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Analytical Methods

Molecularly imprinted polymers for the selective extraction of glycyrrhizic acid from liquorice roots

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ABSTRACT

A new Molecularly Imprinted Solid Phase Extraction (MISPE) protocol was developed for the selective extraction and purification of glycyrrhizic acid (GL) from liquorice roots. Non-covalent MIP were synthesized using methacrylic acid (MAA), 2-(dimethylamino)ethyl methacrilate (DMAEM) or 2-hydroxyethylmetacrylate (HEMA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as crosslinking agent.

After the evaluation of the selectivity of the GL imprinted polymers, the performance of these materials as Solid Phase Extraction (SPE) sorbents was investigated. MIP having HEMA as functional monomer were found to be able to selectively extract almost 80% of GL content in liquorice roots. The proposed MISPE-HPLC procedure has good precision, thus it can be successfully used for the purification of GL from natural sources.

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1. Introduction

Glycirrhiza glabra (liquorice) roots are extensively used in herbal medicines for their emollient, antitussive, anti-inflammatory, antiviral and gastroprotective properties (Chung et al., 2000; Sabbioni et al., 2005). The product called "liquorice" in confectionery manufacturing (i.e. flakes or pastilles of pure liquorice) is obtained by treating dried *G. glabra* roots with boiling water, which is then evaporated to obtain a semi-solid extract (Fenwick, Lutomski, & Nieman, 1990).

The main active compound of *G. glabra* is glycyrrhizin or glycyrrhizic acid (GL), also known as (3*β*, 20*β*)-20-carboxy-11-oxo-30-norlean-12-en-3-yl-2-*o*-*β*-17-glucopyranuronosyl-α-D-glucopyranosiduronic acid. This molecule is present in the root and consists of an aglycon (a pentacyclic triterpenic structure) bound to two glucuronic acid molecules (Andersen, 2007). GL has anti-inflammatory, antiallergenic, antihepatotoxic, antiulcer and antiviral properties (Aly, Al-Alousi, & Salem, 2005; Jo et al., 2005; Ploeger, Meulenbelt, & DeJongh, 2000). Furthermore, GL is one of the leading natural compounds for clinical trials in recent studies on chronic viral hepatitis and human immunodeficiency virus (HIV) infections (Arase et al., 1997; De Simone et al., 2001; Fiore et al. 2008). Chronic consumption of GL prevents the development of hepatic carcinoma from C hepatitis and the antiviral activity of GL against SARS associated corona virus has been demonstrated in vitro (Nassiri-Asl & Hosseinzadeh, 2008). Finally, GL finds application in inhibiting unwanted effects of contraceptive formulations, such as alterations in blood coagulation and thrombosis (Stähli et al., 2007).

Based on these considerations, the determination and the selective extraction of GL form liquorice roots has gained great interest. Several papers report analytical methods for the determination of GL with different techniques such as HPLC, capillary electrophoresis, gas chromatography, and high-performance thin layer chromatography (Ohtakea et al., 2004; Sabbioni et al., 2005; Xie, Wang, Zhang, Bai, & Yang, 2007). In all these techniques, different kind of sample preparation methods are required: plant extracts are, indeed, complex mixtures with a large variety of chemical compounds.

Among the extraction technique, Solid Phase Extraction based on Molecularly Imprinted Polymers (MISPE) is an efficient approach for purification of analytes from complex matrices and the preconcentration of the samples, and it is gaining considerable interest in environmental, clinical, and food analysis (Anderson, Ariffin, Cormack, & Miller, 2008; Benito-Peña, Urraca, Sellergren, & Moreno-Bondi, 2008; Zhang, Ye, & Mosbach, 2006).

Molecular imprinting technique is an attractive synthetic approach to mimic natural molecular recognition, which exhibits a high affinity and selectivity toward a particular target compound named template (Alvarez-Lorenzo & Concheiro, 2006; Puoci, Iemma, & Picci, 2008; Turner et al., 2006). This technique utilises

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template molecules to assemble their own recognition sites by interacting with complementary functional groups of appropriate monomers. In this process, the selected template molecules are first allowed to establish binding interactions with functional monomers in a porogenic solvent. The resulting complexes are then copolymerised with an excess of cross-linkersin the presence of a free radical initiator. Subsequent removal of the template molecules creates specific binding sites with the precise spatial arrangement of the functional groups in the polymer network together with the shape. Thus, reversible re-binding and high selective recognition of the target molecules are realised (Nicholls et al., 2009; Puoci et al., 2009).

MIP can be used as sorbents with selectivity predetermined for a particular substance, or group of structural analogues, and have been used in Solid Phase Extraction for separation or clean-up of target compound in low concentrations or in complex matrixes (Puoci et al., 2007). The improved selectivity of imprinted polymers compared with conventional sorbents may to obtain cleaner chromatographic traces in the subsequent analytical separation (Puoci, Curcio, et al., 2008).

In a previous work, molecularly imprinted Solid Phase Extraction was successfully applied to the selective extraction of Glycyrrhetic acid (GA) from liquorice roots after acidic hydrolysis (Claude, Morin, Lafosse, Belmont, & Haupt, 2008). GA, indeed, is the non sugar component of GL and it is not found in the freed form in the liquorice extracts, in which the main component is GL. In this study, GL imprinted polymers were synthesized by employing methacrylic acid (MAA), 2-hydroxyethylmetacrylate (HEMA) or 2-(dimethylamino)ethyl methacrylate (DMAEM) as functional monomers and Ethylene glycol dimethacrylate (EGDMA) as crosslinker. After the evaluation of the imprinting efficiency, a SPE protocol was optimised and applied for the selective extraction of GL form liquorice root without any preliminary treatment of the sample. A very straight-forward protocol was developed, involving the direct HPLC injection of eluate fractions without any treatment. The pre-treatment of the sample consists only of a crushing step and a soxhlet extraction. In all the steps, no toxic and biocompatible organic solvents were used.

2. Materials and methods

2.1. Reagents and standards

Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2-(dimethylamino)ethyl methacrylate (DMAEM), 2-hydroxyethylmetacrylate (HEMA), 2,2′-azoisobutyronitrile (AIBN), methacrylic acid (MAA), glycyrrhizic acid (GL), glycyrrhetic acid (GA) were obtained from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO). All solvents were reagent grade or HPLC-grade and used without further purification and were provided by Fluka Chemika-Biochemika (Buchs, Switzerland). Monomers were purified before use by distillation under reduced pressure.

2.2. Preparation of molecularly imprinted polymers

The MIP stationary phase was prepared by bulk polymerisation. Two different GL imprinted polymers (MIP) were prepared using MAA, DMAEM or HEMA as functional monomers. Briefly, 0.5 mmol of template glycyrrhizic acid and 16.0 mmol of functional monomers were dissolved in 5 ml of anhydrous DMF in a thick-walled glass tube, and then 25.0 mmol of EGDMA and 0.100 g of AIBN were added. The tube was sparged with nitrogen, sonicated for 10 min, thermo-polymerised under a nitrogen atmosphere for 24 h at 60 °C. The resultant bulk rigid polymers were crushed, grounded into powder and sieved through a 63 nm stainless steel sieve. The sieved MIP materials were collected and the very fine powder, suspended in the supernatant solution (acetone), was discarded. The resultant MIP materials were soxhlet extracted with 200 ml of an ethanol/acetic acid (8/2 v/v) mixture for at least 48 h, followed by 200 ml of ethanol for others 48 h. The extracted MIP materials were dried in an oven at 60 °C overnight. The washed MIP materials were checked to be free of GL and any other compound by HPLC analysis.

Blank polymers (to act as a control) were also prepared when polymerisation was carried out in the absence of GL.

2.3. Binding experiments

The binding efficiency of polymeric matrices towards GL was evaluated by performing re-binding experiments in ethanol and in an ethanol/water (6/4 v/v) mixture. Briefly, 50 mg of polymer particles were mixed with 1 ml GL solution (0.2 mM) in a 1 ml eppendorf and sealed. The eppendorf were oscillated by a wrist action shaker (Burrell Scientific) in a water bath at 37 ± 0.5 °C for 24 h. Then the mixture was centrifuged for 10 min (10,000 rpm) in an ALC® microcentrifugette® 4214 and the GL concentration in the liquid phase was measured by HPLC. The amount of GL bound to the polymer was obtained by comparing its concentration in the MIP samples to the NIP samples.

The same experiments were performed using GA solutions. Each measurement was carried out in triplicate and the data expressed as means (±RSD).

2.4. Molecularly imprinted Solid Phase Extraction conditions

The 500 mg amount of dry particles of MIP and NIP was packed into a 6.0 ml polypropylene SPE column. The column was attached with a stop cock and a reservoir at the bottom end and the top end, respectively. The polymer was rinsed with chloroform, acetonitrile, ethanol and then with the loading solvent.

GL was dissolved in the loading solvent to final concentration of 0.2 mM. After conditioning, dry MISPE column was loaded with GL standard solution. After loading, vacuum was applied through the cartridges for 5 min in order to remove residual solvent. Washing solvent was then passed through the cartridges and finally, after column drying, elution solvent was applied to perform the complete extraction of GL. The loading, washing and eluting fractions was analysed by HPLC to detect the GL amount.

The MISPE protocol was optimised and the best conditions were:

Loading step: 2 ml of ethanol, flow rate ~0.2 ml/min; washing step: 8 ml of ethanol/water mixture (9/1 v/v), flow rate ~0.2 ml/min; eluting step: 2 ml of hot ethanol/water (5/5 v/v) mixture, flow rate ~0.2 ml/min.

In order to evaluate the selectivity of the MIP, optimised protocol was also applied using GA solutions.

Each measurement was carried out in triplicate and the data expressed as means (±RSD).

2.5. Extraction procedure

A liquorice sample was ground to a fine powder and a sample (200 mg) transferred to a 50 mL round bottom flask together 20 mL of ethanol:water (5/5, v/v). The mixture was maintained by thermostat at 60 °C for 25 min with stirring and then centrifuged for 10 min at 3000 rpm (Sabbioni, Ferranti, Bugamelli, Cantelli Forte, & Raggi, 2006).

The supernatant was filtered through a paper filter and an aliquot of the filtrate, suitably diluted with mobile phase, was subjected to HPLC analysis.

2.6. Molecularly imprinted Solid Phase Extraction of vegetable sample extracts

The extract was evaporated to dryness and the reconstituted in solution by adding ethanol. 2 ml of this solution were used to load the MISPE column. The washing solvent consists 8 ml of an ethanol/water mixture (9:1 v/v), while 2 ml of water mixture were used as elution fraction. All the solutions, suitably diluted with mobile phase, was subjected to HPLC analysis.

Each measurement was carried out in triplicate and the data expressed as means (±RSD).

2.7. HPLC analysis

The liquid chromatography consists of a Jasco Model (Tokyo, Japan) consisting of an Jasco BIP-I pump and Jasco UVDEC-100-V detector set at 280 nm. A Tracer Excel 120 ODS-A column particle size 5 μm, 15 × 0.4 cm (Barcelona, Spain) was employed. The mobile phase consists of a methanol/acetonitrile/water/glacial acetic acid mixture 35/35/30/1, by volume, (Sabbioni et al., 2006). The flow rate was 0.5 ml min^{-1}.

2.8. Analytical parameters

In order to evaluate intermediate precision (inter-day precision) and repeatability (intra-day precision), assays were performed by extracting and injecting the same sample six times on the same day and six times over different days. The percentage relative standard deviations (RSD%) of the data thus obtained were calculated.

Accuracy was evaluated by means of recovery assays carried out by adding standard solutions of the analytes to the samples. The amounts of analytes added corresponded to concentrations of 5, 10 and 20 μg/mL. The mean recoveries of the added analytes were then calculated.

3. Results and discussion

3.1. Synthesis and characterised of the imprinted polymers

GL imprinted polymers were synthesized by using the non-covalent imprinting approach, because the rapid recognition kinetics, the simplicity of the process and because of the wide range of functional monomers which can be used (Mosbach, 2001).

When considering the chemical structure of template GL, it is clear that the functionalities suitable to interact with functional monomer are mainly the carboxylic ones. In literature, it is reported that these kinds of template can be successfully imprinted by using acidic, basic or neutral monomers (Beltran, Marcé, Cormack, & Borrull, 2009). Thus, we performed a screening of the most commonly used functional monomer, and in particular MAA was chosen as acidic (MIP1 and NIP1), DMAEM as basic (MIP2 and NIP2) and HEMA as neutral (MIP3 and NIP3) functional monomer. All the polymeric matrices were prepared using the same molar ratio between template, functional monomer and crosslinker. In particular, a template/functional monomer ratio of 1/32 was chosen because GL is a high functionalized molecules with several groups able to form coordination interactions.

The imprinting effect in the synthesized materials was evaluated by binding experiments in which amounts of polymeric particles were incubated with an GL solutions 0.2 mM for 24 h. These preliminary experiments were performed both in ethanol and in an ethanol:water mixture (6:4 v/v) to characterise the materials in eco-compatible environments. In addition, the same binding experiments were performed using GA, which differs from GL because of the absence of the two sugar moieties, in order to test the MIP selectivity (Fig. 1).

The results shown in Table 1, clearly prove the efficiency of the prepared polymers in selectively recognise the template. The chemical and spatial complementarity of the imprinted cavities in the MIP structure, indeed, determines a higher value (%) of bound template by MIP comparing to NIP, as well as a stronger interaction with the template that with the analogue molecule. To better characterise the imprinting effect, the specific parameters, α and ε, should be introduced (Cirillo et al., 2009). α Value is determined as the ratio between the amount (%) of template bound by MIP and NIP and thus represents a quantitative determination of the presence of the imprinted cavities in the polymeric structure. ε Value, which is a quantitative measure of the selectivity within the imprinted cavities, is calculated as the ratio between the amount (%) of GL and GA bound by MIP.

For all the polymers, high α_{GL} values were recorded in all the tested environments, proving the specificity of the interaction between the template and the functional groups on the polymeric

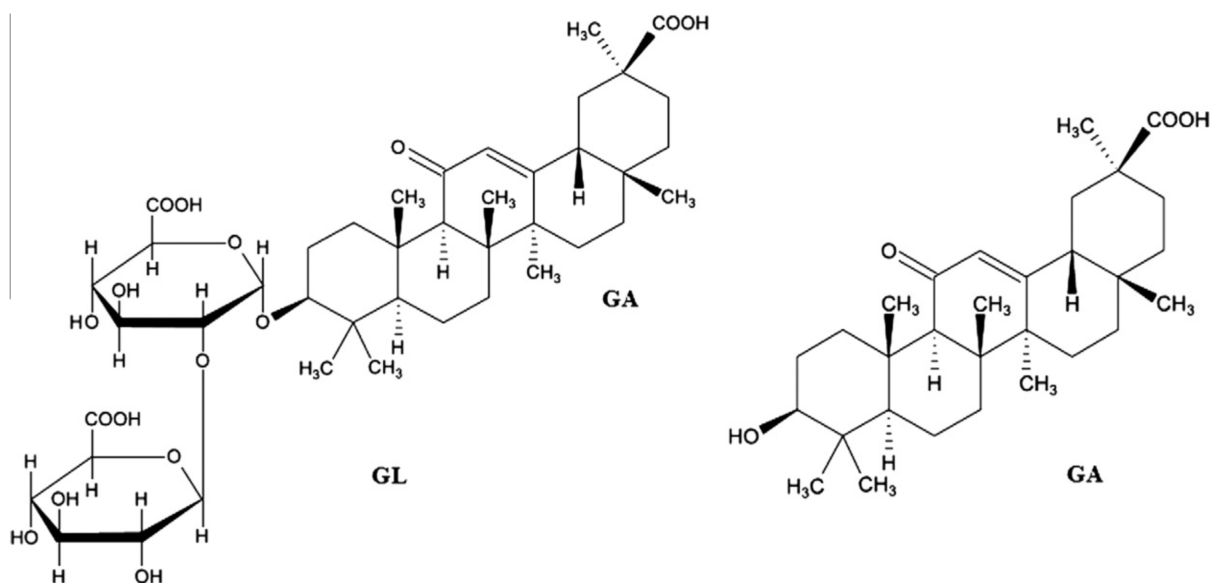

Fig. 1. Chemical structures of glycyrrhizic acid (GL) and glycyrrhetic acid (GA).

Table 1
Percentages of bound analytes after 24 h incubation in ethanol and ethanol/water (6/4 v/v) mixture with GL and GA concentration 0.2 mM. Data are shown as means ± R.S.D.

Polymer	% Bound GL		% Bound GA		α		ε	
	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O	EtOH	EtOH/H_2O
MIP1	40 ± 1.3	38 ± 0.9	18 ± 1.1	32 ± 0.9	3.07	6.33	2.02	1.18
NIP1	13 ± 0.6	6 ± 0.7	19 ± 0.6	33 ± 0.6				
MIP2	73 ± 0.8	90 ± 1.1	1 ± 0.5	16 ± 1.4	4.86	1.50	73.00	5.63
NIP2	15 ± 1.0	60 ± 1.3	1 ± 0.8	14 ± 1.2				
MIP3	27 ± 1.4	17 ± 1.2	20 ± 1.2	25 ± 0.9	2.45	5.00	1.35	1.13
NIP3	11 ± 0.8	3 ± 0.9	19 ± 0.9	24 ± 0.4				

Table 2
Percentages of collected GL in the optimised loading, washing and eluting steps. Data are shown as means ± RSD.

Polymer	Loading	Washing	Eluting
MIP1	6 ± 1.1	15 ± 1.2	79 ± 0.6
NIP1	5 ± 1.4	93 ± 1.5	2 ± 0.3
MIP2	3 ± 0.9	7 ± 0.6	3 ± 1.0
NIP2	5 ± 1.0	6 ± 1.3	2 ± 1.4
MIP3	1 ± 0.8	19 ± 1.4	81 ± 0.9
NIP3	3 ± 0.7	95 ± 1.1	2 ± 1.0

microparticles. In addition, all the obtained ε values are higher than 1.00, confirming that the imprinted cavities are characterised by stronger interaction with the template around which they are formed and lower affinity for its analogue.

By comparing the three different polymers, it is clear that the presence of DMAEM as functional monomer carried out to higher non-specific interactions between analytes and polymeric matrices, as a result of the strong ionic interaction between amine functionality of DMAEM and carboxylic groups of GL and GA.The most efficient polymers were found to be MIP1 and MIP3, containing MAA and HEMA as functional monomer, respectively.

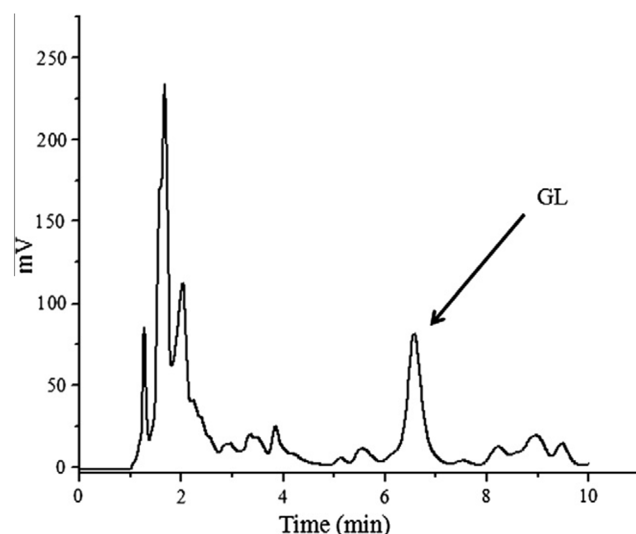

Fig. 2. Chromatogram of the liquorice roots extract. The chromatographic conditions are described under Materials and Methods. Column, Tracer Excel 120 ODS-A (5 μm, 15 × 0.4 cm); detection, 280 nm; flow rate, 0.5 ml min^{-1}. Mobile phase consisted of a methanol/acetonitrile/water/glacial acetic acid mixture 35/35/30/1 by volume.

3.2. Molecularly imprinted Solid Phase Extraction procedure and its optimisation

After the evaluation of MIP efficiency, MISPE cartridges were packed with MIP and the corresponding NIP, and their performance as sorbents for GL SPE was compared.

The cartridges were packed with 500 mg of polymer and the chemical and flow variables, such as composite and volumes of elution solution, sample acidity and loading flow rate were optimised to achieve good sensitivity and precision of this method. It should be considered that the employed solvents (water and ethanol) were chosen for the required eco and bio-compatibility of the process, while the acidification of the elution solutions perturbs the formation of the hydrogen bonds between hydroxyl and carboxyl groups of GL and those of the specific functional monomer.

By considering the loading step, it was found that the complete retention of GL (0.2 mM) on MIP and NIP cartridges was achieve by using ethanol at a flow rate of 0.2 ml min^{-1}, while water and ethanol/water mixtures were not effective. This behaviour is the same when considering polymer form series 1 and 3, while an almost complete retention of the analyte by polymers form series 2 is raised in all the tested environments.

The subsequent washing step was performed to minimise the non-specific interactions between polymeric matrices and template. It was found that the three matrices work in different ways. Neither water and ethanol, as well as ethanol/water mixtures were found able to remove GL from MIP2 and NIP2, and even acidifying the solutions no GL was detected in the washing solvents. This effect clearly prove that these polymers cannot be applied as sorbents in GL SPE protocols. On the contrary, good results were obtained with polymers 1 and 3. It should be considered that, in this step, the employed solvent is required to remove the template form NIP, while it should be retained by MIP. The results show that this effect was obtained by using an ethanol/water (9/1 v/v) mixture in series 3 and HCl (0.01 M) in series 1. In particular, in the washing fraction of MIP3, almost 19% of GL was recovered, while the template was almost completely removed from the corresponding NIP3 (Table 2). When considering the MIP1, the amount (%) of washed GL is almost 15%, while this value is increased to 97% in NIP1 case.

The elution of GL was successfully obtained by using water in MIP3 and in PBS (10^{-3}M, pH 7) in MIP1.

At last, the selectivity of the packing cartridges was evaluated by using GA solution in the optimised condition tested for GL.

In the loading step, MIP1 cartridges retain only 23% of GA, NIP1 cartridges the 19%, while for MIP3 and NIP3 these values are 31% and 35%, respectively.

In the washing fraction of all the MIP and NIP cartridges, all the retained GA was recovered, showing the high selectivity of the synthesized materials for the original template.

Based on these results, MIP1 and MIP3 are very useful as stationary phases for SPE procedure because, besides the adequate selectivity and affinity properties in the recognition of the analyte,

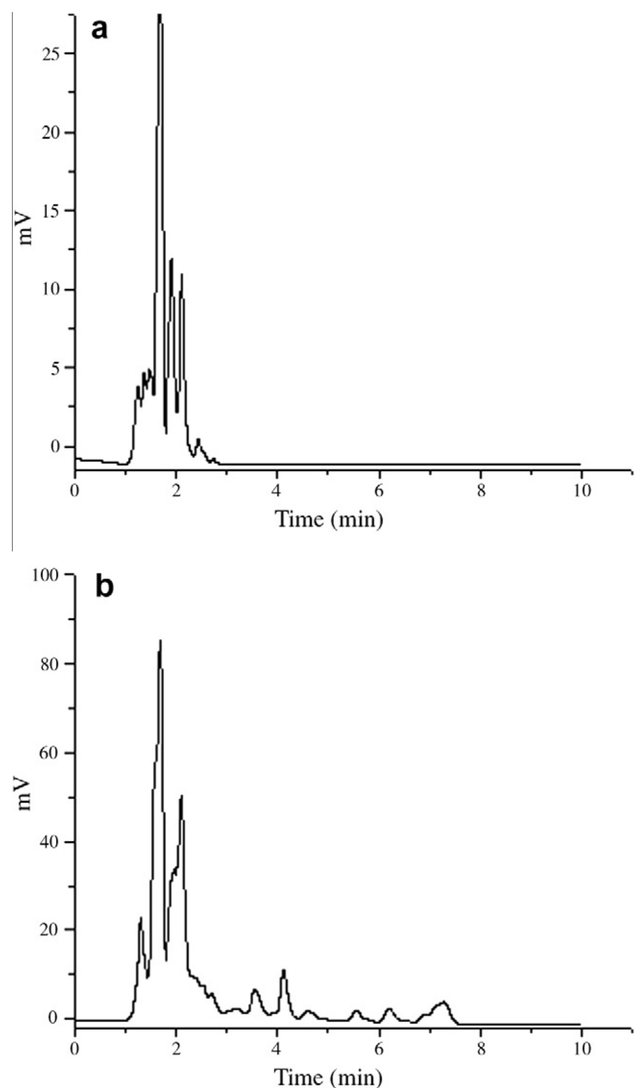

Fig. 3. Chromatograms of MISPE loading (a) and washing step (b). The chromatographic conditions are described under Materials and Methods. Column, Tracer Excel 120 ODS-A (5 µm, 15 × 0.4 cm); detection, 280 nm; flow rate, 0.5 ml min^{-1}. Mobile phase consisted of a methanol/acetonitrile/water/glacial acetic acid mixture 35/35/30/1 by volume.

a low level of template bleeding is raised. MIP2 is not effective because of the high bleeding effect.

3.3. Molecularly imprinted Solid Phase Extraction of liquorice roots

The previous SPE methods have been applied to a liquorice extract for the selective extraction of GL.

First of all, a performing extraction protocol must be applied to the liquorice roots crashed sample. In the development of such a kind of protocol, particular attention should be done to the chemical characteristic of the analytes. As reported in the European Pharmacopoeia, 2005, GL is usually >4% in liquorice root and 3–5% in ethanolic extract. In literature, different solvents were tested for the quantitative extraction of GL from liquorice roots, including water, ethanol, water/ethanol mixtures, ammonium hydroxide and water. A recent work deals with the comparison of these methods and the conclusion of this study is that the optimum extraction solvent is an ethanol/water (1/1 v/v) mixture, and in this study this procedure was adopted for the preparation of the liquorice extract to be loaded on MISPE cartridges. In particular, 200 mg of grounded liquorice sample was extracted with the ethanol/water mixture, dried and then reconstituted to solution by adding ethanol an aliquot of this solution, suitably diluted with mobile phase, was subjected to HPLC analysis (Fig. 2).

Firstly, MIP3 and NIP3 cartridges were tested. Three millilitres of the prepared loading solution was employed to load the cartridges which almost completely absorb GL (Fig. 3a). Thus, in order to obtain a satisfactory clean-up of the template, the washing solvent was applied on the cartridges and the GL amount in the washing fractions determined. In the washing chromatogram of MIP cartridges (Fig. 3b), not so relevant peaks were observed at the retention time of GL, confirming the selective interaction between polymers and template. The GL amount in this fractions represents about the 20% of the loaded one. Furthermore, an important clean-up of the extracted mixture was obtained, as showed by the presence of several peaks referable to the others compounds of the sample.

Finally, the selective elution of GL was obtained by using hot water, obtaining a satisfactory recovery of pure GL in MIP cartridges (about the 80% of the loaded one), confirming that the washing step was able to completely remove all the compounds which co-eluted with the template in the starting extract (Fig. 4).

In the SPE protocol performed on NIP3 cartridges, the loaded GL was almost completely washed out in the washing step and no GL was detected in the eluting fraction.

When MIP1 and NIP1 were employed, the loading step is characterised by the complete retention of GL on the cartridges, but the HCl solution employed in the washing step was not able to clean the cartridges from the compounds which co-eluted with GL in the original extract, which was not found pure in the eluting chromatograms.

3.4. Accuracy and precision

The precision of the method was then evaluated: intermediate precision values were obtained by repeating the SPE extraction of the same sample six times over different days; the RSD% thus calculated was 3.9%. Accuracy was assessed spiking the extract with three different levels of the analyte. The recovery values were between 93% and 107%, thus indicating the satisfactory accuracy of the method.

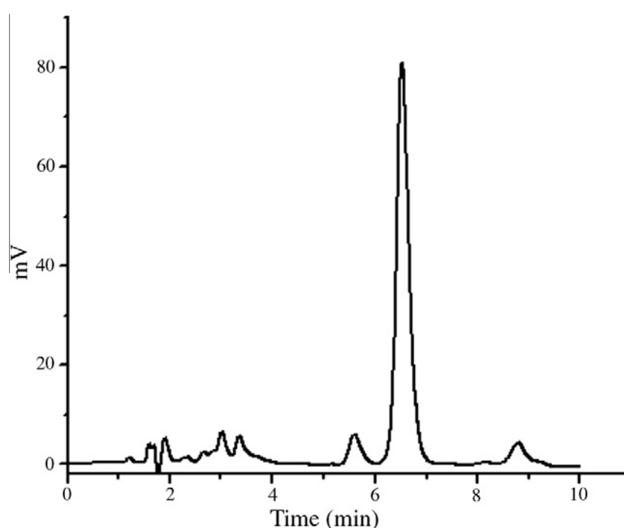

Fig. 4. Chromatogram of MISPE eluting step. The chromatographic conditions are described under Materials and Methods. Column, Tracer Excel 120 ODS-A (5 µm, 15 × 0.4 cm); detection, 280 nm; flow rate, 0.5 ml min^{-1}. Mobile phase consisted of a methanol/acetonitrile/water/glacial acetic acid mixture 35/35/30/1 by volume.

4. Conclusion

In this work, a molecularly imprinted Solid Phase Extraction protocol was developed for the selective extraction of GL form liquorice roots. GL imprinted polymers were synthesized using GL as a template and MAA, DMAEM or HEMA as functional monomers. The imprinting effect and the selectivity of the MIP were evaluated and all the polymers were found to be highly selective for GL.

MIP were then applied as sorbent in SPE to obtain a purification of GL from liquorice roots, and for this purpose, the SPE protocol was optimised. It was found that DMAEM containing materials are not effective, while good results were obtained with HEMA and MAA containing matrices. When the SPE protocol was employed by using liquorice extract, by using HEMA-MIP a selective extraction of 80% of pure GL is raised. On the contrary, MAA-containing materials were not effective. Thus, GL-MISPE protocol using HEMA-MIP offers excellent recovery of the template and is suited to routine use for GL isolation from liquorice.

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