EXAMINATION OF THE CHEMICAL COMPOSITION OF PROPOLIS IX. SOLID PHASE EXTRACTION OF COUMARINS FROM PROPOLIS BY USING MOLECULARLY IMPRINTED POLYMER

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Abstract: The group selective molecularly imprinted polymers (MIPs) for coumarins, including umbelliferone, herniarin, 4-methylumbelliferone, scoparone were developed. Using umbelliferone as the template molecule, methacrylic acid as functional monomer, ethylene glycol dimethacrylate as linking agent, chloroform as porogen and bulk polymerization as synthetic method, the MIPs were synthesized and characterized with rebinding experiments. The characteristics of MIPs were evaluated by chromatographic method and frontal analysis, and demonstrating good selectivity and high binding capacity (269 µg of umbelliferone per 100 mg of polymer). The group selective MIP was used as sorbent for the SPE pre-treatment of coumarins from propolis extracts prior to HPLC analysis. Analysis of the samples showed good recoveries (>70 %). The limits of quantitation (LOQs) for studied compounds were 0.3-10 ng.mL^{-1} (determined for fluorescence detection).

Key words: molecularly imprinted polymer, umbelliferone, solid phase extraction, propolis, HPLC

1. Introduction

Propolis is a bee product which has lots of functional properties. It is a dark-coloured resinous natural substance collected by honeybees from leaf buds and the exudates of various tree species. Propolis's chemical composition is quite variable and complex depending on the provenance of the sample which is closely related with the flora surrounding the hive, the geographic and climatic characteristics at the site, honey bee species, etc.. Raw propolis is composed of resin (consisting of flavonoids, phenolic acids, …), wax, essential oils, pollen and other organic compounds (terpenoids, steroids, aromatic alcohols, aliphatic acids and esters, coumarins, sugars, amino acids, ...). In addition, more than 300 compounds, mainly polyphenols, have been identified as constituents of propolis from different sources. Propolis has been used extensively in folk medicine since it possesses various biological properties, such as antiseptic, antifungal, antibacterial, antiviral, anti-inflammatory, anaesthethic and antioxidant properties. (SILICI *et al*., 2005; SFORCIN *et al.*, 2011).

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Coumarins (benzo-α-pyrone derivatives) constitute an important group of natural compounds. Many of their analogues are found to be biologically active and have been proven to exhibit pharmacological activity. Chemical and pharmaceutical studies have revealed that important compounds in propolis samples are the coumarins, mainly including simple coumarins. Umbelliferone (Fig. 1) has been reported to have antioxidant and antimicrobial properties (JURD *et al*., 1971). Several analytical methods can be used for the determination of simple coumarins. The recent methods for the analysis of coumarins are summarized in Table 1.

The cleaning step prior to analysis is necessary to separate target analytes from the other propolis constituents. The most widely used sample preparation techniques (clean-up and pre-concentration) are Soxhlet extraction, solvent extraction and solid phase extraction (SPE) of the propolis extract obtained. The typical SPE sorbents often lack selectivity towards the target analyte and this is problematic for the selective extraction of analytes from complex matrices. Molecularly imprinted polymers (MIPs) are tailor-made materials with a pre-defined selectivity for a given analyte or closely related compounds for which they were synthesised. These materials are obtained by polymerizing functional and cross-linking monomers around a template molecule, which lead to a highly cross-linked three-dimensional network polymer. The monomers are selected according to their ability to interact with the functional groups of the template molecule. 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 (TAMAYO *et al.*, 2007).

In previous work (HROBOŇOVÁ *et al*., 2013) the liquid-solid extraction with stirring, ultrasonic assisted extraction and accelerated solvent extraction (ASE) methods were tested for sample preparations of propolis. The yields of umbelliferone and scoparone were higher by using of ultrasonic assisted extraction and by using of ASE method in comparison with stirring support extraction.

In the present work the molecularly imprinted polymer was applied as a solid phase sorbent for MIP-SPE cleaning of propolis extracts prior to HPLC analysis. Umbelliferone was used as the template for imprints formation. Methacrylic acid was used as the monomer, and chloroform as the porogen. The binding capacity and the selectivity of polymer, evaluated on the basis of structurally related compounds, were studied.

2. Materials and methods

2.1 Chemicals and samples

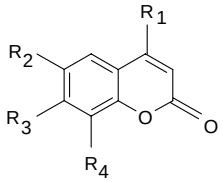
Umbelliferone (7-hydroxycoumarin), 4-methylumbelliferone, herniarin, scoparone, and coumarin (Fig. 1) were purchased from Sigma-Aldrich. Acetonitrile, methanol, ethanol, chloroform (all HPLC grade), acetone, acetic acid, 2,2′-azobis(2-methylpropionitrile) (2,2′-azobisisobutyronitrile, AIBN), methacrylic acid (MAA), and ethylene glycol dimethacrylate (EGDMA) (all analytical grade) were purchased from Merck. Before use, the MAA and EGDMA were distilled at reduced pressure to remove stabiliser from monomers.

Table 1. Summary of the recent analytical methods for separation of coumarins.

Analytical method	Separated compounds	Conditions	LOD [ng·mL^{-1}]	Reference
UPLC	umbelliferone, herniarin	SP: C18/BEH MP: methanol/0.1% formic acid DT: MS/MS	0.1	NOVÁKOVÁ *et al*., 2010
HPLC	esculin, esculetin	SP: C18/Diamonsil; MP: acetonitrile/0.5% acetic acid (12/88 v/v) DT: UV (340 nm)		ZHOU *et al*., 2011
HPLC	daphnetin, umbelliferone, 7-methoxycoumarin	SP: C18/Zorbax SB; MP: acetonitrile/0.5% acetic acid, gradient elution DT: DAD (325 nm), MS-ESI	40-84	SU *et al*., 2009
HPLC	umbelliferone, 4-hydroxycoumarin, scopoletin	SP: ODS-BP/Daisopak-120, MP: methanol/50 mmol·L^{-1} phosphate buffer (pH 5) DT: UV (286, 323 nm), FL (320/450 nm)	12.1-35.6 (UV) 0.06-12.5 (FL)	IKEDA *et al.*, 2009
2D TLC	ubelliferone, aescuketin, coumarin, scopoletin	SP: CN silica MP I: acetonitrile/water (30/70 v/v) MP II: ethyl acetate/n-hexane (35/65 v/v)		CIESLA *et al*., 2009
CE	esculin, esculetin	5 mmol·L^{-1} borate buffer pH 9.4/methanol (80/20 v/v) DT: laser-induced fluorescence	1.8 1.4	WANG *et al.*, 2007
CEC	herniarin, umbelliferone	SP: Hypersil SCX/C18 MP: 50 mmol phosphate buffer pH 2.8 /acetonitrile (50/50 v/v)	0.03	FONSECA *et al*., 2007
MEKC	coumarin	SP: fused-silica capillary, 20 kV MP: 20 mmol·L^{-1} tetraborate buffer pH 9.4 + 20 mmol·L^{-1} sodium dodecylsulfate, DT: DAD (200 nm)	0.01	MICKE *et al.*, 2003
SPT	umbelliferone	λ_{max} 324 nm,	500	MALÍK *et al*., 2012
SF	umbelliferone, 4-methylumbelliferone, coumarin, scopoletin	λ_{excit} 340 nm, λ_{em} 425 nm		FERNÁNDEZ IZQUIERDO *et al*., 2000

UPLC – Ultra pressure liquid chromatography, HPLC – high performance liquid chromatography, 2D TLC - two-dimensional thin layer chromatography, CE – capillary electrophoresis, CEC - capillary electrochromatography, MEKC - micellar electrokinetic chromatography, SPT – spectrophotometry, SF – spectrofluorimetry, SP – stationary phase, MP – mobile phase, DT – detection, DAD – diode array detection, UV – Ultraviolet light, MS – mass spectrometry, ESI – electrospray.

The working solutions were prepared from stock standard solutions of umbelliferone, 4-methylumbelliferone, herniarin, scoparone and coumarin in methanol (c = 1.0 mg·mL^{-1}) by appropriate dilution with water. The solutions were filtered through a 0.45 mm nylon membrane filter.



	R_1	R_2	R_3	R_4
umbelliferone	H	H	OH	H
coumarin	H	H	H	H
4-methylumbelliferone	CH_3	H	OH	H
herniarin	H	H	CH_3O	H
scoparone	H	CH_3O	CH_3O	H

Fig. 1. Chemical structures of umbelliferone and related compounds.

Propolis preparative without alcohol (Sample I) was directly analysed by HPLC or applied on MIP-SPE. For the preparation propolis tincture (Sample II) the portion 1 g of propolis was extracted with 40 mL of pure ethanol for three days at temperature 22 °C. The extract was filtered through a 0.45 μm nylon membrane filter and volume of 20 μL was injected into the liquid chromatograph or applied on MIP-SPE (2 mL of extract was loaded into the conditioned MIP-SPE cartridge and processed as described above for the MIP-SPE experiment).

2.2 HPLC analysis

Experiments were performed on a Hewlett Packard (series 1100) HPLC system equipped with a quaternary solvent delivery system, an injection valve (Rheodyne) with 20 μL injection loop, diode array and fluorescence detector. Chromatographic separations were performed on an analytical column LiChrospher 100 RP18 (250 × 4 mm I.D., 5 μm). The mobile phase for gradient elution consisted of a mixture of acetic acid (0.3 vol %)/acetonitrile (10/90 v/v) (A) and acetonitrile (B). The flow rate was 1.0 mL·min^{-1} and the column temperature kept constant at 22 °C. The monitored wavelength of 323 nm was used for the spectrophotometric detection or 320 nm (λ_{ex}) and 450 (λ_{em}) for fluorescence detection. Gradient profile of mobile phase is shown in Table 2.

Table 2. Gradient profile of mobile phase.

Time [min]	% A	% B
0	100	0
35	44	56
40	0	100
45	0	100

2.3 Preparation of MIPs and NIPs

Umbelliferone (0.1 g, 0.7 mmol), MAA (0.25 g, 2.9 mmol), EGDMA (2.34 g, (11.75 mmol) and AIBN (0.03 g, 0.18 mmol) were added to a glass tube containing 7.5 mL of chloroform. The polymerisation mixture was kept in a water bath at 60 °C for 24 h. The resulting bulk rigid polymers were crushed and sieved through a 40 μm sieve. Fine particles were removed by flotation in acetone and the final product was dried under diminished pressure at 60 °C for 1 h. The template molecule was extracted from the MIP by Soxhlet extraction with 100 mL of methnol/acetic acid (9/1 v/v) for 24 h. The non-imprinted polymer (NIP) was prepared following the same procedure as for MIP but without the template molecules during polymerisation.

2.4 Determination of polymer binding capacity

For the binding capacity studies, 0.3 g of the polymer MIP or NIP was transferred into a glass column and washed with methanol prior to use. The column was equilibrated by washing with umbelliferone solvent and subsequently the solution of umbelliferone was applied. The flow-rate was 0.2 $mL \cdot min^{-1}$. The eluent was monitored by UV detector at 323 nm. The breakthrough curves of each MIP and NIP were measured for umbelliferone solutions prepared in methanol, acetonitrile, methanol/water (50/50 v/v), acetonitrile/water (50/50) and water at a spiking level of 100 $\mu g \cdot mL^{-1}$.

2.5 MIP-SPE experiments

The MIP-SPE column was prepared by packing the SPE cartridge with the MIP or NIP material (0.1 g). The material was fixed by inserting polyethylene frits at the top and at the bottom. The prepared cartridge was conditioned by washing with 3 mL of methanol and 2 mL of water. Next, the working solution of umbelliferone (0.5 mL, c = 10 $\mu g \cdot mL^{-1}$) or 2 mL of propolis tincture was applied at 0.5 $mL \cdot min^{-1}$ flow-rate. The column was washed with 2 mL of water and dried under diminished pressure for 10 min. Finally, elution of the umbelliferone was performed by passing the elution solvent (2 mL of methanol or methnol/acetic acid (90/10 v/v)) through the column at a flow-rate of 0.5 $mL \cdot min^{-1}$. All the fractions (each of the volume of 0.5 mL) from the sample loading, washing and elution step were collected, solvent evaporated to dryness (diminished pressure, 30 °C), dissolved in acetonitrile (250 μL) and analysed by HPLC.

3. Results and discussion

3.1 Preparation of molecularly imprinted polymer

The non-covalent approach is the most frequently used method of preparing MIP, in which the binding sites are formed by self-assembly between the template and the monomer followed by a cross-linked co-polymerization. To perform this non-covalent

MIP approach, we selected the methacrylic acid as monomer. The structure of umbelliferone includes functional groups that may form hydrogen bonds with the monomer. In order to favour hydrogen bonding between the template and the monomer, a nonprotic solvent, chloroform, was chosen as the porogen solvent.

3.2 Characterization of molecularly imprinted polymer

The sorbents were evaluated by determining the sorption capacity of polymers for umbelliferone dissolved in different solvents, by evaluating the imprinting effects for MIP and NIP polymers, by evaluating the selectivity of the polymer, and by optimizing the MIP-SPE procedure.

For determination of binding capacity of polymers the solutions of methanol, methanol/water (50/50 v/v), acetonitrile, acetonitrile/watwer (50/50 v/v) and water spiked with 100 $\mu g \cdot mL^{-1}$ umbelliferone were percolated on the MIP. The same experiment was carried out in parallel on the NIP (non-imprinted polymer) in order to evaluate non-specific interactions during the retention process.

The binding capacities of the prepared polymers were examined by the breakthrough curves method and corresponded to the maximum amount of umbelliferone that can be retained on it without its release monitored by UV detector. Typical break through curves used for the determination of binding capacities of polymers are shown in Fig. 2. In the case of methanol/water (50/50 v/v) as umbelliferone solvent, the breakthrough values were observed at 76 min (MIP) and 18 min (NIP), which corresponds to the polymer binding capacity of 401 µg and 132 µg of umbelliferone per 100 mg of polymer.

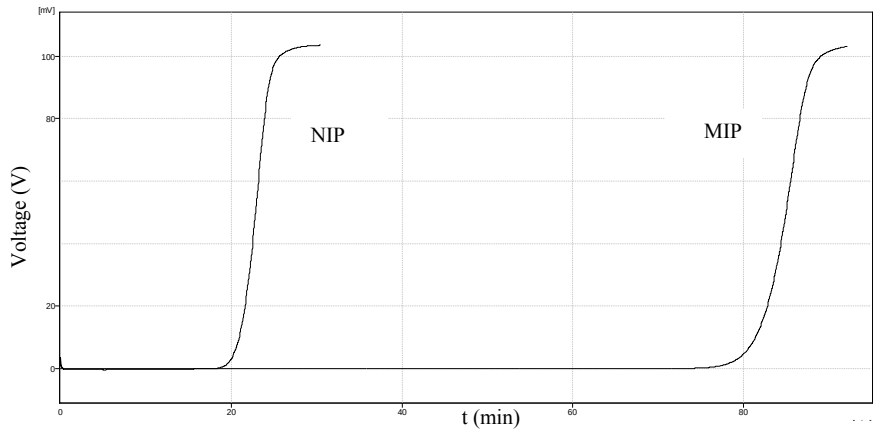


Fig. 2. The break through curves for determination of binding capacities MIP and NIP. Conditions: column – MIP/NIP (0.3 g), solvent used for capacity studies - umbelliferone (concentration 100 $\mu g \cdot mL^{-1}$) in methanol/water (50/50 v/v), flow rate 0.2 $mL \cdot min^{-1}$, spectrophotometric detection at 323 nm.

In the next step, the influence of the nature of the umbelliferone solvent on the binding capacity of prepared polymers was studied. The results presented in Fig. 3

show that the solvent used in the capacity determination process plays an important role in the sorption of umbelliferone on polymer. The nature of the solvent for this step probably influences the relative swelling of the polymer which led to changes in the binding cavities and could also be important for solvation processes. A significant imprinted effect was observed for the mixture methanol/water (50/50 v/v) as umbelliferone solvent (the highest value of specific capacity (MIP capacity minus NIP capacity). The specific binding capacity of MIP prepared in chloroform porogen was 269 μg of umbelliferone per 100 mg of polymer. When the water was selected as the solvent for determining the binding capacity, the umbelliferone was strongly retained on both MIPs and NIPs but no significant difference in binding capacities (data not shown) was observed between MIP and NIP. Retention in this case was probably due to non-specific interactions. Therefore, considering the highest values of specific binding capacity obtained for methanol/water (50/50 v/v) solvent, the experiments with real samples were performed in this medium.

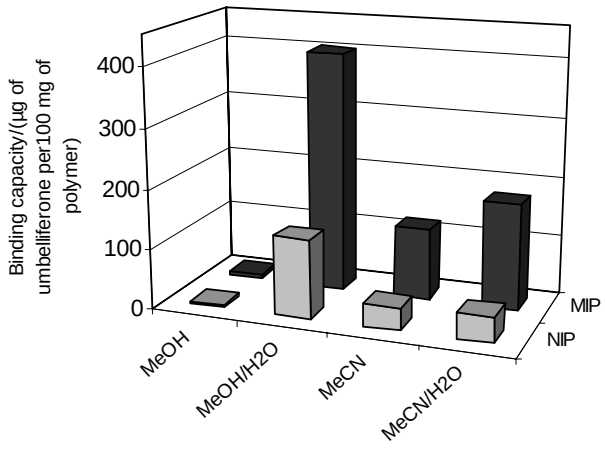


Fig. 3. The binding capacities of MIP and NIP obtained for different umbelliferone solvents. Solvents ratio (50/50 v/v); n=3.

3.3 Retention behaviour of structural analogous on the MIP

The retention of compounds structurally related to umbelliferone, namely coumarin, herniarin, scoparone, 4-methylumbelliferone, was study to evaluate the selectivity of prepared MIP. For this purpose 0.1 g portions of the polymer were stirred in 10 mL of mixture methanol/water (50/50 v/v) (blank) and mixture methanol/water (50/50 v/v) spiked with structural analogous ($c = 10\ \mu g \cdot mL^{-1}$) for 1 h. Subsequently, the polymer was filtered, washed with water and dried. The suspensions were prepared in methanol/acetic acid (90/10 v/v) ($1\ mg \cdot mL^{-1}$ of polymer). The absorption signals at 323 nm in UV spectra of the supernatant, measured in the wavelength interval of 210–400 nm indicated that the polymer prepared for umbelliferone was selective for the template molecule and three structurally related compounds tested (scoparone, herniarin, 4-methylumbelliferone), while the selectivity

for the underivatized molecule of coumarin was low. These results showed that MIP could be used as group recognition sorbent.

3.4 Method development of the MIP-SPE procedure

Since the main aim of this work was to incorporate the MIP prepared to umbelliferone into SPE pretreatment, the polymer was packed into cartridges as described in the experimental section (part 2.5) and used in the off-line procedure. To optimize the extraction, the wash step and the elution step were varied to achieve selective extraction. In the wash step, the clean-up solvent suppressed the non-specific interactions without disrupting the selective interactions between the MIP and the target molecule, and the interfering compounds which were bound non-specifically to the MIP were removed. The washing solvents were optimized to obtain maximal recovery of the analyte (tested for umbelliferone) on the NIP and minimal recovery on the MIP. Solvents including water and methanol/water (1/1 v/v) were tested. The best results were obtained when washing was performed with 2 mL of water, whereas some umbelliferone was released when the mixture methanol/water (1/1 v/v) was used.

The final step was to select a suitable solvent for umbelliferone desorption from the MIP. The elution solvents including methanol and methanol/acetic acid (9/1 v/v) were tested. Mixture methanol/acetic acid (9/1 v/v) gave rise to the best recovery (80 %), when the amount of solvent used being 1.0 mL. Poor recovery (58 %) was found with methanol as elution solvent. An increase in the volumes of these elution solvents to 1.5 mL enhanced the results to 92 % (methanol/acetic acid (9/1 v/v)) or 70 % (methanol). The volume of 2 mL of methanol/acetic acid (9/1 v/v) was used for the elution of umbelliferone from real samples.

3.5 Application of MIP-SPE to propolis samples

To demonstrate the potential of the polymer for the sample clean-up of complex matrices, the molecularly imprinted polymer was applied for the extraction of coumarins from propolis tincture. The MIP-SPE extracts were analysed by HPLC with fluorescent detection.

Three aliquots of Sample I were spiked with 0, 10 and 100 $ng \cdot mL^{-1}$ of umbelliferone and analysed by the proposed MIP-SPE procedure. The recoveries of the extraction on MIP were determined by comparing the results obtained for the elution fractions with the results of the sample analysis without MIP-SPE treatment. The recovery values obtained for the analyses of three replicates of umbelliferone spiked propolis preparative were more than 77 % with RSD less than 10 %. The results demonstrate the suitability of the prepared polymer for the MIP-SPE clean-up of the complex matrix. The propolis tincture prepared from crude propolis material (Sample II) is very complex matrix and the obtained recoveries were about 50 %.

The chromatograms of propolis extracts without and after application of the MIP-SPE procedure are shown in Fig. 4. In the case of the propolis samples tested, the presence of the coumarin derivative scoparone over the LOD was detected along with umbelliferone.

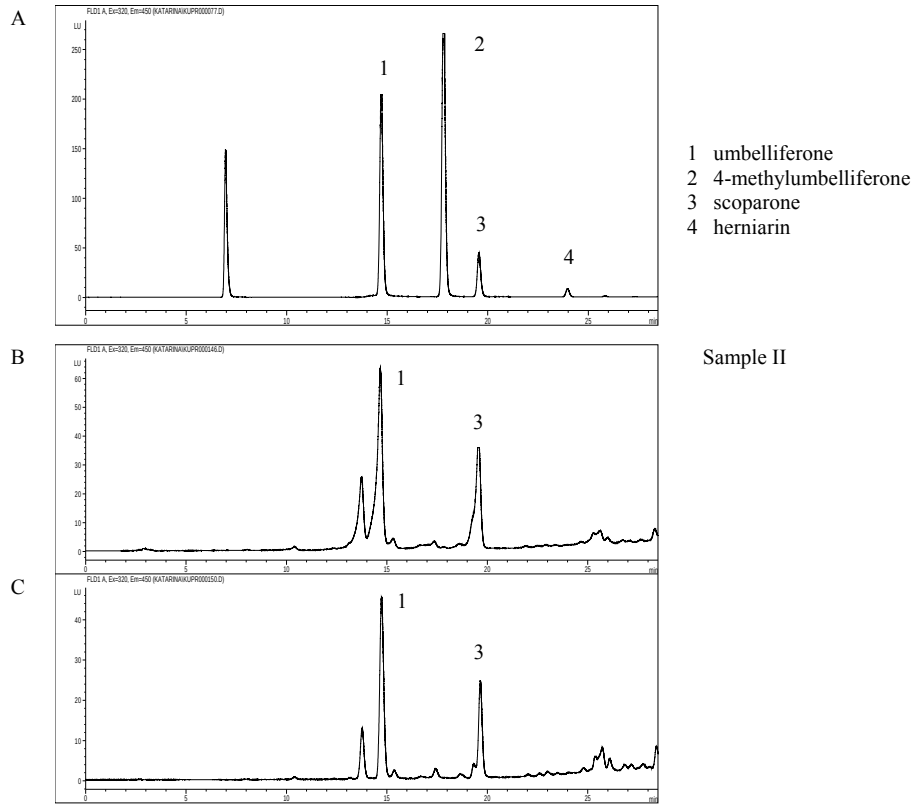


Fig. 4. HPLC chromatograms of reference compounds (A), extract of propolis sample without (B) and after (C) MIP-SPE. Chromatographic conditions: part 2.2

3.6 Assay validation

The method was validated over the concentration range 0.3-1000 ng·mL^{-1}. The regression analysis showed that there was a linear dependence (r = 0.985). Using a signal-to-noise ratio of 3 and 10, the limit of detection (LOD) for umbelliferone and scoparone were 0.1 and 5.0 ng·mL^{-1} and limit of quantification (LOQ) 0.3 and 10 ng·mL^{-1} respectively (determined for fluorescence detection). The values showed that the HPLC method is suitable for the determination of target analytes at the concentration levels presented in the propolis samples. The intra-day and inter-day precision of the MIP-SPE procedure was evaluated by determining umbelliferone in the propolis Sample I. Each assay was repeated three times a day over three different days. The values of the intra-day and inter-day precision expressed by the RSD values were 10% and 18%, respectively. The precision of the HPLC determination of umbelliferone was determined at for a concentration of 100 ng·mL^{-1}. The values of the intra-day and inter-day precision expressed by the RSD were 3.0 % and 5.0 %, respectively. The results showed good repeatability of the method developed.

4. Conclusion

A group selective molecularly imprinted polymer was prepared using umbelliferone as template. The results indicated that the polymer exhibits highly selective binding for coumarins. A specific binding capacity of 269 μg per 100 mg of polymer was determined. The natures of the sample solvent were studied to achieve the maximal sorption capacity. The developed off-line SPE extraction method provided enhanced sample clean-up and adequate recovery from propolis samples.

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