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Effect of solvents on the selectivity of terbutylazine imprinted polymer sorbents used in solid-phase extraction

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Abstract

A solid-phase extraction sample preparation method using a molecularly imprinted polymer (MIP) selective for the triazine type pesticide terbutylazine has been developed. The method involves preconcentration from large volumes of water samples on a C18 disk coupled to selective clean-up on the MIP. The method has been optimised by studying the recovery and retention of terbutylazine and some other structurally related triazine derivates as a function of the selective washing solvent used. The effect of the water content of the selective washing solvent was also investigated on the recovery of the MIP. River water samples were analysed with the coupled technique, and efficient clean-up of the samples was observed.

Keywords: Molecular imprinting; Solid-phase extraction; Water analysis; Terbutylazine; Pesticides

1. Introduction

Molecularly imprinted polymers (MIPs) exhibiting high selectivity and affinity for a predetermined molecule are now seeing a fast growing research interest [1–8]. They owe their attractive characteristics to the way they are synthesised. Binding sites in the polymer are created by polymerizing the monomers in the presence of the template—the target compound. The method involves noncovalent preorganisation, or covalent linkage of the template with functional monomers and fixation of these complexes by creating a highly crosslinked rigid polymer network. The template is removed afterwards, leaving behind binding sites having complementary shape and functionality to the template, allowing its molecular recognition. The preparation technique is simple, cheap and leads to a rugged adsorbent resistant to mechanical stress, heat and harsh chemical environment.

These properties allow MIPs to be used in various fields of analysis like sorbents in separation [9–12], sensing elements in chemical sensors [12–15] or antibody mimics in ligand binding assays [12]. Using MIPs as chiral stationary phases in liquid chromatography was the first widespread attempt towards their analytical application. Although these sorbents in

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many cases showed high selectivity for enantiomers they did not fulfill the requirements of having high enough chromatographic efficiency. This is attributed to the heterogeneous binding site affinity found in these polymers, the slow association–dissociation kinetics and the nonuniform size and irregular shape of the polymer particles.

MIPs have been frequently used as solid-phase extraction (SPE) sorbents [16–28]. There are, however, few studies supporting the design of sample pretreatment strategies using MISPE (molecularly imprinted solid-phase extraction). The present study has been carried out with this goal in mind. We have picked one particular application, that of triazine pesticide analysis in water, as a typical example of MISPE use [29–31].

Aqueous samples can, in principle, be applied to a MISPE column directly. The analyte(s) and the majority of the hydrophobic interfering compounds would be retained. The imprinted selectivity could then be utilised by washing the cartridge with a 'selective' solvent. This solvent would remove the compounds which are bound by hydrophobic forces only, but it would not elute the analyte(s) which are bound by the imprinted sites. Imprinted sites usually bind the analyte by an electrostatic driving force i.e. H-bonds, ionic interactions, $\pi-\pi$ bonds, and therefore the likely 'selective' solvents are aprotic organic solvents [31,32]. The solvent used in MIP synthesis (the 'porogen') is usually a good choice since in this solvent the analyte was effectively forming the sites by the forces mentioned ('template effect'). In some cases selectivity may also be achieved by washing with water containing a minor fraction of organic solvents where instead of the former ones selective hydrophobic forces are operating [31]. Thereafter the template can be easily eluted from the column with a solvent switch or by addition of acid or base. This phenomenon should allow to develop on-line methods based on MIP recognition, where the selective washing solvent has to be compatible with the chromatographic system beyond being highly selective.

In cases utilizing the electrostatic driving force there are two disadvantages in applying aqueous samples directly to the MIP cartridge. One is that the solvent switch from the aqueous sample to the organic 'selective' solvent may cause retention problems due to solvent mixing. One tries to avoid such problems by passing air through the cartridge after sample application to dry the column. We shall show in this paper that this strategy is associated with some drawbacks.

The second problem, which arises particularly with environmental water samples is the large volume of the samples [33–35]. The concentration of the analytes in these samples is typically very low. Depending on the HPLC detector's sensitivity one may need to work up samples of 50–1000 ml volume for a single chromatographic injection. SPE cartridges filled with MIP or any other sorbent suffer from the limitations of restricted flow-rates and plugging when handling environmental water samples. Therefore to pass through a large volume of sample can take a long time especially without prefiltration. We shall show in this paper that this problem can be conveniently overcome by a two stage sample pretreatment. The large volume sample is quickly passed through a C18 disk. The analyte(s) and other substances bound on this disk are then transferred to the MISPE column by the selective wash solvent of the MIP. As this solvent disrupts hydrophobic bonds, it will elute the interferents both from the C18 disk and from the MISPE column. The analyte(s) will be retained on the MISPE column and can be removed subsequently by a suitable solvent, typically methanol. This last eluate is then injected into the HPLC system.

The design of MISPE methods includes also the choice of the selective solvent. We have discussed above the chemistry behind selectivity but did not mention any quantitative aspects. MISPE columns are actually very short chromatographic columns. A good selective solvent should elute the interferents by the first 1–2 ml passing through the MISPE column. The analyte, on the other hand, should not be removed in the same volume. A typical MISPE column is filled with 50 mg of MIP. The dead volume of this column is a few times ten microliters. Thus 1–2 ml of selective solvent is well over 20 dead volumes. This means that the retention factor, k, for the analyte has to exceed 20. Due to the low plate numbers of most MISPE sorbents elution volumes below 100 μl are not practical. This is another important consideration in the optimisation of MISPE protocols.

In view of the foregoing discussion we have studied the elution behaviour with a variety of selective solvents. The effect of water was also investigated, with acetonitrile as the selective solvent. Finally we present the two-stage C18-MISPE sample pretreatment method and its application to river water samples.

2. Experimental

2.1. Materials

Terbutylazine, prometryn, ametryn and atrazine were generously provided by Novartis (Basel, Switzerland). For the molecular structures and log P_{ow} values [31] see Fig. 1 (P_{ow}=octonal–water partition coefficient). Methacrylic acid (MAA) was a product of Aldrich (Milwaukee, WI, USA). Ethyleneglycol dimethacrylate (EDMA) was purchased from Fluka (Buchs, Switzerland). Azobisisobutyronitrile (AIBN) initiator was obtained from Janssen (Geel, Belgium). Acetonitrile, ethylacetate, dichloromethane and toluene were purchased from Romil (Loughborough, UK). Isobutyl methyl ketone was purchased from Merck (Darmstadt, Germany). Methanol was a product of Carlo Erba (Milan, Italy). Milli-Q RG ultrapure water was used to prepare solutions. The C18 extraction disk (Envi™18 DSK, 47 mm diameter) was purchased from Supelco (Bellefonte, PA, USA).

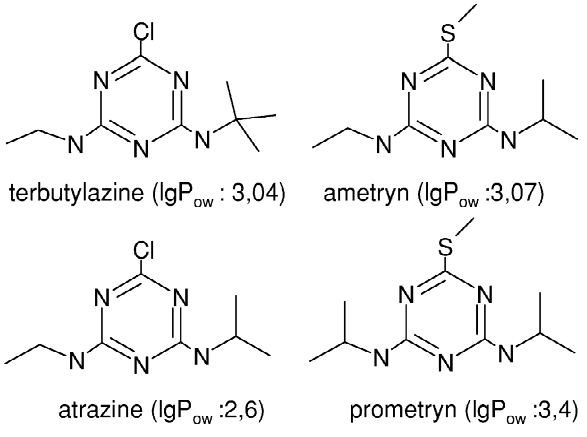

Fig. 1. Chemical structures and log P_{ow} values of the four triazines.

Table 1
Composition of the polymerisation mixture

Terbutylazine	MAA	EDMA	Porogen	AIBN
1 mmol	4 mmol	20 mmol	5.6 ml	40 mg

2.2. Polymer synthesis and work-up

The composition of the polymerisation mixture was as follows (Table 1).

The synthesis of the polymers was performed using MAA as functional monomer, EDMA as crosslinking monomer, terbutylazine as template and toluene as porogen as described elsewhere [29]. The particles were thoroughly washed with water and sieved. The 25–36 μm fraction was collected. The particles were washed with 50 ml portions of MeOH–water (50:50), MeOH–AcOH (90:10) and MeOH, then dried under vacuum overnight.

2.3. MISPE

A 50-mg amount of dry polymer was packed into empty SPE cartridges between two filters. The bleeding of residual template from the polymer was checked by washing the MISPE cartridges with successive methanol fractions (3 ml each). The chromatograms of the second and third fractions were found to be free of terbutylazine at the sensitivity of the UV detector. Two different SPE procedures (Fig. 2) were used during the experiments. Procedure 1 was used to study MISPE behaviour with aqueous samples and procedure 2 with nonaqueous samples.

2.3.1. MISPE drying kinetics measurement after water application

A 50-mg MISPE cartridge was conditioned with 2 ml methanol followed by 5 ml water to wet the polymer completely. Drying was realised by pulling laboratory air through the cartridge using an SPE vacuum manifold. The decrease of water content was followed by weighing.

2.4. SPE with the coupled C18 disk-MISPE system

Steps of the procedure used are shown in Fig. 2

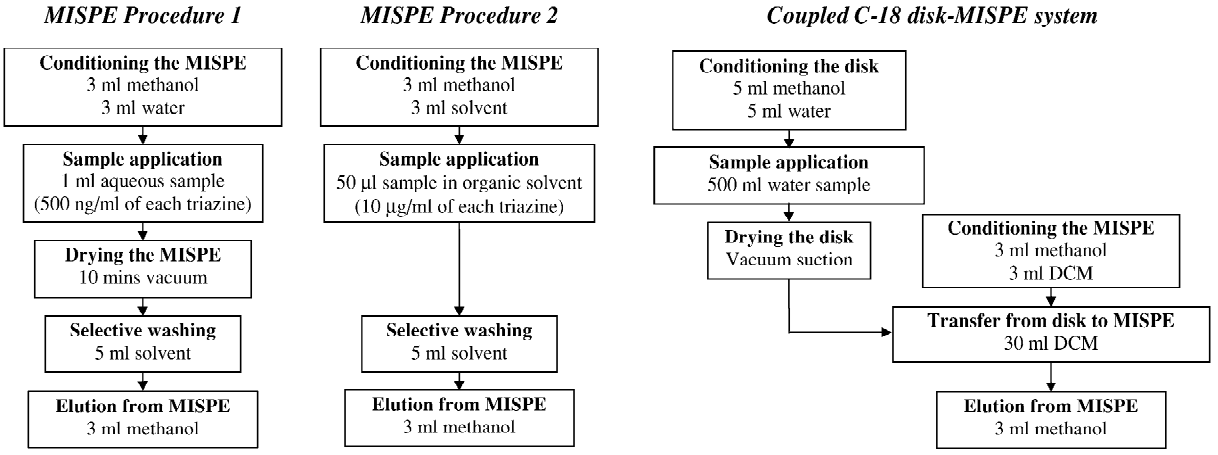

Fig. 2. Solid-phase extraction procedures.

and the coupled C18 disk-MISPE system is shown in Fig. 3.

2.5. Liquid chromatography

Eluates from the MISPE cartridge or the C18 disk were evaporated to dryness under nitrogen stream using a TurboVap system (Zymark, Hopkinton, MA, USA). The residue was dissolved in 1 ml mobile phase and then analysed by HPLC. The mobile phase (30% acetonitrile, 30% methanol, 40% 0.05 *M* phosphate buffer, pH 4) was pumped by a Kontron 420 HPLC pump (Kontron Instruments, Switzerland) through the precolumn (BST ODS Hypersil 5 μm, 20×4 mm) and the analytical column (Hewlett-Packard ODS Hypersil 5 μm, 200× 4.6 mm) at a flow-rate of 1 ml/min. A 50-μl volume sample was injected into the system. The peaks were detected by a Jasco UV-970 type UV–Vis detector (Jasco, Tokyo, Japan) at 233 nm.

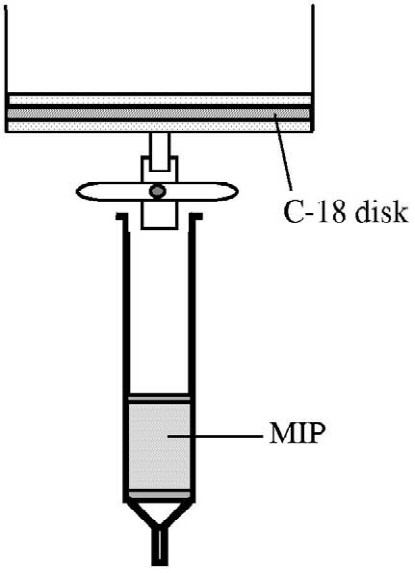

Fig. 3. Coupling of C18 disk and MISPE cartridge in the transfer step.

3. Results and discussion

3.1. Optimisation of the MISPE procedure

The different steps of SPE by the MIPs were studied in off-line SPE mode. Blank and imprinted polymer filled cartridges were used in these experiments.

3.1.1. Sample application

Using Procedure 1 (Fig. 2) the mixture of triazines was applied from aqueous solution after conditioning the MISPE column with methanol and water. The aqueous effluent was checked for triazines but they were completely retained, both by the imprinted and the blank column. As is known from the literature [31] selective recognition by the terbutylazine imprinted polymer is based on H-bonding between the

carboxylic acid group of the polymerised methacrylic acid and a ring nitrogen of the template. These interactions cannot be formed in aqueous medium since water competes for the H-bonding sites; instead binding of triazines from water is dominated by hydrophobic interactions between the polymer and the analytes.

When applying real water samples to the MIP, other hydrophobic molecules from the matrix would also bind with the same mechanism. In order to remove them, a selective washing solvent should be used which disrupts nonselective hydrophobic bonds, but allows selective binding of terbutylazine to the polymer.

3.1.2. Effect of the water content of the selective solvent on the selectivity and recovery of the MISPE

In preliminary experiments various organic solvents were tried as selective washing solvents using Procedure 1: acetonitrile, chloroform, isopropanol, toluene and dichloromethane. Since toluene had been used as porogen in the polymer synthesis, it was expected to give the best recognition i.e. the strongest retention of the template, when applied as washing solvent [32]. The amounts of the structurally similar triazines were determined in the washing fraction using HPLC. In these preliminary experiments the variance of the parallel measurements was unacceptably high and the total recoveries were relatively low.

It was suspected that small amounts of water that could remain on the cartridge after sample application due to incomplete drying can change the binding ability of the columns in the consecutive selective washing step. It was shown by other authors that low proportions of water in the organic mobile phase used in the chromatographic separation of amino acid or peptide enantiomers substantially alter the recognition properties of the imprinted polymer [36,37]. Commonly a decrease in retention and selectivity is seen upon an increase in the aqueous content. Interestingly, at higher aqueous contents many MIPs again regain recognition properties implying that aqueous selective wash solvents may be used [31].

To check these expectations the following experiments were carried out. Firstly we determined the 'drying profile' of the MISPE cartridge by following the decrease of the mass of adsorbed water during the drying process. We found that the removal of adsorbed water takes at least 30 min and the application of organic solvent before complete drying might cause the high variance observed in the preliminary experiments.

In order to avoid the presence of ill-defined water residues on the cartridge we switched to Procedure 2 (Fig. 2). After conditioning the MISPE column with acetonitrile the sample—a mixture of four triazines dissolved in acetonitrile—was applied. Subsequently the cartridge was washed with fractions of an acetonitrile–water mixture. The triazines were determined in each wash fraction. The experiment was repeated at different acetonitrile-to-water ratios and the results were plotted against the water content of acetonitrile.

The results for terbutylazine are shown in Fig. 4. From this picture we see indeed that small concentrations of water (up to 10%) in acetonitrile drastically decrease the retention on the imprinted polymer. However, further increasing the water content of acetonitrile an increased retention of terbutylazine can be observed. Therefore in water-free acetonitrile and at high aqueous contents of acetonitrile the binding of the analyte to the polymer is the strongest and a minimum binding strength prevails in between. No such dependence can be observed with the blank polymer. At up to 10% water the blank cartridge basically does not retain any of the analytes; the effect of water appears above 10% water content, where the retention of the triazines starts to increase due to the hydrophobic effect and correlates with the respective log P_{ow} values.

The data obtained for all four triazine compounds in the same experiment are shown in Fig. 5. In Fig. 5a the cumulative recoveries of the triazines are shown in consecutive 0.5-ml acetonitrile fractions. The MIP retains terbutylazine and atrazine almost completely up to 2 ml of acetonitrile. Ametryn and prometryn are similarly, although less strongly bound to the SPE cartridge. It was shown earlier that polymers imprinted with chlorotriazines preferentially bind their template over *s*-triazines and vice versa [31,38]. Our data support these earlier findings. Presumably in water-free solvent the main inter-

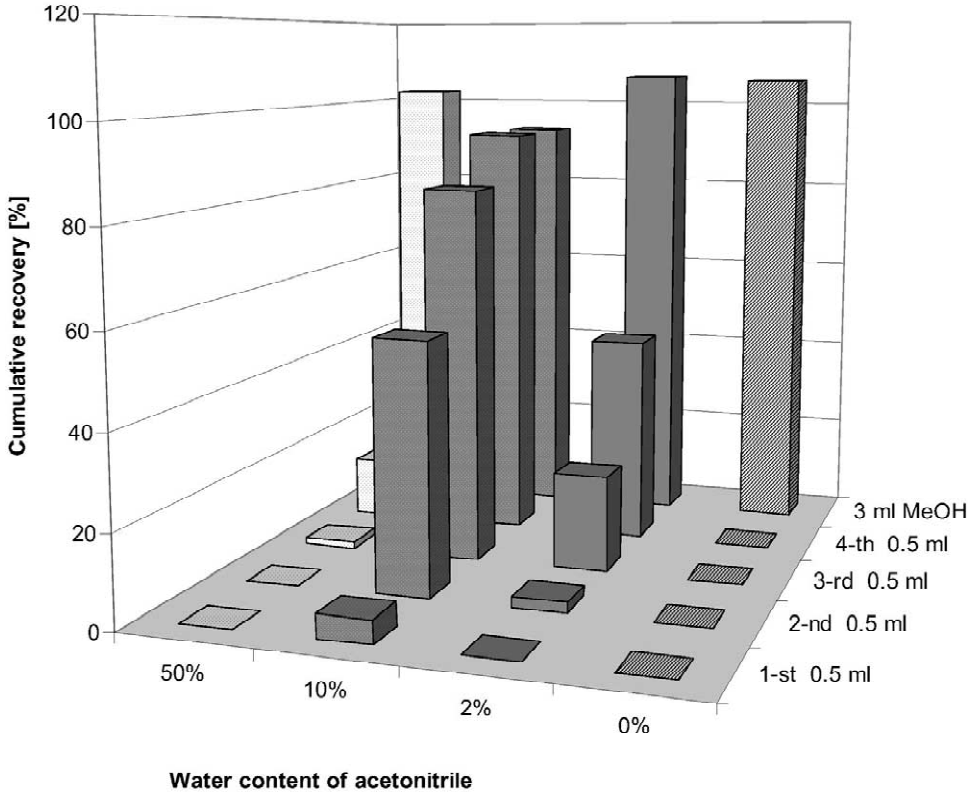

Fig. 4. Cumulative recovery of terbutylazine on a MISPE in fractions of different acetonitrile–water mixtures.

action between the analytes and the polymer is cooperative H-bonding.

Adding 2% water to the acetonitrile washing solvent (Fig. 5b) drastically decreases the H-bonding interactions between the triazines and the recognition sites of the polymer. The retention decreases for all four compounds, although for terbutylazine in a smaller degree. In Fig. 5c, i.e. at 10% water content the retention further decreases and the differences between the four structurally similar analytes almost disappear. In this medium the binding to the MIP occurs almost exclusively by nonselective hydrophobic forces. A striking phenomenon can be observed in Fig. 5d which shows the data for 50% water content. The retention ability of the polymer for the four triazines starts to increase here, but not to the same extent. The retention of terbutylazine is considerably higher than that of the more hydrophobic prometryn. This shows that the retention of terbutylazine is not merely based on a nonspecific hydrophobic driving force and agrees with results previously published by Dauwe and Sellergren [31]. On the other hand since terbutylazine is much better retained than atrazine, and terbutylazine differs from atrazine only in the presence of a tertiary butyl group, we can conclude that the specific retention of terbutylazine in the 50% aqueous eluent is based on a specific hydrophobic interaction of the *tert.*-butyl group with the polymer. The above experiment has proved that minor aqueous residues can drasctically change the binding ability and selectivity of the MIP in the selective organic washing step. Therefore, in the following study of organic solvents as selective washing solvents Procedure 2 was used to avoid contamination of the cartridge with water. The results shown here also raise the possibility of utilizing wash solvents with high aqueous content to achieve novel selective patterns with the polymers used. We have not followed this trail further in the present work.

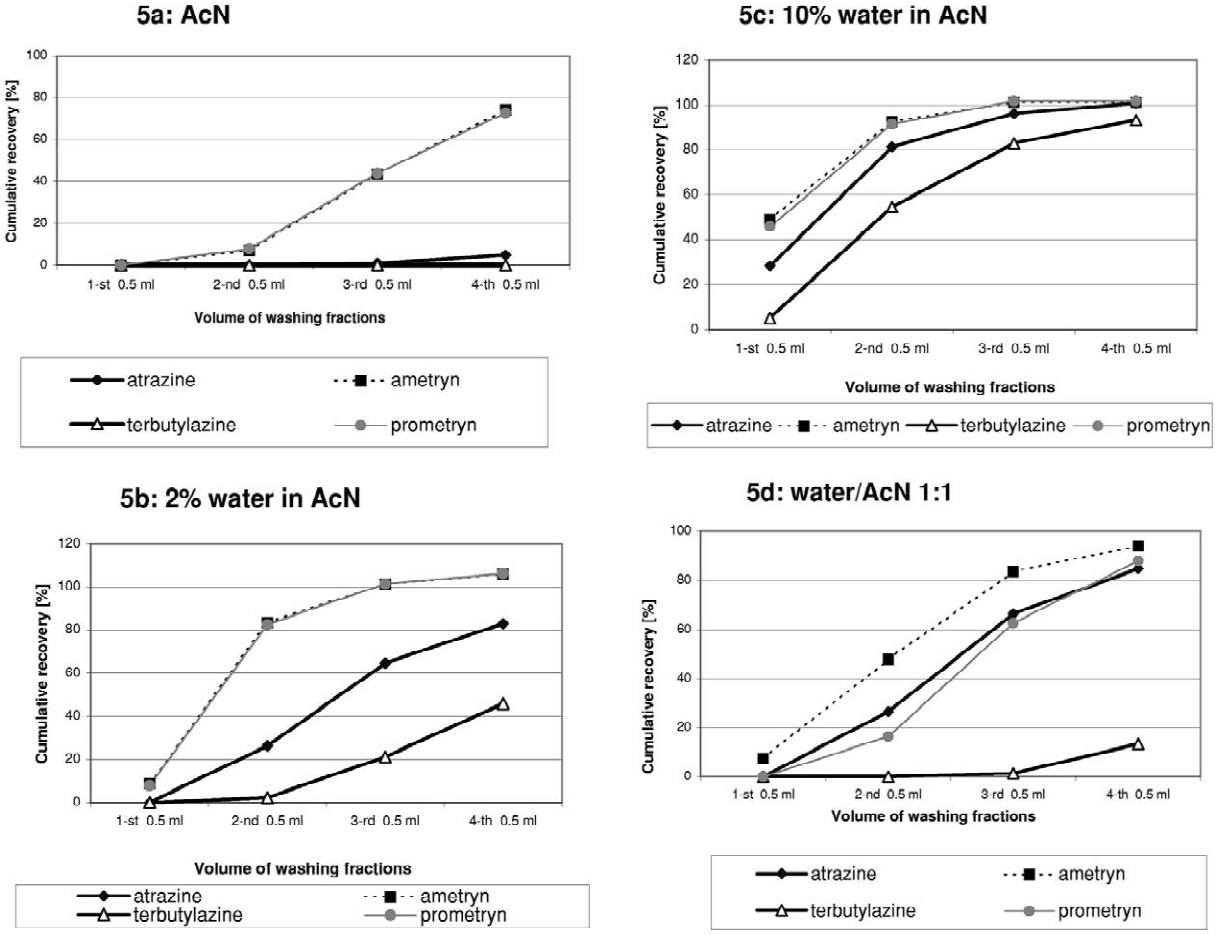

Fig. 5. Cumulative recovery of triazines on a MISPE in acetonitrile (a); in acetonitrile with 2% water (b); in acetonitrile with 10% water (c) and in acetonitrile with 50% water (d).

3.1.3. Choice of the selective washing solvent

A selective washing solvent is used after sample application to remove virtually everything else than the analyte from the MIP. This implies that the selective washing solvent should disrupt the binding forces between the MIP surface and interfering substances while it should not disrupt the forces binding the analyte to the MIP. In other words the distribution coefficient of the interferents toward the MIP should be low whereas that of the analyte should be high. One can think of a MISPE cartridge as a short liquid chromatography column with low separation efficiency. The difference in distribution coefficients and consequently in the retention factors should be considerable and in fact much larger than generally required for good HPLC separations. Practical samples may contain many interfering substances and it is therefore difficult to design a suitably general model experiment. Thus we decided to give the MIP a rather difficult task: to separate the analyte from closely related compounds using the MISPE column. In this study we investigated a MIP imprinted for terbutylazine for its separation capability against other triazines: prometryn, ametryn and atrazine.

Ethyl acetate, acetonitrile, dichloromethane, iso butyl methyl ketone and toluene were chosen to test their selectivity as wash solvents of the MIP in the SPE format (MISPE). A blank polymer was also tested as reference in the same way. The dielectric

Table 2
Dielectric constants (ϵ) and hydrogen bond parameters (HBP) of various organic solvents used for selective washing

Solvent	ϵ	HBP
Acetonitrile	36.2	7.0
Dichloromethane	9.1	2.2
Ethyl acetate	6.0	6.5
Isobutyl methyl ketone	13.1	7.7
Toluene	2.5	3.6

constants (ϵ) and the hydrogen bond parameters (HBP) of the solvents used are shown in Table 2 [39].

To avoid the disturbing effects of water the samples were always prepared in the tested selective washing solvent, i.e. Procedure 2 was used. This time a mixture of compounds was applied to the MISPE column as sample and then the column was washed with a series of aliquots of the solvent. Finally all remaining adsorbed material was washed off the column with methanol. It was established by experiments not shown here that 2–3 ml of methanol completely removes all the triazines bound to the cartridge. Therefore in our experiments the amount of each substance in the methanol fraction is equal to the amount still retained after the selective wash. The wash fractions were analysed by HPLC as described in Section 2.5. The recoveries in the selective washing fractions and in the final methanolic elution step are plotted in Fig. 6 for dichloromethane and toluene, respectively.

The blank polymer does not retain any of the test compounds in most solvents except in toluene. In toluene, which is the least polar solvent in the row, all investigated triazines are eluted quite slowly. The elution order is the reverse order of their hydrophobicity i.e. the most hydrophobic prometryn elutes first and less hydrophobic terbutylazine and ametryn elute later (see Fig. 6b). In this apolar solvent the hydrophobic interactions between the short alkyl chains of the polymer and the alkyl groups of these compounds are not able to give any retention but this medium allows nonselective H-bond interactions with the fairly polar ester and acid functional groups of the polymer network. In this case the blank polymer behaves like a normal stationary phase.

The imprinted polymer shows quite different selectivity behaviour in different solvents. The weakest binding occurs in isobutyl methyl ketone. There is practically no difference between the blank and imprinted polymer using this solvent. Less than 3 ml isobutyl methyl ketone elutes the ametryn and prometryn, and 4 ml elutes the terbutylazine too.

A somewhat stronger binding, at least for the imprinting template terbutylazine can be observed in ethyl acetate. While ametryn and prometryn practically elute from the cartridge in the first 2–3 ml, terbutylazine's elution curve is rather broad (from 1 to 10 ml).

In acetonitrile the amounts in the washing fractions show a similar pattern to ethyl acetate, however with somewhat higher retentions and broader elution profiles.

In dichloromethane (Fig. 6a) terbutylazine is completely retained by the cartridge even after washing with 30 ml of solvent. The two other triazines are slowly eluted in this volume.

The binding of terbutylazine in toluene (Fig. 6b) is as strong as in dichloromethane, however, the two other compounds show similar affinity to the polymer as terbutylazine, in contrast to the dichloromethane case.

Based on these results dichloromethane—which has the lowest hydrogen bond parameter among the solvents studied—is the best choice as selective washing solvent in the SPE method since it shows the best selectivity as well as good retention.

3.2. Method for the measurement of environmental water samples

The preparation of water samples on the C18 disk-MISPE system was realised as in Fig. 2. The different phases of the procedure were optimised and the resulting optimal procedure is presented here.

3.2.1. Sample application

To demonstrate the applicability of the method a medium (0.5 μg/l) concentration was chosen for spiking, considering the measurement ranges of the international literature and the European directives [40] which declare that the environmental limit is 0.1 μg/l for natural waters. A 500-ml water sample spiked with 250 ng of each triazine (0.5 μg/l) and

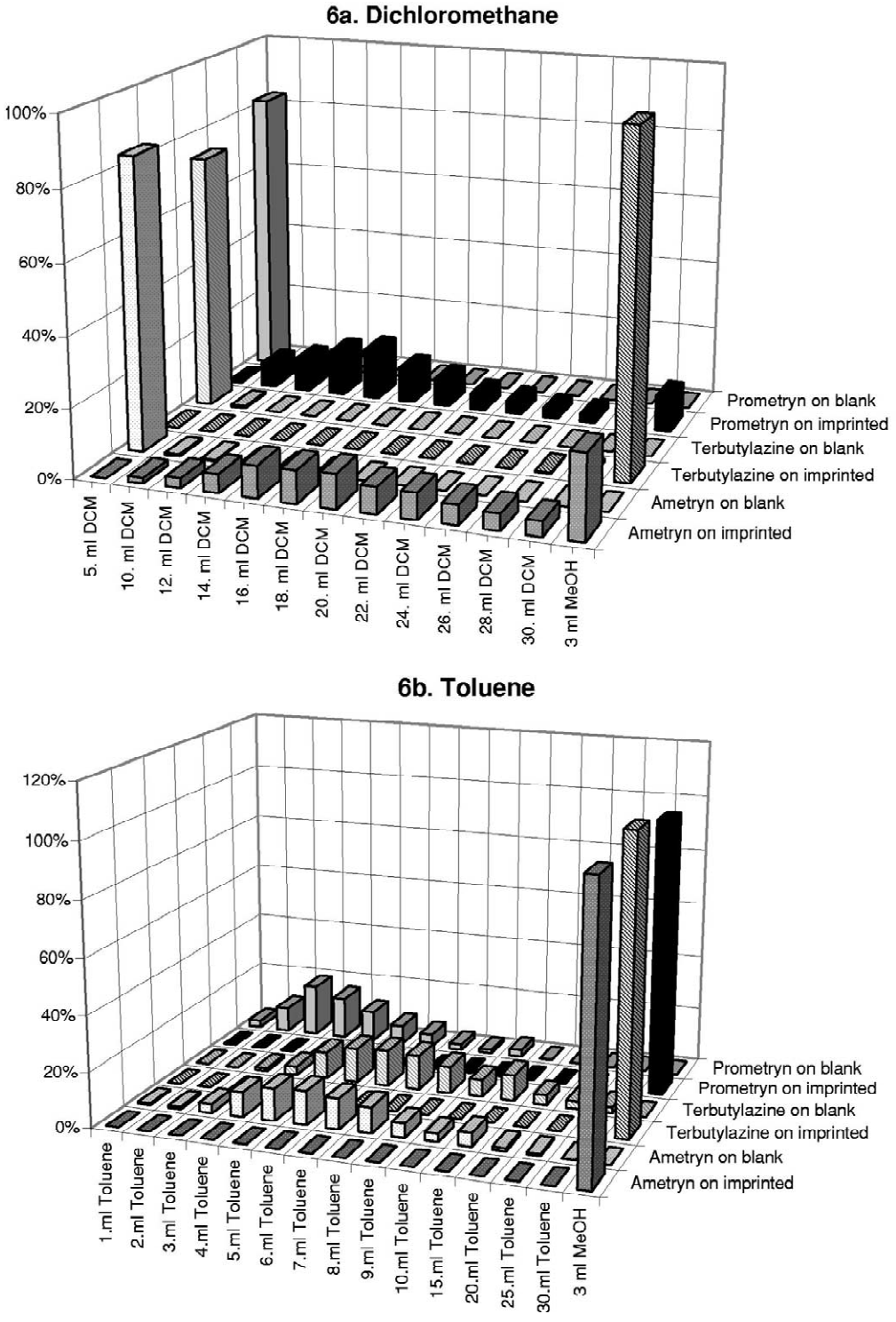

Fig. 6. Elution profile of triazines on a blank and imprinted polymers using different organic media as selective washing solvent: dichloromethane (a); toluene (b).

0.5% methanol was passed through the C18 disk. The triazines were bound on the disk almost completely (94.5–97%). This procedure took 5 min.

3.2.2. Elution from the C18 disk onto the MIP cartridge—the transfer step

For elution dichloromethane was chosen, since this solvent is a good selective washing solvent of the MIP and it was also suggested by the manufacturer of the C18 disk as a possible elution solvent [41]. The amount of dichloromethane that quantitatively elutes the triazines was determined by measuring the recovery in successive fractions. It can be concluded that 30 ml of this solvent almost quantitatively (86–91%) desorbs the analytes of interest from the disk. In addition, it had been established previously that this amount of dichloromethane does not desorb terbutylazine from the MIP and can be safely used as a selective washing solvent.

3.2.3. Elution from the MIP

After the sample components bound on the C18 disk were washed with 30 ml of dichloromethane from the C18 disk directly to the MIP cartridge, the two sorbents were disconnected and 3 ml of methanol were used to elute terbutylazine from the MIP. This purified sample was then further analysed on the HPLC system as described in Section 2.5.

3.2.4. Real sample analysis

To demonstrate the applicability of the coupled C18 disk-MIP system surface water samples from the river Danube were analysed. To show the difference in clean-up efficiency between a sample

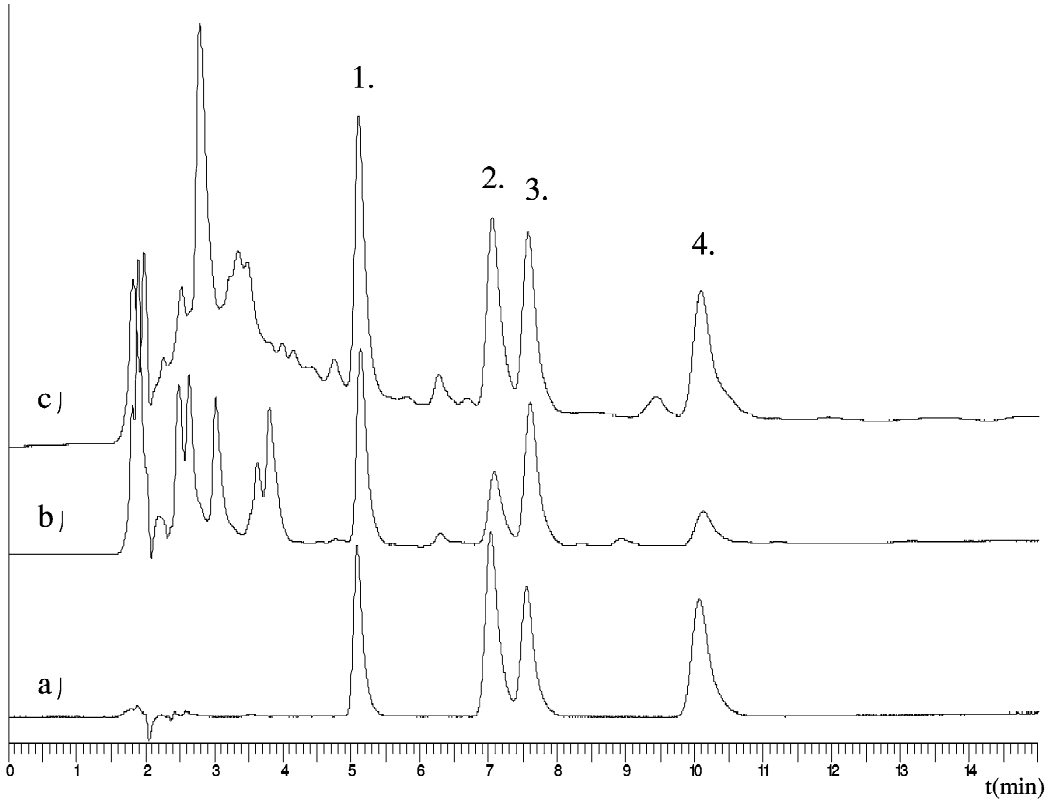

Fig. 7. Chromatogram of a standard solution (500 ng/ml in eluent) of triazines: 1=atrazine (t_R=5.08), 2=ametryn (t_R=7.03), 3=terbutylazine (t_R=7.55), 4=prometryn (t_R=10.06) (a); spiked Danube water (500 ng/l) of each compound using C18 disk-MIP system (b); spiked Danube water (500 ng/l) using only C18 disk (c).

pretreatment using only the C18 sorbent and the coupled system, chromatograms are shown of spiked river water samples pretreated in either way (Fig. 7). Chromatogram 7b shows a good clean-up for the imprinted compound terbutylazine and for the other triazines, too. The recovery for terbutylazine was 70% (n=4 SD=16%), for atrazine 76% (n=4, SD=6%), for amertyn 45% (n=4, SD=14%) and for promeryn 38% (n=4, SD=25%). It should be stressed here again that our method has been developed for terbutylazine only; the other triazines were included only to study method selectivity against compounds extremely similar to the analyte.

4. Conclusion

It has been shown that a molecularly imprinted polymer prepared using terbutylazine as template, methacrylic acid as functional monomer and toluene as porogen exhibited high affinity for terbutylazine. The selectivity of the polymer was investigated with the template and closely related triazine compounds in MISPE format using different organic solvents and acetonitrile–water mixtures as selective washing solvents. It was demonstrated that a small water content in acetonitrile can dramatically decrease the selective binding ability, but higher water contents lead to a hydrophobic selectivity which phenomenon needs further evaluation. The results obtained for the different organic washing solvents indicate that the most selective interactions between the MIP and the template terbutylazine can be achieved in dichloromethane. Finally, based on these results, a coupled C18 disk-MISPE sample pretreatment technique was developed and an example for surface water clean-up was shown.

Acknowledgements

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