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Investigation of matrix effects of urine on a molecularly imprinted solid-phase extraction

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Abstract

This study investigates matrix effects on a molecularly imprinted solid-phase extraction (MISPE) method developed for the clean-up of diphenyl phosphate (a hydrolysis product of the commonly used flame retardant and plasticizer, triphenyl phosphate) in urine samples. The influence of potentially interfering compounds that naturally occur in urine was examined with respect to extraction recovery, repeatability and selectivity. The components tested were NaCl, urea, creatinine and hippuric acid. The imprinted polymer was prepared using 2-vinylpyridine as the functional monomer, ethylene glycol dimethacrylate as crosslinker and a structural analogue of the analyte as the template molecule. The recovery of diphenyl phosphate from water standards was over 90% using MISPE, compared to less than 25% using a non-imprinted SPE (NISPE) counterpart. The selectivity of MISPE compared to NISPE was achieved in a wash step with a basic modifier in methanol. The recovery and repeatability of the MISPE method were affected most by NaCl in the tested concentrations, while urea, creatinine and hippuric acid had no significant influence. NaCl most likely weakens the binding during the loading of the sample. This effect could be suppressed by diluting the sample with a citrate buffer at pH 4.0.

Keywords: Molecularly imprinted polymers; Solid-phase extraction; Matrix effects; Urine samples

1. Introduction

The analysis of complex samples, like biofluids, places high demands on sample preparation prior to analysis. Solid-phase extraction (SPE) is a well-established method for sample clean-up and pre-concentration for aqueous samples at trace levels. Nevertheless, the method often lacks the ability to extract target compounds selectively, potentially leading to the co-extraction of matrix interferences. Methods based on molecular recognition, such as the use of immunoaffinity extraction (IAE) sorbents and molecularly imprinted polymers (MIPs) allow both high affinity and selectivity. IAE sorbents exploit biological tools, such as antibodies, for selective extraction and concentration of individual compounds or classes of compounds. Immunoextractions were first described for large molecules, such as enzymes, proteins, viruses, peptides and hormones because of the ready availability of antibodies for these types of compounds [1]. Molecularly imprinted polymers possess levels of affinity that can be comparable to those of natural antibodies, and they are often called synthetic antibody mimics. Since the development of immunosorbents is both time-consuming and expensive, the use of MIPs can be a valuable alternative or complement to IAE methods, particularly for smaller molecules.

The MIP approach is based on a highly cross-linked copolymer network synthesized in the presence of a template compound. Extraction of the template leaves imprints with binding sites that have both steric and chemical affinity for the compound. The first study of the use of MIPs as sorbents in SPE, MISPE, was presented in 1994 by Sellergren [2]. Since then, MISPE has been shown to be a promising technique, with applicability, inter alia, in bioanalyses [3–6]

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and environmental studies [7–9]. Several recent reviews of the technique have been published [10–12].

Most MIPs are synthesised in organic solvents, and studies on imprint rebinding often utilize organic solvents as incubation media. The establishment of strong and selective binding to the imprints under these conditions is relatively well understood. However, current MIP technology often fails to generate polymers for use in pure aqueous environments. Because of the hydrophobic character of the MIP, non-selective adsorption to the lipophilic surface commonly occurs when processing aqueous samples. The total binding to the polymer is the sum of the selective binding to the imprints and the non-selective binding to the polymer. To use a MIP most effectively, it is important to suppress the non-selective binding. One solution to this problem was presented by Andersson et al., who investigated the influence of detergents in the buffer during a MISPE of local anaesthetics from human plasma [13]. They found that three different neutral detergents were able to eliminate non-selective adsorption to the polymer and leave selective imprint-analyte binding essentially unaffected. A modification of this MISPE method in pure aqueous systems has recently been presented by Dirion et al. [14]. By modifying the original MIP composition via the incorporation of a hydrophilic co-monomer, 2-hydroxyethyl methacrylate, the non-specific binding was reduced, especially during the loading of the plasma sample. The MISPE cartridge can also be washed with selective solvents that are capable of disrupting the non-selective (but not selective) interaction with the polymer, when extracting aqueous samples. The selectivity then occurs in the washing rather than the loading step, leading to selective desorption rather than selective extraction [10].

To help cope with current demands for more rapid sample preparation methods, combinatorial methods have recently offered valuable tools in the development of MIPs [15]. Since many variables in the imprinting process influence the selectivity and capacity of the resulting MIPs, optimisation can be very time-consuming, especially if it is done by trial-and-error. However, a high-throughput synthesis and screening system for large libraries of MIPs was recently presented by Dirion et al. [14], which allowed rapid optimisation and fine-tuning of the recognition properties for extracting plasma samples. Another recent study presented by Piletsky et al. [16] describes the fast design and synthesis of a MIP with high affinity for the template in aqueous solutions, using a computational method.

Matrix effects from biofluids are well-known problems, especially in LC/ESI-MS analysis [17]. In these cases, the effect is on the ionization of the target analyte, causing suppression or enhancement of the analyte response. Matrices may also affect chromatography or other separation methods. We have recently presented a study of a MISPE method for a flame retardant hydrolysis product, diphenyl phosphate (the chemical structure of which is shown in Fig. 1), in human urine. The matrix was shown to affect the recovery, selectivity and repeatability of the method [18]. It was found that a large breakthrough occurred during the loading of the urine samples. When the urine was diluted with a low pH buffer this breakthrough was avoided, but the non-selective interactions increased in strength, probably due to a non-selective ionic interaction with the polymer that became stronger when the basic monomer was more highly charged. The objective of the present study was to investigate more thoroughly these matrix effects.

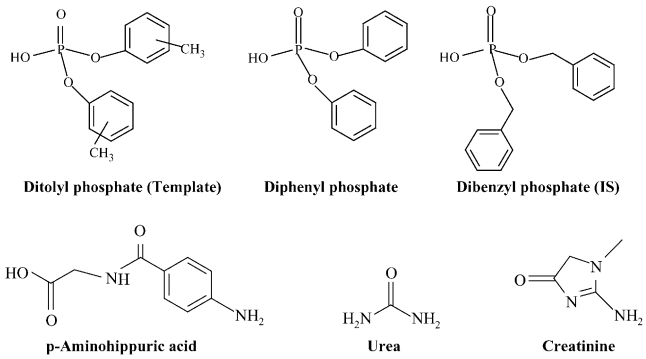

Fig. 1. Structures of the compounds used in this study: template, analyte, internal standard and urine components.

2. Experimental

2.1. Chemicals

Diphenyl phosphate (97%) was obtained from Sigma-Aldrich (Steinheim, Germany) and dibenzyl phosphate (99%) from Lancaster (Morecambe, UK). Acetic acid, ammonia solution (NH_3, 25%), sodium chloride (NaCl), 2-vinyl pyridine (2-Vpy), ethyleneglycol dimethacrylate (EGDMA), basic aluminium oxide and triethylamine (TEA) were purchased from Merck (Darmstadt, Germany). HPLC grade methanol was obtained from BDH (Poole, UK) and HPLC grade acetonitrile (ACN) from Merck. Urea, trisodium citrate dihydrate, creatinine and *p*-aminohippuric acid were obtained from Sigma-Aldrich. Citric acid and 2,2-azobisisobutyronitrile (AIBN) were obtained from Acros Organics (Geel, Belgium). Chloroform was purchased from Riedel-de Haën (Seelze, Germany). Water was purified using a Millipore system, Milli-Q PLUS 185, from (Molsheim, France). All the chemicals were used with no further purification except for EGDMA and chloroform, which were passed through basic aluminium oxide before use.

2.2. Preparation of MIPs

The template, ditolyl phosphate, was synthesized from a pure enantiomer, *m*-tritolyl phosphate. This procedure has been described in a previous paper [19]. Synthesis of the molecularly imprinted polymers was based on the method reported by Andersson [6].

Ditolyl phosphate (template, 0.37 mmol) and AIBN (initiator, 0.34 mmol) were weighed into a flask, dissolved in 6 ml chloroform and briefly ultrasonicated. EGDMA (21.8 mmol) and 2-Vpy (4.33 mmol) were added to the flask and briefly ultrasonicated. The clear solution was poured into glass tubes, cooled on ice and sparged with nitrogen for 5 min. The tubes were then sealed, placed under a UV-source (365 nm) at 5–7 °C for 24 h, and rotated periodically to ensure homogenous polymerization. The tubes were then smashed and the hard polymers were soaked in methanol for 4 h to remove unreacted monomers. The hard polymers were ground manually with a mortar and pestle and sieved, under water, through 36 and 25 μm sieves and the particles between 25 and 36 μm were collected. For the polymerisation reaction a UVL-56 long-wave 365 nm UV-lamp from UVP (Upland, CA) and polymer sieves from Retsch (Haan, Germany) were used. The imprinted polymer particles were transferred to glass filter funnels and washed with three cycles of 3 × 100 ml methanol:acetic acid (4:1, v:v) and 3 × 100 ml methanol, to remove the template. The washed polymer particles were then dried under vacuum and stored in a desiccator at ambient temperature until use. Non-imprinted polymers (NIPs) were synthesized simultaneously under the same conditions except for the addition of template.

2.3. Evaluation of selectivity in the wash steps

MIP and NIP cartridges were prepared by packing 40 mg of the respective polymer suspensions into empty 3 ml SPE cartridges (Isolute SPE, IST-International Sorbent Technology, Mid Glamorgan, UK) and secured by polyethylene frits at the top and bottom. The polymer particles, 25–36 μm in diameter, were suspended in a solution of methanol:water (1:1). The standard solution used for extraction contained 0.25 μg diphenyl phosphate in 1 ml water. The cartridges were conditioned before extraction with 3 ml methanol and 2 ml water. Duplicate cartridges of both MIP and NIP were used for each extraction. The standard solution was passed by gravity through both MIP and NIP cartridges at an approximate flow rate of 0.5 ml/min. The cartridges were then washed with 1 ml water, 1 ml methanol and 1 ml of 5 mM NH_3, containing different percentages of methanol. Elution was performed by passing 2 × 1 ml of a solution containing 1% TEA in methanol. The last wash and the elution fractions were collected and 1.6 μg dibenzyl phosphate was added as an internal standard. The fractions were then evaporated until dryness at 40 °C under a stream of N_2 and re-dissolved in 250 μl ACN:water (5:95). All collected fractions were analyzed by LC/ESI-MS in SIM mode and the recoveries were determined by comparing the analyte/internal standard peak area ratios with those of an external standard.

2.4. Investigation of matrix effects on MISPE

MISPE and NISPE cartridges were packed with 40 mg polymer as described above. Standard solutions were prepared containing 0.25 μg diphenyl phosphate with differing amounts of NaCl, urea, creatinine or hippuric acid in 1 ml water. Duplicate extractions were performed for each standard with both MIP and NIP cartridges. The amounts of NaCl in the standards were 2.5, 7.5, 12.5, 17.5 and 22.5 mg. The urea standards contained 8.0, 16.0, 24.0, 32 and 40 mg. The creatinine and hippuric acid standard was a mixture of both compounds and contained 1.3 mg of creatinine and 0.68 mg of hippuric acid, respectively, in 1 ml aqueous solution. The same procedure for extraction and final MS analysis as described in Section 2.3 was used, except that 1 ml of 5 mM NH_3 in methanol was used in the last wash step.

2.5. Extraction from a NaCl/urea solution: optimization of recovery and selectivity

An aqueous standard solution containing 13 mg/ml NaCl and 24 mg/ml urea was prepared, then 10 μl diphenyl phosphate, 25.4 ng/μl in water, was added to 1 ml of the solution. MISPE and NISPE cartridges were packed with 40 mg polymer as described in Section 2.3. Conditioning, loading, washing and elution of the cartridges and final MS analysis were performed as described in Section 2.3. To investigate the effects of pH, standard solutions were diluted with 1 ml of 10 mM citrate buffer and the pH was adjusted to pH 3.0, 4.0, 5.0 and 7.0, respectively. Duplicate extractions were performed at each pH on both MISPE and NISPE cartridges. To optimise the selectivity of the extraction of the dilute standard solution, the effects of adding varying amount of methanol (50%, 75% and 100%), with 5 mM NH_3 in each case, to the last wash solution was tested.

2.6. MISPE from spiked human urine samples

Empty SPE cartridges were packed with 60 mg suspensions of MIP and NIP, as described in Section 2.3. Human urine (1 ml) was spiked with a solution containing 0.25 μg DPhP in 10 μl water, and diluted with 1 ml citrate buffer (50 mM, pH 4.0) and then vortex mixed for a few seconds. The same extraction procedure as described in Section 2.3 was then applied, except that 1 mM aqueous NH_3:methanol (1:1) was used for the final wash step. The last wash fraction and the elution fraction were collected and 0.7 μg dibenzyl phosphate in 10 μl ACN was added. Evaporation, LC/ESI-MS and determination of recovery were then performed as described in Section 2.3.

2.7. Chromatographic conditions and MS detection

Chromatographic separation of the MISPE eluate was performed on a C_{18} X-Terra reversed phase column (2.1 mm × 150 mm i.d., 3.5 μm particle size, Waters, Milford, MA, USA). The HPLC system consisted of a Rheos Model 4000 pump (Flux Instruments, Switzerland) connected to an autoinjector with a 5-μl loop. The mobile phase flow rate was 200 μl/min. A linear gradient was used for separation and the

mobile phase consisted of ACN and water containing 10 mM NH_3. The gradient started with 10% of acetonitrile and increased to 60% over 15 min.

A Finnigan LCQ ion-trap mass spectrometer (Thermoquest, San Jose, CA, USA) equipped with an electrospray ionisation source was used for detection, as follows. The instrument was operated in negative-mode using the following settings: spray voltage, 3.5 kV; capillary temperature, 250 °C; capillary voltage, −20 V; tube lens offset, 10 V; sheath gas flow (N_2), 80 (arbitrary units); auxiliary gas flow (N_2), 20 (arbitrary units). Detection was carried out in SIM mode and the quasi-molecular ions $[M - H]^-$ were monitored, i.e. *m/z* 249 ± 2 for diphenyl phosphate and *m/z* 277 ± 2 for dibenzyl phosphate.

3. Results and discussion

3.1. Evaluation of the selectivity of MISPE from aqueous standards

We have previously shown the importance of optimizing the wash step in MISPE, to achieve selective extraction and to obtain acceptable recoveries of diphenyl phosphate when extracting the compound from low pH buffered urine [18]. In the present study, the effects on extraction of using a basic modifier, NH_3, and a varying content of methanol in the last wash step were investigated. The selectivity was evaluated by comparing the recoveries from MISPE and NISPE. The results showed that the methanol in the wash step had an important concentration-dependent, disruptive effect on the non-selective interaction (see Fig. 2). When no methanol was added to a 5 mM NH_3 solution, there was almost no difference in recovery between the MIP and NIP. However, a selective wash was achieved for MIP when more than 50% methanol was used. For the NIP, the breakthrough of diphenyl phopshate was then detected in this wash step. A wash solution with this composition seems to be able to disrupt the strong non-selective adsorption to the NIP (which has both ionic and hydrophobic components). However, no reductions

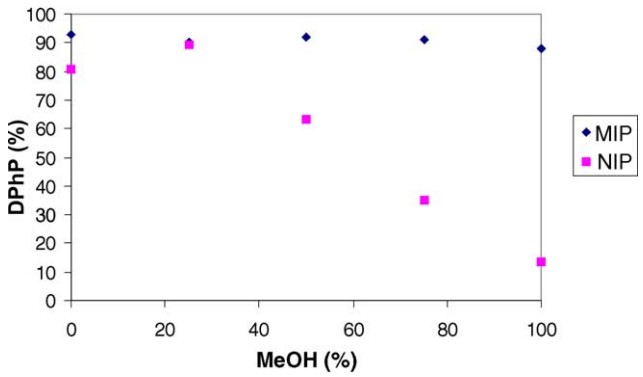

Fig. 2. Recoveries of diphenyl phosphate extracted by MIP and NIP cartridges from 1 ml water washed with a 5 mM NH_3 solution containing different percentages of methanol. Each value represents an average of duplicate samples.

of the recovery for the MIP cartridges were observed, demonstrating the imprinting effect. A higher concentration of NH_3, 10 mM, in methanol was also tested, but then substantial breakthrough was detected in the wash fraction for the MIP (results not shown). The recovery from MISPE when extracting 1 ml aqueous solution of diphenyl phosphate was 90.6% with an R.S.D. of 4.3% (n = 10).

3.2. Investigation of matrix effects from individual naturally occurring components in urine

The effects of potential, naturally occurring, interferences on the selectivity of the MISPE method for recovering diphenyl phosphate from urine were examined. Urea and NaCl are the most abundant compounds in normal human urine. Therefore, standards with diphenyl phosphate in aqueous solutions with varying concentrations of urea or NaCl, were extracted by MISPE and NISPE. It was suspected that the presence of urea in the urea standards would reduce the capacity of the MIP, since urea is a highly acidic compound (pK_a 0.1) like diphenyl phosphate (pK_a 0.3) and may, therefore, compete for the selective sites by ionic interactions. However, results from extractions with urea standards demonstrated that urea did not affect the selectivity or the recovery of diphenyl phosphate. The recovery obtained using MISPE in the presence of urea was in the same range as extraction from water, and selectivity was achieved in the last wash, even for standards with a concentration as high as 40 mg/ml (results not shown). In contrast, the extractions with NaCl standards showed that NaCl affected both the recovery and repeatability (Fig. 3). When the salt content increased, the recovery from NISPE decreased. Leakage occurred not just in the last wash step with NH_3, but also in the second step with methanol. Thus, both types of non-selective adsorption, i.e. ionic and hydrophobic, were suppressed. For MISPE, the recovery was non-repeatable and no clear trend was observed with increasing salt contents. Substantial leakage was detected in the last wash step with NH_3. An explanation for the observed phenomena is that an electrostatic interaction may be formed between the acidic, negatively charged analytes and the positively charged Na^+ ions. Such a complex would make the molecule more neutral, thereby, hindering the ionic interaction with the polymer. A similar hypothesis, suggesting that cation–analyte interactions can suppress adsorption to the polymer, has recently been proposed by Chapuis et al. [20]. The cited authors observed reduced extraction recoveries for aqueous samples, caused by the presence of cations in the matrix. The effect was explained as being due to an ion-exchange between the proton of the carboxylic acid functionality of the polymer and the divalent cations, removing the hydrogen bond donor groups necessary for selective retention on the MIP.

To investigate the effects of more hydrophobic components than urea or NaCl, standards of diphenyl phosphate in aqueous solutions of creatinine and *p*-aminohippuric acid were also tested. The concentrations of creatinine and *p*-

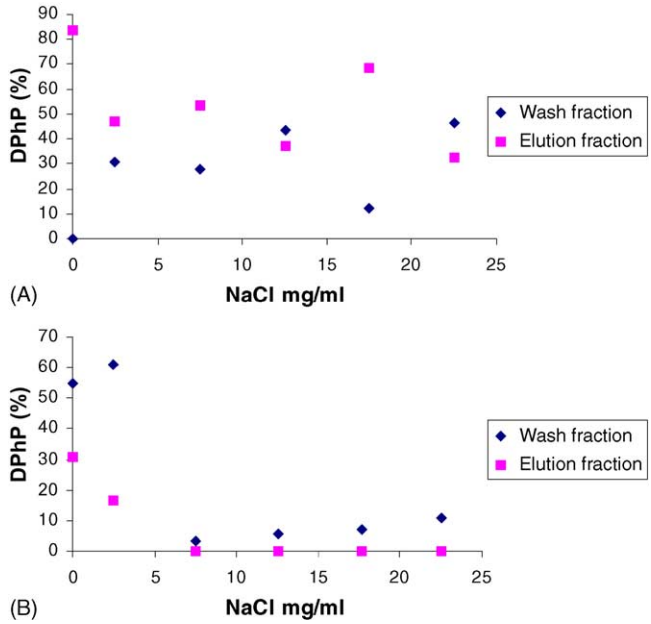

Fig. 3. Recoveries of diphenyl phosphate from water containing different amounts of NaCl. Extraction on (A) MIP cartridges and (B) NIP cartridges. The breakthrough from the last wash fraction, 1 ml MeOH:H_2O (1:1) 5 mM NH_3, and the recoveries from the elution fraction, 2 × 1 ml 1% TEA in methanol, are presented as two series. The values are means for duplicate samples.

aminohippuric acid were in the same range as those usually found in normal urine. As shown in Fig. 1, these components have some structural similarities to diphenyl phosphate that might interact with the imprints, such as an acidic functionality, carbonyl groups and the aromatic moiety. Results from duplicate extractions from creatinine and *p*-aminohippuric acid standards are presented in Table 1. The recoveries were as high as when extracting from pure aqueous standards of diphenyl phosphate, and the selectivity obtained, in terms of the difference in recovery between MISPE and NISPE, was also similar.

3.3. Optimization of recovery and selectivity of MISPE from a salt (urine mimic) standard

To further investigate the effects of the salt content on MISPE, extractions were performed with an aqueous standard containing 24 mg/ml urea, 13 mg/ml NaCl and 0.21 μg/ml diphenyl phosphate. The aim was to increase the strength of the interactions in the loading step, while retaining the selectivity achieved in the wash step.

Table 1
Recoveries of diphenyl phosphate from the extraction of standards containing a mixture of creatinine and *p*-aminohippuric acid

	MIP	NIP
Recovery (%)	97.3	22.1
R.S.D. (%)	7.7	18.9

$N = 4$.

Table 2
Recoveries of diphenyl phosphate from 1 ml urea/NaCl standard diluted with buffer at different pH

pH	Recovery (%)	
	MIP	NIP
3	93.6 ± 3.9	79.7 ± 3.3
4	77.6 ± 6.0	11.8 ± 4.2
5	52.4 ± 12.3	11.9 ± 23.7
7	63.7 ± 21.7	n.d.

Duplicate extractions for each pH. n.d., not detected.

When extracting from an undiluted NaCl/urea standard the recoveries from MISPE were low, 20–30%, and leakage of diphenyl phosphate was detected in the wash steps with methanol and NH_3. No recovery at all was detected from NISPE under these conditions. Since leakage was detected in the last wash, the wash composition in this step was modified from 1 ml 5 mM NH_3 in methanol to 1 ml MeOH:H_2O (1:1) with 5 mM NH_3. The urea/NaCl standard was diluted with 10 mM citrate buffer at four different pH values (3.0, 4.0, 5.0 and 7.0). The results from the extractions under these conditions showed that the highest recoveries were achieved at low pH (Table 2). However, a lower pH also leads to a stronger interaction with the NIP, which is difficult to disrupt. A pH of 4.0 was found to be optimal for the selectivity. At this pH, the recovery was higher than 77% from MISPE, while it was as low as 12% from NISPE. The improvement in recovery at lower pH is most likely due to increases in the density of positively charged sites in the polymer, which suppress the complex formation with Na^+. The functional monomer, 2-Vpy, has a pK_a value of 5.8.

3.4. MISPE from human urine

Extraction from spiked human urine samples was performed using the same conditions as described for extracting the standards in Section 3.3. Each urine sample was spiked with 0.25 μg diphenyl phosphate and then diluted with citrate buffer at pH 4.0. However, it was found that the citrate concentration had to be increased from 10 to 50 mM to avoid leakage in the loading step, probably due to insufficient buffer capacity at 10 mM. Urine was also shown to decrease the capacity of the MIP, since it induced a large breakthrough in the wash step with NH_3. Therefore, the amount of polymer was increased to 60 mg for the urine extractions. When washing with a solution of 5 mM NH_3 in water:methanol (1:1), the recovery from MISPE was only 44% and from NISPE 9%. By fine-tuning the final wash step, the recovery and selectivity could be optimized. The highest recovery, over 90%, was achieved with 0.5 mM NH_3, but the selectivity was low. The selectivity was improved when the wash solution was changed to 1 mM NH_3. Under these conditions, the recovery from MISPE was decreased to 67%, while it decreased for NISPE to less than 30%, as shown in Table 3.

The MS-SIM chromatogram of a urine extract, obtained using the optimised MISPE method, is shown in Fig. 4. Al-

Table 3
Extraction recoveries of diphenyl phosphate from human urine diluted with 50 mM citrate buffer adjusted to pH 4.0

	MIP	NIP
Recovery (%)	67.3	29.2
R.S.D. (%)	9.6	18.4

$N = 4$.

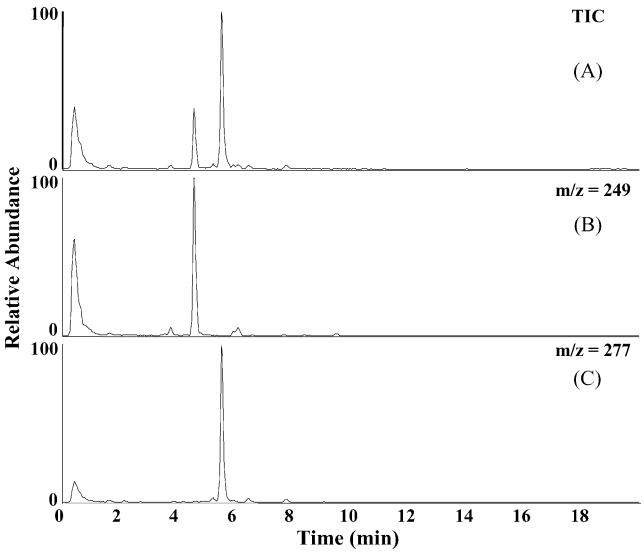

Fig. 4. (A) Total ion chromatogram (TIC) from an extract of 1 ml urine sample spiked with 0.25 μg diphenyl phosphate and diluted with 1 ml citrate buffer pH 4.0 from MISPE. Reconstructed ion chromatogram (RIC) of (B) $m/z = 249$ for diphenyl phosphate and (C) $m/z = 277$ for dibenzyl phosphate.

though only specific ion intervals were monitored, the chromatogram demonstrates the clean-up efficiency of the MISPE method. Only minor interferences from the matrix can be observed.

4. Conclusions

This study has highlighted the importance of identifying the effects from the sample matrix on MISPE. A method to suppress matrix effects for urine samples has been presented. By simply diluting the urine in buffer, the influence from Na^+ ions on the repeatability and recovery was suppressed. The results contribute to the knowledge of MIP recognition in aqueous media that still in many cases needs to be improved. In this study the selectivity of the MIP was achieved in the wash step, and was explained by a mechanism based on both ionic and hydrophobic interactions. The importance of fine-tuning the final wash step to optimize recovery and selectivity was also demonstrated.

Although there is a compromise between selectivity and recovery, the developed method should be useful for exposure studies of organophosphate triesters in human urine. Since the repeatability is acceptable and the clean-up is efficient, a recovery of 67% of the target analyte should be sufficiently high.

Acknowledgement

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