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Pacific Northwest
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Ultraselective Sorbents
Task 2: Molecularly Imprinted Polymers (MIPs)/Stabilized Antibody Fragments (STABs)
Final Report -- Fiscal Year (FY) 2005

S. D. Harvey

September 2005

Prepared for the National Nuclear Security Administration
Office of Nonproliferation Research and Engineering, NA-22
Prepared for the U.S. Department of Energy
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EXECUTIVE SUMMARY

Trace analysis of signature compounds is usually accomplished by concentrating the target signature compound from a large volume environmental sample on an appropriate sorbent. Unfortunately, the organic matrix components also become concentrated, which necessitates separating the signature compound from this complex mixture in sufficient purity to allow identification and quantification. Due to the complexity of the mixture, sophisticated, laboratory-based multidimensional instrumentation often is required to provide adequate separation.

The goal of this research is to develop highly selective sorbents that will enable collection of relatively pure analyte fractions during the sampling step. Due to the high purity of the initial fraction, subsequent analytical steps leading to high-integrity identifications at trace concentrations can be greatly simplified. The required analytical instrumentation can reflect this simplification by being made more compact, lightweight, and field portable. This advancement is made possible by the high degree of matrix discrimination accomplished during the sampling step.

Two different selective sorbents were developed and evaluated in this research. Both sorbents must be capable of operation in nonaqueous environments to protect hydrolytically sensitive analytes during analysis. The approaches investigated were nonaqueous immunochromatography featuring the use of stabilized antibody fragments (STABs) and molecularly imprinted polymers (MIPs). Both approaches were pursued in Fiscal Year (FY) 2003, during the course of which it became clear that the MIPs approach had clear advantages over STABs. Further research efforts during FY 2004 and beyond focused exclusively on the development of MIPs.

Our MIP studies have had two distinct thrusts: one thrust targeted volatile organic signatures, and the other thrust targeted nonvolatile phosphate or methylphosphonate half-acid esters. The first studies targeted the volatile signatures. By nature these volatile compounds do not contain polar functionalities in their chemical structures. Since most imprinting is based on polar functional group interactions, these compounds proved difficult to imprint. Nonetheless, we were successful in making MIPs that targeted tributyl phosphate (TBP) and diisopropyl methylphosphonate (DIMP). Selective interactions were not pronounced, as expected due to the weak interactions; however, these sorbents were capable of impressive selective capture of the target analyte with near-quantitative recovery from extremely complex environmental samples. These results were summarized in the manuscript submitted to the *Journal of Separation Science*. During the publication review process, we were asked to provide additional experiments. The experiments demonstrated that 1) our MIP sorbents had the expected cross reactivity toward structurally related analytes and, 2) our MIP selectivity was superior to traditional normal-phase sorbents, *e.g.*, silica or alumina. We also calculated typical matrix discrimination factors for MIP capture of targets from complex samples to

put the MIP selectivity in perspective. The results were included in the MIP manuscript published in May 2005 (see Appendix A).

Further MIP studies have focused on preparing MIPs specific toward polar nonvolatile phosphate or methylphosphonate half-acid esters. Synthesis of MIPs specific toward these compounds proceeded by advanced suspension polymerization techniques. In some cases, MIP preparation first required custom synthesis of half-acid ester products that were not available commercially for use as templates and analytical standards. We devised a simple novel synthesis that was capable of preparing gram quantities of pure (>95 percent) G-series nerve agent hydrolysis products within a day without specialized equipment or glassware. The approach was a significant improvement over more complicated and time-consuming literature procedures. A manuscript detailing this work was published in 2005 in *Sulfur, Phosphorus, and Silicon,* Volume 180 (pp 1885-1891).

During FY04, we designed a MIP-based instrument for the analysis of half-acid phosphate and methylphosphonate esters from aqueous samples. The instrument was presented in our FY04 Task 2 Final report. The instrument operates by concentrating lipophilic contents on an octadecyl silica (C-18) sorbent. Water is then removed by a stream of dry gas followed by transfer of the organics from the C-18 to the MIP sorbent with acetonitrile. The target compound is selectively sorbed on the MIP from the acetonitrile mobile phase, while the other organics pass through the MIP as waste. The final step is to elute the target compound from the MIP with water and detect it using a conductivity detector. The instrument performed well during initial assessment using pinacolyl methylphosphonic acid (PMP) as the test analyte. During these proof-of-principal experiments, a simple injection loop was used for sample introduction. A surface microlayer skimmer and sample pump could be added later to further concentrate analytes. After reasonable concentration values are chosen for the surface microlayer enhancement and the C-18 preconcentration stages, detection limit estimates are in the part-per trillion (ng/L) range. Lower detection limits would be possible with alternative, more sensitive detection strategies such as an enzyme-based amperometric or electrospray high-resolution ion mobility spectrometry (IMS) detection schemes.

Our principal focus during FY05 was to provide an extensive characterization of custom MIPs we prepared that were specific toward bis(2-ethylhexyl) hydrogenphosphate (B2EHP) or pinacolyl methylphosphonic acid (PMP). B2EHP is an organic signature associated with uranium processing, whereas PMP is the hydrolysis product of the nerve agent Soman. These MIPs were intended to be used in the novel MIP-based instrument described above. Sorbent evaluation studies were performed by injecting analyte on short HPLC columns, packed with either MIPs or a control sorbent, while collecting eluate fractions. The analyte in these fractions was analyzed by negative ion electrospray mass spectrometry. The first studies (Phase 1) showed that each of these MIPs selectively captured their targets relative to a nonimprinted control sobent. In this case, interactions were far stronger than those observed during the study of volatile signatures due to the polar functionalities contained in the half-acid ester templates. Phase 2 studies examined cross reactivity of the MIPs toward structurally related methylphosphonic half-acid esters, phosphinic acids, and alkylphosphonic acids.

Reasonable cross reactivity and selective retention relative to controls verified a strong imprinting effect in the MIP polymers we had prepared. Phase 3 studies were planned to investigate the matrix discrimination capabilities of the MIP sorbents. We anticipated using analyte-fortified environmental waters, soil extract slurries, and physiological samples, *e.g.*, saliva and/or urine, to assess whether the selectivity of the MIP is adequate for use with a relatively nonselective conductivity detector. If selectivity is not high enough to ensure delivery of a pure analyte to the detector, a more selective detection strategy could be substituted.

Although completion of these studies, as well as initiation of several new research directions, were proposed for Task 2 in the FY06 Ultraselective Sorbents lifecycle plan, the project has been put on hold until such time as the NA-22 FY06 budget can be more fully evaluated. The last section of this report provides specific recommendations for further research in case NA-22 funding does not resume. We have already published a complete study for the volatile organic signature compounds. The recommendation section of this report provides suggestions for furthering these studies. MIP studies that address the half-acid phosphate and methylphosphonate esters are completed through Phase 2. Phase 3 studies need to be performed to fully assess the matrix discrimination capabilities of the MIP sorbents and their suitability for incorporation into the current novel trace enrichment instrument design. Recommendations are presented for improving the detection selectivity and sensitivity for this instrument, as well.

Rapid progress has been made during this project toward our goal of synthesizing and assessing the analytical utility of MIPs for selective capture and analysis of organic signatures. The project has established a solid foundation for further development of MIP-based analytical instrumentation for the selective analysis of both volatile and nonvolatile signature compounds. Although substantial work remains in this area, we expect further development to result in compact field portable instruments that deliver high-integrity results. This streamlined analysis approach is made possible by the selective capture of a relatively pure fraction on the selective MIP sorbent during an initial stage of the analysis, either during sampling or immediately thereafter.

INTRODUCTION

Objectives

Several approaches are available for analyzing ultratrace concentrations of signature compounds in complex environmental samples. Analysis often involves initial concentration of the signature compounds on a sorbent. The sorbent usually collects and concentrates other organic matrix components along with the target species, resulting in a concentrated complex organic mixture that requires further separation to isolate the target species in a pure enough form for identification and quantification.

The concentrated organic matrix mixtures can be extremely complex. Single separation stages may not provide adequate separation power to resolve the target compound from the matrix background, even under ideal high-resolution conditions. For this reason, complex multidimensional instruments that combine two or more high-resolution separations may be necessary for successful analysis [1]. As the effective peak capacity is multiplicative rather than additive for multidimensional systems, the separation power is extremely high. However, these instruments are large, complex, and laboratory based. Although they can provide impressive separations, multidimensional systems tend to be unreliable, and require a skilled chromatographer to maintain efficient operations.

The goal of this research is to significantly improve the present state-of-the-art by applying highly selective sampling of signature compounds from the environment rather than by relying on traditional nonspecific sorbent sampling. The pure fraction that results will lower the subsequent analytical requirements for determining signature compounds at trace concentrations.

The highly selective sorbents developed during this research should provide enormous matrix discrimination such that only the signature compound of interest is collected during sampling while the interference compounds pass through the sorbent and become waste. Accordingly, a large volume environmental sample can be processed resulting in a pure and concentrated zone of signature compound that is retained on the sorbent. If the high degree of matrix discrimination can be obtained, the subsequent analysis steps leading to high-confidence identifications at trace concentrations can be greatly simplified. It should be possible to construct more compact, lighter-weight, and field-portable systems that provide similar performance as the nonselective sampling approach combined with laboratory-based multidimensional separations. The long-term goal of this research, therefore, will be the development of a selective sorbent-based instrument that, due to highly selective sampling, will feature streamlined analysis and enable high-confidence identification at ultratrace concentrations in a field-portable format.

Due to the hydrolytic sensitivity and limited aqueous solubility of many volatile signature compounds, a system that operates with nonaqueous mobile phases is highly

desirable. Such a system can transfer analytes using organic solvents or supercritical fluids. These mobile phases promote stability of hydrolytically sensitive signature compounds, *e.g.*, many of the nerve agents. Unfortunately, while fostering target compound integrity, the requirement of operating in a nonaqueous environment places severe restrictions on the types of molecular recognition mechanisms that can be explored.

Ideally, large-volume environmental samples could be directly sampled on the selective sorbent. However, this may not be practical for gas-phase sampling because many organics will interact by nonspecific mechanisms or be collected as part of the aerosol particulates. If direct sampling with the selective sorbents proves unfeasible, the selective sorbent can be positioned immediately behind a more traditional air sampling sorbent such as XAD-2 or Tenax GC. The atmospheric organics in this arrangement generally will be collected on the traditional nonspecific sorbent during sampling. The sorbed organics will then be transferred as a complex organic mixture that contains the signature compound from the traditional sorbent to the selective sorbent with an organic solvent or a supercritical fluid mobile phase. During this process, the signature compound is retained on the selective sorbent while the matrix organics pass through this sorbent unhindered. Figure 1 illustrates formats where the selective sorbent is incorporated as a primary or a secondary sampling sorbent.

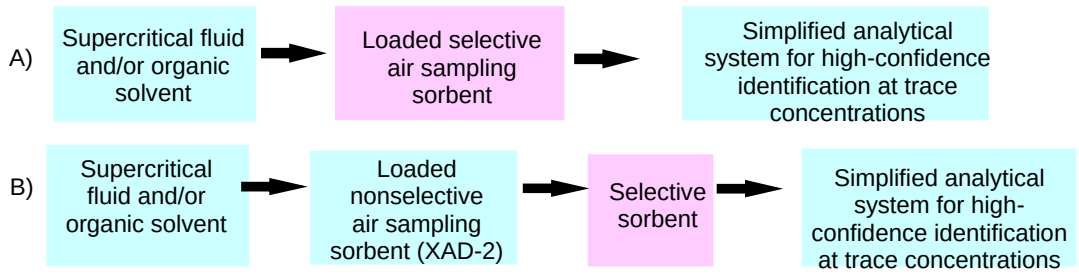

Figure 1. Two proposed formats for implementing selective sorbent sampling. The selective sorbent can be used as A) a primary air sampling sorbent or B) a secondary sorbent situated immediately behind a traditional nonspecific air sampling sorbent.

The sorbent in our system will operate on one of two different selective molecular recognition mechanisms. Our studies will focus on immunorecognition (antibodies or antibody fragments) or molecularly imprinted polymers (MIPs). Sorbents based on these recognition mechanisms have the potential to provide highly selective sampling. These two approaches are described below.

Nonaqueous Immunochromatography

The antibody/antigen interactions are strongest under physiological conditions [2-4], which is understandable given the evolutionary origins of the immune system. Therefore, antibody/antigen interactions are optimal within defined limits of ionic strength, pH, and temperature. Immunointeractions tend to be strongest for large polar molecules. In fact, a molecule must be larger than the critical size of approximately 2500 Daltons to illicit an immune response, otherwise it must be conjugated to a large protein carrier molecule before an immune response is observed [5,6]. Antibodies can be covalently immobilized on chromatographic stationary supports to yield stable immunosorbents. Covalently immobilized antibodies are more stable when exposed to extreme conditions than the free antibody.

Analysis based on antibody/antigen interaction in aqueous buffers is well established. Many of these assays have reached a high degree of sophistication. For example, miniaturized mosaic immunoassays have been described that use microfluidics to pattern lines of antigen on a surface and then flow antibodies at 90° in another microfluidic network across the antigens. This allows reliable combinatorial antibody screening at high sensitivity in a highly miniaturized format that exhibits minimal reagent consumption [7]. Gas-phase analytes are usually sampled by an impinger and trapped in an aqueous buffer. The analytes are then determined by an enzyme linked immunosorbent assay or other immunochemical technique [8]. Immunochromatographic preconcentration has been extensively described for aqueous systems [9,10].

A handful of studies exist that describe active antibody/antigen interactions in organic solvents or in the gas phase [11-15]. Much of the interest in nonaqueous immunointeraction was prompted by the unique attributes observed for enzymatic catalysis performed in organic solvents [16-21]. Guilbault, Luong and Ngeh-Negwainbi *et al.* described antibody-coated piezoelectric sensors that selectively detect analytes in the gas phase [11-12]. In contrast, Rajakovic *et al.* failed to demonstrate selective immunochemical binding in the gas phase [13]. In an extensive study conducted by Russell *et al.* [14], antibody/antigen binding was characterized using radiolabeled analytes in anhydrous organic solvents. The authors reported that immunoassociation in organic solvents can be strong and highly specific. The work of Pinalva *et al.* also reported immunointeractions in organic solvents, a finding that is consistent with the research of Russell *et al.* [14,15]. Many authors, however, find that the antibody/antigen interactions diminish with increasing organic content of the surrounding media [2,3]. One study showed that antibodies can function in alcohol concentrations of up to 50 percent [2]. Higher concentrations are thought to potentially denature the antibody resulting in diminished immunointeractions.

A convincing demonstration of analytically useful nonaqueous immunochromatography would be a novel development that would allow selective preconcentration of hydrophobic analytes, *e.g.*, compounds that display limited aqueous solubility or analytes that might be hydrolytically sensitive (including many of the nerve agents). Additionally, novel instrument designs that feature nonaqueous

immunoconcentration could be developed possibly for gas-phase collection or in a format where analytes are collected on a nonselective preconcentration stage and then transferred to an immunosorbent by using either organic solvents or supercritical fluids. Progress along these lines would necessitate definitively resolving the literature contradictions regarding nonaqueous antibody activity.

One drawback of the immunochromatographic approach is the limited number of antibodies specific toward small molecules that are commercially available. Even when commercially available, the antibodies must be purified from animal serum before sorbent preparation can commence. For our studies, however, custom antibody preparation is required since antibodies toward relevant signature compounds are not commercially available.

Custom antibody production using traditional methods involves hapten complex synthesis, an immunization protocol, titer monitoring, animal bleeding, and antibody purification [22]. This approach is time consuming and may take up to a year before the antibody is available. Alternatively, one may create a hybridoma cell line from the immunized animal or use an existing hybridoma cell line. Hybridoma cell lines produce monoclonal antibodies by either tissue culture or ascites production techniques [23]. The tissue culture approach yields approximately 20 to 50 mg of monoclonal antibody per liter of medium [22]. Ascites production yields between 0.9 and 9 mg antibody per milliliter of ascites fluid, with each mouse yielding approximately 3 mL of ascites fluid. Collection of ascites can occur about four weeks after inoculation [22].

Methods based on genetic engineering are gaining favor in recent studies. For example, a yeast display library can be used to produce single-chain antibodies for a wide variety of relevant antigens [24]. Clones selected from the yeast display library are capable of producing 1 to 10 mg of antibody per liter of culture media under ideal conditions. In another approach, genetically engineered organisms are designed to produce stabilized antibody fragments (STABs) [3, 25-27]. These fragments are similar to single-chain antibody fragments except that an additional inter-chain disulfide linkage is included to provide additional stability. Literature reports describe STABs as having exceptional stability toward exposure to organic solvents [3]. Production techniques giving rise to STABs yielded only microgram quantities of material [27].

Molecularly Imprinted Polymers

The concept of molecular imprinting dates back to experiments conducted by Dickey where a silica surface was imprinted with an organic dye molecule [28]. Studies of the imprinted silica revealed an enhanced binding capability on an unmodified silica surface. Work by Gunter Wulff in 1972 was the first to imprint an organic polymer [28]. These studies incorporated a template molecule that would undergo a reversible covalent interaction with the polymer monomers. Several examples include 1) boronic acid ester formation by reaction between a vicinal diol and boronic acid, 2) Schiff's base formation by reaction of an amine and an aldehyde, 3) ketal formation by reaction of a diol and a

ketone, and 4) acetal formation by reaction of a hemiacetal and an alcohol [29,30]. These phases, although extremely useful for many purposes, had limited use for chromatography due to slow template binding kinetics [31].

A seminal development in molecular imprinting was the advent of noncovalent molecular imprinting techniques introduced by Mosbach in 1988 [28,29]. This approach was based on a noncovalent association between a monomer and the template. During polymerization, a rigid polymer formed around the noncovalently bound template. The polymer was then grounded, sized, and the template removed by extensive extraction. Template removal resulted in a complementary molecular imprint in the polymer that was ideal for future recognition of the template molecule. The advantage of this approach over the covalent interaction approach was that imprinted polymers could be formed, with simple polymerization systems that are specific toward diverse templates, without having to plan specific covalent binding strategies. Importantly, the association/dissociation kinetics were fast making these molecularly imprinted polymers (MIPs) fully compatible with chromatographic separations [30]. The relative ease of preparation placed the synthesis of custom imprinted polymers within the reach of most research laboratories.

A control polymer is always prepared along with an imprinted polymer using identical reagents and reaction conditions except the template is not added to the control reaction mixture. The resulting polymers are identical except the control material is not imprinted. Comparison of the chromatographic behavior of the two polymers provides evidence for the imprinting effect. Often, despite extensive washing, the template molecule cannot be entirely removed from the imprinted polymer and will diffuse into the mobile phase during analysis [31]. This can become problematic if the polymer is used for trace analysis. A possible solution is to imprint with a structural analog and depend on analyte cross-reactivity for analysis [31]. More complete removal of the template from the imprinted polymer may be possible by extracting the template from the polymer with supercritical fluids rather than organic solvents. The enhanced matrix permeability of, and analyte diffusion in, supercritical fluids should allow more complete removal of the template resulting in less template bleed from the polymer.

Bulk cast polymers that are grounded and sieved result in irregular particles having a range of sizes. These particles are not ideally suited for chromatographic applications. Several methods are available to form monodisperse spherical particles that are ideal for chromatographic studies [32]. This development represents a significant advancement in MIP technology. Various other approaches have been described including imprinting of membranes [33] and sol-gels [34], as well as the formation of monolithic imprinted polymers [33,34,36] and imprinted polymer films that can be deposited on surfaces [33,37].

A diverse number of MIP applications have been reported for a wide range of chemical classes including carbohydrates, peptides, proteins, xanthines, vitamins, nucleotides, steroids, pesticides, herbicides, drugs, and antibiotics [28,29,38]. A particular area of interest is the use of MIPs for enantiomeric separations since MIPs

offer predicable elution of antipodes (the enantiomer used for the template will be retained more strongly on the column) [28,39,40]. MIPs also exhibit sufficient sample capacity for preparative separations [41].

Molecularly Imprinted Polymers Compared to Immunosorbents

Antibodies and MIPs can be used in similar analytical formats. MIPs are sometimes referred to as antibody mimics or "plastic antibodies" [31]. Both terms emphasize the similarity between antigen/antibody affinity and the attraction of a template for its imprinted polymer. MIPs and antibodies can be used in competitive binding assays [42] and incorporated in sensors and membranes [33]. However, important differences exist between MIPs and antibodies. MIPs operate optimally in nonpolar organic solvents although there are also examples of MIP-based separations that operate efficiently in aqueous systems [43]. Antibody interactions, on the other hand, are optimal in aqueous buffers. As only a handful of studies report antibody activity in nonaqueous media, the area of nonaqueous immunochromatography remains controversial. MIPs are ideally suited for recognition of small organic or inorganic templates, although MIPs also can be prepared for large proteins as well. Antibodies are most suited for recognition of relatively large molecules; however, many examples also exist of antibody affinity toward small organic molecules. MIP technology has an undeniable advantage over antibody techniques in the ease of sorbent preparation [33,44]. As previously mentioned, custom antibody production may take up to a year using traditional methods. On the other hand, it is reasonable for a custom MIP to be synthesized, its affinity toward the template verified, and the selective polymer incorporated into experimental protocols within several months.

RESEARCH PROGRESSION AND REPORT ORGANIZATION

From the experimental results obtained during FY 2003, it became clear that MIPs were showing more promise than the STABs approach. Accordingly, our FY 2004 resources were allocated to advance the MIPs research. The decision was reinforced during the academic project review held at PNNL in May 2004, in which the review committee recommended that resources be focused exclusively on the MIPs approach. Therefore, the research described for FY 2004 and beyond focuses exclusively on MIPs. The two areas we emphasized in FY 2005 were 1) publishing the MIP manuscript we had submitted to *Journal of Separation Science* that describes MIP-based capture of volatile organic signature compounds from complex samples, and 2) providing a detailed characterization of MIPs specific toward nonvolatile half-acid phosphate and methylphosphonate signature compounds.

The following pages briefly describes the two areas of emphasis. The first area discussed are studies associated with the completion of the MIP manuscript. These studies involved cross reactivity studies performed on the DIMP- and TBP-specific MIPs along with a demonstration showing that the MIP selectivity was superior to that obtained on traditional normal-phase sorbents. We also defined, during these studies, the matrix rejection factors for analysis of complex samples using selective cature on the MIP sorbents. The second area of research was the characterization of half-acid ester MIPs. We provided detailed studies that demonstrated selective capture relative to a nonimprinted control sorbent. We then performed cross reactivity studies to examine the MIP affinity toward structurally related compounds. The final section of this report lists suggestions and recommendations to complete the studies in progress and to further build upon the research foundation accomplished during Task 2 activities.

COMPLETION OF MIP STUDIES FOR VOLATILE ORGANIC SIGNATURES

Matrix Rejection Factors for TBP- and DIMP-specific MIPs

Separate experiments characterized matrix discrimination factors achievable on the TBP- and DIMP-specific MIP sorbents. For these experiments, the summed integrated peak areas obtained from capillary gas chromatography (GC) separations of complex mixtures (with allowances provided for solvent peak subtraction) were compiled. The peak areas were compared to peak areas for an easily detectable quantity of analyte (either TBP or DIMP) in the retained MIP fractions. Air extract concentrates, gasoline, and diesel fuel were used as the complex mixtures. Discrimination factors were calculated as follows: *Discrimination Factor = Total Integrated Matrix Peak Area/Area of an Easily Detected Quantity of Analyte Within the MIP-retained Matrix Fraction*. Discrimination factors calculated in this fashion typically approach a value of 10^5.

As the desired discrimination factors would be on the order of 10^6, we are falling just short of the target discrimination goal. Slightly larger MIP columns that provide additional matrix discrimination would be expected to bring the matrix rejection into the desired range. For specific applications, it is likely that the MIP sorbent bed size will be optimized to provide the required discrimination while minimizing the amount of MIP sorbent required.

Additional Studies Performed for the TBP- and DIMP-specific MIP Manuscript

During the external peer review associated with publishing our MIP manuscript, *Journal of Separation Science* reviewers requested we perform additional studies with the DIMP- and TBP-specific MIPs prior to accepting the manuscript. The reviewers requested cross reactivity studies and a demonstration that the MIPs provided superior matrix discrimination compared to a traditional normal-phase sorbent such as silica or alumina.

Both the cross reactivity and normal-phase separation studies were included in the final manuscript that was published by the *Journal of Separation Science* on May 9, 2005 [45]. The studies are summarized below. For study details, see Appendix A of this document.

Cross reactivity studies

Cross reactivity of TBP and DIMP on the vinylpyridine-based TBP- and DIMP-specific MIP sorbents were investigated. The studies revealed that both DIMP and TBP were retained on the TBP-specific MIP, whereas the DIMP-specific MIP only retained DIMP but not TBP. The results suggest that the complementary MIP cavity for DIMP does not effectively accommodate the larger TBP molecule due to stearic hindrance. The results, along with selective retention of the target compounds on their respective MIPs compared to control sorbents, provide extremely strong evidence for a molecular imprinting effect. The cross reactivity experimental results are summarized in Table 2 of the MIP manuscript (see Appendix A).

MIP Compared to Traditional Normal-phase Fractionation

Experiments were conducted to compare matrix discrimination obtained on traditional normal-phase sorbents (silica or alumina) to those obtained on the vinylpyridine-based, TBP-specific MIP. The studies were performed in an HPLC format by injecting a diesel fuel sample on either a silica or alumina column that had identical dimensions as the MIP column and collecting the pentane mobile phase fraction that corresponded to TBP elution on the TBP-specific MIP. The complexity of the retained fractions was then compared to the retained fraction from the TBP-specific MIP by capillary GC analysis. The results indicate that the retained fraction from the MIP was far cleaner than the corresponding fractions from either silica or alumina. This result was expected because normal-phase sorbents provide compound-class separations where the more polar compounds elute with increasing retention. Because most complex mixtures

contain a variety of different chemical classes that have differing polarities, matrix interferences can be expected to elute from normal-phase sorbents well into the chromatographic run. The results of our study verified that the components contained in diesel fuel eluted from the normal-phase sorbents over an extended retention range. Chromatographic results of this study are shown in Figure 4 of the MIP manuscript (see Appendix A).

MIPS TOWARD NONVOLATILE HALF-ACID PHOSPHATE OR METHYLPHOSPHONATE ESTER SIGNATURES

Background

In past research (see Ultraselective Sorbents, Task 2, FY2004 Final Report), we prepared a novel MIP-based trace enrichment system that selectively collects half-acid ester signatures out of aqueous samples [45]. An invention disclosure (PNNL #14442) was prepared that described this instrument [46]. Our MIP characterization studies evaluated both bis(2-ethylhexyl) hydrogenphosphate (B2EHP)- and pinacolyl methylphosphonate (PMP)-specific MIPs. Initial HPLC studies that used refractive index detection indicated that although both sorbents showed selective retention of their intended templates compared to the control, a more robust response was observed from the PMP-specific MIP. Our rationale was to perform the preliminary trace enrichment work with the PMP-specific MIP because this sorbent appeared to display superior performance. Our intent was to return to the B2EHP once we completed a preliminary proof-of-principal study of the trace enrichment instrument.

The MIP-based trace enrichment system was designed to analyze aqueous samples by initially concentrating lipophilic organics from aqueous media on an octadecyl silica (C-18) sorbent cartridge. Samples can be introduced by injection loop, a sample pump for large volumes, or from a surface microlayer skimmer. After initial non-selective capture, water is removed from the system by a flow of dry gas leaving the lipophilic components sorbed on the dry C-18 sorbent. The enriched components are then transferred to a MIP sorbent using an organic solvent as a mobile phase. This allows introduction of the target analyte to the MIP in a solvent that will optimize selective capture of the target. Subsequent elution of pure analyte from the MIP with a water-containing mobile phase and transfer to a conductivity detector completes the analysis. This analysis progression is illustrated in Figure 2.

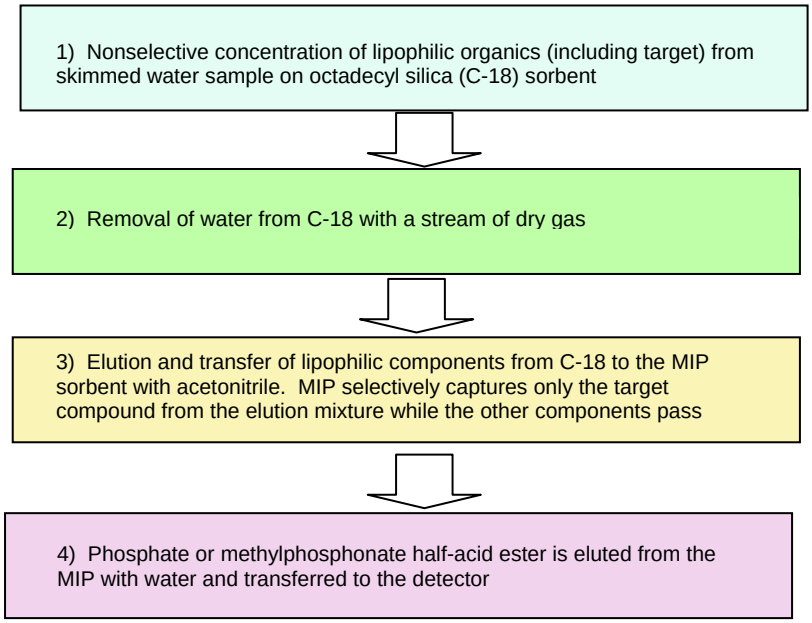

Figure 2. Flowchart showing the steps for analyzing an aqueous sample on the MIP-based trace enrichment system.

FY05 Study Goals and MIP Sorbent Evaluation Experimental Design

Our first FY05 objective was to synthesize and completely characterize MIP sorbents that were specific toward the target species. These sorbents will be incorporated into a novel MIP-based trace enrichment instrument that will enable analysis of aqueous samples. Because the performance of our proposed instrument will be highly dependent on MIP sorbent selectivity, a thorough study was needed to characterize MIP selectivity relative to controls as well as cross reactivity toward structurally related compounds. After completion of these studies we will perform discrimination challenges using complex matrices. Finally, complete characterization of the performance of the novel MIP-based trace enrichment system will be performed. For matrix studies, we planned on investigating fortified aqueous soil slurries, Columbia River water, and human urine and saliva samples. We realized at the onset that this research progression would extend well into FY06. Our plan was to progress as far as we could during FY05.

The experiments to characterize the PMP- and B2EHP-specific MIP sorbents proceeded in three phases. The first phase examined selective retention of target analytes on the MIP sorbents relative to the nonimprinted control. The chromatographic trials were conducted in triplicate to highlight errors associated with typical HPLC fraction recoveries and overall analyte mass balance. Phase two experiments examined sorbent cross reactivity with structurally similar analytes. In these experiments, the retention characteristics of structurally related compounds were determined on the control and the two different MIP sorbents. Cross reactivity was indicated by significant retention of a

related compound on one of the MIPs relative to the control sorbent. If time permits, phase 3 studies will be pursued to investigate the matrix discrimination capabilities of the sorbents by applying complex environmental or physiological samples and verifying the purity of the MIP-captured fraction. After all these experiments were completed, the MIP sorbent was incorporated into a novel trace enrichment system and the performance of this system characterized.

Experimental

The synthesis of methylphosphonates that are not commercially available has been described in our manuscript published in *Phosporus, Sulfur, and Silicon* [48]. Our suspension polymerization synthesis of methacrylic acid-based MIPs specific toward the half-acid esters also has been described [45].

HPLC analysis: Gilson HPLC pumps (Models 305, 306, and 307, Middleton, WI), operated at a flow of 1.0 ml/min, were used for the studies. The components were introduced to the control or MIP HPLC columns through a Rheodyne Model 7125 valve (Alltech, Deerfield, IL, USA) fitted with a standard 20-µl injection loop. Accurate injection volumes (24.3 µl) were determined gravimetrically from mercury injections. Two fractions were collected for further analysis. The first fraction (F1) consisted of acetonitrile collected during the first 4.0 min after injection. The second fraction (F2) was eluted from 4.0 to 8.0 min with a mobile phase that was strong enough to completely removed retained analyte. For most samples 100 percent water was used for elution of F2; however, a water:acetonitrile mixture (50:50, v/v) was used in cases where B2EHP was the analyte. The acetonitrile/water mixture was used for B2EHP rather than pure water to enhance analyte solubility in the mobile phase. Fraction F2 is referred to below as the retained HPLC fraction.

The collected HPLC fractions were processed for analysis by electrospray ionization MS. The first step in this preparation was neutralization of the acids with a slight molar excess of 0.1 M ammonium hydroxide. This step was performed to produce the ammonium salts and limit the loss of some of the more volatile acids. The acetonitrile samples were evaporated under a stream of dry nitrogen. Any excess base immediately evaporated leaving behind the ammonium salt of the acid. For the acetonitrile:water (50:50, v/v) samples, the acetonitrile also was removed by a stream of nitrogen, leaving the aqueous portion. These samples, as well as the 100 percent water fractions, were then frozen and lyophilized overnight, leaving the nonvolatile acid salts in the dry form. In preparation for MS analysis, all samples were reconstituted with 1.0 mL of a methanol:water solution (50:50:, v/v) that also contained 15.0 ppm of the internal standard, ethylphosphonic acid.

Electrospray MS Analysis: HPLC fractions were analyzed using a ThermoFinnigan TSQ Triple-Quadrupole Mass Spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source operated in the negative ion mode. A Harvard Apparatus Model 22 syringe pump (South Natick, MS) operated at 20 µl/min was used for direct infusion sample introduction. Experimental conditions were as follows: ESI source (3kV); quadrupole manifold temperature (100°C); capillary temperature (250°C);

scan range and speed (from 90 to 500 amu in 1.0 sec). Sheath and auxiliary flows were set by adjusting nitrogen pressures to 40 and 5 psi, respectively. For selected ion monitoring experiments, the $(M-H)^-$ ions for the internal standard (ethylphosphonic acid, m/z = 109) and the analyte of interest were monitored with a dwell time of 1.0 sec/ion using a mass window of 0.5 amu. Data collected from 3 to 5 min were averaged for quantification. To calculate the amount of analyte in each fraction, the ratios of analyte to internal standard signals were compiled and compared to the ratio obtained for a 100 percent analyte standard.

Results and Discussion

Phase 1, Selective Retention Over Controls. Phase 1 studies clearly show that the PMP-specific MIP selectively retains PMP, compared to the nonimprinted control sorbent (Table 1). The amount of unretained analyte passing through the columns decreased from 19 percent to 0.2 percent when comparing the control to the PMP-specific MIP, indicating that the MIP selectively and efficiently captures PMP from acetonitrile. An examination of the retained fractions also highlights the selective retention with 78 ± 15 percent of the analyte being retained on the MIP, whereas only 61 ± 15 percent was retained on the control. Mass balances from this experiment were reasonable (see Table 1). The water elution alone seems adequate to regenerate the sorbent for the next run, at least when simple standard solutions are being analyzed.

Table 1. Percent recovery in HPLC fractions on control and MIP columns. A propagation of error was applied to determine uncertainties.

Percent Recovery/ Injected analyte/Sorbent	% Analyte in acetonitrile fraction[a]	% Analyte retained on MIP[b]	Mass Balance[c]
PMP Injection			
Control	19 ± 14	61 ± 15	80 ± 21
PMP-specific MIP	0.2 ± 0.3	78 ± 15	78 ± 15
B2EHP Injection			
Control Sorbent	65 ± 11	32 ± 9	97 ± 14
B2EHP-specific MIP	48 ± 36	66 ± 43	114 ± 56

[a]Percentage of injected compound collected in the first 4.0 ml of acetonitrile
[b]Percentage of injected compound eluted from MIP with water
[c]Percentage of injected compound recovered in either the acetonitrile or water fractions

Phase 1 studies for B2EHP are slightly more complex. This sorbent also shows preferential retention on the MIP over the control sorbent; however, the errors associated with the percentage of analyte retained on the MIP are much higher than were seen for the corresponding PMP-specific MIP studies. The reason for the larger variance becomes obvious when examining recoveries for each individual injection. The first run on the

B2EHP-MIP showed strong selective capture of the target with retention of about 95 percent of the analyte. This value diminished with subsequent injections. This change in successive runs is responsible for the large variance observed. The retained B2EHP was effectively recovered from the B2EHP-specific MIP with an acetonitrile:water wash (50:50, v/v) for each of the three trials, as verified by mass balances for the individual runs (data not shown), and by the composite mass balance average (see Table 1). The error in the mass balance remains high due to propagation of the large variance in percentage of analyte retained on the MIP. The results indicate that the B2EHP-specific MIP does exhibit selective retention over the control sorbent; however, the effect is variable and tends to be less prominent with runs performed in rapid succession. One probable explanation for this behavior is that the sorbent may require more extensive regeneration, such as rinsing with an acetic acid solution, to obtain optimal performance.

Previous work performed in other laboratories has shown activity of a MAA-based PMP-specific MIP prepared by bulk polymerization [49]. The authors demonstrated cross reactivity of this MIP with several related methylphosphonates and demonstrated crude solid-phase extraction capabilities of this sorbent for isolating the target from serum. As discussed further below, the studies we present here are significantly more detailed and advanced than previous literature reports. We present a more extensive selective MIP capture study (relative to controls) along with the associated errors for both PMP- and B2EHP-specific MIPs. Our cross reactivity studies examine not only a more extensive list of methylphosphonate half-acid esters, but also phosphinic acids and alkylphosphonic acids. Additionally, our studies are based on a more sophisticated material that was prepared by suspension polymerization rather than block polymerization. The block polymerization process results in irregular particles with a relatively large size distribution, whereas suspension polymerization yields macroporous, spherical, and monodisperse particles that are ideal for chromatographic applications. Finally, our MIP-based, on-line trace enrichment approach is far more sophisticated than the solid-phase extraction approach presented in previous literature.

Phase 2 Studies, Cross Reactivity, Nonspecific Interactions. An examination of the percentage of compound retained on the control sorbent indicates an interesting trend. The retention/recognition mechanism of these sorbents is based on hydrogen binding between the methacrylic acid and the basic phosphoryl oxygen group contained in the analytes. The degree of interaction can be determined by the electron density localized on the phosphoryl oxygen, which, in turn, is largely dictated by electronic induction effects around the phosphorus atom. Of the compounds studied, the electron-donating effects of the two methyl groups contained in dimethylphosphinic acid would be expected to place the highest electron density on the phosphoryl oxygen. The basicity of the methylphosphonate half-acid ester compounds would be expected to be less than the phosphinic acids due to the electron-withdrawing effect of the ester oxygen. Larger alkyl groups would lead to higher basicity within the methylphosphonate half-acid ester group due to greater electronic induction. The half-acid phosphate ester, B2EHP, would be expected to be the least basic due to the electron-withdrawing effects resulting from two ester oxygens. Therefore, the expected basicity decreases in the following order: dimethylphosphinic acid > PMP = CMP > IMP > B2EHP, an order that exactly reflects

the binding strength (percentage anayte retained) on the control sorbent, *i.e*., 99 percent, 80 percent, 73 percent, 34 percent, and 29 percent, respectively, as shown in Table 2.

Phase 2 Studies, MIP Cross Reactivity. MIP Interactions. Results from the cross reactivity studies are presented in Table 2. The mass balance values observed for valeric acid during the study were very low indicating that, although we neutralized the acids to reduce volatility and minimize losses, the ammonium salt of valeric acid was sufficiently volatile to be lost during sample processing. Valeric acid data is omitted from Table 2 for this reason. Mass balances for the remaining analytes fell within a reasonable range of 70 percent to 126 percent indicating that analytes were not irreversibly bound to the sorbents and, to a first approximation, were quantitatively recovered in fractions F1 and F2. We present cross reactivity results in Table 2 as relative percentages recovered in each fraction, assuming 100 percent recovery, to facilitate comparison of the data.

Several compounds were very strongly retained on both the MIP and control sorbents. Butylphosphonic acid and dimethylphosphinic acid were quantitatively retained on both the MIPs as well as the control sorbent. Conclusions regarding cross reactivity for these compounds on the MIP sorbents are, therefore, not possible.

The remaining compounds in the Phase 2 study are half-acid phosphate or methylphosphonate esters. The PMP-specific MIP shows significant cross reactivity toward these related analytes. This difference can be semi-quantitatively visualized by taking the difference between the relative percentage of analyte found in F1 (or alternatively in F2) between the control and the MIP sorbents. For example, ethyl methylphosphonic acid (EMP), isopropyl methylphosphonic acid (IMP), cyclohexyl methylphosphonic acid (CMP) and B2EHP display 64, 51, 17, and 23 percent higher capture on the PMP-specific MIP relative to the control. These values can be compared to the 24 percent enhanced capture observed for the PMP template. Interestingly, the smaller half-acid methylphosphonic acid compounds (EMP and IMP) display a larger differential binding between the MIP and the control sorbent than the original PMP template.

For the B2EHP-specific MIP, cross reactivity is observed only for EMP (21 percent). This value can be compared to the 25 percent enhanced binding of the B2EHP template on the B2EHP-specific MIP. The strongest cross reactivity on the PMP-specific MIP was also with ethyl methylphosphonate. The larger methylphosphonic half-acid esters, IMP and CMP, do not display cross reactivity as indicated by percent-binding values that are approximately the same as the control sorbent (see Table 2). One can speculate that the cavity geometry does not present an optimal geometrical fit for methylphosphonates such that stearic exclusion from the imprint site occurs for compounds in this class larger than EMP. Interestingly, far less PMP is captured on the B2EHP-specific MIP compared to the control sorbent (the difference is -38 percent). Although more difficult to explain, the lower binding seen with pinacolyl methylphosphonate relative to the control sorbent may reflect cavity exclusion along with a lower density of accessible nonspecific binding sites on the MIP compared to the control sorbent.

Table 2. Relative percentage of analyte in each HPLC fraction.

Sorbent	Analyte	% Analyte in acetonitrile fraction[b]	% Analyte retained on MIP[c]
Control[a]	PMP	24	76
PMP-specific MIP[a]	PMP	0	100
Control[a]	B2EHP	67	33
B2EHP-specific MIP[a]	B2EHP	42	58
Control	B2EHP	71	29
PMP-specific MIP	B2EHP	48	52
Control	PMP	20	80
B2EHP-specific MIP	PMP	58	42
Control	EMP	84	16
PMP-specific MIP	EMP	20	80
B2EHP-specific MIP	EMP	63	37
Control	IMP	66	34
PMP-specific MIP	IMP	15	85
B2EHP-specific MIP	IMP	62	38
Control	CMP	27	73
PMP-specific MIP	CMP	10	90
B2EHP-specific MIP	CMP	29	71
Control	Butylphosphonic acid	0	100
PMP-specific MIP	Butylphosphonic acid	0	100
B2EHP-specific MIP	Butylphosphonic acid	1	99
Control	Dimethylphosphinic acid	1	99
PMP-specific MIP	Dimethylphosphinic acid	0	100
B2EHP-specific MIP	Dimethylphosphinic acid	1	99

[a]Values derived from Table 1

[b]Percentage of injected compound collected in the first 4.0 ml of acetonitrile

[c]Percentage of injected compound eluted from MIP with water

CONCLUDING COMMENTS

Funding Considerations and Recommendation for Further Work

An FY06 lifecycle continuation plan that covered completing the ongoing studies as well as initiating several new areas of research within Task 2 was submitted to NA-22. The NA-22 review recommended that the Ultraselective Sorbents project be put on hold until such time as more complete FY06 budget information is available. Should the FY06 budget allow, and project continuation be granted, the studies outlined below will receive priority. Otherwise, the comments below are intended as concluding remarks and recommendations for further work.

Analysis of Volatile Signatures with Vinylpyridine-based MIPs

Our MIP study targeting volatile organic signatures is one of only a few studies that address analysis of nonpolar volatile target analytes. These compounds are extremely difficult to imprint due to the lack of polar imprinting handles on which to base the imprint process. Sorbents that we prepared were imprinted, as evidenced by selective retention of the targets compared to controls, and by reasonable cross reactivity toward related compounds. Further, we were able to demonstrate highly selective capture of target analytes from relevant and extremely complex environmental samples.

Weak interactions between the MIP sorbents and the target analytes are at least partially responsible for some complications that should be mentioned. Selective retention, although definitely present, does not have a large effect compared to MIPs prepared with more polar templates. The weak interactions partially impact sorbent production reproducibility. During the course of this work, we prepared many batches of sorbents by various different techniques. We were able to demonstrate activity in the majority of batches; however, some batches appeared to be inactive. We observed the imprint effect enough times to know the phenomenon is real. The fact that we could not verify activity in all synthetic batches reflects the weak selective interactions as well as other synthetic subtleties that we do not fully understand. We expect that further work will resolve these issues and allow for consistent preparation of active MIP sorbent batches toward these relatively nonpolar volatile signature compounds.

Methacrylic Acid (MAA)-based Polymers for Volatile Signatures.

One of our research thrusts involved preparing MAA-based MIPs for the analysis of volatile signatures. Our initial studies could not observe selective retention on these sorbents because the analytes were completely retained from pentane on both the control and MIP sorbents. This behavior is a direct consequence of strong nonspecific interactions between the polymer matrix and the analyte. In this case, we could completely elute the analytes with acetone, a very strong solvent for this type of MIP. The lack of observing selective retention does not mean the MIP sorbents that we prepared were not imprinted. There is a strong possibility that a mobile phase could be

found that is intermediate in strength between pentane and acetone that allows observation of differential retention on the MIP compared to the control sorbent. This research activity was a major thrust outlined in our FY06 lifecycle continuation proposal.

This research should be given high priority because a strong hydrogen-bond interaction is anticipated between the MAA functional monomer and the analytes studied. Research that defines a mobile phase that gives selective retention would be very important because analyte-MIP interactions would be reasonably strong. If stronger interactions are obtained, it would be easier to define experimental conditions to achieve maximum matrix discrimination. Also, sorbent batch reproducibility would be much higher when based on the stronger analyte-MAA interactions. Pursuing this line of research would be one of our strongest recommendations for future MIP research that targets volatile organic signature compounds.

MIPs Toward Nonvolatile Half-acid Ester Signatures

The experimental results provided in this report represent a solid foundation for continuing the MIP-based trace enrichment work. We demonstrated preparation of MIPs that show strong selective retention of the B2EHP and PMP targets relative to their controls. The results, along with reasonable cross reactivity based on the expected imprint cavity size, proves that we have an active molecular imprint cavity in the MIPs we prepared. The B2EHP sorbent seems particularly selective showing cross reactivity only toward ethyl methylphosphonate (of the compounds we studied). In addition, an interesting nonspecific retention effect was observed. We noticed retention of analytes on the nonimprinted control sorbent correlated with anticipated basicity of the phosphoryl oxygen as predicted by the expected electronic induction effects. This retention order can be explained by the strength of the hydrogen-bond recognition mechanism between the anayte phosphoryl oxygen and the MAA functional monomer.

Unfortunately, project funds and time were depleted before we could move into phase 3 studies. Completion of this work is critical since performance of the current MIP-based trace enrichment instrument depends on the MIP fraction being pure. For future studies we propose challenging the MIP with a variety of analyte-fortified complex samples including Columbia River water, aqueous soil extract slurries, urine, and saliva. These matix challenges will be performed on the trace enrichment instrument and, therefore, the sample will first undergo enrichment on an octadecyl silica sorbent prior to being introduced to the MIP in an acetonitrile mobile phase. The purity of the retained MIP fraction will be carefully scrutinized to indicate whether the purity is consistent with using a relatively nonspecific conductivity detector.

Although we anticipate that MIP fraction will be pure, it still remains desirable to consider other detection modes that may offer higher selectivity and sensitivity. One possibility is an interface with ESI/high resolution IMS detection [50]. This could be used in the negative ion mode to selectively detect the analyte in the MIP eluates at low part-per-billion (ng/L) concentrations. Compared to MS, the IMS arrangement is more compact and has the potential to be made field portable.

Other arrangements also should be considered. One possibility is a simple potentiometric detection of the half-acid ester (rather than conductivity). Further possibilities include additional electrochemical detection approaches. For example, work by Ilya Elashvili at Army Edgewood Chemical Biological Center (ECBC) has isolated a phosphate ester hydrolase enzyme that cleaves the ester portion of the methyphosphonate half-acid esters to yield methylphosphonic acid and an alcohol [51]. This enzyme could be incorporated into an electrochemical detector that detects the alcohol produced. The same enzyme may also work for phosphate half-acid esters such as B2EHP. Alternatively, an enzyme immobilized ISFET potentiometric detector could be developed to monitor the decrease in pH as the half-acid ester is converted to the full acid (either methylphosphonic or phosphoric acids for PMP or B2EHP, respectively).

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TASK 2 ACCOMPLISHMENTS

The publications, presentations, and invention disclosures that have resulted from Task 2 of the Ultraselective Sorbent Project are listed as follow:

Publications:

Harvey, SD, BR Valenzuela , and JA Campbell. July 2005. Molecularly Imprinted Polymers for Selective Analysis of G-series Nerve Agent Hydrolysis Products in Complex Environmental Matrices, in preparation .

Harvey, SD. 2005. Molecularly Imprinted Polymers for Selective Analysis of Chemical Warfare Surrogate and Nuclear Signature Compounds in Complex Matrices, *Journal of Separation Science*, **28**:1221-1230.

Mong, GM, SD Harvey, and JA Campbell. 2005. Synthesis of Alkyl Methylphosphonic Acid Esters, *Phosphorus, Sulfur, and Silicon*, **180**:1885-1891.

Harvey, SD and T Zemanian. 2005. Ultraselective Sorbents for Special Nuclear Materials (U), Trace Effluent Detection and Analysis –R&D Portfolio 2004, NNSA/NA22/TEDAP –2004. (S/NSI)

Harvey, SD, GM Mong, JS McLean, SM Goodwin, DA Atkinson, and NB Valentine. 2005. *Imprinted Media for the Highly Selective Capture of Chemical and Biological Threats*, PNNL-SA-45133, paper submitted to the Proceedings of the Homeland Security Meeting, Working Together: Research & Development Partnerships in Homeland Security, April 27-28th, Boston, MA. Conference Proceedings published on September 1, 2005.

Skaggs, R, T Straub, B Wright, C Bruckner-Lea, and SD Harvey. 2004. *Development of the Next Generation Microbial and Chemical Detection Capabilities for Water Supplies*, Chapter 18, Water Supply Systems Security, Larry Mays, Ed., McGraw Hill, New York pp.18.1-18.21.

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Presentations:

Harvey, SD. 2005. Ultraselective Sorbents, Task 2, MIPs/STABs, Oral presentation given to two separate groups at DOE Headquarters for the NNSA/NA-22, 2005 Trace Effluent Detection & Analysis Portfolio Review, Washington DC, March 25, 2005.

Harvey, SD. September 11, 2003. Ultraselective Sorbents, Task 2: MIPs/STABs, Oral presentation given at the NNSA/NA-22 Program Review held at Sandia National Laboratory, Albuquerque, New Mexico.

Harvey, SD, GM Mong, JS McLean, SM Goodwin, DA Atkinson, and NB Valentine. 2005. *Imprinted Media for the Highly Selective Capture of Chemical and Biological Threats*, Poster Presented at the Working Together: Research & Development Partnerships in Homeland Security, Sponsored by the Department of Homeland Security, PNNL-SA-44314, April 27-28th, Boston, MA.

Harvey, SD, GM Mong, JS McLean, SM Goodwin, DA Atkinson, and NB Valentine. September 14-16, 2004. *Imprinted Media for Highly Selective Capture of Chemical, Biological, and Organic Nuclear Signature Threats*, PNNL-SA-42219, Poster presented at the Detector/Sensor and Technology for Homeland and National Security Conference, Gatlinburg, TN.

Zemanian, TS, SD Harvey, RS Addleman, GE Fryxell, and A Gutowska. April 11-15, 2005. *Ultraselective Sorbents*, Poster presented at the NA-22 Technical Information Exchange (TIE) Conference, Sandia National Laboratory, Albuquerque, New Mexico.

Invention Disclosures:

Harvey, SD and M Yan. 2004. *Scaffold Imprinting for Preparing Polymers Imprinted with Nitro-containing Explosives*, Invention Disclosure #14482-E, Pacific Northwest National Laboratory, Richland, WA.

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Appendix A

Published Manuscript

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Molecularly imprinted polymers for selective analysis of chemical warfare surrogate and nuclear signature compounds in complex matrices

This paper describes the preparation and evaluation of molecularly imprinted polymers (MIPs) that display specificity toward diisopropyl methylphosphonate (DIMP) and tributyl phosphate (TBP). Polymer activity was assessed by solid-phase extraction and high-performance liquid chromatography experiments. Both DIMP- and TBP-specific vinylpyridine-based MIPs selectively retained their targets relative to a non-imprinted control. Proof-of-principle experiments demonstrated highly selective analysis of the targets from fortified complex matrix samples (diesel fuel, gasoline, and air extract concentrate). The retained MIP fractions gave near quantitative recovery of the target analytes with very low matrix background content. The same fraction from the control sorbent recovered only about half of the analyte and tended to be less pure.

Key Words: MIPs; Selective analysis; Signature compounds

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1 Introduction

Chemical warfare and nuclear signature analytes are often non-polar and hydrolytically sensitive. Non-aqueous analysis is, therefore, advantageous to foster the stability and solubility of these compounds during analysis. Multi-dimensional trace enrichment seems ideally suited for this analysis, particularly if the initial preconcentration stage can incorporate a highly selective sorbent. Effective matrix discrimination achieved during selective preconcentration can result in significant simplification in the overall analytical instrumentation required to obtain high-confidence identifications. The selective sorbent could serve as either a primary air-sampling stage or a secondary stage situated after a traditional non-selective sampling sorbent (i.e., Tenax GC or XAD-2). For the latter arrangement, an organic solvent or supercritical fluid could be used to transfer analytes to the secondary selective sorbent.

A number of sorbents could potentially be used for selective samplings, although very few are capable of effective non-aqueous operation. Stationary phases based on β-diketonates chelated to rare earth elements can be applied for selective gas-phase sampling of nucleophilic volatile compounds from complex samples such as cigarette smoke. These chelate phases are fine powders and, as such, cannot be directly packed into chromatographic columns. Instead, these phases are usually coated on inert supports that contain a thin layer of a non-polar gas-chromatographic stationary phase coating, such as SE-30. The purpose of the SE-30 is to deactivate the support material, provide media for uniform distribution of the chelate powder, and shield the chelate polymer from exposure to water [1–3]. This format has the disadvantage of requiring elevated temperatures and leads to a mixed-mode retention of analytes based on SE-30 retention superimposed on the metal β-diketonate complex interaction. One particularly promising technology for selective analyte retention in non-aqueous environments is the use of molecularly imprinted polymers (MIPs) [4–7]. These phases can rival antibody-antigen selectivity, and, in fact, MIPs are often referred to as antibody mimics or plastic antibodies [8]. Unlike antibodies, however, MIPs display optimal recognition of their targets in non-aqueous systems, a property that makes these selective sorbents ideal for our studies [4, 9, 10].

MIPs are polymers that are synthesized in the presence of a target molecule template. Conditions can be chosen such that the functional monomer is associated strongly, yet non-covalently, with the template during polymerization. After a rigid polymer is formed, the template molecule is washed from the polymer matrix, leaving a complementary cavity that has an ideal topography for template recognition [4–7]. The approach has enormous versatility because the same basic polymerization conditions can be used to produce polymers active toward a large number of target analytes. A limitation of the MIP approach is the difficulty in completely removing entrapped template from

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the polymer matrix. This difficulty can result in low-level residual template bleed that becomes problematic during trace analysis. One solution involves imprinting with a close structural analog that can be chromatographically resolved from the target analyte during subsequent separations [8–12].

The objectives of the study are to 1) synthesize MIPs toward several signature compounds related to national security concerns, 2) evaluate the activity of these sorbents in non-aqueous environments, and 3) conduct selectivity challenges by analyzing for target compounds in the presence of complex matrices. These goals will be pursued by synthesizing MIP sorbents and evaluating selective retention relative to a non-imprinted control polymer using either solid-phase extraction (SPE) or more sophisticated and versatile high-performance liquid chromatography (HPLC) experiments. Finally, analyte-fortified matrix samples (diesel fuel, gasoline, and air extract concentrate) will be analyzed to evaluate the matrix discrimination achieved on the MIP sorbents. These experiments are conducted within a proof-of-principle framework with the objective of determining potential for further, more detailed studies.

2 Experimental section

2.1 Reagents

Trimethylolpropane trimethacrylate (TRIM); 4-vinylpyridine; 2,2′-azobisisobutyronitrile (AIBN); tributyl phosphate (TBP); acetone (99.9+% HPLC grade); perfluoro(methylcylcohexane); methacrylic acid; and basic alumina (Brockmann Activity I, 150 mesh) were all obtained from Aldrich Chemical (Milwaukee, WI, USA). Diisopropyl methylphosphonate (DIMP) was purchased from Lancaster (Pelham, NH, USA), whereas acetic acid was procured from J.T. Baker (Phillipsberg, NJ, USA). Polyfluoro-acrylate, PFAC-1 was obtained from Oakwood Products (West Columbia, SC, USA) and poly(ethylene glycol) 1000 monomethyl ether monomethacrylate was purchased from Polysciences, Inc. (Warrington, PA, USA). Solvents were obtained from various manufacturers; hexane and pentane from Burdick & Jackson (Muskegon, MI, USA), chloroform from EM Science (Gibbstown, NJ, USA), and ethanol from Quantum Chemical (Tuscola, IL, USA). U.S. Environmental Protection Agency diesel fuel was provided by the Internal Revenue Service (reference # 0034847), and gasoline was regular, unleaded grade obtained from a local Chevron service station.

2.2 Polymer synthesis

Vinylpyridine-based polymers were synthesized according to methods described by Bruggemann *et al.* [13]. Immediately before reaction, inhibitors were removed from 4-vinylpyridine and TRIM by passing through a SPE cartridge (SPICE^{TM} cartridges, Analtech, Newark, DE, USA) packed with basic alumina. Likewise, the ethanol stabilizer was removed from chloroform immediately before use by passing over basic alumina. The control polymer reaction mixture consisted of 0.530 mL of 4-vinylpyridine (4.91 mmoles), 4.70 mL of TRIM (14.7 mmoles), 62 mg of AIBN (0.38 mmoles), and 8.0 mL of chloroform. The MIP reaction mixtures contained 1.23 mmoles of template molecule in addition to the control polymer starting materials listed above. This quantity corresponded to 211 μL DIMP and 334 μL TBP. Control sorbent preparation was performed at the same time, with the same reagents, and under the same conditions as the MIPs.

Reaction mixtures were placed in 25 mm × 150 mm Pyrex tubes (#9826 25, Corning Inc., Corning, NY, USA), vigorously mixed, degassed in an ultrasonic bath followed by purging with nitrogen for 10 min, and sealed with PTFE-lined screw caps. Polymerization was achieved by submerging in a 60°C water bath for 7 h. The vinylpyridine-based bulk cast polymers were then removed from the tubes and ground in a mortar and pestle to pass a 74-μm sieve. The sieved polymer was sequentially washed with methanol:acetic acid (7:3, *v/v*) followed by methanol. Fines were removed by repetitively suspending the material in acetone, allowing the particles to settle, and decanting the fines.

For comparison with the vinylpyridine-based polymers described above, polymers based on methacrylic acid were synthesized according to a suspension polymerization method described by Mayes and Mosbach [14]. Minor deviations from the literature procedure reflected the use of synthetic starting materials that were readily available in the United States. First, a custom surfactant was synthesized by combining 4.0 g of polyfluoro-acrylate (PFAC-1), 0.38 g of poly(ethylene glycol)1000 monomethyl ether monomethacrylate, 10.0 mL of inhibitor-free chloroform, and 24 mg of AIBN in a reaction tube. The solution was purged with nitrogen, the tube sealed, and the polymerization performed at 60°C for 48 h. After reaction, the solvent was removed under vacuum. Prior to suspension polymerization, the inhibitor was removed from methacrylic acid by vacuum distillation. The suspension polymerization reaction mixture contained 4.6 g of chloroform, 20.0 mL of perfluoro(methylcyclohexane), 0.40 g of methacrylic acid, 1.57 g of inhibitor-free TRIM, and 11.4 mg of custom surfactant. The MIP reaction contained 1.16 mmole of template in addition to the above reagents. Reaction mixtures were stirred at 2000 rpm for 5 min, purged with nitrogen for an additional 5 min, and polymerized by exposing the mixture to ultraviolet irradiation (366 nm) while stirring at 500 rpm for 4 h. Spherical 12-μm beads were isolated by filtration and extensively washed with acetone.

2.3 Preparation of SPE cartridges and HPLC columns

Dry sorbent materials were packed into SPICE™ SPE cartridge blanks in preparation for target recognition experiments. Cartridges were extensively conditioned with hexane before use. Sorbent materials also were sent to Higgins Analytical (Mountain View, CA, USA) for packing into 37 × 3.0 mm stainless steel HPLC cartridges. In addition to MIP and control materials, silica and alumina sorbents obtained from commercially available SPE cartridges (Supelco, Bellefonte, PA, USA) also were packed into HPLC cartridges. For testing, cartridges were fitted into Hewlett-Packard cartridge holders (part number 820311–001, Palo Alto, CA, USA) after machining and threading a substitute stainless steel cylindrical sheath of the appropriate length.

2.4 Solid-phase extraction

Solid-phase extraction proceeded using successive application of stronger solvents to the MIP or control cartridges. The first fraction (F1) resulted from application of 6.0 mL of hexane. This fraction gave an indication of the residual template bleed from the polymer [8–12]. The second fraction (F2) contained eluate from application of the analyte sample followed by a solvent rinse. The analyte (200 μg) was applied in 1.0 mL of hexane followed by a 6.0-mL hexane:ethanol (99.25:0.75, *v/v*) rinse. The small proportion of ethanol in the rinse solvent served to reduce weak non-specific interactions of analyte with the polymer. The third and last fraction (F3) was eluted from the polymer with 6.0 mL of acetone. Acetone is a strong solvent capable of stripping all analyte from the polymer. After elution of fraction F3 with acetone, the sorbent received an additional acetone rinse followed by extensive conditioning with hexane.

Each fraction was collected into a 10.0-mL volumetric flask that contained 1.0 mL of an injection volume standard dissolved in hexane. The 1.0-mL standard used for DIMP studies contained 152 μg of *n*-tetradecane, whereas the standard used for TBP studies contained 200 μg of naphthalene. After eluate collection, the total volume for each fraction was adjusted to 10.0 mL with hexane, and aliquots were taken for analysis by capillary gas chromatography. The percentage of recovery for each fraction was referenced to a 100% standard that was prepared by diluting 1.0 mL of injection volume standard along with 1.0 mL of analyte standard to 10.0 mL with hexane.

2.5 Analyte-spiked matrix challenge samples

Based on initial SPE experiments indicating activity of the vinylpyridine-based MIPs, these sorbents were further evaluated in HPLC matrix challenge experiments. The samples for evaluating TBP-specific MIP performance consisted of 20.0 μL of diesel fuel along with 2.0 μL of TBP diluted to 10.0 mL with pentane. For DIMP, the matrix solution consisted of 36.0 μL of gasoline and 2.0 μL of DIMP diluted to 10.0 mL with pentane.

An air extract concentrate was prepared as an additional matrix challenge sample. A Soxhlet extraction thimble that contained 60 g of XAD-2 sandwiched between two polyurethane foam plugs was used to collect the air sample. The thimble contents were first cleaned by Soxhlet extraction with methylene chloride followed by pentane before drying under vacuum. Sampling proceeded by passing a total of 732 m^3 of laboratory air through the sorbent bed using a vented ring compressor pump. The thimble was extracted for 24 h with 500 mL of pentane. Preparation of the challenge solution involved reducing the volume of the final pentane Soxhlet extract a factor of 80 times under a gentle stream of nitrogen and fortifying the air extract concentrate with TBP to a concentration of 160 μg/mL.

2.6 HPLC analysis

Waters Model 616 pump and Model 600S controller (Milford, MA, USA) were used for mobile phase delivery. The pentane mobile phase (or acetonitrile for the methacrylic acid-based polymers) was delivered at a flow rate of 0.5 mL/min. Samples were introduced to the control, MIP, or comparison HPLC columns through a Rheodyne Model 7125 valve (Alltech, Deerfield, IL, USA) fitted with a standard 20-μL injection loop. Accurate injection volumes (24.3 μL) were determined gravimetrically from mercury injections as previously described [15]. Separated components were detected either by a Waters R401 Differential Refractometer or an Isco CV^4 absorbance detector (Lincoln, NE, USA) operating at 230 nm and recorded on a Hewlett-Packard Model 3393A integrator. Fractions of the column eluate were collected for further analysis by capillary gas chromatography. The first fraction consisted of the pentane eluting during the first 2.5 min of the chromatographic run, whereas the second fraction contained the column eluate collected from 2.5 to 6 min after injection. The second fraction is referred to below as the retained HPLC fraction. Before gas-chromatographic analysis, the first fraction was brought to the same volume as the retained fraction (1.75 mL). For comparison, 100% standards were prepared by diluting 24.3 μL of the HPLC standard solutions to 1.75 mL with pentane.

2.7 Capillary gas-chromatographic analysis

A Hewlett-Packard 5890 Series II gas chromatograph (GC) was used for these studies. For separation of SPE fractions, chromatography was conducted on a 15-m × 250-μm ID column that contained a 0.5-μm film of 78% cyanopropyl methylpolysiloxane (#007–23, Quadrex,

New Haven, CT, USA). The column head pressure of helium was adjusted to give a linear flow of 30 cm/s at 40°C as judged by injecting methane as a dead volume marker. The split was adjusted to a flow of 100 mL/min. Samples (2.0 μL) were injected in the splitless mode with the split valve remaining closed 30 s after injection. For analysis of DIMP, the column was held at 40°C for 2 min before programming to 125°C at 6 K/min. The temperature program for TBP held the column at 40°C for 2 min before ramping to 140°C at 10 K/min. Both temperature programs held the final column temperature for 10 min before the column was recycled to the initial temperature. Compound elution was monitored by flame ionization detection and recorded on a Hewlett-Packard Model 3395 integrating recorder. For all GC traces reported in this study, a full-scale recorder deflection corresponded to a 16-mV electrometer output.

Analysis of fractions collected during HPLC evaluation of MIP sorbents required large-volume GC injections to compensate for dilution that occurred during the condensed-phase separation [16, 17]. For these studies, a valve fitted with a 20-μL sample loop delivered sample injections to a 15-m × 250-μm ID non-polar deactivated retention gap (Supelco) that was connected to different GC capillary separation columns, depending on the analysis, using a J & W (Folsom, CA, USA) press-fit column connector. Again, the helium head pressure was adjusted to achieve a linear velocity of 30 cm/s. Injections were accomplished by switching the helium carrier gas through the sample loop for 75 s before returning to the loop bypass position. The temperature program was initiated upon return of the recorder pen to the on-scale position after elution of the solvent peak.

The separation column used for TBP-spiked diesel fuel was a 30-m × 250-μm ID, d_f = 1.0 μm, Rtx-1 column (Restek Corp., Bellefonte, PA, USA). The initial column temperature of 40°C was held for 2 min before a linear ramp at 8 K/min was initiated. Once 275°C was reached, this final temperature was held for 30 min. For DIMP-spiked gasoline separations, a Restek Corporation Rtx-200 (trifluoropropylmethyl polysiloxane) column (15-m × 250-μm ID, d_f = 1.0 μm) was used. For the TBP-spiked air extract concentrate study, a 15-m Restek XTI-5 column (250-μm ID, d_f = 1.0 μm) was employed. Both Rtx-200 and XTI-5 columns used the same temperature program that started with an initial 2 min hold at 40°C followed by a linear ramp at 6 K/min to a final temperature of 225°C. The final temperature was held for 15 min before the column was cycled to its original temperature. Finally, for comparison of retained diesel fuel fractions from silica and MIP columns, a 15-m Restek XTI-5 column (250-μm ID, d_f = 1.0 μm) was again used; however, for these studies the temperature program started with an initial 2 min hold at 30°C followed by a linear ramp at 8 K/min to 275°C with a final temperature hold of 30 min.

3 Results and discussion

3.1 Off-line SPE results

Evaluation of SPE cartridges packed with methacrylic acid-based polymers revealed that analytes were quantitatively recovered in fraction F3 for both the MIP and control polymers. These results indicate strong non-specific interaction of the analytes with the polymer.

Table 1 summarizes the results of the off-line SPE studies for the vinylpyridine-based polymers. Analyte was not detected in fraction F1 indicating that template bleed from the MIPs was not a factor at the analyte concentrations used for this study. The material found in the fraction F2 eluate is indicative of the analyte that was not retained on the SPE cartridge. The MIP results for fraction F2 compared to the control gives an indication of the selective retention obtained. For TBP, fraction F2 eluate analysis indicated a 12% preferential retention on the MIP relative to the control, whereas 28% preferential retention was observed for DIMP on the DIMP-specific MIP. These values suggest a retention mechanism based on imprinting, a contention that is supported by further studies

Table 1. Percentage of applied TBP or DIMP that appeared in each SPE fraction for both the vinylpyridine-based, analyte-specific MIPs and control polymers along with the total mass balance. Values reported are averages along with the associated standard deviations.

Experiment SPE packing	Fraction			Mass balance
	F1	F2	F3	
TBP experiment (n = 4)				
TBP-specific MIP	0 ± 0	37 ± 5	62 ± 3	99 ± 6%
Control	0 ± 0	29 ± 8	49 ± 4	98 ± 9%
DIMP experiment (n = 3)				
DIMP-specific MIP	0 ± 0	56 ± 7	59 ± 6	115 ± 9%
Control	1 ± 2	84 ± 12	25 ± 4	110 ± 13%

described below. The material that was retained on the SPE cartridges was eluted in fraction F3. Mass balances ranging between 98% and 115% verified that analyte recovery was acceptable and furthermore, at the concentrations studied, irreversible adsorption to the polymers was negligible. These results were consistent with earlier preliminary SPE studies that were conducted with different vinylpyridine-based MIP synthetic batches (data not shown). Overall, these experiments indicate that the vinylpyridine-based MIPs were active and capable of analytically useful separations. Further experiments to determine the analytical utility of these MIP sorbents were performed in the HPLC format described below.

3.2 On-line HPLC evaluation

HPLC studies allowed continuation of MIP sorbent evaluation in a more sophisticated and versatile format. Use of a refractive index detector was preferred for monitoring the column eluate over low-wavelength UV detection due to the relative UV transparency of the test compounds. HPLC eluate fractions were collected for further analysis by capillary GC. To compensate for the dilution that occurred during the HPLC separation, relatively high initial analyte concentration and large-volume GC injections were used [16, 17].

Like the SPE experiments, HPLC studies always assessed MIP activity relative to a non-imprinted control polymer. **Figure 1** shows HPLC chromatograms of a TBP standard injected on the vinylpyridine-based control column (bottom) compared to the same standard injected on the corresponding TBP-specific MIP column (center). The downward pen deflection at 0.75 min represents the column dead volume. A retention time of 2.40 min for TBP on the control column reflects weak non-specific interactions between the analyte and the polymer. TBP was more strongly retained (3.40 min) when the MIP cartridge was substituted. The TBP retention time difference between the control and the MIP columns was possibly due to a molecular imprint retention mechanism. HPLC peaks obtained on the polymer columns were characteristically broad [18, 19].

Similar results were obtained for DIMP on the vinylpyridine-based, DIMP-specific MIP. For these experiments, the control gave a DIMP retention time of 2.44 min, whereas DIMP retention on the DIMP-specific MIP was 3.40 min. Both TBP- and DIMP-imprinted polymers displayed a difference in retention between the control and MIP columns of about 0.98 min.

Similar HPLC studies also were performed with the methacrylic acid-based polymers. Acetonitrile mobile phase was substituted for pentane due to the strong non-specific retention that was observed during the SPE studies. Acetonitrile has previously been used as a weak

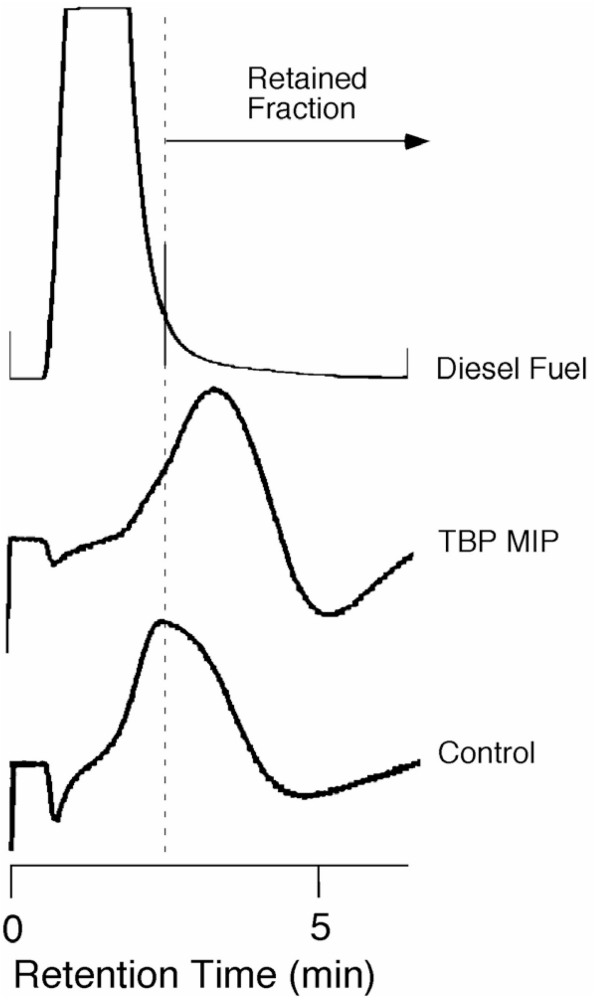

Figure 1. HPLC experiments with TBP. Center and bottom chromatograms result from TBP standard injections with refractive index detection on the vinylpyridine-based, TBP-specific MIP (center) and the control (bottom) columns (see text for details). The top chromatogram represents an injection of TBP-spiked pentane that contains diesel fuel on the TBP-specific MIP with UV absorption detection at 230 nm. The retained fraction elutes between 2.5 and 6.0 min. The mobile phase is pentane delivered at 0.5 mL/min.

mobile phase for methacrylic acid-based MIP sorbents [20]. HPLC studies of our sorbents showed minimal analyte retention with no evidence for selective retention on the MIP compared to the control when using an acetonitrile mobile phase. The studies do not conclusively demonstrate the lack of imprinting since a mobile phase composition that is intermediate in strength between a hydrocarbon (either pentane or hexane) and acetonitrile may facilitate selective retention on the MIP. The fact that selective retention was not observed on the methacrylic acid-based MIP was surprising due to the expected interaction between the acidic functional monomer and the

basic analytes. Further studies aimed at testing additional mobile phases seem warranted.

3.3 Matrix challenges of MIP selectivity

A variety of matrices were examined by HPLC on the vinylpyridine-based MIP columns using UV detection at 230 nm to monitor compound elution. Matrices examined included diesel fuel (20.0 μL diluted to 10.0 mL with pentane), gasoline (36 μL diluted to 10.0 mL with pentane), and an air extract concentrate (concentrated 80 times after Soxhlet extraction). For example, an injection of TBP-spiked diesel fuel on the TBP-specific MIP is presented at the top of Fig. 1. As with the other matrices studied, the vast majority of components eluted well before 2.5 min. Arbitrary assignment of a retained HPLC fraction as being greater than 2.5 min was made based on matrix component elution as well as MIP retention of the target analyte. Based on our results, the retained fraction on the MIP would be expected to contain almost all the target analyte while at the same time offering maximum discrimination against matrix interferences. The corresponding retained fraction on the control polymer also would discriminate against matrix components; however, this fraction would contain only a portion of the target analyte.

Limited preliminary experiments also were performed to investigate cross reactivity of the analytes on the vinylpyridine-based MIP sorbents. The selectivity results are presented as capacity factor (k') values in **Table 2**. These initial studies revealed that both DIMP and TBP were retained on the TBP-specific MIP, whereas the DIMP-specific MIP only retained DIMP but not TBP. The results suggest that the complementary cavity for DIMP does not effectively accommodate the larger TBP molecule due to steric hindrance. Although these results warrant further, more definitive investigation, the results obtained provide strong evidence for an imprinting effect.

Table 2. Capacity factor (k') values resulting from analysis of TBP and DIMP on either the TBP- or DIMP-specific MIPs.

Sorbents	Capacity factor (k')	
	k' for TBP	k' for DIMP
TBP-specific MIP[a)]	3.5	3.4
TBP-specific MIP[b)]	3.5	–
DIMP-specific MIP[a)]	2.5	3.6
DIMP-specific MIP[b)]	–	3.5
Control[b)]	2.2	2.3

[a)] Calculated from retention data performed during the cross reactivity experiment.

[b)] Calculated from data presented in the on-line HPLC evaluation section.

3.4 Gas-chromatographic analysis of HPLC fractions

Preliminary studies performed GC analysis of the vinylpyridine-based MIP column eluates to verify that residual template bleed was acceptably low. In general, template bleed into the mobile phase resulted in a signal that was barely discernible from the baseline under the chromatographic conditions used (conservatively, a concentration of < 2 ng/mL). The matrix challenge experiments involved injecting the spiked matrix sample onto either the control or MIP column and collecting two HPLC eluate fractions. The first fraction was collected during the first 2.5 min, whereas the second retained fraction was collected between 2.5 and 6.0 min. These pentane fractions were then subjected to capillary GC analysis after adjustment of the first fraction to 1.75 mL.

The first proof-of-principle experiment analyzed TBP-spiked diesel fuel on both the vinylpyridine-based control and the TBP-specific MIP columns. **Figure 2** shows capillary gas chromatograms that compare the total TBP-forti-

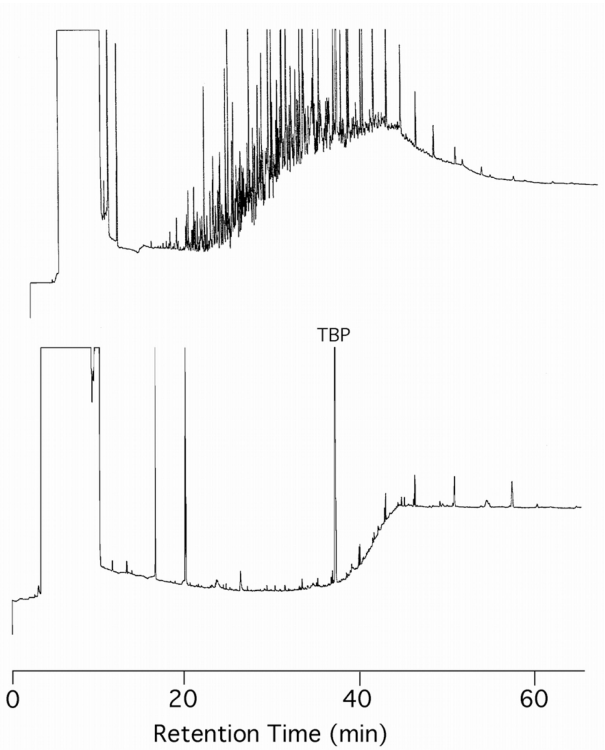

Figure 2. Capillary gas chromatograms illustrating the total TBP-spiked diesel fuel sample (top) compared to the retained HPLC fraction of this sample collected from the vinylpyridine-based, TBP-specific MIP (bottom). Conditions: 20 μL injections on a 15-m × 250-μm ID non-polar deactivated retention gap attached to a 30-m × 250-μm ID, d_f = 1.0-μm, Rtx-1 column; temperature program started at 40°C for 2 min followed by an 8 K/min ramp to 275°C with a 30 min hold at 275°C; helium carrier gas was used with flame ionization detection.

fied diesel fuel sample (top) to the retained fraction from the TBP-specific MIP (bottom). The upper chromatogram in Fig. 2 reflects the presence of literally thousands of individual compounds, many of which coelute in the chromatogram. It would be extremely difficult to identify, much less quantify, an individual component, such as TBP, in this matrix due to the sample complexity. A chromatogram of the retained fraction from the TBP-specific MIP sorbent is presented at the bottom of Fig. 2. This chromatogram shows an extremely pure fraction that reflects quantitative recovery of TBP. Identifying and quantifying TBP in the retained MIP fraction was straightforward. There are a few other peaks in the chromatogram that elute between 17 and 22 min. These peaks were absent from the total sample chromatogram but were present in the system eluate blank. These components may be oligomers that leach into the mobile phase from the sorbents.

Chromatographic comparison of the retained fraction contents from the analysis of TBP-fortified diesel fuel on the TBP-specific MIP and the control sorbent yielded a TBP recovery of 101% in the retained MIP fraction and only 51.3% in the retained control fraction. Both sorbents effectively discriminate against the diesel fuel matrix components. One would expect to obtain similar matrix discrimination between the control and the MIP; however, the MIP fraction appeared to be purer than the control fraction. An explanation of this phenomenon remains elusive. The result could be explained if the control column contained slightly more sorbent and, therefore, exhibited slightly more non-specific retention; however, this explanation is unlikely since sorbent bed volumes, packing procedures, and column dead volumes were identical between the control and MIP columns. Column dead volumes can be visualized as negative deflections in the HPLC chromatograms that occur at 0.75 min (see Fig. 1). Indistinguishable dead volumes offer strong evidence for consistent packing between the control and MIP columns and lend further credibility to the selective retention observed on the MIP compared to the control.

Similar experiments were performed using a vinylpyridine-based, DIMP-specific MIP to analyze a DIMP-fortified gasoline matrix. Again, impressive matrix discrimination against the gasoline components was obtained on the MIP column, resulting in a highly purified fraction that contained little besides DIMP. Recovery of DIMP in the retained fraction from the DIMP-specific MIP was 88%. Similar to the TBP studies, the retained fraction from the control column gave a lower recovery of DIMP (56%), and the fraction was not as pure as that obtained on the MIP. Based on the structural similarity between DIMP and the nerve agent Sarin (GB), the possibility exists that the DIMP-specific MIP will show significant cross reactivity with GB [21, 22]. This is important because GB would be too chemically reactive to serve as an effective template for direct polymer imprinting. Studies investigating the potential use of the DIMP-specific MIP for selective analysis of GB (based on analyte cross reactivity with the sorbent) are currently under way.

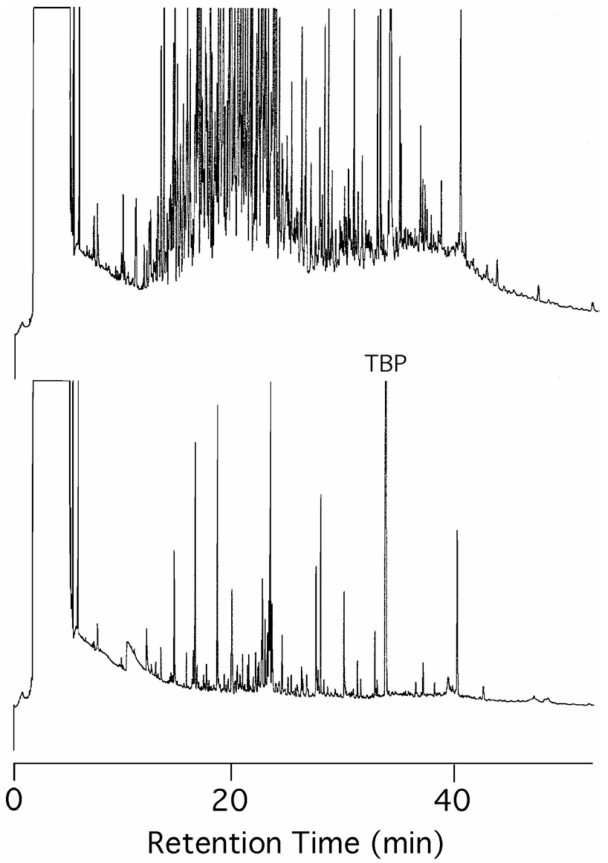

Figure 3. Capillary gas chromatograms illustrating the total TBP-spiked air extract concentrate sample (top) compared to the retained HPLC fraction of this sample collected from the vinylpyridine-based, TBP-specific MIP (bottom). Conditions: 20 μL injections on a 15-m × 250-μm ID non-polar retention gap attached to a 15-m × 250-μm ID, d_f = 1.0-μm, XTI-5 column; temperature program started at 40°C for 2 min followed by a 6 K/min ramp to 225°C with a 15 min hold at 225°C; helium carrier gas was used with flame ionization detection.

As a final proof-of-principle experiment, a TBP-fortified air extract concentrate was analyzed on the vinylpyridine-based sorbents. The experiment is relevant because it highlights an extremely difficult matrix that corresponds to a realistic national security application. An effort was made during this study to apply sufficient matrix material to overwhelm the discriminatory capacity of the short MIP column. The top chromatogram in **Fig. 3** presents the total TBP-spiked air extract concentrate sample. As can be seen from the chromatogram, the sample is highly complex and contains compounds that cover a large volatility range. In addition, the sample is expected to contain a

wide diversity of compound types. In common with the diesel fuel sample, the air extract concentrate chromatogram exhibited an elevated baseline throughout most of the elution range due to numerous coeluting compounds. With the exception of a few peaks, the chromatographic complexity of the non-retained HPLC fraction from the TBP-specific MIP (data not shown) looks similar to the total TBP-spiked air extract concentrate (top of Fig. 3), demonstrating that nearly all the matrix material elutes from the MIP column without being retained. The bottom chromatogram in Fig. 3 shows the retained fraction from the TBP-specific MIP. Although some of the matrix components carried over into the retained HPLC fraction from the TBP-specific MIP, the chromatogram (Fig. 3 bottom) still illustrates impressive matrix discrimination. MIP columns packed with larger quantities of sorbent would be expected to provide more efficient discrimination against the matrix components in these complex concentrated samples. Consistent with previous results, the recovery of TBP in the retained TBP-specific MIP fraction was near quantitative (90%), whereas the recovery of TBP in the retained fraction from the control column was lower (46%).

The impressive matrix discrimination achieved on the MIP sorbents could not have been obtained using traditional normal-phase supports such as silica or alumina. These materials give compound class separations based on polarity and, given the enormous diversity of compound types contained in the challenge matrices, the retained fraction from these traditional sorbents would be expected to contain considerable matrix interference. This expectation was experimentally verified (see **Fig. 4**) by comparing the gas-chromatographic profiles of the initial portion (2.5 to 4.5 min) of the retained diesel fuel HPLC fraction separated on silica (top chromatogram) to a corresponding fraction separated on the vinylpyridine-based, TBP-specific MIP (bottom chromatogram). An equivalent fraction from alumina was also analyzed and was found to be similar in complexity to the silica fraction. Although the studies described here do not quantitatively address sorbent sample capacity, matrix challenge experiments were conducted well below the capacity limit based on estimates obtained from the SPE experiments and the near quantitative recoveries that were observed.

The active MIPs used in this study were based on bulk cast polymers constructed with a 4-vinylpyridine functional monomer. The polymers yielded heterogeneous ($< 74\ \mu m$) irregular particles upon mechanical grinding and sieving. This material is not ideal for chromatographic studies due to the inability to efficiently close-pack the particles. Substitution of homogeneous macroporous spherical particles would be expected to enhance the performance over the MIP materials used in this study. Although spherical particles based on 4-vinylpyridine have been

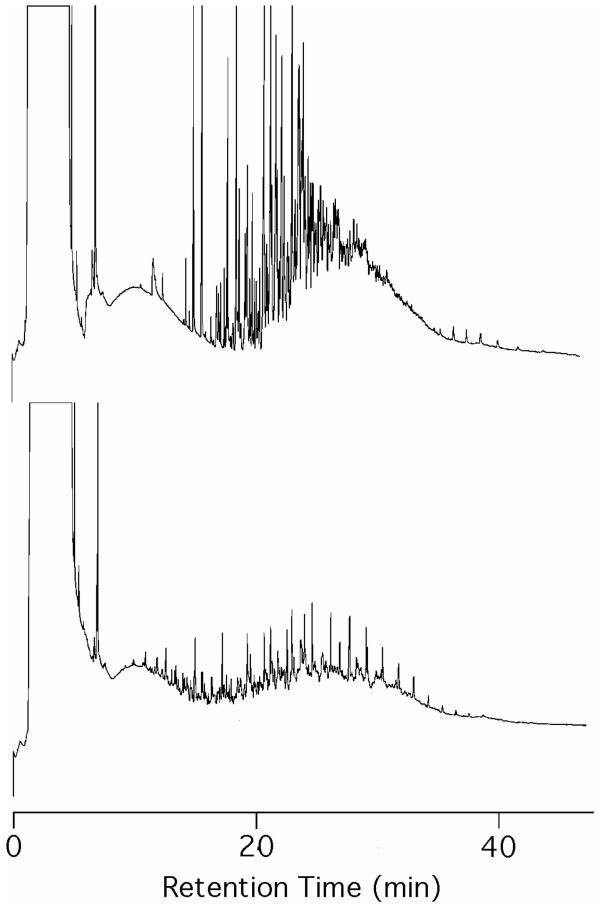

Figure 4. Capillary gas chromatograms comparing the initial portion (2.5 to 4.5 min) of the retained HPLC fraction from diesel fuel collected from silica (top) and the vinylpyridine-based, TBP-specific MIP (bottom) columns. Conditions: 20 injections on a 15-m × 250-μm ID non-polar deactivated retention gap attached to a 15-m × 250-μm ID, d_f = 1.0-μm, XTI-5 column; temperature program started at 30°C for 2 min followed by an 8 K/min ramp to 275°C with a 30 min hold at 275°C; helium carrier gas was used with flame ionization detection.

prepared by Mosbach and co-workers [23, 24], these particles are too small for convenient chromatographic applications. Further research in this area can be expected to result in homogeneous spherical particles in the low micrometer size range that would display enhanced performance for applications similar to those discussed above. Another limitation of the MIPs described in this study is that ultratrace applications may be limited by residual template bleed. To achieve the desired detection limits, it may be necessary to synthesize MIPs using close structural analogs of the target species.

Several MIP sorbents were synthesized and found to be inactive relative to the control sorbent when evaluated by HPLC. These polymers were synthesized with a variety of

templates, including 2-chloroethyl ethylsulfide (CEES) and 1-iodobutane. Several polymers changed color during reaction, indicating that the templates had undergone transformation during polymerization. The results serve to emphasize that, although the non-covalent imprinting approach is very versatile, many polymers prepared will not be active for a variety of reasons, including template reactivity [25].

4 Concluding remarks

This proof-of-principle study demonstrated that MIP sorbents can be applied to extremely complex samples to obtain impressive discrimination against matrix interferences in non-aqueous analytical systems. Examples presented use bulk cast MIP sorbents prepared with a 4-vinylpyridine functional monomer. These polymers were effectively imprinted toward the chemical warfare surrogate compound DIMP, or the nuclear signature compound TBP, as indicated by selective retention versus the non-imprinted control polymer. In addition, selectivity studies showed TBP was only retained on the TBP-specific MIP, whereas both DIMP and TBP were retained on the DIMP-specific MIP. These results were readily explained based on stearic exclusion of the larger TBP molecule from the DIMP imprint site. Syntheses of MIPs toward several other templates were attempted but yielded inactive polymers, emphasizing the fact that some reactive analytes are not suitable for MIP preparation.

Activity toward TBP and DIMP was first demonstrated by off-line SPE experiments followed by on-line studies using short HPLC columns. Matrices examined with the TBP-specific MIP included diesel fuel and an air extract concentrate. Gasoline was used as a matrix to challenge the DIMP-specific MIP. In general, the retained HPLC fractions from analyte-specific MIPs displayed enormous matrix discrimination, resulting in highly purified fractions, while yielding near-quantitative recovery of the target analyte. Matrix discrimination also was observed in the retained fraction from the control sorbent; however, analyte recovery was far lower, and the fractions tended to be less pure.

To our knowledge, this is one of the few reports of MIPs that target small, non-polar volatile compounds. Both DIMP and TBP contain minimal chemical functionalization on which to base imprinting. As a consequence, we observed a relatively small differential retention between the control and MIP sorbents in comparison to studies that target less volatile analytes that contain polar functional groups. For example, MIPs specific toward pinacolyl methylphosphonate (PMP, the hydrolysis product of Soman) were prepared [20, 26]. This imprint was easier to achieve than targeting the parent nerve agent because the polar acid functionality facilitated imprinting [20, 22, 26]. Although this PMP-specific MIP was extremely useful for selective analysis of the hydrolysis product of Soman, detection of this compound was, at best, indirect evidence of nerve agent use. An important outcome of the work described herein was the demonstration that moderate differential selective retention observed for MIPs imprinted with volatile non-polar organics (compared to their control polymers) was analytically useful, suggesting that these sorbents can be effectively applied for direct analysis of non-polar gas-phase signature compounds.

Short MIP HPLC columns were found to provide an impressive ability to discriminate against complex matrix backgrounds while maintaining high recovery of target analytes. This technology could be implemented as an initial concentration/separation stage for analysis of complex samples for specific signature compounds. The pure fraction obtained from the MIP stage would greatly simplify the subsequent analysis required to obtain high-confidence analyte identification at trace concentrations. The ability to provide effective matrix discrimination at an initial sampling stage should enable the development of compact field-portable instrumentation that can maintain high performance when analyzing difficult matrices.

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