		301/244	−110 ± 1	−103 ± 1	−110 ± 1
		301/179	−108 ± 1	−103 ± 1	−110 ± 1
		301/151	−127 ± 1	−102 ± 1	−111 ± 1
	Hesperidin	609/343	−151 ± 2	−109 ± 7	
		609/325	−103 ± 3	−108 ± 1	
		609/174	−101 ± 3		
		609/151	−98 ± 4	−96 ± 2	
	Quercetin	301/179	−87 ± 2	−77 ± 3	−95 ± 3
		301/151	−85 ± 3	−74 ± 4	−93 ± 3
		301/107	−85 ± 3	−76 ± 5	
		301/97	−85 ± 2	−76 ± 4	
Honey	Hesperetin	301/286	−89 ± 2	−5 ± 6	
		301/244	−80 ± 2	−5 ± 7	
		301/179	−85 ± 1	−6 ± 5	
		301/151	−84 ± 2	−4 ± 5	
	Hesperidin	609/343	−15 ± 4	−46 ± 8	
		609/325	−19 ± 4	−42 ± 5	
		609/174	−15 ± 3	−48 ± 4	
		609/151	−12 ± 3	−44 ± 2	
	Quercetin	301/179	−76 ± 5	−8 ± 4	
		301/151	−74 ± 4	−8 ± 4	
		301/107	−76 ± 5	−8 ± 4	
		301/97	−75 ± 5	−7 ± 4	
	Kaempferol	285/256	−63 ± 3	−13 ± 6	
		285/243	−63 ± 2	−14 ± 7	
		285/228	−63 ± 4	−12 ± 7	
		285/125	−62 ± 4	−12 ± 8	
Red onion	Hesperetin	301/286	−72 ± 2	−94 ± 1	−87 ± 1
		301/244	−74 ± 1	−96 ± 1	−85 ± 1
		301/179	−75 ± 1	−95 ± 2	
		301/151	−76 ± 2		−87 ± 1
	Hesperidin	609/343	−64 ± 3		−63 ± 4
		609/325	−64 ± 2		−69 ± 1
		609/174	−64 ± 5		
		609/151	−68 ± 6		
	Quercetin	301/179	−71 ± 3	−52 ± 5	−32 ± 3
		301/151	−71 ± 4	−53 ± 4	
		301/107	−71 ± 3	−52 ± 5	−31 ± 3
		301/97	−71 ± 4	−51 ± 5	
	Rutin	609/301	−37 ± 1	−96 ± 4	−63 ± 2
		609/273			−65 ± 2
		609/257	−30 ± 6	−99 ± 5	−66 ± 9
		609/179	−34 ± 6		
	Kaempferol	285/256	−64 ± 4	−38 ± 6	−49 ± 2
		285/243	−64 ± 5	−38 ± 6	
		285/228	−61 ± 4	−39 ± 6	−47 ± 1
		285/125	−65 ± 4		

extract solution may vary between samples. This means that the degree of signal suppressions by coeluting substances also varies from sample to sample. It is concluded that it will be difficult and impractical to remove co-eluting substances completely for the reduction of the signal suppression. Thus, as the method of choice in our case is standard addition method, that is, analyzing extract solution with added known quantity of standard solution; the calculation procedure is as follows:

$$X_i = \frac{I_x}{I_{s+x}}(S_f + X_f)$$

where X_i is the amount of bioflavonoids in the extract solution; S_f is the amount of bioflavonoids spiked into the extract solution; I_x is the signal intensity of bioflavonoids in the extract solution and I_{s+x} is the signal intensity of bioflavonoids in the spiked solution.

This method requires at least two LC–MS runs per analysis—the run of the extract sample and the run of the extract samples spiked with a known quantity of bioflavonoids.

3.3.1. Method performance and validation

After having developed a purification procedure for a given compound, a specific mass spectrometric measurement for a standard solution submitted to the procedure is expected to produce a satisfying signal, indicating a good recovery of the analyte. An identification and quantification processes based only on the target analyte signal can be very critical in case of ion suppression, but a systematic use of spiked extracted samples for calibration curves instead of standard solutions is clearly preferable.

The food samples were analyzed for bioflavonoids content by applying the method of standard addition. Prior to analysis, the performance of the method was checked by analyzing standard solutions of analytes prepared in methanol as solvent. Calibration curves for each analyte were constructed by plotting the peak area of the analyte against corresponding concentration. The curves were linear in the concentration range 0.05–10 μg/mL with regression parameters given in Table 5.

The trueness expressed as recovery ($=C_{found}/C_{added} \times 100$) and precision, expressed as relative standard deviation ($=SD \times 100/\bar{x}$) were calculated by analyzing 8 solutions with known concentration of analytes. Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from the calibration curves for sufficiently low concentration of analytes (0.05–1.00 μg/mL) using the formula $k \times (S_b/a)$ where k is 3.3 for LOD and 10 for LOQ. S_b is the calculated standard deviation in intercept of calibration curve and a is its gradient. The obtained results are given in Table 6.

The standard addition method was performed by adding the unknown solution to 125 μL of standard solution, in a 5 mL volumetric flask, to the mark. Thus, no dilution with solvent was employed. The unknown concentration was assayed by plotting the corrected signal against the added concentration of the analytes. The unknown concentration was read as the x-intercept of the graph. The uncertainty of the intercept was calculated as

$$SD_x = \frac{s_y}{|a|}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{a^2 \times \sum (x_i - \bar{x})^2}} \quad (3)$$

where s_y is standard deviation in y—readings, a is the slope of least—squares line, n is a number of data points, x is concentration and y chromatographic response. The confidence interval was calculated as t x SD_x where t is a Student t for $n-2$ degrees of freedom.

The detection limits of rutin, hesperetin, hesperidin, quercetin and kaempferol in food samples were determined from the standard addition curves based on the definition of the concentration of analyte yielding a signal equivalent to three times the standard deviation of the non-spiked sample ($n=5$). The limit of detection was in the range 0.014–0.063 μg/mL indicating that the method has satisfactory performance for the determination of chosen bioflavonoids in real food samples.

The orange peel, red onion and honey samples were analyzed for quercetin, rutin, hesperetin, hesperidin and kaempferol content by using the standard addition method to the sample extracts. In Fig. 5, standard addition curve was plotted together with calibration curve obtained in methanol for hesperetin determination in orange peel. Large difference indicates a strong matrix effect. The linear standard addition curves were obtained in the concentration range of the added standard solutions of bioflavonoids 0.5–5.0 μg/mL (four concentration levels). Linearity

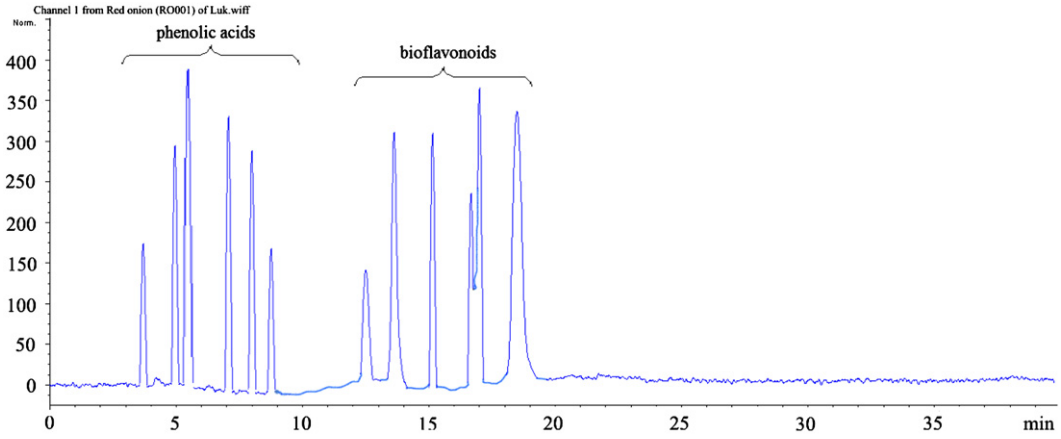

Fig. 4. HPLC/UV chromatograms of red onion extract after SPE.

Table 5
Regression equation ($Y=a+bX$; Y=area of the signal, X=concentration of analyte, μg/mL) for calibration curves at most intensive SRM transition.

Compound	SRM transition	Number of points, (N)	Slope (b) ($\times 10^5$)	Intercept (a) ($\times 10^4$)	Standard error ($\times 10^4$)	Correlation coefficient (r^2)
Hesperetin	301/286	8	3.07 ± 0.08	4.4 ± 0.3	7.9	0.9948
	301/244	8	1.82 ± 0.05	2.6 ± 0.2	4.8	0.9945
Hesperidin	609/325	8	4.83 ± 0.04	0.24 ± 0.02	0.4	0.9995
	609/174	8	9.64 ± 0.09	1.3 ± 0.3	8.6	0.9994
Quercetin	301/179	8	2.96 ± 0.09	1.93 ± 0.08	2.6	0.9987
	301/151	8	1.84 ± 0.06	1.64 ± 0.09	4.8	0.9931
Rutin	609/301	8	4.61 ± 0.07	3.1 ± 0.3	6.7	0.9984
	609/273	8	1.37 ± 0.02	0.73 ± 0.08	1.9	0.9986
Kaempferol	285/256	8	7.64 ± 0.08	3.65 ± 0.05	5.6	0.9941
	285/243	8	3.64 ± 0.09	2.31 ± 0.02	3.7	0.9976

Table 6
Statistical parametars of method validation for the LC–MS/MS analysis of bioflavonoids in food.

Conc added (μg/mL)		0.05	0.10	0.25	0.50	1.00	2.50	5.00	10.00	LOD	LOQ
Hesperetin	301/286	0.044	0.087	0.253	0.518	1.014	2.338	4.987	9.953	0.055	0.167
	Recovery (%)	88.0	87.0	101.2	103.6	101.4	93.5	99.7	99.5		
	RSD (%)	4.76	3.02	2.11	2.54	1.23	1.45	1.31	0.87		
	301/244	0.049	0.107	0.270	0.516	0.925	2.399	5.018	9.930	0.046	0.139
	Recovery (%)	98.0	107.0	108.0	103.2	92.5	95.96	100.4	99.3		
	RSD (%)	5.04	3.64	2.35	2.78	1.67	1.34	1.55	0.93		
Hesperidin	609/325	0.047	0.075	0.226	0.477	1.009	2.517	5.174	9.909	0.045	0.136
	Recovery (%)	94.0	95.0	90.4	95.4	100.9	100.7	103.5	99.1		
	RSD (%)	4.56	4.59	2.23	2.28	0.14	1.72	5.05	0.29		
	609/179	0.063	0.091	0.238	0.468	0.986	2.445	5.209	9.913	0.049	0.148
	Recovery (%)	112.0	91.0	95.2	93.6	98.6	97.8	104.2	99.1		
	RSD (%)	4.98	3.59	0.88	2.74	0.81	1.19	0.98	0.74		
Quercetin	301/179	0.0505	0.101	0.2525	0.505	1.01	2.626	5.02	10.3	0.043	0.130
	Recovery (%)	101.0	101.3	98.6	97.3	102.6	103.3	100.2	103.2		
	RSD (%)	3.87	4.62	4.03	2.69	3.98	4.67	2.36	2.47		
	301/151	0.0491	0.102	0.2613	0.523	1.136	2.587	5.13	10.35	0.041	0.124
	Recovery (%)	98.2	102.0	104.5	104.6	113.6	103.5	102.6	103.5		
	RSD (%)	3.64	4.25	3.81	3.97	5.79	2.38	4.67	2.64		
Rutin	609/301	0.049	0.093	0.245	0.483	0.965	2.621	5.218	10.025	0.076	0.229
	Recovery (%)	98.0	93.0	98.0	96.6	96.5	104.8	104.4	100.3		
	RSD (%)	4.61	2.57	0.98	3.07	1.49	1.14	1.12	0.46		
	609/273	0.048	0.092	0.241	0.481	0.955	2.615	5.206	10.033	0.062	0.189
	Recovery (%)	96.0	92.0	96.4	96.2	95.5	104.6	104.1	100.3		
	RSD (%)	8.24	3.71	0.88	1.94	1.03	0.39	0.38	1.23		
Kaempferol	285/256	0.057	0.114	0.285	0.57	1.14	2.964	5.31	11.4	0.039	0.118
	Recovery (%)	114.0	112.4	111.2	110.9	109.8	108.6	109.6	106.2		
	RSD (%)	3.56	4.67	4.59	4.37	3.28	3.67	2.89	2.57		
	285/243	0.056	0.124	0.264	0.567	1.15	2.56	5.31	10.6	0.04	0.121
	Recovery (%)	112.0	106.3	102.6	103.4	105.6	107.3	108.3	101.3		
	RSD (%)	4.57	4.69	4.78	3.85	3.67	2.94	2.68	2.47		

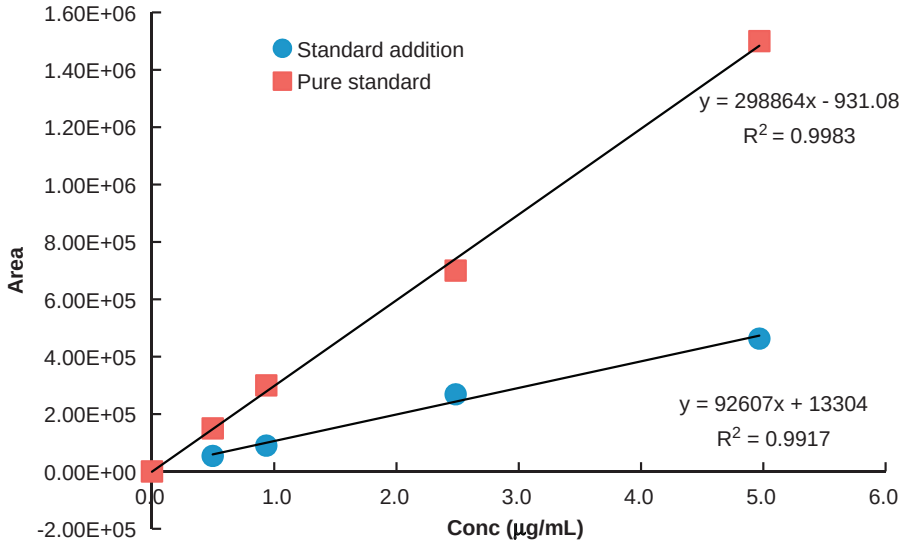

Fig. 5. Calibration curve for hesperetin in methanol and standard addition curve for hesperetin in orange peel extract.

Table 7
Flavonoid content in food samples, compared to other studies.

Food	Compound	Contents (mg/100 g fw) this study	CV (%)	Recovery (%)	x-intercept $\pm$ SD_x	t-test	Contents (mg/100 g fw) other study
Orange peel	Hesperetin	36.4	5.87	91.5	0.14 ± 0.08	0.2535	31–41.4 [29,30]
	Hesperidin	24.3	3.42	103.5	0.06 ± 0.01	0.1205	18–66.5 [29,30]
	Quercetin	0.65	2.56	93.47	0.10 ± 0.11	0.1758	0.00–2.20 [31,32]
Red onion	Hesperetin	0.0068	2.36	108.7	0.19 ± 0.01	0.2402	0.0064–0.0076 [33–35]
	Hesperidin	0.016	3.59	87.6	0.04 ± 0.04	0.0932	0.001–0.03 [33–35]
	Quercetin	56.3	3.68	99.37	0.24 ± 0.02	0.2873	0.00–191.7 [35–41]
	Rutin	0.16	4.73	93.4	0.62 ± 0.11	0.2663	0.17–0.27 [41]
	Kaempferol	1.63	5.61	92.4	0.41 ± 0.11	0.1873	0.00–4.5 [42–44]
Honey	Hesperetin	0.072	3.38	105.6	0.63 ± 0.23	0.2617	0.028–0.084 [45,46]
	Hesperidin	14	6.48	106.4	0.13 ± 0.02	0.2822	12–26 [46]
	Quercetin	0.67	3.56	91.4	0.19 ± 0.02	0.3014	0.02–1.3 [45–50]
	Kaempferol	0.14	4.1	87.65	0.38 ± 0.06	0.1489	0.05–0.17 [45–50]

Fw-fresh weigth, CV-coefficient of variation, SD_x-standard deviation of intercept.

was confirmed by the values of the regression coefficient higher than 0.98 and Cohran's test for homoscedasticity ($G_{max}=s^2_{max}/\Sigma s_i^2$ was compared with tabulated value, null hypothesis about equality of individual point standard deviation, accepted if $G_{max} < G_{table}$) indicated homogenous distribution of standard deviations.

The results of bioflavonoids determination in the chosen samples are given in Table 7.

Our values were compared with literature data for the bioflavonoid contents in food (see Table 7). Reasonably good agreement was obtained with published data using other detection modes.

4. Conclusion

A new LC–MS/MS procedure for the determination of bioflavonoids in food samples using the ultrasonic extraction method was developed to avoid hydrolysis of their glycosides and to have an insight into the real composition of the food. Excellent selectivity and sensitivity in determination of bioflavonoids were achieved by ESI ionization technique in SRM detection mode. During the method development the matrix effect accompanying the determination was evaluated. SPE and LC–ESI–MS/MS provide a novel method to determine levels of bioflavonoids in food. The advantage of the described method is quantitative extraction without the need for excessive sample clean-up steps. LC–ESI–MS/MS in addition to being fast and specific provides sensitivity in the low µg/mL range. Thus, the main advantage of the method is the rapid separation and specific detection. The standard addition method to quantify the bioflavonoids by calibration with the standard bioflavonoids solution added in the extract solution can serve as a very promising and practical approach to overcome matrix effects and has a great potential to be applicable to other matrices where the LC–ESI–MS/MS technique is used. Thus, the suppression of the ionization efficiency which occurs due to co-eluting substances and causes variation in LC–MS responses was overcame by the successful use of standard addition method.

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