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Journal of Chromatography A, 948 (2002) 163–169

JOURNAL OF CHROMATOGRAPHY A

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Selective solid-phase extraction sorbent for caffeine made by molecular imprinting

G. Theodoridis*, P. Manesiotis[1]

Laboratory of Analytical Chemistry, Chemistry Department, Aristotle University of Thessaloniki, GR-54006 Thessaloniki, Greece

Abstract

A molecularly imprinted polymer (MIP) was prepared with caffeine as the template molecule. Thermal polymerisation (60°C) was optimised, varying ratios of monomer, cross linker and template. The polymer was used as a solid-phase extraction (SPE) sorbent, for selective trapping and pre-concentration of caffeine. Caffeine was loaded on the MIP-SPE cartridge using different loading conditions (solvents, pH value). Washing and elution of the caffeine bound to the MIP was studied utilising different protocols. The extraction protocol was successfully applied to the direct extraction of caffeine from beverages and spiked human plasma.

Keywords: Molecular imprinting; Solid-phase extraction; Caffeine; Theophylline; Theobromine; Xanthines

1. Introduction

Solid-phase extraction (SPE) is established as a foremost sample pre-treatment technique with a broad application area. SPE offers significant advantages such as ease of operation, high load (up to semi-preparative) abilities, high recoveries, a wide spectrum of stationary phases available, automation capabilities, enhanced reproducibility and so forth. The authors have extensively used SPE as a powerful tool for the pre-treatment of biological fluids, plant tissue culture, and forensic samples [1–6]. However, despite its attractive features, SPE suffers from a major problem common in conventional chromatographic methods, namely generic selectivity. Controlling and adjusting the separation mechanism on conventional HPLC materials has been a major issue for decades. Unfortunately non-specific interactions, which often govern retention in chromatography, may prove too large an obstacle to overcome.

A solution to the above problem is the coupling of molecular recognition mechanisms with separation procedures. This coupling combines the advantages of both modes: specificity, selectivity and sensitivity of molecular recognition mechanisms with the high resolving power and ruggedness of separation methods. Furthermore, such a combination suppresses the limitations of the relevant modes such as the cross-reactivity of molecular recognition modes. Immunoaffinity chromatography has been the major tool in that regard and has proven a potent alternative to complex extraction protocols [7,8]. However, such an approach presents significant difficulties: the need for animal experiments, the long and tedious coupling and conjugation schemes which are required for most analytes, the long and costly immunisation schemes, and so forth.

*Corresponding author. Tel.: +30-31-997-718; fax: +30-31-997-719.

E-mail address: gtheodor@chem.auth.gr (G. Theodoridis).

[1]Present address: Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg Universität Mainz, Duesbergweg 10-14, D-55099 Mainz, Germany.

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01457-1

Over the past decade molecularly imprinted polymers (MIPs) have been one of the strongest trends in separations. Straightforward development of specific ''tailor media'' is a feature long desired by practitioners. Several other qualities of MIPs such as rigidity and tolerance of extreme pH values and organic environments, have brought imprinting technology to the forefront in contemporary chemical research [9–13]. Sellergren first described the use of MIPs as sorbents for SPE [14]. Since then MIPs have been used as selective sorbents for a wide range of analytes of biological, pharmaceutical and environmental interest and a comprehensive review has very recently covered the topic [15].

Molecularly imprinted polymers to be used in separations have been prepared against several analytes. Xanthines represent a group of templates of timely interest and thus MIPs against theophylline have been incorporated in separations [16–18], membranes [19–22], sensor devices [23,24], binding assays [25,26] and pharmaceutical applications [25]. Caffeine has also served as the template for the production of sensors [23,27,28] and microspheres used in radioligand bioassay [29]. Pulsed elution of xanthines from theophylline imprinted polymers recently developed by Mullet and Lai signifies an interesting approach [30,31]. The described method accomplished a selective retention of the template on the polymer and a subsequent elution with the injection of 20 μl of methanol.

Herewith we report our preliminary investigations into the manufacturing of an imprinted polymer and its use as a solid-phase extraction sorbent for caffeine. Of the various application areas of MIPs, separations are by far the broadest and most rapidly expanding. Exploitation of the molecular recognition properties of the imprinted polymers in sample pretreatment offers intrinsic advantages for the selective enrichment and clean-up of various samples. Caffeine represents one of the most common analytes in modern instrumental analysis. For years there has been a continuous interest from various starting points (pharmaceutical industry, clinical chemistry, food industry) for the development of analytical methods for the determination of caffeine in varying samples.

A methacrylic acid polymer was made with caffeine as the template molecule. Polymerisation parameters that were studied included different ratios of monomer, cross-linker and template. The produced polymers were packed and evaluated as selective solid-phase extraction cartridges. Extraction conditions (conditioning, loading, washing, eluting) were altered in order to achieve the highest recovery and at the same time suppress non-specific interactions and binding. The extraction protocol was successfully applied to the direct extraction of caffeine from beverages and spiked human plasma.

2. Experimental

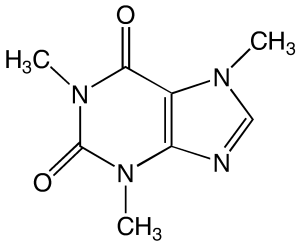
2.1. Chemicals

Caffeine (**1**), theophylline (**2**) and theobromine (**3**) were from Sigma (St. Louis, MO, USA) (Fig. 1). Ethylene glycol dimethacrylate, methacrylic acid and azo-*N*,*N*′-diisobutyronitrile (AIBN) were from Fluka (Buchs, Switzerland). All solvents used (acetonitrile, methanol, ethanol) were of HPLC quality and were obtained from Riedel-de Haën (Seelze, Germany). Water was double de-ionised and filtered though a 20-μm filter (Schleicher and Schül, Dassel, Germany).

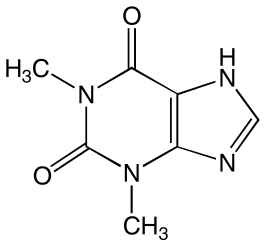
2.2. Polymer preparation

Polymerisation was based on the method reported by Andersson et al. [32]. AIBN (initiator, 15 mg) and (**1**) (0.50 mmol for P1 or 0.25 mmol for P2) were dissolved in 2 ml of acetonitrile in 20-ml glass tubes. Methacrylic acid (MAA, 2 mmol) and ethylene glycol dimethacrylate (EDMA, 6 mmol) were added to solution and the mixture was degassed by N_2 sparging for 2 min. Next the tubes were sealed and heated in a water bath at 60°C for 16 h. Then 4 h after the initiation, the first polymer formations emerged in the pattern of flakes. At the end of the heating, a dense white coloured rigid polymer network was obtained. The tubes were then smashed and the monolithic polymers obtained were ground in a laboratory mortar and pestle.

In order to remove remains of the monomers and the template the polymers were extracted in a Soxhlet apparatus using a mixture of methanol–acetic acid (9:1, v/v). Extraction lasted for 16 h,



(1)



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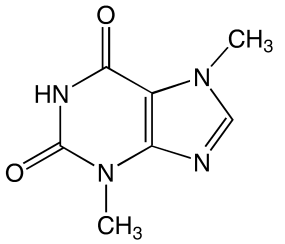

(3)

Fig. 1. Structures of caffeine (**1**), theophylline (**2**) and theobromine (**3**).

resulting from a sum of 30–35 solvent cycles (each cycle ~30 min). To determine the effectiveness of the template removal, the concentration of caffeine in the extraction solvents was determined by HPLC. At the end of the 16-h period no more template could be identified in the Soxhlet extract.

Next the polymers were ground in a laboratory mortar and pestle to reduce their particle size and the particles were sieved through 71- and 20-μm sieves with water. Particles that passed through the 71-μm sieve were collected, while retained particles were reground, until all the material could pass through the sieve. Grinding the polymer with water resulted in numerous fine particles and a large population of particles with diameter between 25 and 30 μm. The rest of the polymer mass remained in bigger particles (larger than 200 μm in diameter), so more than five repeated grinding and sieving cycles were necessary to reduce the size of the particles and obtain an adequate amount of particles of the desired size. Transmission electron microscopy (Zeiss Axio Lab A) of the 20–71-μm fraction revealed an irregularly shaped particles ranging mostly between 30 and 40 μm. Fine particles were removed from the polymer by repeated sedimentations in methanol–water (1:1, v/v). Finally the particles were dried under vacuum and stored at ambient temperature until use.

In order to verify that retention of caffeine was due to molecular recognition and not due to non-specific binding, a control (non-imprinted polymer) was prepared following the same procedure (Soxhlet extraction included), but with omission of caffeine.

2.3. HPLC determinations

HPLC analyses were performed using a Spectra-Physics (San Jose, CA, USA) HPLC system employing a Model SP 8800 ternary HPLC pump, a Spectra Chrom 100 variable-wavelength detector operating at 272 nm, an SP 4490 integrator, and a Rheodyne (Cotati, CA, USA) 7125 injection valve. The injection volume was 10 μl throughout the study. Separations were carried out on a 15 cm×4.6 mm, 5 μm Supelco (Bellefonte, PA, USA) Discovery C_{18} column. The mobile phase was a mixture of methanol–0.05 *M* aqueous CH_3COONH_4 (20:80, v/v) and the flow was maintained at 0.8 ml/min. To facilitate quantitative determinations in extracts, a calibration curve was obtained. Reference standard solutions of the three xanthines at concentrations: 0.25, 0.5, 1, 5, 20 and 50 μg/ml, were analysed five consecutive times and the peak areas were plotted versus the concentration.

2.4. MIP-SPE

Commercial SPE cartridges (which had already been used in the authors' laboratory) were emptied from their packing material. Next the cartridge tube and frits were thoroughly cleaned and dried. A 200-mg sample of the corresponding polymer was packed dry in the cartridge and the upper frit was placed on top. Extraction experiments consisted of loading the MIP-SPE column with 50 μg/ml of xanthine **1**, **2**, **3** or mixtures thereof. Then 2 min after each sample/

solvent loading the MIP-SPE cartridge was centrifuged at 3000 rpm for 5 min to facilitate passing of the solvent through the bed. Different protocols were applied (as described below), utilising different solvents during conditioning, loading, washing and eluting the MIP-SPE column. All the applied fractions were collected and evaporated to dryness (at 45°C under a stream of N_2). The residues were reconstituted to solution with 200 μl of mobile phase, and an aliquot was analysed on HPLC. Extraction recoveries were calculated using the constructed calibration curve

2.5. Extraction of spiked human plasma

To 1 ml of human plasma, 200 μl of a caffeine solution (50 μg/ml in methanol) were added and next 200 μl of cold acetonitrile were added. The mixture was vortex mixed for 1 min and centrifuged at 3000 rpm for 5 min. The supernatant was diluted to 2 ml with addition of water and the mixture was applied on the MIP-SPE cartridge. The SPE protocol was applied and the resulting solution was analysed by HPLC. Each sample was assayed (MIP extraction and HPLC) four consecutive times.

2.6. Extraction of beverages

A 200-μl sample of a cola beverage was diluted ten and 100 times with water and the resulting solutions were applied on the MIP-SPE. The cartridge was treated as described above and the final solution was analysed by HPLC. Each sample was assayed (MIP extraction and HLPC) four consecutive times.

3. Results and discussion

The aim of the work was to evaluate the feasibility of using MIP sorbents for SPE of caffeine. The scheme desired should accomplish molecular recognition and selective clean-up of real samples containing caffeine. Thus the experimental procedure employed a generic SPE protocol with the following general steps: (i) conditioning the column to an appropriate environment (solvent, pH), (ii) loading the sample in a solvent identical to the solvent employed in the last conditioning step, (iii) washing off possible interferences and/or substances non-specifically bound on column, (iv) applying the best solvent to specifically elute caffeine from the column.

As a general rule MIPs exhibit better molecular recognition in the solvent used as porogen during polymerisation. It is theorised that selective binding of the template to the MIP is enhanced in conditions similar to those occurring during the molecules self-assembly in the polymerisation mixture. Therefore it was anticipated that best binding would occur with acetonitrile as the loading solvent, since the MIPs were prepared with acetonitrile as the porogen. However loading on acetonitrile resulted in weak binding. The major portion of caffeine was not retained by either the MIPs (60.0% for P1 and 65.8% for P2) or the control polymer (82.8%). Nevertheless slightly higher affinity of the MIP towards the template molecule was obtained. The weak retention observed was attributed to the strong eluting power of acetonitrile in both reversed- and normal-phase chromatography.

In order to overcome the low extraction recovery an investigation of the best loading conditions was necessary to gain insight on retention mechanism. Retention of the template on the SPE column can occur either in normal-phase chromatographic conditions or in reversed-phase conditions. Initially a less polar solvent (dichloromethane) was utilised in the loading step. Only slightly higher recovery (~15%) was obtained on both MIPs, but this was not found to be satisfactory. Furthermore there was no evidence of selective binding of caffeine on the polymer.

Thus in a second series of experiments, aqueous loading (reversed-phase conditions) was tested in the search for adequate analyte retention. Loading in aqueous environment resulted in strong retention of caffeine in all polymers. Recovery of up to 80% was achieved on the MIPs, but this was attributed to non-specific binding since comparable recoveries were found in the control polymer.

Trapping of the template in aqueous environment and subsequent implementation of a selective ''desorption step'' has recently been described by Berggren et al. for the extraction of clenbuterol [33] and Ferrer et al. [34] and Matsui and co-workers

Table 1
Recovery of caffeine (50 μg) using aqueous loading, basic washing and acidic elution

Fractions	Recovery (%)					
	Control polymer		P1		P2	
	1st	2nd	1st	2nd	1st	2nd
Loading (water, 1 ml)	4.2	4.9	0.4	1.6	1.0	2.0
Washing (ACN–TEA 1%, 1 ml)	53.1	56.7	43.6	36.6	27.5	27.0
Elution (ACN–CH_3COOH 1%, 1 ml)	17.5	16.1	54.7	60.5	57.9	55.7
Total	74.8	77.7	98.8	98.7	86.4	84.7

Results of two experiments. ACN, acetonitrile.

[35,36] for the extraction of triazine herbicides. In order to facilitate selective binding of the template, a ''molecular recognition step'' was included in the extraction procedure. Optimisation of the washing steps is critical in such an approach. The aim of the additional step is to disrupt non-specific interactions between the analytes and polymeric stationary phase. In such a case analytes non-selectively bound to the MIP would be eluted, whereas the template would remain trapped in the polymer by selective interactions. In contrast, in the control polymer, quantitative elution of the template is expected due to the disturbance of the hydrophobic interactions retaining the molecule. Hence subsequent to loading in aqueous conditions, 1 ml of acetonitrile containing 1% TEA was applied on column and was allowed to pass through the bed by the force of gravity alone. It was anticipated that during this step, elution of caffeine non-specifically bound on the control polymer would occur. Concerning the imprinted polymer stronger binding of caffeine was expected on imprinted active sites. The first case was actually observed (Table 1), however as regards the second, washing the cartridge with acetonitrile 1% TEA eluted a significant portion of caffeine, revealing the heterogeneity of the binding sites.

Finally an alkaline buffer (0.05 *M* CH_3COONH_4–$NH_{3(aq.)}$, pH 9) was employed for conditioning, loading and washing the columns. The role of the buffer was to suppress non-specific interactions, by masking the reactive acidic moieties on the surface of the polymer. In such an environment it was expected that specific binding would be enhanced. As can be seen in Table 2, this scheme resulted in the largest differences in recovery between the MIPs and the control polymer. Recovery of caffeine from the MIPs during the last elution step was thus clearly attributed to molecular recognition of the template molecule. The overall performance of polymer P1 is superior to polymer P2 in conditions of selective recognition (Tables 1 and 2). This was actually expected since polymer P1 was prepared with a

Table 2
Recovery of caffeine (50 μg) using aqueous loading and conditioning in alkaline buffer, double basic washing and acidic elution

Fractions	Recovery (%)					
	Control polymer		P1		P2	
	1st	2nd	1st	2nd	1st	2nd
Loading (0.05 *M* CH_3COONH_4–$NH_{3(aq.)}$, pH 9)	0.0	0.1	0.0	0.0	0.0	0.1
1st Basic washing (0.05 *M* CH_3COONH_4–$NH_{3(aq.)}$, pH 9)	1.1	1.3	0.0	0.0	16.4	14.0
2nd Basic washing (ACN–TEA, 1%)	78.0	74.6	21.9	23.3	28.2	29.0
Elution (ACN–CH_3COOH 1%, 1 ml)	4.9	4.3	84.0	87.6	57.0	61.8
Total	84.0	80.1	105.9	110.9	101.6	104.8

Results of two experiments.

double template mass compared to P2. Hence a much larger number of active sites is expected to exist in P1.

To evaluate cross-reactivity of the MIP against analogous molecules, theophylline and theobromine solutions were extracted on both the MIP and the control polymer. A mixture of the three xanthines (5 μg/ml each) was processed using the finally selected protocol and recoveries obtained are summarised in Table 3. It can be seen that the MIP exhibits moderate cross-reactivity recognising analogue xanthines, but to a lower extent compared to caffeine. In contrast, the control polymer shows similar behaviour and low recovery for all three xanthines.

Concerning the analysis of real samples, the matrix of the tested samples provided no difficulty in the analysis. Hence a straightforward determination of caffeine in a cola beverage and human blood was feasible. Satisfactory sample clean-up was achieved by the MIP extraction, thus no difference was observed between reference standard solutions and real samples. This can be seen in Fig. 2, where the chromatograms of blank and spiked blood plasma after extraction on MIP are illustrated. It was observed, however, that the recovery diminished if more than four consecutive extractions of blood plasma were performed in the same cartridge. This was attributed to the accumulation of matrix components non-selectively retained on the MIP. The sorption of these components could block the active sites of the polymer or modify its retention properties. In such a case, washing the MIP cartridge with an excess of the elution solvent (5 ml) sufficed for its re-generation. With regard to extraction recovery, all values were calculated using the constructed calibration curve (see Experimental, HPLC determinations section). For evaluation of the recovery from beverages, the caffeine content in the beverage was calculated by HPLC. MIP-extraction recoveries were reproducible and in good agreement

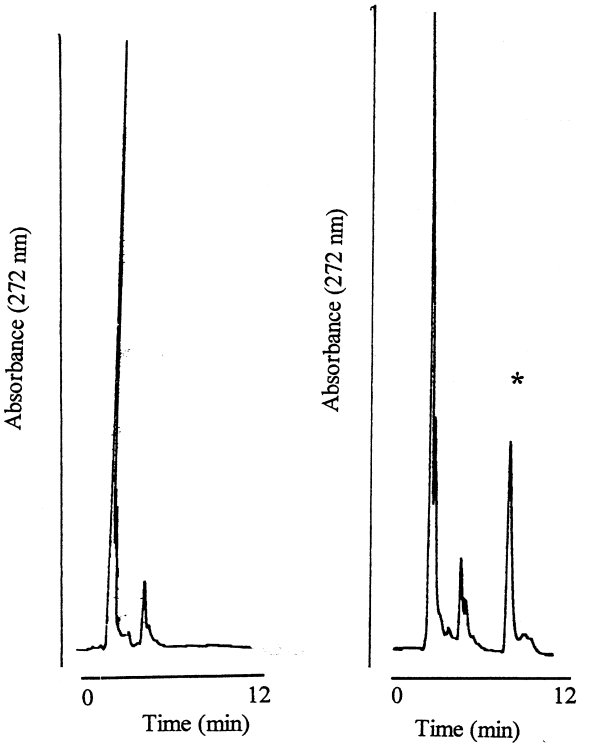

Fig. 2. HPLC analysis of blood plasma extracted on the polymer P1 with the developed method. Analysis of the sample prior to caffeine addition (left) and after the addition of 10 μg caffeine (right). HPLC conditions as described in the Experimental section. Caffeine peak at 8.09 min.

Table 3
Recovery of xanthines (50 μg each) with the selected extraction scheme (as described in Table 2) on polymer P1

Fractions	Recovery (%)											
	Control polymer						Imprinted polymer					
	Caffeine		Theophylline		Theobromine		Caffeine		Theophylline		Theobromine	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Loading	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.4
1st Basic washing	0.0	0.0	1.8	0.6	1.2	1.3	1.1	1.1	0.0	0.0	1.6	0.7
2nd Basic washing	99.0	94.7	89.9	94.2	97.9	90.0	29.4	27.0	52.2	58.0	60.2	64.8
Elution	4.0	5.1	1.0	2.0	2.0	2.1	72.9	68.3	42.0	44.4	39.8	33.2

Results of two experiments.

Table 4
Recovery of caffeine from spiked blood samples (50 μg/ml) and beverage extracts extracted on polymer P1

Sample	Recovery (%)	RSD (%)
Spiked blood	82.6	6.90
Beverage[a]	81.0	5.22
Beverage[b]	83.4	4.35

Results of four experiments.
[a] Diluted 1/10=13 μg/ml.
[b] Diluted 1/100=1.3 μg/ml.

with the recoveries of reference standard solutions (Table 4).

This work shows that a methacrylic acid imprinted polymer exhibited retention of the template molecule in aqueous conditions. The polymer retained selectively the template in subsequent washing steps, whereas the control polymer did not. This work represents the first application of a caffeine imprinted polymer in SPE. Despite the employment of an additional step in the SPE scheme, such an approach facilitates direct loading of aqueous samples on the extraction column, thus speeding up processing of samples. The latter is a parameter of special value in industrial quality control and quality assurance procedures.

Acknowledgements

Dr B. Sellergren is acknowledged for helpful suggestions and careful reading of the manuscript.

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