CHEMIJA. 2013. Vol. 24. No. 3. P. 217–222

Optimization of matrix solid-phase dispersion extraction for the chromatographic analysis of flavones in cranberries

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A matrix solid-phase dispersion (MSPD) extraction method was applied for three flavones in cranberry (*Vaccinium macrocarpon* Ait.) matrix for the first time. The optimized MSPD procedure required a small amount of the sample (0.25 g), C18 bonded silica gel (1.0 g) as a dispersant solid-phase sorbent and small volumes of methanol using its aqueous solutions (10 mL) as eluting solvents. Identification and quantification of myricetin, quercetin and kaempferol were performed by HPLC-DAD-MS. The limit of detection varies from 0.04 μg/g to 0.08 μg/g with linear regression coefficients ($r^2 > 0.999$) for each compound. The recoveries of the analytes ranging from 59% to 111% with relative standard deviations less than 9.5% were checked using spiked freeze-dried cranberry samples. The MSPD method is simple, rapid and efficient for the extraction of flavones from cranberry samples.

Key words: matrix solid-phase dispersion (MSPD), cranberry, myricetin, quercetin, kaempferol, HPLC-MS

INTRODUCTION

The cranberry (*Vaccinium macrocarpon* Ait.) is of growing public interest as a functional food because of potential health benefits linked to phytochemicals in the fruits [1]. These berries are a rich source of diverse phenolic compounds that includes six different classes of flavonoids (flavanones, flavanonols, leucoanthocyanidins, flavones, anthocyanins and anthocyanidins) [2], catechins, hydroxycinnamic and other phenolic acids, and triterpenoids [3]. Commonly among the fruits cranberries accumulate some of the highest concentrations of flavonoid compounds which have antioxidant, antiviral and anticancer properties [1, 4–6].

Most of the methods for qualitative and quantitative analyses of the cranberry flavonoids have been developed based on high-performance liquid chromatography (HPLC) with a diode array detector (DAD) in UV-Vis range and mass spectrometry (MS) using atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) [2, 7–13]. The procedures of sample preparation are usually labour-intensive, time-consuming and need large volumes of solvents. The isolation of the phenolic compounds includes repeated extractions and / or liquid-liquid extractions of the analytes from the cranberries matrix, the concentration, the fractiona-

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tion and / or the preparative HPLC purification procedure of the extracts. All these procedures require specific equipment or materials (e. g. Sephadex LH-20 sorbent column) and large amounts of organic solvents (i. e. acetone, hexane, ethyl acetate and methanol) [8–14].

A promising alternative, recently introduced for the sample preparation of the complex matrices, is matrix solid-phase dispersion (MSPD) [15, 16]. This method has been successfully applied for the isolation of different types of analytes from plants and food stuff, such as the phenolic compounds in white grapes or in red wine [17, 18], the isoflavones in *T. pratense* [19], the labdane diterpenoids in *Coleus forskohlii* [20] and the lignin in *Sinopodophyllum* [21]. The principles of MSPD method have been published in several papers [15, 16, 22]. Basically, MSPD is a sample preparation methodology that combines a sample disruption with a sorbent, followed by desorption with a small amount of solvent [15] and often combines extraction and clean-up into a single step.

The aim of this study was to develop an analytical procedure which combines the MSPD extraction and HPLC-DAD-MS method for qualitative and quantitative analyses of the flavonoids in cranberry berries. Three commonly existing flavones, myricetin, quercetin and kaempferol [2], were selected for this research. The effects of several extraction parameters, such as the selection of the dispersant solid-phase and its quantity, eluting solvent and its volume, were tested in order to improve recovery and sensitivity.

EXPERIMENTAL

Chemicals and reagents

Methanol, acetonitrile, acetone were purchased from Sigma-Aldrich (Steinheim, Germany), ethanol from Merck KGaA (Darmstadt, Germany), formic acid from Scharlau Chemie (Geel, Belgium). All organic solvents were of HPLC grade. The water was treated with the NANOpure Infinity Water Purification System (Barnstead, USA).

The flavone standard myricetin (CAS-No. 529-44-2; purity ≥ 96.0%), quercetin (CAS-No. 117-39-5; purity ≥ 98.0%) and kaempferol (CAS-No. 520-18-3; purity ≥ 90%) were purchased from Sigma-Aldrich (Steinheim, Germany). The stock solutions (0.5 mg/g) of myricetin, quercetin and kaempferol were prepared in methanol, stored at 4 °C, protected from light. Working standard solutions were prepared daily by diluting the stock solution with methanol-water (9 : 1, *v/v*).

Two sorbents tested for MSPD included C18 bonded silica gel (particle size 40–63 μm) and were from Sigma-Aldrich (Milwaukee, WI, USA), and silica gel (particle size 40–63 μm) was from J. T. Baker (Deventer, The Netherlands). Polypropylene solid-phase extraction (SPE) syringe barrels (15 mL capacity) fitted with a single bottom frit were obtained from International Sorbent Technology (Hengoed, UK). Syringe filters (Millex GV, 13 mm, and 0.22 μm) were obtained from Millipore (Billerica, MA, USA).

Working standard solutions of myricetin and quercetin, as model flavone solutions, were used for the method development studies. Each sample was prepared in a 90% aqueous solution of methanol at a concentration of 0.5 mg/g by appropriate dilution of an aliquot of the stock solution.

After the method validation it was applied for the analysis of a freeze-dried cranberry powder (Healthy Supplies, UK).

HPLC-DAD and APCI-MS analysis of flavones

The HPLC analysis was performed on the Agilent 1100 Series liquid chromatography system (Agilent Technologies, USA) equipped with a quaternary pump, a vacuum degasser module, a manual injector with a 20 μL sample loop, a temperature controlled column compartment and a diode array detector (DAD) set at 370 nm and 380 nm. The analytes were separated using a Zorbax Eclipse XDB–C18 (5 μm, 150 × 4.6 mm) reversed phase column (Agilent Technologies, USA) coupled with an Eclipse XDB–C18 (5 μm, 12.6 × 4.6 mm) guard column (Agilent Technologies, USA). Separations were carried out in a binary solvent system: solvent A, 1% formic acid in water; solvent B, 1% formic acid in methanol. A linear gradient of 30–50% B from 0 to 7 min; 50–60% B from 7 to 12 min; 60–70% B from 12 to 16 min and an isocratic elution with 70% B from 16 to 18 min at a flow rate of 1 mL/min were used.

Atmospheric pressure chemical ionization (APCI) in the negative ion detection mode was obtained using an Agilent 1100 Series LC/MSD Trap XCT Plus mass spectrometer (Agilent Technologies, USA). Zorbax Eclipse XDB–C18 (5 μm, 150 × 4.6 mm) reversed phase column and a mobile phase of methanol / formic acid / water at a flow rate 1 mL/min, as described above, were used. The typical parameters were as follows: corona, 3.5 kV; capillary voltage, 3.5 kV; skimmer voltage, 40 V; source temperature, 315 °C; APCI probe temperature 350 °C; dry gas (nitrogen) flow rate 6 L/min. Spectra were scanned over a mass range of m/z 50–1 200 at 1.0 s per cycle.

The calibration curves for quantification of flavones were prepared using corresponding standards dissolved in methanol at a concentration of 0.5 mg/g and stored at 4 °C as stock solutions. Concentrations of each analyte ranged from 0.25 to 50.0 μg/g. Identification of myricetin, quercetin and kaemp-

Table 1. **Retention times, target ions and linear regression coefficients, limits of detection (LODs) and quantification (LOQs) for the three flavones (n = 3)**

Standards	t_R, min	Regression equation	r^2	LOD[b], μg g^{-1}	LOQ[b], μg g^{-1}	Target ion, m/z
Myricetin[a]	9.79	$y = 0.026x - 0.001$	0.999	0.08	0.1	317 $[M-H]^-$
Quercetin[a]	12.56	$y = 0.027x - 0.001$	0.999	0.08	0.1	301 $[M-H]^-$
Kaempferol	15.46	$y = 0.021x + 0.002$	0.999	0.04	0.07	285 $[M-H]^-$

Note. [a] The standards chosen for the model flavones samples are reported in bold;

[b] LOD and LOQ calculated for DAD detection.

ferol was performed by matching their retention times, spectroscopic characteristics measured at 370 nm for quercetin and kaempferol, 380 nm for myricetin, spectrometric m/z values of $[M-H]^-$ of flavones in standard solutions (Table 1).

MSPD procedure

The influence of the most important parameters, such as selection of the dispersant solid-phase and its quantity, eluting solvent and its volume, were studied in order to determine the optimum experimental conditions for the analysis of flavones in cranberry powder.

For this reason, 0.5 g of the chosen dispersant solid-phase with 100 μL of the model flavones solution (0.05 mg/g myricetin and quercetin) were placed in an agate mortar and carefully grounded together using an agate pestle. After grinding (2 min) the sample was packed into an empty disposable plastic 15 mL volume syringe containing a frit at the bottom. The second frit was placed on the top of the sample by careful compression with a syringe plunger. The cartridge packed in this way was attached to a vacuum manifold coupled with a small vacuum pump. The analytes were eluted from the cartridge with 5 mL of suitable H_2O/MeOH solution, which flow was adjusted to 2.5 mL/min. All eluates were evaporated to dryness at room temperature under the stream of nitrogen. The residues were redissolved in 1 mL of methanol and 20 μL of the prepared samples were analyzed using HPLC-DAD-MS.

Each experiment was repeated 3 times.

After optimization the procedure was applied for flavones extraction from natural matrix (the freeze-dried cranberry powder) samples.

RESULTS AND DISCUSSION

Optimization of HPLC-DAD-MS conditions

Cranberry flavonols, anthocyanins and proanthocyanidins have been previously characterized by HPLC-MS [4, 8–12]. In the present study, the C18 reverse phase columns and the methanol-water-formic acid system were chosen for the flavone separation procedure. The gradient procedure permitted the full separation of cranberry flavonoids with different polarity. It would be difficult to achieve the same result if isocratic elution would be applied. Therefore, gradient elution optimization has been performed resulting in a good resolution with reasonable retention times and good peak shapes for myricetin, quercetin, and kaempferol.

The flavones were determined at two wavelengths by using the diode-array detector (DAD). The best results were obtained by detection of quercetin and kaempferol at 370 nm, and myricetin at 380 nm. All three compounds could be analyzed in a single chromatographic run but at 370 nm the peak of myricetin has been overlapped by two other compounds from the real cranberry sample extract. The myricetin was detected at a wavelength of 380 nm and detection sensitivity remained almost the same. APCI-MS analysis in the negative ionization mode was employed to identify the mass spectra of flavones. Both DAD and APCI-MS detection could be used for the quantitative analysis, but DAD was more sensitive and offered a more stable baseline measuring the real cranberry samples. Therefore, DAD was selected for the quantification. The obtained results are summarized in Table 1.

Optimization of MSPD method

The type of the sorbent and polarity of the extraction solvent are the most important factors in MSPD extraction since both influence the efficiency of the extraction and the purity of the final extract [18–20].

The sorbent used in MSPD is not only for adsorption and separation of compounds but also for the disruption of plant matrix and the blend of materials and supports.

In this study silica gel and C18 were tested since previous publications of MSPD application described these sorbents as suitable for the adsorption of phenolic compounds from plant matrix [17–19]. The results of the dispersant solid-phase screening showed that myricetin and quercetin were lost within the first fraction recoveries (35–52%) when silica gels were used (Fig. 1a). C18 gave very low recoveries (~0.5%) for both compounds in fraction 1 and the best recoveries (71–82%) in fraction 3. It was crucial to separate foreign compounds and to get extracts of only analytes of the interest. Consequently, C18 sorbent has been chosen for the subsequent studies.

The eluting solvent was also optimized. Ideally, extraction solvent is supposed to have good solubility for target compounds but not for unwanted ones. According to this principle, four solvents with a wide range of dielectric constants (ε), namely acetone (ε = 20.7), ethanol (ε = 24.35), methanol (ε = 33.1) and acetonitrile (ε = 37.5), were studied. Figure 1b indicates that ethanol and methanol had much greater capacities (70–81%) in recovering the flavones than the two other solvents under study. As expected, acetone, a polar aprotic solvent, did not efficiently elute polar compounds such as phenolic compounds. Ethanol has demonstrated good results but 5–15% of analytes were lost in fraction 2. Consequently, a methanol and methanol / water solution was chosen as the eluting solvent. The extraction of the specific classes of the compounds with different solubility from cranberries was achieved by using methanol / water solutions in the agreement with previous studies [4, 8, 10].

To evaluate the effect of the volume of the eluting solvent, assays were also performed on the model flavones solution with different volumes (2.0, 5.0, 7.0, 10.0, 15.0 mL) of aqueous MeOH solutions. Figure 1c shows that the highest recoveries for myricetin (97%) and quercetin (83%) were obtained when 10 mL elution solvent was used. Using smaller volumes of the solvents resulted in smaller amounts of desorbed analytes, i. e. the recoveries were from 85 to 91% for myricetin and from 72 to 77% for quercetin, respectively. In addition, too large volumes of the elution solvent could remarkably reduce detection sensitivity or prolong the sample preparation procedure (without preconcentration by evaporation of the extract) [23].

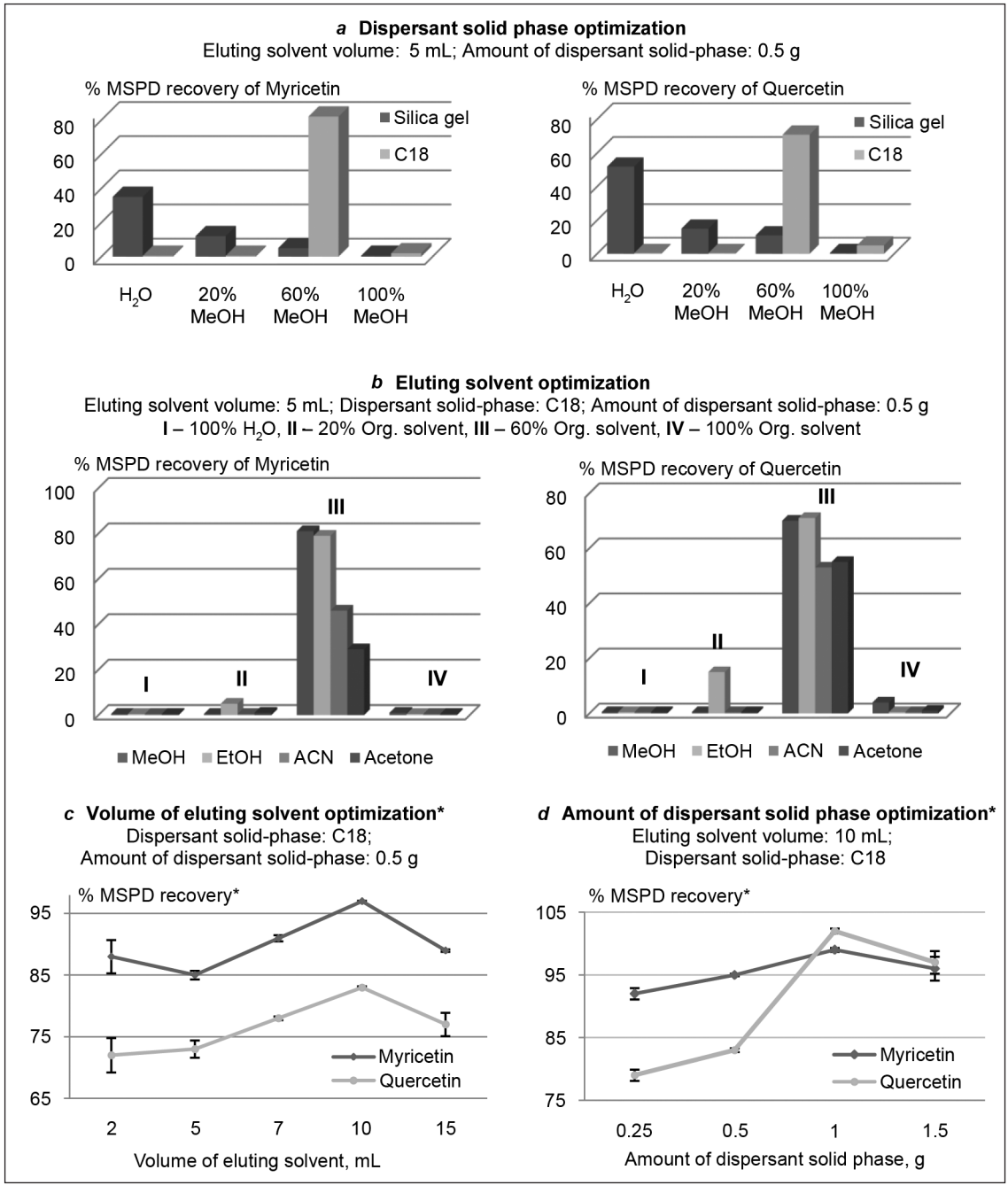

Fig. 1. Effect of the dispersant solid-phase (*a*), eluting solvent (*b*), volume of the eluting solvent (*c*) and amount of the dispersant solid phase (*d*) for the recoveries of myricetin and quercetin in a laboratory-model flavones sample, followed by HPLC/MS analysis. * – Recovery was calculated according to data of fraction 3, i. e. 60% MeOH. The error bars represent relative standard deviations (n = 3)

In literature, sample / sorbent ratios varied from 1 : 1 to 1 : 4 [17]. Therefore, the amount of the sorbent was optimized using the model flavones solutions and four different quantities (0.25, 0.5, 1.0 and 1.5 g) of the C18 sorbent. The analytes were eluted from the cartridge with 10 mL aqueous MeOH solutions. Figure 1d illustrates the influence of the amount of the dispersant solid-phase on the recoveries using MSPD extraction. The highest recoveries (99–102%) were obtained using 1.0 g of the C18 sorbent. The optimum ratio depends on the nature of the sample matrix. So, we carried out quantitative optimization of the freeze-dried cranberry sample in order to make sure that our choice was correct. The amount of lyophilized cranberry powder was optimized changing the sample / sorbent ratios from 1 : 3 to 1 : 6 (0.16, 0.2, 0.25 and 0.33 g of freeze-dried cranberry powder with 1.0 g of the C18 sorbent). In this step the cleanliness of the final extracts was checked visually and by number and intensity of the peaks in the chromatographic profiles to evaluate the further cleaning or dilution of the extracts was essential.

Table 2. **Recoveries and RSD obtained with the MSPD method for spiked samples, analyzed using HPLC diode array detection at 380 nm for myricetin and 370 nm for quercetin and kaempferol (n = 5)**

Compound	Recovery ± RSD, % (n = 5)	
	2.5 μg g^{-1}	10.0 μg g^{-1}
Myricetin	87.9 ± 3.4	98.7 ± 9.5
Quercetin	111.5 ± 6.6	102.2 ± 6.3
Kaempferol	59.1 ± 8.2	73.4 ± 4.9

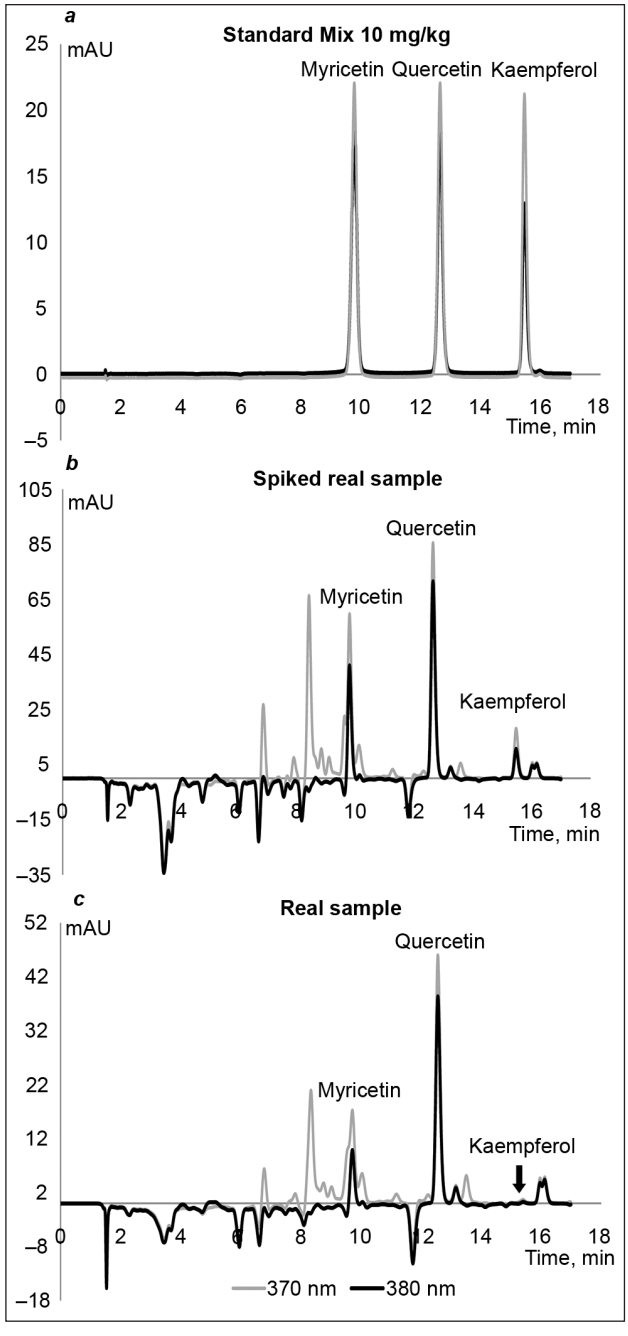

Fig. 2. HPLC-DAD chromatograms of (*a*) a standard mixture of three flavones (10 mg/kg), (*b*) spiked (10 mg/kg) and (*c*) unspiked MSPD extracts of freeze-dried cranberry powder (as could be seen, there were 13.1 ± 1.2 μg/g of myricetin, 53.5 ± 8.6 μg/g quercetin and kaempferol <LOQ in the freeze-dried cranberry sample). For peak identification see Table 1. For chromatographic conditions see Experimental (section 2)

Results based on the tests provided optimal conditions for MSPD extraction of flavones from cranberries: 0.25 g of the freeze-dried cranberry sample, 1.0 g of C18 bonded silica gel as the dispersant sorbent and 10 mL of methanol and its aqueous solutions as the eluting solvents.

Method validation

Once the MSPD extraction conditions on the model solution samples were optimized, the procedure was also performed on freeze-dried cranberry powder spiked with the known amount of analytes. Figure 2 represents the chromatograms of (a) flavones from standard and the MSPD extracts of (b) the freeze-dried cranberry powder spiked with the three flavones and (c) the same unspiked sample.

Each phenolic compound was quantified using eight-point calibration of mixed standard solutions covering a concentration range from 0.25 to 50.0 μg/g as described above. All compounds showed good linearity ($r^2 > 0.999$) over the tested concentration ranges. The limits of detection (LOD, S/N = 3) of the three flavones ranged from 0.04 to 0.08 μg/g, and the limits of quantification (LOQ, S/N = 10) ranged from 0.07 to 0.1 μg/g. The results are summarized in Table 1.

To assess the accuracy and the repeatability of the method the recovery test was applied by adding standards with the known content of the three analytes (at two fortification levels, i. e. 2.5 and 10 μg/g) to 0.25 g freeze-dried cranberry powder samples. Then the spiked samples were extracted and analyzed by the proposed method. The recoveries of the flavones (myricetin, quercetin and kaempferol) were calculated, as 100% × (the determined amount in spiked samples – the original amount in unspiked samples) / (the spiked amount). The recoveries of myricetin, quercetin and kaempferol were 87.9%, 111.5% and 59.1%, respectively, when spiked with 2.5 μg/g, and 98.7%, 102.2% and 73.4% when spiked with 10 μg/g (Table 2). The repeatability of the method was evaluated three times repeating the extraction procedure of five replicates. The RSDs values ranged from 3.4% to 9.5%. Therefore, this method can be used efficaciously for the quantitative analysis of the wide range of the flavonoids in plant material matrices.

CONCLUSIONS

The MSPD method conditions were developed and validated for the reliable separation of three flavones in cranberry berries. The myricetin, quercetin and kaempferol analytes were determined and identified by HPLC-DAD-MS. The MSPD procedure allows doing extraction and clean-up of extracts in one single step, requiring small amounts of the sample (0.25 g) and sorbent (1.0 g), low consumption of solvents (10 mL) and short extraction time (30 min). The main advantages of the proposed method are less time and materials consuming extraction, and simplicity compared to commonly used classical methods.

Received 23 May 2013
Accepted 5 June 2013

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KIETAFAZĖ DISPERGUOJAMOJI EKSTRAKCIJA FLAVONŲ IŠSKYRIMUI IR NUSTATYMUI SPANGUOLĖSE CHROMATOGRAFINIU METODU

S a n t r a u k a

Pirmą kartą kietafazės disperguojamosios ekstrakcijos (KDE) metodas buvo pritaikytas flavonų išskyrimui iš spanguolių (lot. *Vaccinium macrocarpon*). Optimizuotai KDE procedūrai reikia mažo bandinio kiekio (0,25 g), oktadecilsilikagelio (1,0 g), kuris atlieka disperguojančio sorbento funkciją, ir nedidelio tūrio eliuento – vandeninio metanolio tirpalo (10 mL). Miricetino, kvercetino ir kaempferolio identifikavimas ir kiekybinis nustatymas atliekamas efektyviosios skysčių chromatografijos su fotodiodinės matricos ir spektrometriniu detektoriumi. Flavonų aptikimo ribos yra nuo 0,04 μg/g iki 0,08 μg/g, koreliacijos koeficientai kiekvienai analitei didesni nei 0,999. Analičių išgavos iš liofilizuotų spanguolių svyruoja nuo 59 % iki 111 %, o santykinis standartinis nuokrypis mažesnis nei 9,5 %. KDE metodas yra paprastas, greitas ir lengvai pritaikomas flavonų ekstrakcijai iš augalinės kilmės objektų.