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Application of solid-phase extraction for isolation of coumarins from wine samples

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Abstract

Coumarins can be as a result wine storage and aging in wood drums and they can affect organoleptic characteristics of wine. The aim of this work was to determine the content of coumarins in wine samples originated from Slovak Tokaj wine region. The HPLC method with high specificity, accuracy, precision, and recovery was proposed. SPE sorbents of C18 type, styrene-divinylbenzene copolymer and molecularly imprinted polymers were compared for extraction of six coumarins, coumarin, aesculin, scoparone, scopoletin, 4-methylumbelliferone, and herniarin. Higher recoveries (above 89 %; except aesculin – recoveries higher from 68 %, RSDs less than 6 %) were obtained with selective polymeric sorbent laboratory prepared by molecularly imprinted technology. The results showed that content of coumarins in wine samples are in ng.mL^{-1} concentration levels and depend on the age and wine puttony.

Introduction

Coumarins, a class of compounds that contain a 1,2-benzopyrone skeleton, occur as secondary metabolites mainly originated from shikimic acid pathway. These compounds are widely distributed in the seeds, roots and leaves of many plants of *Apiaceae*, *Rutaceae*, *Asteraceae* and *Fabaceae* families. The source of coumarins (glycosylated or aglycone forms) in wine could be wood of drums in which it is matured. Wood releases the coumarins and other compounds (e.g. phenolic acids) into the wine, which contribute to wine taste (e.g. glycosides are bitter, aglycones are acidic). The content of coumarins in wood changes through the degradation of the wood lignin during the heat treatment of the barrel and also depends on the type of the wood (Ribéreau-Gayon *et al*. 2006; De Rosso *et al*. 2009; Canas 2017). The main coumarins in wine are aesculetin (6,7-dihydroxy-coumarin), aesculin (6,7-dihydroxy-coumarin-6-glucoside), scopoletin (7-methoxy-6-hydroxy-coumarin), and scopolin (scopoletin-7-glucoside). In some wines, 4-hydroxycoumarin and umbelliferone (7-hydroxycoumarin) were identified (Liberatore *et al*. 2010; Canas 2017). Coumarins can be used for evaluation of wine, as a possible markers of storage of wines in wood barrels. In addition, the coumarins exhibit a wide range of biological effects, e.g. anti-inflammatory, antithrombotic, antimicrobial, antifungal, antiviral (including anti-HIV), anticonvulsant, antioxidant, and antitumor (Kubrak *et al*. 2017).

Wine is very complex matrices, often components of very low concentrations are of interest (concentrations of ng.mL^{-1} for coumarins; Salagoity-Auguste *et al.* 1987). Therefore, it is necessary to use sample preparation and analytical

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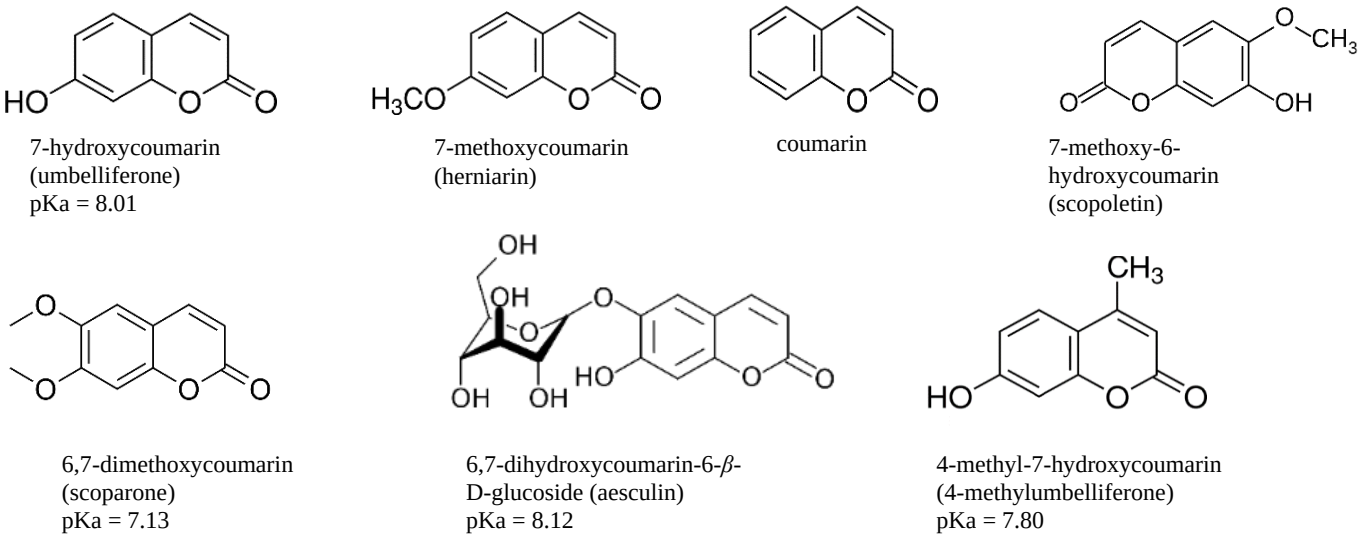

Fig. 1. Names, structural formulas and pKa values of simple coumarins.

methods for determination of coumarins that are characterised by high selectivity and sensitivity.

Sample preparation is an essential part of chemical analyses. The main goal of sample preparation for subsequent HPLC analysis is removal of matrix interferences and to obtain sample aliquot or final analytes extract in solvent which is compatible with the HPLC mobile phase used. Sometimes, depending on detection sensitivity, pre-concentration of target analytes is needed. The most widely and routinely used sample preparation technique (clean-up and pre-concentration) for the extraction of analytes from a complex matrix (including wine samples) is solid-phase extraction (SPE). This technique is rapid, easy to perform, can be automated, and low amount of solvents is handled (compared with liquid-liquid extraction).

A broad range of sorbents based on electrostatic, hydrophobic or polar interactions exists for SPE. These traditional SPE sorbents are widely available, well characterized and have high binding capacity, but only a few types (e.g. C18 type) are used in the majority of SPE sample preparations, and they are the solvents in wash and/or elution steps which are varied according to the applications. Sometimes their selectivity is limited and the target analyte often co-elutes with interfering compounds from matrix. There are several publications, which present applications of different sample preparation techniques in the wine analysis (Lorrain *et al.* 2013), mainly for extraction of selected phenolic compounds. Research in solid-phase extraction techniques is focused on development of sorption materials with increasing selectivity for target analytes.

A novel type of SPE sorbets are molecularly imprinted polymers (MIPs) – synthetic tailor-made materials with a pre-defined selectivity for a target analyte or structurally related compounds based on specific interactions between analyte and binding sites on MIP. This technology enables to reduce the presence of interferences during the extraction procedure and obtain higher recoveries even at low concentration levels. MIPs are obtained by polymerising functional and cross-linking monomers around a template molecule, which lead to a highly cross-linked three-dimensional network polymer. After polymerisation, the template molecule is extracted and binding sites having their shape, size, and functionalities complementary to the target analyte are established. The resulting imprinted polymers are stable, robust, and resistant to a wide range of pH, solvents, and temperature. The MIP solid-phase extraction (MISPE) was applied in wine analysis for determination of quercetin (Molinelli *et al.* 2002), ochratoxin A (Giovannoli *et al.* 2014), phthalates (Barciela-Alonso *et al.* 2017), organophosphorous herbicides (Sanagi *et al.* 2011), catechin (Büyüktuncel *et al.* 2018), umbelliferone (Machyňáková *et al* 2017a).

In this paper the content of coumarins in wine samples were determined. Different SPE sorbents, C18 bonded silica, styrene-divinylbenzene

copolymers, and MIP based sorbents have been tested and compared for extraction of simple coumarins (Fig. 1) the most important natural coumarins. HPLC method was used for determination of the extracted analytes.

Experimental

Chemicals and samples

The standards of 7-hydroxycoumarin (99 %), 7-methoxycoumarin (98 %), 4-methyl-7-hydroxy-coumarin (98 %), 6,7-dimethoxycoumarin (98 %), 7-methoxy-6-hydroxycoumarin (98 %), 6,7-dihydroxycoumarin-6-*β*-D-glucoside (98 %), and coumarin (99 %) used for purpose of this study were purchased from Sigma-Aldrich (St. Louis, USA). Solvents for SPE extraction and preparation of HPLC mobile phases, methanol and ethanol of HPLC gradient grade and acetic acid (99 %) were purchased from Merck (Germany). HPLC grade water was prepared by AquaMax ultra (series 370) water purification system.
The Tokaj wine (sample I: puttony 3 (2002), sample II: puttony 4 (2004), sample III: puttony 5 (2002), sample IV: puttony 6 (2003) was a product of Slovak winery.

Preparation of standard solutions

Stock standard solutions of each compound (aesculin, scopoletin, coumarin, scoparone, 4-methylumbelliferone, herniarin,) were prepared in methanol/1 % acetic acid (20/80; v/v) to reach concentration of 0.1 mg.mL^{-1} and stored in freeze at –20 °C. The mixed working solutions were prepared by diluting the stock solutions obtaining lower concentrations.

Sample preparation

The SPE cartridge (C18 Hydra (I), HR-X (II), HR-P (III) (all cartridges purchased from Chromabond, Macherey-Nagel, Germany), AFFINIMIP Phenolic (IV) (purchased from Affinisep, France) and cartridge with 0.1 g of laboratory prepared MIP-umbelliferone adsorbent) was preconditioned with 3 mL of methanol and subsequently with 3 mL of 12 % ethanol. The appropriate volume of sample (5 mL) was passed through cartridge followed by washing with 3 mL of water. Finally, the analytes were eluted with 1 mL of methanol/acetic acid (9/1; v/v). Extract was filtered through a 0.45 μm nylon membrane filter and used for the injection into HPLC. The extraction procedure was repeated three times for each kind of tested sorbent material. For solid-phase extraction the vacuum manifold was used.

HPLC analysis

A HPLC Agilent Technologies (series 1200) consisting of a binary pump, an autosampler, a column oven, a diode array detector, and fluorescence detector was used. HPLC analyses were performed on a Kinetex C18 (100 mm x 4.6 mm I.D., 5 μm particle size) analytical column. The column was kept at 23 °C. An injection volume was 20 μL. The mobile phase consisted of methanol/acetic acid (99/1; v/v) (A) and 1 % aqueous solution of acetic acid (B) was mixed by the gradient program as follows, 0 – 12 min linear gradient of A from 20 to 45 %, 12 – 12.5 min linear gradient of A from 45 to 100 %, 12.5 – 14.5 min 100 % of A, 14.5 – 15 min a reverse gradient of A from 100 to 20 %, and 15 – 19 min 20 % A. The flow rate was 1.0 mL min^{-1}. The diode array detector was operated in the wavelength range of 190 – 400 nm and chromatograms were recorded at 280 nm for coumarin and 330 nm for aesculin, scopoletin, scoparone, 4-methylumbelliferone, herniarin. Fluorescence detector was set at the wavelengths 330 nm (λex) and 450 nm (λem) and fluorescence spectra were scanned in the wavelength range of 340 – 500 nm.

Results and Discussion

Different types of SPE sorbents were selected for purpose of this study, i) alkyl – bonded silica, ii) styrene-divinylbenzene copolymeric sorbents, and iii) selective MIP based polymeric sorbents.
In this work, all selected cartridges and laboratory prepared MIP-umbelliferone cartridge were used in the same SPE procedure with optimal extraction conditions included conditioning with methanol and 12 % ethanol, washing with water, and analyte eluting with methanol/acetic acid (9/1; v/v).

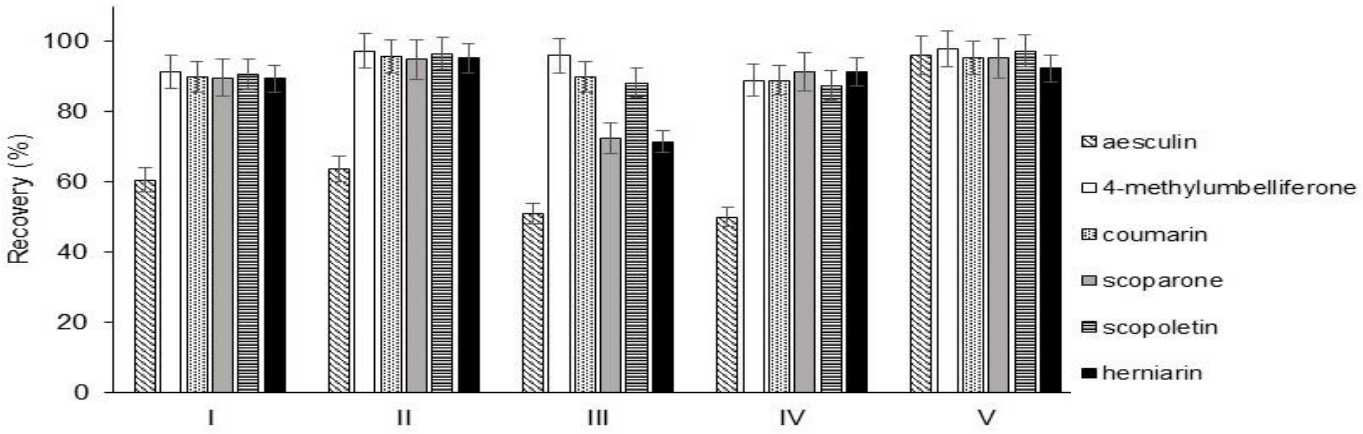

Fig. 2. Recovery values of coumarins on different SPE sorbents.

Efficiencies of SPE sorbents evaluated by recovery values were calculated and compared (Fig. 2).

Coumarins under study represent less polar type of compounds and from this reason the reversed phase approach was selected for SPE. Reversed phase mode involves a polar or moderately polar sample matrix and a nonpolar adsorbent. Octadecyl silica type of sorbent (I) is most frequently used for isolation of less polar compounds. The retention of analytes from polar matrices is due to the Van der Waals forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. To elute a compound from adsorbent, a nonpolar solvent is used (the disruption of the forces analyte-packing adsorbent). The hydrophobic (II) and high porous (III) polymeric polystyrene-divinylbenzene sorbents were also used for purpose of the study.

The advantage of these adsorbents is higher resistance in whole pH range, which leads to applicability of the SPE methods for acidified samples to. They are suitable for extraction of compounds from complex, food, pharmaceutical or biological samples (II) and from water samples (III). The efficiency values of SPE for sorbents I and II are approximately the same and higher than 89 %, except aesculin, where sorption recovery was significantly lower (less than 64 %). The lover recoveries were obtained for some of compounds for adsorbent III. The best extraction performance of adsorbent II, in comparison with I and III adsorbents, is related to aromatic functional groups and hydrophobic moieties of the sorbent which produce π-π and hydrophobic interactions for retention of analytes.

MIP sorbents, on contrary to the traditional SPE sorbents (e.g. silica-based sorbents) are characterised by higher selectivity and specificity

Table 1. Parameters of the MISPE-HPLC method[a].

Compound	t_r [min]	Regression equation	Concentration range	LOD	LOQ
aesculin	2.51	*y = 131.1x – 3.3* R^2 = 0.987	3.0 – 100 ng.mL^{-1}	1.0 ng.mL^{-1}	3.0 ng.mL^{-1}
scopoletin	6.50	*y = 1219.7x + 13.2* R^2 = 0.998	3.0 – 100 ng.mL^{-1}	1.0 ng.mL^{-1}	3.0 ng.mL^{-1}
coumarin	8.38	*y = 3650.8x + 5.3* R^2 = 0.995	0.5 – 100 µg.mL^{-1}	0.2 µg.mL^{-1}	0.5 µg.mL^{-1}
scoparone	8.98	*y = 82.8x + 1.3* R^2 = 0.998	10.0 – 100 ng.mL^{-1}	3.0 ng.mL^{-1}	10.0 ng.mL^{-1}
4-methylumbelliferone	9.45	*y = 306.3x + 6.2* R^2 = 0.997	0.5 – 100 ng.mL^{-1}	0.2 ng.mL^{-1}	0.5 ng.mL^{-1}
herniarin	11.30	*y = 25.5x – 0.2* R^2 = 0.998	12.0 – 100 ng.mL^{-1}	4.0 ng.mL^{-1}	12.0 ng.mL^{-1}

[a] coumarin – parameters for UV spectrophotometric detection; other coumarins – parameters for FL detection.

Table 2. Accuracy and precision of the MISPE-HPLC method [a].

Compound	Recovery (%) ± SD		
	C1	C2	C3
aesculin	68.7±0.8	70.9±0.9	72.1±0.9
scopoletin	91.4±3.2	94.7±3.8	96.0±3.3
coumarin	90.0±3.1	89.8±2.6	89.0±2.9
scoparone	94.9±1.1	91.3±1.1	95.4±1.4
4-methylumbelliferone	91.3±4.5	95.3±5.1	92.3±4.5
herniarin	95.2±2.3	97.3±2.3	92.3±2.0

[a] coumarin – C1 1.0 μg.mL^{-1}, C2 5.0 μg.mL^{-1}, C3 50.0 μg.mL^{-1}; other coumarins – C1 0.01 μg.mL^{-1}, C2 0.05 μg.mL^{-1}, C3 0.1 μg.mL^{-1}; n = 6.

to target analyte, leading to effective elimination of matrix interferences. MIP selectivity is related to recognition cavities in polymeric matrices which are complementary to the target molecule in size, shape, and arrangement of the functional groups. Two types of MIPs were tested for purpose of this study, MIP selective for phenolic compounds (IV) and coumarins (V). Although the sorbent IV was designed for phenolic compounds, the recovery values (higher than 88 %) indicated, that it was suitable for isolation of investigated coumarins. The lowest recovery of aesculin (about 50 %) results from incompatibility of MIP imprinted cavity with shape and functionality of analyte. The laboratory synthesised MIP (V) by imprinting of umbelliferone molecule was selective for template and also for other structural analoges (Fig. 1) (Machyňáková *et al*. 2017b). This sorbent shows complete retention of all investigated coumarins with highest values of recovery, 92 – 98 %, compared with traditional and polymeric sorbents I-IV. The advantage of MIP based sorbents is alongside increased selectivity also reusability. Sorbent can be reused up to fifty times without losing extraction capacity.

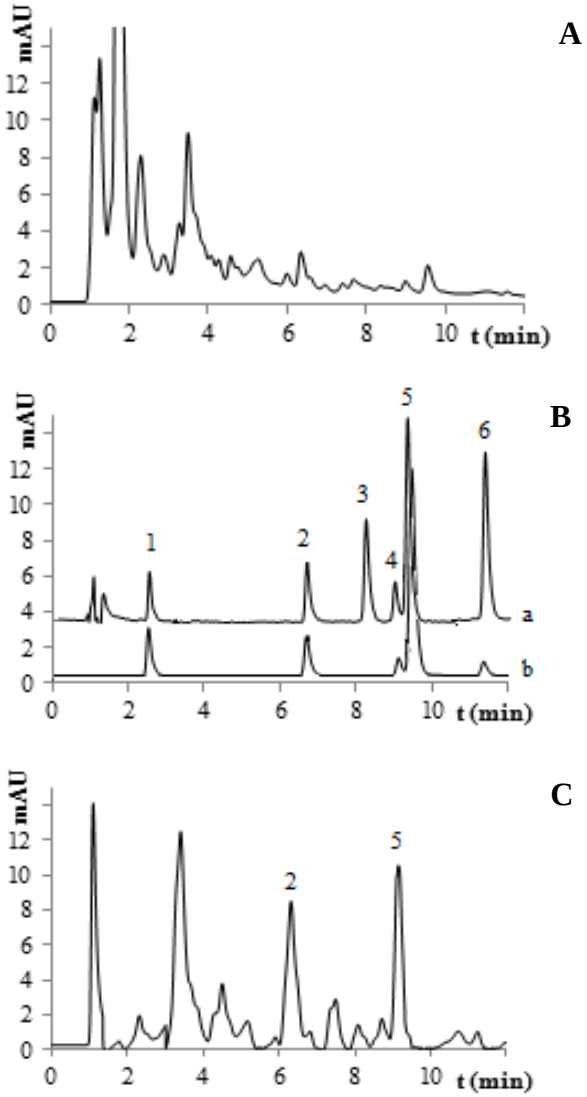

Fig. 3. Chromatograms of coumarins mixture (**A**; line *a* – UV spectrophotometric detection, *b* – fluorescence detection), wine sample III extract without (**B**) and after (**C**) MISPE extraction. The peaks indicate for aesculin – 1, scopoletin – 2, coumarin – 3, scoparone – 4, 4-methylumbelliferone – 5, herniarin – 6.

The real sample of wines were selected in order to demonstrate the applicability for the MISPE method. Parameters including LOD, LOQ, linear range, correlation coefficient, precision and accuracy were examined to validate the developed MISPE method coupled with HPLC-DAD/FLD. By using HPLC method, acceptable asymmetry (A_S ~ 0.9), good peaks resolution (R_S ≥ 2.1), and analysis time lower than 15 min were obtained (Fig. 3A). The RSDs repeatability of retention times varied from 0.2 to 0.5 %. LODs and LOQs (Table 1) were calculated using 3 s_a and 10 s_a criteria (s_a is the standard deviation of the intercept of calibration curve). The calibration curves were linear in working ranges from LOQ to 100 μg.mL^{-1}

for coumarin detected with UV detection and in the LOQ – 100 ng.mL^{-1} concentration range for other coumarins detected with fluorescence detector. The precision and accuracy (expressed as the recovery) of the developed method was determined for spiked sample at three concentration levels of each analyte (Table 2). The intra-day precision expressed as RSD (%) ranged from 1.2 to 5.4 %. The obtained recoveries ranged from 68.7 to 96.0 %. Two of coumarins, scopoletin and 4-methylumbelliferone, were determined in wine samples using MISPE-HPLC method in ng.mL^{-1} concentration levels (sample I: 5.4±0.3 ng.mL^{-1} of scopoletin, sample II: 9.2±0.1 ng.mL^{-1} of scopoletin, sample III: 10.1±0.5 ng.mL^{-1} of scopoletin and 12.0±0.3 ng.mL^{-1} of 4-methylumbelliferone, sample IV: 17.2±0.3 ng.mL^{-1} of scopoletin). Chromatograms of wine extracts (Fig. 3) documented that MISPE was suitable for extraction and pre-concentration of coumarins from wine samples. The results showed that content of coumarins in wine samples depended on the age and wine puttony.

Conclusions

In this study, the solid-phase extraction with different types of sorbents, C18-silica, styrene-divinylbenzene copolymeric sorbents, and selective MIP based sorbents were compared for isolation and pre-concentration of six coumarins. MIPs displayed higher selectivity and reusability. The obtained recoveries for target analytes ranged from 68.7 to 96.0 %. The results obtained demonstrated the appropriateness of solid-phase extraction with MIP-umbelliferone sorbent in wine analysis. Developed MISPE coupled with HPLC method was applicable for determination of simple coumarins in real samples and exhibited good specificity, accuracy, precision and recovery. The results showed that content of coumarins in wine samples are in ng.mL^{-1} concentration levels and depend on the age and wine puttony. For this purpose, further research on identification and quantification of coumarins is needed. Alternatively, this study could provide a promising method for the selective separation and determination of coumarins in other samples of beverages and sample extracts too.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflict of interest.

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