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Pacific Northwest
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Ultraselective Sorbents
Task 2: Molecularly Imprinted Polymers (MIPs)/Stabilized Antibody Fragments (STABs)
Final Report FY 2004

S.D. Harvey

September 2004

Prepared for the National Nuclear Security Administration
Office of Nonproliferation Research and Engineering, NA-22
U.S. Department of Energy
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Richland, WA 99352
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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 task is to develop highly selective sorbents that will allow 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 task. Both sorbents must be capable of operation in nonaqueous environments to protect hydrolytically sensitive analytes during analysis. The approaches investigated were 1) nonaqueous immunochromatography featuring the use of stabilized antibody fragments (STABs) and 2) molecularly imprinted polymers (MIPs). Both approaches were pursued in 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 focused exclusively on the development of MIPs.

A major emphasis of FY 2004 studies was the preparation of MIPs specific toward explosives. Our strategy utilized two functional monomers known to have specificity toward nitro-containing explosives. The monomers were previously incorporated into polymers; however, the polymers have never been prepared in an imprinted format. The monomers, vinyl pyrrolidone and styryl hexafluorodimethylcarbinol, were incorporated into a styrene divinylbenzene polymer matrix. Polymers imprinted with RDX and corresponding nonimprinted control polymers were prepared. When tested by high-performance liquid chromatography (HPLC) relative to the control, the MIPs were expected to show selective retention of RDX as a result of interaction between the analyte and the specific molecular imprint cavities contained within the polymer. Surprisingly, however, the experiments showed that RDX was retained more strongly on the control polymer than the MIP. After various causes for the results were considered, we concluded that the presence of the explosive in the MIP reaction mixture interfered with the polymerization process, giving rise to a polymer that was not as highly cross-linked as the control. This arises because the nitro groups contained in the explosive quench free radicals that are used to initiate polymerization. Since the template is not present in the controls, these materials polymerize more completely. (See Appendix 1.)

Alternative strategies for preparing MIPs that were independent of free radical initiation (or for that matter, polymerization) were devised. We proposed to continue preparing imprinted variants of polymers that contained either vinyl pyrrolidone or styryl hexafluorodimethylcarbinol as the functional

monomer. The new strategy involved incorporating the monomers into a polymer that is soluble in dimethyl sulfoxide. For the MIP "reaction", the solubilized polymer is allowed to pre-associate with an explosive in solution. Then, the polymer is precipitated by exposure to water. This precipitation "freezes" the polymer into a configuration that recognizes the explosive. The control polymer is formed the same way, except that RDX is not present and the resulting polymer precipitate is not locked into an imprinted configuration. The beauty of the approach is that the polymer already is formed before exposure to the template and, therefore, the template cannot interfere with the polymerization. The approach also offers significant advantages for imprinting labile analytes since the conditions are extremely mild. (See Appendix 2.)

MIPs specific toward the G-series nerve agent hydrolysis products were prepared during FY 2004. MIP preparation first required preparation of several half–acid ester hydrolysis products by custom organic synthesis. Synthesis was required because most of these compounds are not commercially available. 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. This approach was a significant improvement over more complicated and time-consuming literature procedures. The manuscript resulting from our synthetic work has been accepted for publication in the journal, *Sulfur, Phosphorus, and Silicon* (See Appendix 3). The half-acid ester hydrolysis products produced by these synthetic methods were used as templates for preparing MIP stationary phases. MIPs specific towards isopropyl methylphosphonate (IMP), pinacolyl methylphosphonate (PMP), and cyclohexyl methylphosphonate (CMP) were prepared. In addition, we prepared a MIP with specificity toward bis(2-ethylhexyl) hydrogenphosphate, an organic signature compound associated with uranium processing. Although this compound is not a methylphosphonate, the chemical structure is similar to the nerve agent hydrolysis half-acid ester compounds.

Evaluation of the nerve agent half-acid ester MIP sorbents is described in detail in the invention disclosure contained in Appendix 4. This invention disclosure describes a novel MIP-based instrument that is capable of performing analysis of aqueous samples. Although the instrument has general applicability for water-soluble compounds that have hydrophobic properties, the specific example used for the proof-of-principle experiments is an analysis for the nerve agent hydrolysis compound, PMP. The instrument also is expected to be applicable to bis(2-ethylhexyl) hydrogenphosphate as well as other water-soluble organic compounds that have some lipophilic character.

The instrument is designed to use two stages of nonspecific trace enrichment. The first stage is achieved by skimming the aqueous surface microlayer. This layer often contains concentration enhancements of approximately three orders of magnitude relative to the bulk solution concentration. In the second enrichment stage, the lipophilic contents are concentrated by passing the skimmed surface layer through 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, whereas 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 conductivity.

In preliminary studies with PMP, the instrument performed well. The actual prototype instrument did not contain the surface microlayer sampler but the component could be added later.

Detection limits were estimated from 20-μL aqueous sample injections onto the C-18 stage. After reasonable concentration values are chosen for the surface microlayer enhancement and the C-18 preconcentration stages, detection estimates are in the part-per trillion (ng/L) range. Lower detection limits would be possible with alternative sensitive detection strategies (such as an enzyme-based amperometric detection).

The final studies in this report expand on previous MIP studies by interfacing MIP separations with ion mobility spectrometric detection. In FY 2003, MIPs specific toward tributyl phosphate (TBP) and diisopropyl methylphosphonate (DIMP) were synthesized. Evaluation by HPLC showed these MIP sorbents were active. Further studies challenged the MIPs with complex mixtures that contained the target compounds. Examination of the retained MIP fractions showed enormous matrix discrimination with near-quantitative recovery of the target in a relatively clean fraction. These studies were described in the FY2003 NA-22 Final Report and in a manuscript that was submitted to *Journal of Separation Science*.

Research conducted in FY 2004 expanded the MIP studies by interfacing the separation with ion mobility spectrometry (IMS). The research showed that IMS is extremely compatible with these separations and that the MIP/IMS combination has great potential for providing highly selective analysis of trace signature compounds. At present, this research is considered high priority and is being actively pursued by Pacific Northwest National Laboratory. Our preliminary MIP/IMS studies are presented in the last section of this report.

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 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 since 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.

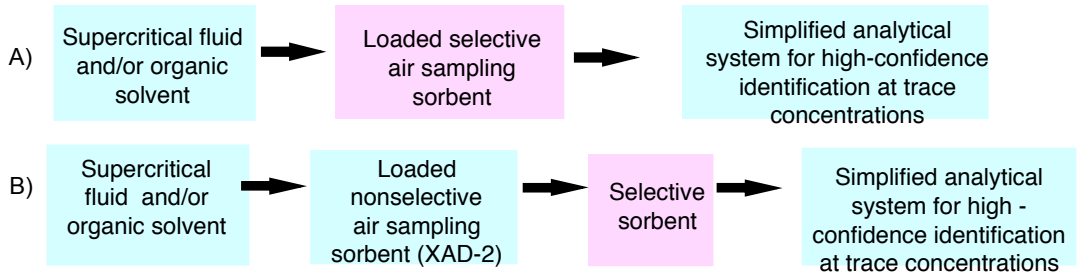

Figure 1. Two proposed formats for implementing selective sorbent sampling. The selective sorbent can be used as A) a primary air sampling sorbent or B) a secondary sorbent situated immediately behind a traditional nonspecific air sampling sorbent.

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 and 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 about 0.9 to 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 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 is 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 weeks.

RESEARCH PROGRESSION AND REPORT ORGANIZATION

From the experimental results obtained during FY 2003, it became clear that MIPs was 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 will only discuss MIPs. The three areas we emphasized in 2004 were 1) the preparation of MIPs toward explosives, 2) the development of an instrument for the analysis of the G-series nerve agent hydrolysis compounds, commonly referred to as the half-acid esters, and 3) preliminary interfacing of MIP separations with ion mobility spectrometry (IMS).

The following pages provide brief descriptions of the above topics. The first topic for discussion is the preparation of explosive-specific MIPs, and includes the general research strategy and initial results that were obtained, and the synthetic strategies that we believe hold a high probability of success for preparing MIPs imprinted with explosives. The second topic is the construction of MIP-based instrumentation for analysis of the G-series nerve agent hydrolysis products and chemically related compounds. The third section describes interfacing MIP separations with ion mobility spectrometry (IMS) detection. Finally, several concluding paragraphs will describe tangible project accomplishments and research acknowledgements.

Each of the first two major sections will refer to material that is contained in one of four appendices. These appendices contain detailed information regarding the rationale for the experiments and sufficient experimental details to reproduce the studies. Since the appendices contain all the detailed information, this section comprises the bulk of this report; whereas the front text serves as a brief synopsis and orientation. The first two appendices contain PNNL invention disclosures that describe explosive-specific MIPs. The second of these invention disclosures describes phase-inversion precipitation techniques that we feel hold the most potential for success in this endeavor. Appendix 3 is a manuscript that has been recently accepted for publication in *Sulfur, Phosphorus, and Silicon*. This manuscript describes a novel synthesis of the G-series nerve agent hydrolysis compounds. We used these techniques to produce signature compounds that are not commercially available for use as imprint templates. Appendix 4 describes the design, construction, and evaluation of a novel MIP-based instrument that analyzes aqueous solutions for trace quantities of G-series nerve agent hydrolysis compounds.

EXPLOSIVE-SPECIFIC MIPS

Research Rationale and Preparation of Explosive-specific MIPs

Past research on preparing MIPs that are specific toward explosives has been limited. Although two strategies have been presented in the literature, neither the manuscript nor patent publications give experimental evidence that these strategies actually work. Most work related to the topic originated from George Murray's group at Johns Hopkins University. The group created imprinted sensor

coatings specific for certain explosives. These coatings incorporated both molecular recognition capabilities and a transduction mechanism to detect explosives. Murray's group proposed two approaches. The first approach, described in the peer-reviewed literature, is based on formation of a charge-transfer complex between a tertiary amine functional monomer unit and a nitroaromatic explosive. The second approach is only described in the patent literature and involves interaction of a nitroaromatic explosive with a porphyrin functional monomer. Both techniques detect the explosive based on an absorption wavelength shift that occurs upon interaction with the explosive. The approaches are limited in scope since they apply only to nitroaromatic compounds. Nitroaromatics comprise only a small proportion of the explosives class.

We propose a more general approach that targets nitro-containing explosives, a class that is far more extensive than the nitroaromatics targeted by Murray. A broader approach is possible with chromatographic studies since downstream detection is allowed rather than sensor transduction. Our approach is based on the demonstrated selective sorption of explosives on either Porapak R or strong hydrogen bond acidic polymers. Extensive work has been done with polymers based on these functional monomers; however, imprinted variants of these polymers have not been described. The new imprinted polymers should have significantly higher selectivity than the nonimprinted variants.

The functional monomers investigated were 1-vinyl-2-pyrrolidone (used in Porapak R) and styryl hexafluorodimethylcarbinol (a strong hydrogen bond acidic functional monomer). The monomers were incorporated into a styrene/divinylbenzene polymer matrix. Other types of polymers, such as acrylate-based materials, were avoided since the hexafluorodimethylcarbinol functional monomer would preferentially interact with the carbonyl group in the acrylate rather than any explosive, thereby preventing effective imprinting. Polymers were prepared using standard 2,2'-azobisisobutyronitrile (AIBN) free radical initiation techniques. RDX was included in the imprint reaction mixtures as the explosive template, whereas the control reaction mixtures did not contain an explosive. A high concentration of divinylbenzene crosslinker (9-12 percent) was required to prepare imprinted polymers.

The resulting polymers were carefully evaluated by HPLC with an acetonitrile mobile phase. Retention of RDX on the various columns was referenced to a dead volume marker to correct for any small differences in column packing efficiencies. The results obtained were surprising. The 1-vinyl-2-pyrrolidone- and the styryl hexafluorodimethycarbinol-based MIPs displayed lower retention for RDX compared to the appropriate control polymers. The result was exactly opposite from what was expected.

Several possible explanations were proposed to account for the unusual results. The most probable explanation is that free radical quenching properties of nitro groups contained in the template explosive inhibited the polymerization process for the MIP polymers. Radical quenching by nitro groups is a known phenomenon. This led to less efficient polymerization for the MIPs compared to the controls. This also fit the experimental observation that unusually high crosslinking was required to obtain brittle polymers for the explosive-templated MIPs.

Detailed results of our studies up to this point are presented in Appendix 1. Clearly, to obtain consistent polymers between controls and explosive-templated MIPs, free radical initiation mechanisms cannot be used. At the end of Appendix 1 we propose a few ideas, based on Lewis acid

initiated polymerization, that deviate from the free radical polymerization approach. During our academic project review, we reviewed the MIP work up to this point and discussed possible solutions to the polymerization consistency issue. The alternative approaches that emerged are discussed below.

Proposed Scaffold Imprinting for Explosives-specific MIPs

In planning alternative approaches, we felt that our choice of functional monomers was scientifically sound and, therefore, this aspect of the strategy was retained. Alternate methods for MIP preparation could not involve free radical initiation for reasons discussed above and, ideally, would not involve polymerization at all. In a procedure termed phase-inversion polymerization, a preformed polymer is dissolved in solution and allowed to associate with a template. After association, another solvent is added that causes precipitation of the polymer. If a template is present, the precipitated polymer is locked into a specific configuration. Once the template is washed out of the polymer matrix, a MIP is created that contains a complementary cavity that will recognize the template. The control polymer is prepared from the same dissolved polymer solution, except the template is not added. In this case, precipitation results in a randomly oriented polymer that is not imprinted. Consistency is not an issue since there is no polymerization in the presence of the template.

This technique is advantageous for our studies since we can synthesize a polymer that contains our specialized functional monomers using standard free radical initiation techniques. Typically, the specialized monomers would be co-polymerized with acrylonitile monomer units. The resulting polymer would be prepared in a solvent like dimethyl sulfoxide under conditions where polymerization is limited and the relatively low molecular weight (roughly 1.5×10^5 Da) polymer product remains in solution. The polymer-containing solution would then be used for preparation of both the MIP and the control polymers. Since polymerization has already occurred, the control and MIP polymers will be consistent. Additionally, this is an extremely mild technique since there is a complete absence of covalent reaction chemistry. Because conditions are so mild, extremely labile analytes may be imprinted using these methods.

The invention disclosure in Appendix 2 describes the phase-inversion imprinting technique in detail. In addition to this approach, other techniques based on scaffold imprinting are presented as possible approaches that would result in explosive-specific MIPs. Some of these approaches depend on condensation polymerization and other approaches consist of hybrids between phase inversion precipitation and condensation polymerization.

During the academic project review, the committee suggested that we focus more strongly on organic signature compounds and, due to the difficulty associated with the explosive-specific MIP work, that we curtail research in this area. The research we have conducted in this area has resulted in two invention disclosures and numerous proposals and white papers to user agencies and clients including the Department of Homeland Security and the Defense Intelligence Agency. We are confident that the advancements made possible through this project have provided a sufficient foundation for further successful research under user agency funding.

NOVEL MIP-BASED INSTRUMENT FOR ANALYSIS OF AQUEOUS SAMPLES

Synthesis of the G-series Nerve Agent Hydrolysis Compounds

Improved synthetic methodologies are needed for G-series nerve agent hydrolysis compounds. Only one of these compounds, pinacolyl methylphosphonate (PMP), is available commercially. The other related compounds, isopropyl methylphosphonate (IMP) and cyclohexyl methylphosphonate (CMP), are accessible only through synthetic methodologies that are described in the literature; however, these syntheses are complicated, time consuming, and require specialized facilities and glassware, e.g., for vacuum distillation. Gram quantities of these pure compounds are needed as templates for MIP preparation and for use as analytical standards. Our initial objective was to define a synthetic strategy that would rapidly provide a highly pure product in gram quantities and be simple enough to perform in most research laboratories. Such a methodology would greatly facilitate analytical research aimed at developing trace analysis strategies for the relatively stable environmental hydrolysis products of the G-series nerve agents.

Our procedure involved addition of an equivalent molar amount of alcohol (cyclohexanol or isopropanol) to methylphosphonic dichloride in toluene at 105°C. After 15 minutes of reflux, the reaction mixture was cooled to 80°C, and two equivalents of water were added. The mixture was then refluxed for an additional 10 minutes. After cooling, the reaction consisted of a yellow organic phase and a denser aqueous layer. The principal reaction products were dialkyl methylphosphonate (mostly in the toluene layer) and a mixture of methylphosphonic acid and the desired monoalkyl methylphosphonate (located primarily in the aqueous layer). The product was purified by removing the toluene layer, and hence the dialkyl methylphosphonate. Next, the desired product was extracted from the acidic aqueous layer into chloroform. Washing the chloroform layer several times with water completely removed the methylphosphonic acid. Evaporation of the chloroform left the pure product (98+ percent). Synthesis was rapid and the product was pure enough that vacuum distillation was not necessary. The reaction and purification procedures gave the desired product with yields greater than 30 percent. Our studies characterized the methyl and trimethylsilyl ester derivatives by capillary gas chromatography. Temperature programmed retention indices and mass spectral data for the derivatives are presented to aid other researchers.

See Appendix 3 for a detailed description of this synthesis. This manuscript has been accepted for publication in the journal, *Sulfur, Phosphorus, and Silicon*.

Novel MIP-based instrument of the analysis of G-series Nerve Agent Hydrolysis Products and Related Compounds

MIP sorbents operate at peak efficiency in nonaqueous environments. Water usually competes with the template recognition mechanisms and, therefore, is considered a strong mobile phase for these sorbents. A current research trend is the development of methodologies to create MIPs that directly capture analytes from aqueous solution. The research is driven by the need for economical and stable antibody alternatives. Usually MIPs that operate successfully in aqueous solutions have strong interactions between the functional monomers and the template.

An alternate approach for performing MIP-based aqueous analysis is to first non-specifically concentrate the lipophilic organics by traditional techniques. One such approach involves capturing analytes on an octadecyl silica (C-18) sorbent bed. The water then can be removed from the system by a stream of dry gas, leaving the lipophilic components sorbed to the dry C-18 material. The enriched components then can be 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 the selective interaction of the target with the MIP.

MIPs were prepared for our studies by advanced suspension polymerization techniques using methacrylic acid as the functional monomer. Reagents were added to a perfluoro solvent, which is a poor solvent for the reactants. Upon mixing, a stable emulsion is formed. The size of the emulsion droplets is precisely controlled by the quantity of a special fluorinated surfactant that is added to the system. Once the emulsion is formed, polymerization is initiated. The advantage of this technique is that spherical, homogenous, macroporous beads are produced that are easily isolated by filtration. The size of the beads is readily controlled by the amount of surfactant added. The properties of these beads are quite different from the 4-vinylpyridine-based bulk polymers described in our previous work (MIPs specific toward TBP and DIMP) because of the different functional monomer. For suspension polymerization polymers described in this section, acetonitrile rather than pentane was used as the organic mobile phase.

When designing the MIP-based instrument for the G-series nerve agent hydrolysis compounds, we adhered to the analysis progression given in the flowchart shown in Figure 2. The instrument was constructed to be compatible with a surface microlayer sampler. This skimmer provides the first stage of nonspecific trace enrichment. The surface microlayer often concentrates analytes relative to the bulk aqueous concentration. Concentration factors of 10^3 are common, although this is highly dependent on the specific compound. The next stage of the instrument provides further nonspecific enrichment by passing the surface microlayer aqueous sample through an octadecyl silica (C-18) sorbent. The C-18 sorbent retains all components that have a lipophilic character. Although the nerve agent hydrolysis compounds and bis(2-ethylhexyl) hydrogenphosphate are reasonably polar phosphoric acid derivatives, the alkane chains on the ester portion of these molecules impart sufficient lipophilic character that the compounds are captured by the C-18 sorbent. Next, all traces of water are removed from the system with a flow of dry inert gas, which leaves the organic compounds adsorbed on the dry C-18 sorbent. The organics then are transferred using acetonitrile to the MIP where the target compound is selectively captured while the matrix components pass through the sorbent unaffected. The final step is to transfer the half-acid ester to a conductivity detector with a water mobile phase. Details of the suspension polymerization preparation of MIP sorbents, instrument construction, valving, and timing diagrams are presented in Appendix 4. A photograph of the instrument used to collect data presented in Appendix 4 is shown in Figure 3. The instrument sits in a cart to emphasize its portability; however, many of the components could easily be made much smaller. A simple improvement would be to substitute smaller HPLC pumps, such as the pump shown in Figures 5 and 7.

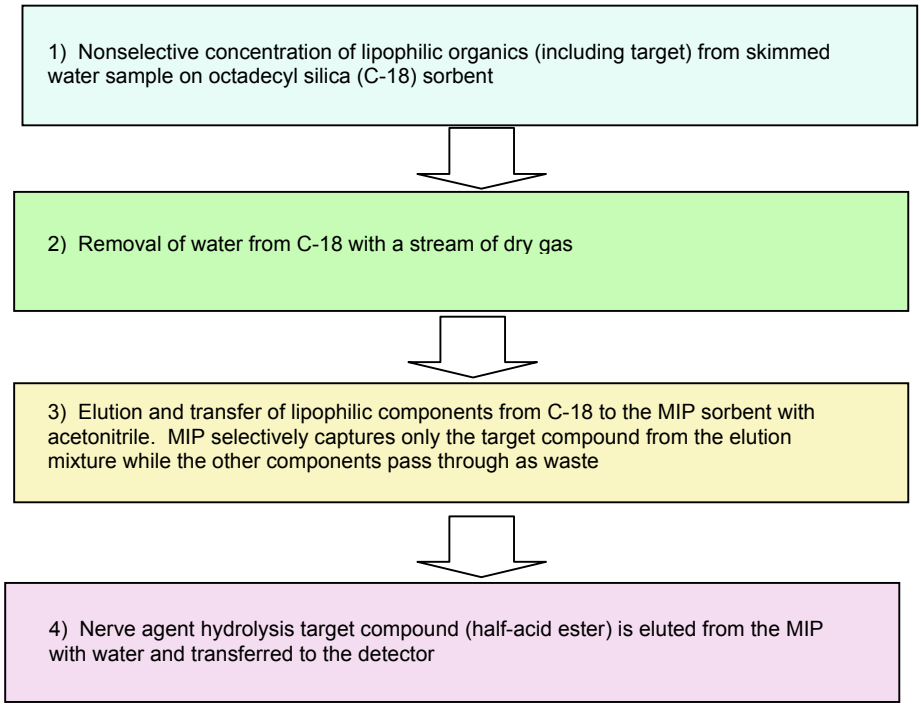

Figure 2. Flowchart showing the steps for analyzing an aqueous sample on the MIP-based system.

Figure 3. The MIP-based instrument was developed for the analysis of G-series nerve agent hydrolysis compounds.

Performance of this instrument using conductivity detection without nonspecific trace enrichment showed the feasibility of easily detecting low microgram quantities of PMP. Given the enormous preconcentration of the two nonspecific enrichment stages, the overall instrument detection limits are projected to be in the part-per-trillion (ng/L) concentration range.

In our design, we used a conductivity detector for analyte detection. Other, potentially more sensitive detection techniques are highly desirable and are being considered. For example, it should be possible to use an enzyme to cleave the ester bond and detect the resulting alcohol electrochemically. The attractive attributes of this detection technique is its applicability towards phosphate ester analytes, including bis(2-ethylhexyl) hydrogenphosphate, the G-series nerve agent hydrolysis compounds, and compounds like tributyl phosphate. One approach proposed in the past was to use an enzyme called organic phosphate hydrolase (OPA). Our preliminary studies showed that OPA did not hydrolyze either tributyl phosphate or diisopropyl methylphosphonate. The result was not surprising, as OPA is known to cleave the halogen off nerve agents, leaving the half-acid ester product that contains the intact ester bonds.

Researchers elsewhere are investigating the use of enzymes to cleave the ester bonds of methylphosphonate esters using the enzyme phosphonate ester hydrolase (PEH). Ilya Elashvili and Joseph DeFrank at Army ECBC have demonstrated that cell-free extracts from *Burkholderia caryophilli* (strain PG2982) cleave ester bonds from the hydrolysis products of G- and V-series nerve agents resulting in the formation of an alcohol and methylphosphonic acid. More recently, methods for the partial purification of PEH have been described. Immobilization of PEH on an electrode would allow electrochemical detection of the alcohol liberated from the half-acid esters, resulting in highly selective and sensitive detection. This detection mode would significantly enhance the performance of the MIP-based half-acid ester instrument described above. It is possible that this, or a related enzyme, would allow similar detection of nuclear signature compounds such as tributyl phosphate or bis(2-ethylhexyl) hydrogenphosphate.

Proof-of-principal experiments (See Appendix 4) were performed using PMP standards in water. The actual instrument performance with real samples depends on how effectively the MIP sorbent discriminates against complex matrices. We are presently conducting matrix challenge experiments with the PMP-specific MIP. We are examining the MIP's ability to selectively retain PMP from complex acetonitrile soil extracts. The soil extract matrix is more highly complex than the mixture transferred to the MIP in the actual instrument since the C-18 will retain only the lipophilic components from the soil extract. This challenge, therefore, is quite realistic and rigorous because the challenge mixture is more complex than is likely to be encountered under actual operation.

This instrumentation development was originally intended for the analysis of bis(2-ethylhexyl) hydrogenphosphate. However, the initial evaluation of the MIP specific toward this compound indicated that it was not active. Later, this same MIP sorbent was retested using a more sensitive and stable refractive index detector. The repeated studies showed the bis(2-ethylhexyl) hydrogenphosphate-specific MIP to have moderate activity; however, the activity of MIPs specific toward G-series nerve agent hydrolysis compounds proved to be much higher. We proceeded with proof-of-principal experiments using the PMP-specific MIP for this reason. Preparation of MIPs having higher activity toward bis(2-ethylhexyl) hydrogenphosphate would allow analysis of this

compound as well. As this instrument stands, it is a general methodology that allows MIP-based analysis of compounds out of aqueous samples at extremely trace concentrations. Therefore, the analysis technique is generally applicable to a wide range of anaytes in a broad cross section of disciplines.

Interfacing MIP Separations With Ion Mobility Spectrometry

Research conducted in FY 2003 synthesized MIPs that are specific toward tributyl phosphate and diisopropyl methylphosphonate. High-performance liquid chromatographic evaluation of these sorbents using a pentane mobile phase showed selective retention of the target compounds relative to a nonimprinted control polymer. These sorbents displayed preferential retention toward their intended target and gave little retention for gasoline, diesel fuel, or air extract concentrate matrices. When complex matrices containing target compound spikes are introduced to the appropriate MIP columns, the retained fraction exhibits excellent matrix discrimination, is relatively clean, and gives near-quantitative recovery of the target analyte. The research formed the basis for a manuscript submitted for publication in the *Journal of Separation Science*.

An example of the matrix discrimination obtained by the MIP separation is presented in Figure 4. The top portion of this figure shows a capillary gas chromatogram of a large-volume air extract concentrate that has been spiked with diisopropyl methyphosphonate (DIMP), a Sarin surrogate. When this complex mixture is applied to the DIMP-specific MIP, DIMP is selectively retained on the sorbent, and the vast majority of the matrix components pass through unaffected. A chromatogram of the retained MIP fraction is shown in the bottom chromatogram in Figure 4. This chromatogram illustrates the enormous matrix discrimination that is achieved on the MIP stage, as well as the near-quantitative recovery for DIMP. Further discrimination can be obtained using larger MIP columns, if desired.

Gas chromatographic analysis was used to demonstrate the composition of the retained MIP fraction. For field-portable instrumentation, we wish to incorporate simpler and more compact detection devices. Ion mobility spectrometry (IMS) has many desirable characteristics for integrating with MIP separations. To probe the utility of IMS, we challenged an IMS instrument with the same complex DIMP-spiked air extract concentrate sample as profiled in the top of Figure 4. To frame this experiment in a meaningful context, DIMP standards and MIP fractions also were analyzed. Samples were introduced directly to the IMS by use of a microliter syringe (Figure 5). Figure 6 presents the results from this experiment. The top IMS plasmagram (light blue trace) is the instrument background signal. The signal at 7.8 msec is due to ammonia reagent gas that is added to suppress interferences. The second plasmagram (red trace) results from an injection of a DIMP standard. This spectrum shows the 13.2 msec IMS drift time for DIMP at a signal intensity that represents 100 percent recovery. The bottom trace (dark blue) shows a direct introduction of the DIMP-spiked air extract concentrate; the same complex sample that was profiled in the top chromatogram of Figure 4. IMS analysis of this complex sample is inadequate due to the attenuated DIMP signal, the ion clustering indicated by longer drift times, and complete suppression of the reagent gas signal. In short, direct IMS analysis is not capable of providing adequate analysis for a sample of this complexity. The green trace represents an

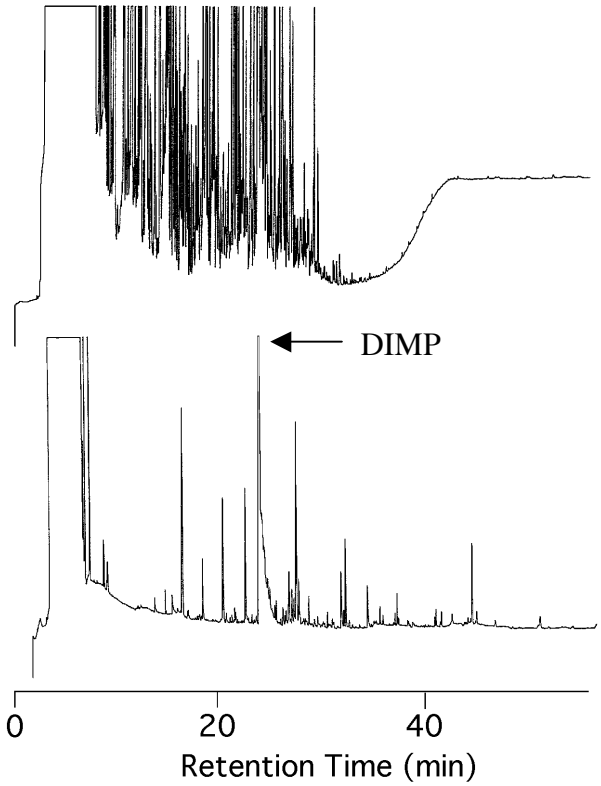

Figure 4. Capillary gas chromatograms illustrating the total DIMP-spiked air extract concentrate (top) compared to the retained fraction from the DIMP-specific MIP (bottom).

Figure 5. Off-line introduction of standards and MIP fractions into IMS instrument.

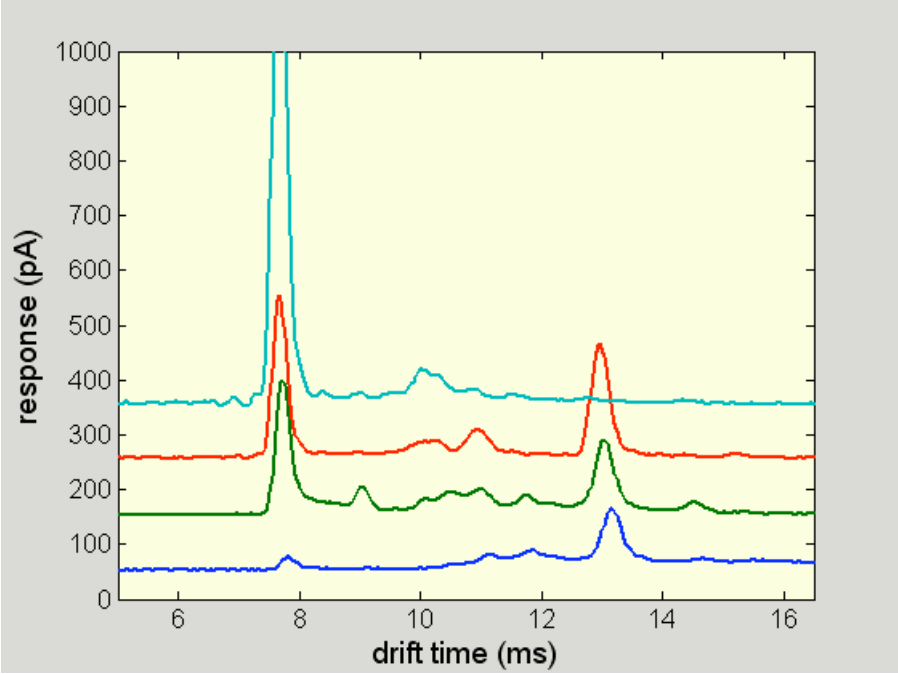

Figure 6. From top to bottom, IMS plasmagrams showing instrument background, DIMP standard, retained MIP fraction of DIMP-spiked air extract concentrate, and DIMP-spiked air extract concentrate without chromatographic processing. See text for discussion.

analysis of the retained MIP fraction from the DIMP-spiked air extract concentrate. Although a few extraneous signals can be seen in this plasmagram (consistent with the chromatogram in the bottom of Figure 4), the analysis is acceptable and further shows that the majority of DIMP was recovered in the retained MIP fraction. This set of experiments emphasizes the value added for combining MIP separations with IMS detection.

Manual introduction of samples to the IMS is useful for demonstrating the utility and limitations of this detection technique. However, what is needed is an on-line feed of the MIP column eluate into the IMS detector. The instrument shown in Figure 7 shows a simple interface between a MIP column and an IMS. The interface consists of a flow splitter that reduces the pentane flow from 0.5 mL/min at the end of the MIP column down to 20 μL/min for introduction to the IMS. The reduced flow is directed through a capillary that is positioned directly in the throat of the heated IMS inlet. This simple interface proved adequate to perform on-line experiments with DIMP standards. A contour plot (Figure 8) shows the on-line chromatogram of a DIMP standard using IMS detection. The DIMP peak elutes around scan number 232 and has the characteristic IMS drift time of roughly 13 msec. IMS has some desirable characteristics for these studies. Insensitivity of the IMS toward the pentane mobile phase allows a relatively large amount of this mobile phase solvent to be introduced without perturbing the signal baseline. Also, IMS is extremely sensitive toward the types of compounds we wish to detect.

Typically, low picogram to high femtogram quantities of the phosphate, phosphonate, organic nitro, organic nitrate, and halogenated alkane compounds can be detected.

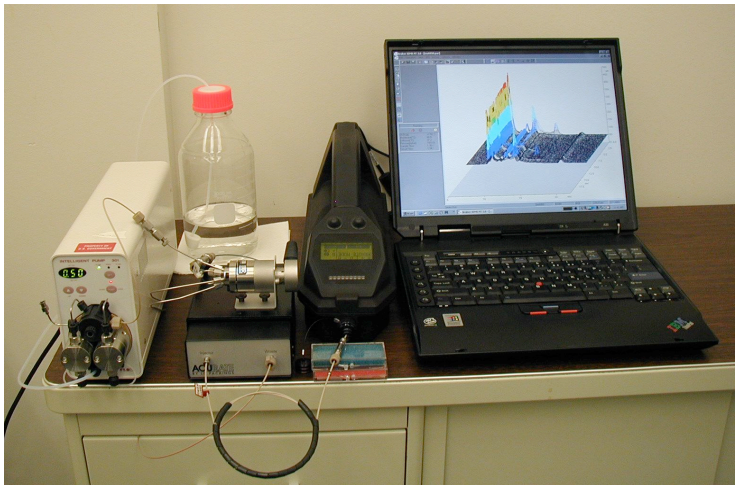

Figure 7. Photograph of on-line interface between the MIP separation and IMS detection.

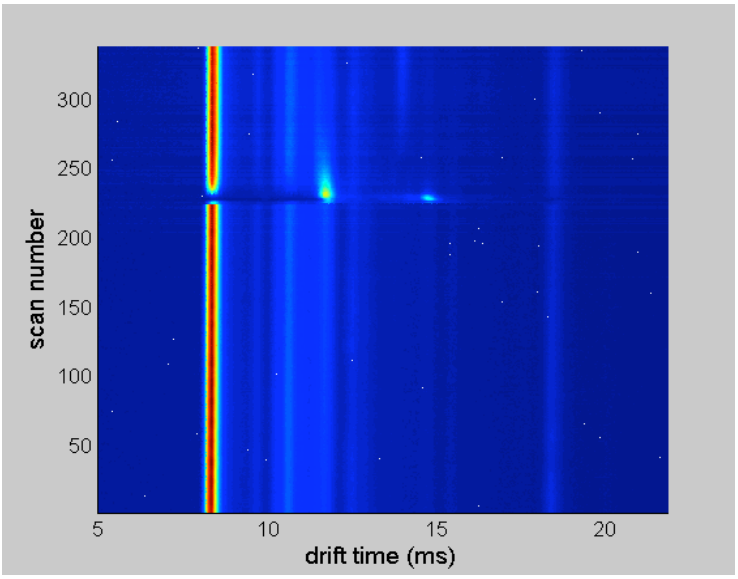

Figure 8. Contour plot showing an on-line separation of a DIMP standard. DIMP signal is at the intersection of the characteristic retention time (scan number 230) and IMS drift time (13 msec).

This work is being expanded to address real-world analysis problems such as the analysis of TBP in complex environmental extracts. The Raid-M Basic (Brucker Daltonics, Billerica, MA, USA) was used for the MIP/IMS DIMP analysis proof-of-principal experiments. One limitation of this IMS instrument is that inlet heating has a temperature limit of about 60°C. Higher interface temperatures would enable analysis of semivolatile analytes, such as TBP and explosives. Recently, we gained access to several IMS instruments that are on loan from the Transportation Security Administration–two Plastec Explosives Detector instruments (Graseby Security, Hertfordshire, England) and an Ionscan instrument (Barreinger Instruments, Warren, NJ, USA). One of these instruments, depending on which proves most suitable for the studies, will be dedicated to the MIP-IMS interface studies. Although not as portable as the instruments shown in Figures 5 and 7, the TSA spectrometers are capable of operating at elevated temperatures, making them suitable for TBP studies. We are presently incorporating the spectrometers with LabView software to allow instrument control and data acquisition. The advancement of the MIP/IMS interface and a meaningful demonstration with TBP are a top priority activity.

RESEARCH CONTRIBUTION ACKNOWLEDGEMENT

Several researchers provided significant input to the studies. Gary M. Mong's contributions to the synthetic preparations of styryl hexafluorodimethylcarbinol and the G-series nerve agent hydrolysis products, as well as general problem solving discussions, helped advance many of the studies. Mindi Yan, Portland State University, provided insights during the academic project review that led directly to the proposal to use the phase inversion precipitation approach for synthesizing explosive-specific MIPs. Richard N. Lee is currently contributing to matrix challenge experiments using the G-series nerve agent hydrolysis instrument. Finally, David A. Atkinson's contribution to the MIP/IMS interface studies is appreciated.

PROJECT ACCOMPLISHMENTS

Below is a list of publications, presentations, and invention disclosures that have resulted from Task 2 of the Ultraselective Sorbent Project:

Harvey, SD, GM Mong, JS McLean, JK Fredrickson, SM Goodwin, DA Atkinson, NB Valentine, and CE Petersen. *Imprinted Media for Highly Selective Capture of Chemical, Biological, and Organic Nuclear Signature Threats*, a poster presented at the Detector/Sensor Research and Technology for Homeland and National Security Workshop, Gatlinburg, TN, September 14-16, 2004.

Mong, GM, SD Harvey, and JA Campbell. 2004. *Simplified Synthesis and Purification of the G-Series Nerve Agent Hydrolysis Products: Cyclohexyl Methylphosphonic Acid and Isopropyl Methylphosphonic Acid*, Phosphorus, Sulfur and Silicon (in press).

Harvey, SD. 2003, *Molecularly Imprinted Polymers for Selective Analysis of Chemical Warfare Surrogate and Nuclear Signature Compounds in Complex Matrices*, J. Sep. Sci., (submitted October) .

Skaggs, R, T Straub, B Wright, C.Bruckner-Lea, and S Harvey. 2004. *Development of the Next*

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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.

Harvey, SD. 2004. *Novel Trace Enrichment Instrument Based on Molecularly Imprinted Polymers (MIPs) for Analysis of G-series Nerve Agent Hydrolysis Compounds (and Related Phosphoric Acid Half-acid Esters) in Aqueous Samples*, Invention Disclosure #14442-E, Pacific Northwest National Laboratory, Richland, WA.

Harvey, SD and GM Mong. 2004. *Preparation and Use of Molecularly Imprinted Polymers (MIPs) and Related Sorbents With Specificity Toward Nitro-containing Explosives*, Invention Disclosure #14397-E, Pacific Northwest National Laboratory, Richland, WA .

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APPENDIX I

PNNL Invention Disclosure #14397-E

Preparation and Use of Molecularly Imprinted Polymers (MIPs) and Related Sorbents With Specificity Toward Nitro-containing Explosives

Inventors: Scott D. Harvey and Gary M. Mong, Witnesses: Bob W. Wright and David A. Nelson,
Submitted on 5-11-2004.

Preparation and Use of Molecularly Imprinted Polymers (MIPs) and Related Sorbents With Specificity Toward Nitro-containing Explosives

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Invention Summary:

A variety of important applications depend on the selective concentration and detection of explosives. This invention describes preparation of molecularly imprinted polymers that are capable of highly selective capture of nitro-containing explosives. The novel polymers developed in this invention report are based on polymer coatings or sorbents that are known to exhibit selectivity toward explosives; however, materials described here are prepared for the first time as imprinted polymers. These polymers are imprinted with a specific explosive and are expected to have an affinity for the target explosive beyond that exhibited by the nonimprinted polymer. The exceptional affinity of the imprinted polymer will allow selective retention of specific explosives from strong organic solvents. The non-imprinted polymers will display only minimal retention under these conditions. Therefore, a novel property of the imprinted polymers described here is the ability to capture explosives with exceptional selectivity directly from organic extracts (or organic eluates from traditional sorbent clean-up columns) that originate from complex environmental or biological samples.

A secondary area that is conceptually addressed in this invention report (without experimental involvement) is the extension of functional monomers we propose to incorporate into MIPs (or slight chemical variations thereof) for surface derivatization of self-assembled monolayers on mesoporous supports (SAMMS). These materials should exhibit selectivity toward explosives; however, the selectivity will not be as high as the MIPs since the SAMMS materials are not imprinted. SAMMS are characterized by an extremely high surface area (700 m^2/g), associated high sample capacities, and low sorbent bed pressure drops. All these properties make SAMMS ideal for moderately selective large-volume air sampling [1-3].

Although we anticipate performing proof-of-principle experiments on chromatographic sorbent beds using high-performance liquid chromatography (HPLC), the materials described in this invention can also be used as sensor coatings [*i.e.,* surface acoustic wave (SAW) or microcantilever sensors].

Proof-of-principle experiments for the MIPs will demonstrate selective retention of the explosive RDX on two different MIPs relative to the nonimprinted control polymers. RDX is used as a representative nitro-containing explosive. One of the polymers is based on a strong hydrogen bond acidic functional monomer unit that has been incorporated into a styrene/divinylbenzene copolymer. The other polymer is based on the chromatographic sorbent Porapak R. Porapak R is a styrene/divinylbenzene copolymer that also contains 1-vinyl-2-pyrrolidone as a functional monomer.

Intended Use:

As further described below, the intended use for these high affinity materials will be either as selective sampling sorbents or as selective coatings for sensors. Both approaches have numerous applications that are summarized below.

If implemented as a selective sampling sorbent, the MIP can be used for direct air sampling or, alternatively, as a secondary sorbent to be situated immediately behind a more traditional nonselective air sampling sorbent (such as XAD-2 resin) [4]. In the latter case, the trace explosive, along with the organic matrix interferences from the air sample, are transferred to the MIP using an organic solvent or a supercritical fluid. In this case, selective capture of the target explosive occurs on the secondary MIP stage. In either arrangement, the matrix interferences are not retained on the MIP and can be diverted to waste. The end result is that enormous matrix discrimination takes place on the MIP resulting in a highly purified sample. The high purity of the sample allows simplification of subsequent analytical instrumentation required to perform high integrity analyses at extremely low detection limits. The enormous matrix discrimination, obtained up-front during sampling or immediately thereafter, makes possible the construction of compact field-portable instrumentation.

Once selective capture has been accomplished, the explosive is released from the MIP sorbent and either directly detected or processed first through a single-stage chromatographic column, if required, to separate out minor impurities before being detected. Suitable detectors will have a selectivity that is orthogonal to the MIP, have a rapid response time, and be highly sensitive toward the explosive. Possible

detectors include ion mobility spectrometry, electron capture, mass spectrometry, electrochemical, or thermal energy analysis detection.

The two functional monomers used for MIP preparation, or close structural analogs, could also be used for preparation of self-assembled monolayers on mesoporous supports (SAMMS). Surface functionalization requires that the monolayers self assemble (usually thorough van der Waals forces) and have the necessary functionalization to form covalent bonds with surface silanol groups on the SAMMS. Therefore, structural analogs of the MIP functional monomers described above, that contain an appropriate reactive group for covalent attachment to the SAMMS surface, could be used. Silane functionalities have been used for covalent bonding through hydrosilylation reactions [2], although a diverse repertoire of well-defined surface chemistries designed for preparation of silica-based bonded HPLC supports would also be applicable. The combination of a SAMMS primary air sampling stage with a MIP secondary stage would be expected to be particularly powerful since this instrumental arrangement exploits the high sample capacity and low pressure drop of SAMMS sorbents with the enormous selectivity of the MIPs stationary support.

Alternatively, the polymers can be placed on the sensor surfaces to allow selective detection of specific explosives. Possible sensors include SAWs or microcantiliever devices [5,6]. These devices may be most effective when operated as part of a sensor array that contains other coatings having differing selectivity toward the target analyte [7].

This invention addresses numerous applications that are critical for national and homeland security. One of the high priority applications is to exploit the high selectivity of these materials for developing bomb detection instrumentation. This instrumentation could be used for detecting explosives in luggage, cargo holds, and shipping containers. Additional applications involve detection of unexploded ordnance in mine fields and artillery ranges.

Environmental applications involve analysis of vegetation and crops that have been exposed to munitions [8,9,10]. Plant exposure occurs through growth in munitions-tainted soil or by irrigation with munitions-contaminated aquifer water. Plant uptake can result in bioaccumulation of the parent explosive and/or possible formation of toxic metabolites. Detection of trace level munitions in plants requires selective separations due to the extreme complexity of the biological matrix. MIPs should be

particularly suited for isolating explosives from complex plant extracts due to their high selectivity. For example, one would expect to obtain selective retention of explosives even in extremely strong mobile phases that will not allow retention of most organic compounds. This provides an effective strategy to clean up highly complex samples that contain RDX; one simply needs to inject the sample and collect the retained fraction that will contain all the RDX and very little matrix background interferences. Explosive-specific MIPs would also be useful for the monitoring pollution plumes of explosives in aquifers at ultratrace concentrations [11]. Additional applications would exploit the high sample capacity of SAMMS for environmental remediation of explosives from soil or water streams.

Current State-of-the-art and Approach:

Very little research has focused on preparing MIPs that are specific toward explosives. Most work related to this topic has originated from George Murray's group at Johns Hopkins University [12,13]. This group created imprinted sensor coatings specific for certain explosives. These coatings incorporated both molecular recognition capabilities and a transduction mechanism to serve as a basis for detection. Murray's group has proposed two approaches. The first approach is described in the peer-reviewed literature and is based on formation of a charge-transfer complex between a tertiary amine functional monomer unit and a nitroaromatic explosive [12]. The second approach is only described in the patent literature and involves interaction of a nitroaromatic explosive with a porphyrin functional monomer [13]. Both techniques detect the explosive based on an absorption wavelength shift that occurs upon interaction with the explosive. These approaches are very limited in scope since they apply only to nitroaromatic compounds. Nitroaromatics comprise only a small proportion of the explosives class.

Our approach will not be as restrictive as Murray's due largely to our chromatographic emphasis that allows for downstream detection. In other words, our polymers do not need to incorporate a concurrent transduction mechanism. Therefore, we have more flexibility in the design of the imprinted polymers. A significant advantage over Murray's work is that our polymers are designed to generally recognize nitro-containing explosives (*i.e.*, aliphatic and aromatic nitramines, nitroaromatics, aliphatic nitro compounds including polynitro cyclical cage explosives, and organic nitrates). As previously mentioned, this is a much broader group than the narrowly focused nitroaromatic explosives that Murray's work addresses. Our approach is based on the demonstrated

selective sorption of explosives on either Porapak R or strong hydrogen bond acidic polymers. Extensive work has been done in both these areas [11,14-17]; however, imprinted variants of these polymers have not been described. The new imprinted polymers will have significantly higher selectivity than the nonimprinted variants. This enhanced selectivity can be exploited for designing compact field-portable analytical instrumentation for highly selective trace analysis.

Synthesis of Polymers:

For every MIP prepared, a control polymer is made in parallel at the same time using the same reagents. The difference between the control and the MIP is that the control reaction mixture lacks the explosive template molecule and, therefore, the resulting control polymer cannot be imprinted. Since both the control and the MIP contain the same polymer matrix, any differenced in analyte interaction should be due to the presence of the imprint on the MIP.

Strong hydrogen bond acidic polymer

Styryl hexafluorodimethylcarbinol synthesis: This functional monomer material was prepared according to the methods described by Snow et al. [15]. Briefly, a Grignard reagent was prepared from chlorostyrene in tetrahydrofuran and then reacted with hexafluoroacetone gas. This reaction was conducted under reflux conditions using a cryogenic condenser held at –78°C. The product was purified from the reaction mixture by adding hydrochloric acid and extracting the product-containing oil into chloroform. Next, the sodium salt of the product was formed by adding sodium hydroxide. The organic solvents were then stripped from solution by rotary evaporation. At this point the product was then protonated by making the solution strongly acidic (HCl added until pH < 3.0) and the product extracted into chloroform. Crude product was obtained after removal of chloroform by rotary evaporation. The last step of purification involved vacuum distillation. Unreacted chlorostyrene starting material distilled first followed by the styryl hexaflurodimethylcarbinol product (68°C at 2 torr). Purity of the final product was estimated at 99% by GC/MS.

Several polymer synthesis reagents required purification before use. RDX was used as the template explosive. This material was obtained from a standard military source and purified by several recrystallizations from acetone. Since three functional monomer units are expected to interact with each molecule of RDX, the quantity of explosive was one-third the molar amount of the stryryl hexafluorodimethylcarbinol. 2,2'-Azobisisobutyronitrile (AIBN) was used as the polymerization initiator and was recrystallized from methanol prior to use. Polymerization inhibitors were removed from styrene

and divinylbenzene by passing through a commercially available column designed for this purpose. Inhibitor was removed from 1-vinyl-2-pyrrolidone by vacuum distillation.

Polymer synthesis: Initial attempts at making polymers used a 3% weight of divinylbenzene cross linker with 8.6% weight of styryl hexafluorodimethylcarbinol functional monomer. The resulting RDX-imprinted and control polymers were rubbery. From literature references [10,15], as well as the known properties of Amberlite ion-exchange resins, one would expect a brittle polymer using 3% cross-linking. We hypothesize that the styryl hexafluorodimethylcarbinol has a lower polymerization reactivity compared to styrene due to the fluorine electron withdrawing properties that leave comparatively little electron density in the vinyl functionality. Therefore, the styryl hexafluorodimethylcarbinol functional monomer unit may not be as readily incorporated into the polymer. To compensate for this possible lower reactivity, we increased the cross linking to 12% by weight.

For the MIP polymer the following reagents were added to a reaction tube: 1) 131 mg RDX [0.590 mmole], 2) 485 mg styryl hexafluorodimethylcarbinol [0.353 ml or 1.80 mmole], 3) 4.5 g styrene [4.97 ml or 43.2 mmoles], 4) 680 mg divinylbenzene [0.744 ml or 5.22 mmole], 5) 5.0 ml of acetonitirile, and 6) 60 mg AIBN. The control reaction mixture contained all the materials listed above except the RDX template. After thoroughly mixing, the solutions were purged with nitrogen for 5 min, the tube sealed, the mixture subjected to ultrasonic agitation for several hours, and polymerization performed by heating at 60°C for 24 hours.

Porapak R based MIP

Because we did not expect to have the same reactivity problems with the vinylpyrrolidone polymer, we dropped the crosslinking to 9% divinylbenzene by weight. Again, due to the expected interaction stoichiometry, a 3:1 functional monomer to RDX molar ratio was used for the imprinted polymer.

For the MIP polymer the following reagents were added to a reaction tube: 1) 131 mg RDX [0.590 mmole], 2) 200 mg 1-vinyl-2-pyrrolidone [0.192 ml or 1.80 mmole], 3) 4.5 g styrene [4.97 ml or 43.2 mmoles], 4) 465 mg divinylbenzene [0.508 ml or 3.57 mmole], 5) 5.0 ml of acetonitirile, and 6) 60 mg AIBN. Again, the control contained all the materials except the RDX template. Polymerization was accomplished at 60°C as described in the previous section.

Sorbent Preparation and HPLC column preparation

Polymers that contained 9 to 12% divinylbenzene cross linker were very hard materials that were not rubbery. The bulk polymers were processed to sorbents by freezing in liquid nitrogen to make brittle and then grinding in a mortar and pestle. These materials were difficult to grind and, because of material loss associated with extended processing and manual manipulation, polymers for proof-of-principle experiments were ground only to relatively large particles ($d_p < 210$ μm). In contrast, we normally prefer to continue the grinding until the sorbent particles pass a 80-μm screen sieve. Once sorbent particles less than 210 μm were obtained, they were repetitively washed with acetone and the fines decanted to waste. The particles were then dried under house vacuum (approximately 100 torr) for several days, again sieved through a 210-μm sieve, and sent to Higgins Analytical (Mountain View, CA) to be packed into 30-mm x 4.6-mm i.d. columns.

HPLC Evaluation:

Columns were installed on an HPLC system that utilized an Intelligent pump (Model 301-S, Japan) to deliver pure acetonitrile mobile phase at flow rate of 0.5 ml/min. An RDX standard (23 ppm) was introduced to the columns through a Rheodyne (Model 7125, Alltech, Deerfield, IL) injection valve (24.3 μl) and RDX detected by UV absorption at 254 nm (Isco Model CV4 Detector, Lincoln, NE). Chromatograms were recorded on a HP-3390A (Agilent, Palo Alto, CA) integrating recorder.

Chromatographic comparison of a MIP and its corresponding control sorbent should be a straightforward experiment. Assuming that uniform polymerization takes place in the formation of both the MIP and control polymers, differences between target compound retention can be attributed solely to the imprint retention mechanism. Figure 1 presents chromatograms of RDX on the strong hydrogen bond acidic control and MIP columns. The retention time of RDX is 40.0 and 55.3 seconds on the control and MIP columns, respectively. The presence of an additional 15.3 seconds of retention on the MIP provides apparent evidence for an active imprint retention mechanism. However, several inconsistencies were noted during this experiment including difficulties in packing the columns and a broader RDX peak at lower retention time on the control compared to the MIP column (see Figure 1). These inconsistencies prompted a more thorough evaluation of RDX using k' values. The advantage of using k' is that these values are independent of column packing efficiencies. The k' value is calculated by subtracting the column dead time (determined by injections of 1,1,2-trifluortrichloroethane) from RDX retention and dividing this value by the dead time.

The k' values are listed in Table 1. This experiment yielded several important and interesting results. First, the positive k' values for RDX on the control sorbents (about 0.3 for both columns) indicate an active retention mechanism for both polymers relative to a non-retained compound. Given that the experiments were conducted in an extremely strong mobile phase, these values indicate a substantial interaction between RDX and the functional monomer units. This experiment also gave the surprising result that the MIPs retain RDX less than the corresponding control sorbents. Several possible explanations have been proposed to account for these results including the question of whether 1,1,2-trifluorotrichloroethane is an appropriate non-retained dead volume marker. Since this Freon exhibited the least retention out of a series of additional compounds tested (including toluene, chloroform, and 1-chlorooctane) it appears it is an appropriate marker. Another possibility was that the template may not have been completely removed from the MIP and, therefore, the MIP sorbent had recognition cavities that were obscured and unavailable for interaction. This explanation is unlikely since both acetone, used to remove the fines during sorbent processing, and the acetonitrile mobile phases are both excellent solvents for RDX. Yet another possibility is that pre-association of the template and the functional monomer could decrease the reactivity of the vinyl group, resulting in less efficient incorporation of the monomer into the MIP polymer compared to the control. This explanation is not likely, as this specific interaction would be expected to increase, not decrease, the vinyl reactivity, (if it alters vinyl reactivity at all). The final explanation is based on the fact that nitro groups are known to quench free radicals. Since polymerization with AIBN is based on a free radical mechanism, it seems likely that the presence of RDX in the MIP reaction inhibits polymerization resulting in a polymer that has less efficient incorporation of the functional monomer, and a different overall structure, than the control.

Several solutions to the MIP polymerization problem are being considered. One possible approach is to use an initiator that is not based on free radicals. Since initiation using gamma irradiation proceeds mostly by a free radical mechanism, this approach was not considered a viable alternative. One possibility is to initiate polymerization with a Lewis acid (boron trifluoride diethyl etherate), an approach that is used in the commercial polymerization of polystyrene. The presence of nitro groups does not affect this Lewis acid initiation mechanism, and both the template and reagents should be chemically stable in the presence of this acid. The end result should be a uniform polymerization achieved between the control and MIP polymer. One concern, that at present remains unresolved, is that interactions between the Lewis acid and the basic nitro groups may diminish pre-association of the functional monomer with the explosive. Other approaches are also under consideration. Given the strong interactions achieved between the functional monomer units and RDX (see Table 1), we feel confident that a uniformly polymerized and imprinted material will offer selectivity above that available from the control materials.

Extensions of Invention:

Strong hydrogen bond acidic polymers are capable of selectively interacting with analytes that have a basic character [7,15-17,19]. This compound category includes organic compounds that contain nitro, nitrate ester, phosphoral, or ketone groups. It is anticipated that compounds that contain these functionalities can be imprinted using the techniques described herein. The phosphoral group is of particular importance to national security as this encompasses the nerve agent and insecticide classes of compounds. Further work is planned to prepare and evaluate imprinted polymers specific towards the G-series nerve agents based on the strong hydrogen bond approach. Other compounds that contain basic functionalities likewise have fundamental importance to national security. It is also anticipated that other strong hydrogen bond acidic functional monomer units, besides styryl hexafluorodimethylcarbinol, would be useful for preparing imprinted polymers toward basic analytes [7,15-17,19].

Research in our group has demonstrated that Porapak R has selectivity toward nitro groups [11,20], as well as nitroso and nitrosamine functionalities [21,22]. It has also been hypothesized that this recognition mechanism would be active for organic nitrate and nitrite ester compounds [23]. It is expected that many of these compounds could be imprinted with the techniques described in this invention report by using vinylpyrrolidone as the functional monomer.

Funding Support and Division of Intellectual Property:

These intellectual property (IP) concepts were developed entirely through DOE funding. Specific project support for this IP was partly through the Imprinted Media project (F52301) funded by internal laboratory-level Laboratory Directed Research and Development funds (10%) as well as through the Ultraselective Sorbents (F45028) NA-22 project (90%). Scott D. Harvey is the Principal Investigator of both these projects. IP contributions to the concept are divided as follows: Scott D. Harvey (80%) and Gary M. Mong (20%).

Concept Origin Information:

Concept origin can be traced to inclusion in two different proposals written by S.D. Harvey. The first is the Imprinted Media FY04 LDRD proposal for the Homeland Security Initiative that

proposes creation of an imprinted variant of Porapak R, using 1-vinyl-2-pyrrolidone as the functional monomer, for selective isolation of explosives. This proposal was submitted on July 11, 2003. The general idea of using Porapak R based sorbents came from a series of previously submitted invention reports [17-20] and a literature publication [8]. The general concept of making explosive-specific MIPs was again mentioned in the Task 2 portion of the FY04 NA-22 Lifecycle plan for the Ultraselective Sorbents project. The specific concept for preparing explosive-specific MIPs using strong hydrogen bond acidic functional monomer units was first detailed in an e-mail to Jay Grate on October 17, 2003 (memo is attached). This memo also discusses the Porapak R approach. Both imprinted polymer designs were further elaborated during an on-site NA-22 project review presentation given by S.D. Harvey to Les Pitts on January 20, 2004. Numerous PNNL staff members were in attendance for this presentation including Robert Clemmer, Jay Grate, and Thomas Zemanian. A hard copy of the 1/20/04 PowerPoint presentation is attached to document the level of conceptual development. The first formal written description of this invention is this invention report. The first experimental data that provides support for the concepts presented herein are signed and dated chromatograms collected by S.D. Harvey on 3/8/04.

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Table 1. k' values for RDX on the MIP and control columns using acetonitrile as a mobile phase.

Polymers	k' for RDX
Vinylpyrrolidone	
Control	0.275 ± 0.006
RDX-specific MIP	-0.0050 ± 0.0001
Hexafluorodimethylcarbinol	
Control	0.318 ± 0.005
RDX-specific MIP	0.177 ± 0.002

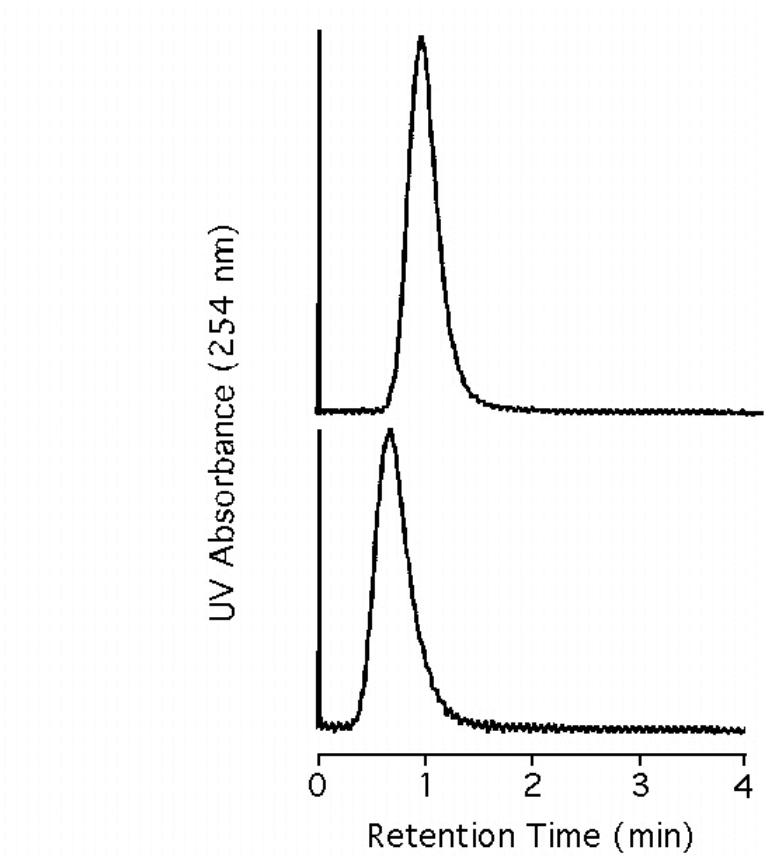

Figure1. Chromatograms of RDX on a column packed with a styryl hexafluorodimethylcarbinol-based MIP imprinted with RDX (top) compared to the corresponding nonimprinted control polymer (bottom). Mobile phase was pure acetonitrile delivered at 0.5 ml/min. RDX was detected by UV absorbance at 254 nm. Similar selective retention of RDX relative to the corresponding control was observed on a different RDX-specific MIP that was based on a vinylpyrrolidone functional monomer (see text for details).

APPENDIX II

PNNL Invention Disclosure #14482-E

Scaffold Imprinting for Preparing Polymers Imprinted with Nitro-Containing Explosives

Inventors: Scott D. Harvey and Mingdi Yan, Witnesses: Michael A. Lind and Bob W. Wright, Submitted on 8-13-2004.

B. 2

Scaffold Imprinting for Preparing Polymers Imprinted with Nitro-Containing Explosives

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Pacific Northwest National Laboratory[1] and Portland State University[2]

Introduction:

Molecularly imprinted polymers (MIPs) display highly selective interactions toward their templates that can rival antibody/antigen interactions [1,2]. In many ways the MIP/template interaction is complementary to antibody/antigen interactions. While antibodies operate best in aqueous buffers, MIPs display optimal efficiency in nonaqueous solvents [3,4]. The relative ease of preparation and the storage stability of MIPs are distinct advantages of using MIPs over antibodies. This invention report focuses principally on non-covalent imprinting techniques for chromatographic analysis. Imprinting using reversible covalent recognition is also possible although this approach is rarely used for chromatographic studies due to the sluggish association/disassociation kinetics. An extension of the work presented below would include covalent or hybrid covalent/non-covalent recognition mechanisms for applications that do not require fast kinetics.

Several publications describe preparation of MIPs towards explosives; however, no experimental data has been presented [5,6]. Definitive proof-of-principle experiments must demonstrate enhanced capture of the target explosive relative to a non-imprinted control polymer. The conceptual approaches developed in the literature have exclusively targeted the nitroaromatic group of explosives, a small proportion of the entire explosives class.

Previous work at PNNL focused on preparing imprinted variants of polymers known to have selectivity toward explosives [7(PNNL Invention Report #14397-E)]. Two types of polymers were prepared, one was based on Porapak R (a styrene/divinyl benzene polymer that contains vinylpyrrolidone), and a second polymer that consisted of a styrene/divinylbenzene polymer that contained a strong hydrogen bond functional monomer (styryl hexafluorodimethylcarbinol). MIPs imprinted toward RDX and the corresponding controls were synthesized. Chromatographic evaluation of these sorbents gave the surprising result that the MIPs actually provided less retention than the controls.

This phenomenon was traced to the free radical quenching properties of the nitro group in the template that resulted in less efficient polymerization in the MIPs compared to the control polymers.

The purpose of this invention report is to explore alternative polymerization methodologies for synthesizing MIPs that will yield uniform polymers between the MIP and control. We feel the choice of functional monomers used in previous work was scientifically sound and, provided that consistent polymerization could be achieved, we are confident that MIPs prepared with these monomers would display selective capture over the control polymers. Toward this end, we propose to explore other scaffold imprinting techniques to achieve consistent polymerization between the MIP and control polymers.

As discussed above, consistent polymerization between MIPs imprinted with nitro-containing explosive templates and controls cannot be based on free radical initiation. A previous invention report addresses this issue and suggests polymerization of the vinyl functionalities using Lewis acid initiation as a possible solution [7]. The present invention report describes other methods based on the use of preformed polymers as scaffolds. Chemical condensation cross-linking methods may be used in concert with this approach. One resounding advantage for the preformed polymer approach is that explosives are not subjected to free radical initiation or the harsh chemical conditions associated with polymerization. As an example of the preformed polymer imprinting technique, we will discuss at length the phase inversion polymerization methodology. This discussion is intended as a specific example of a much broader approach that utilizes preformed polymers. Specific additional approaches will be discussed in the end of the "Concept Extension" section of this disclosure. Besides the preformed polymer approach, another emphasis of this disclosure deals with preparation of polymers using condensation polymerization reactions. As this technique can be used to cross-link existing polymer chains to give an insoluble polymer, condensation reactions can serve a hybrid function within the preformed polymer approach context. Chemical condensation cross-linking methods provide an alternative to the free radical initiation; however, success with this technique depends on the reaction conditions being sufficiently mild that the template explosive is not chemically altered.

A good example of how the preformed polymer approach can be implemented is given by the phase inversion precipitation methodology. In this approach, a relatively

low molecular weight polymer (about 1.5 x 10^5 Da) is synthesized using standard free radical initiation techniques in dimethy sulfoxide (DMSO) [8-12]. Initial descriptions of this work focused on co-polymerization of acrylonitrile with acrylic acid [8]. Later work demonstrated the feasibility of substituting vinylpyridine isomers or styrene for the acrylic acid functional monomer [9]. Inclusion of polyacrylonitrile in these polymers promotes polymer cohesion through the strong dipole interactions between the nitrile groups. Once formed, the polymer was split into two portions, one served as the control, and the other was allowed to pre-associate with a template. Both polymers were then placed into a water environment to induce precipitation. Precipitation essentially locked the MIP into a preconfigured confirmation dictated by association with the template, whereas the control, since no template was present, was not locked into a specific conformation. The template was removed leaving behind specific recognition sites in the MIP that had high specificity and affinity toward the compound used to imprint. The chemical, physical, and mechanical properties of the films produced by this technique are highly dependent on the original molecular weight of the polymer.

Proposed Approach and Concept Extension:

Early research demonstrated the use of preformed biological polymers for molecular imprinting [13,14]. These studies showed that proteins [13] and dextrans [14] can be imprinted with target molecules by lyophilizing in the presence of template. Freeze drying locks the conformation of the biological polymer such that it selectively recognizes the template. This conformation is stable provided that the polymer is not exposed to water. Water causes the polymer to revert to the preferred configuration normally exhibited in aqueous solution. More recently, imprinted biological polymers have been prepared that have a stable imprint configuration in water. These polymers are formed by associating the template with chitosan followed by cross-linking with glutaraldehyde [15].

Preformed polymers have other advantages in addition to avoiding complications of polymerizing in the presence of template. Multiple template binding sites are available resulting in stronger interactions than is possible using a traditional functional monomer. Although each of the individual interactions may be fairly weak, the overall interaction obtained with multiple recognition sites can be quite strong. Synthetic polymers offer advantages over biological polymers due to the greater diversity and flexibility of recognition entities that can be design into a synthetic polymer.

Other examples of imprinting with preformed synthetic polymers are given below. A variety of MIPs have been prepared with cross-linked poly(allylamine) [16,17]. For example, an oral pharmaceutical designed to capture bile acids is based on imprinted poly(allylamine) [16]. Polymers capable of recognizing glucose have also been prepared with this technique [17]. Synthetic hydrogen-bonded networks of synthetic polyamides can be used for imprinting [18]. Yet another example is an imprinted polyethersulfone polymer that recognizes templates through the formation of donor-acceptor complexes [19]. Further examples exist; however, free radical cross-linking of vinyl groups is required and, therefore, these approached are probably not appropriate for preparation of explosive-specific MIPs.

Under certain circumstances, chemical condensation cross-linking may be desirable. For example, one approach involves preassociation of a template with a macromolecular monomer in DMSO followed by cross-linking with diisocyanate with subsequent precipitation of the resulting polymer from acetone [20]. This example is presented because it involves a hybrid methodology featuring initial template association with a multifunctional macromolecular monomer, cross-linking to form a polymer in solution, followed by phase inversion precipitation to yield the MIP. In addition to diisocyanate, glutaraldehyde, diisocyanate, and epichlorohydrin crosslinkers have all been used in similar applications.

We propose to use similar techniques as described above to overcome polymerization consistency problems encountered with our previous work [7]. Initial studies will continue to probe vinylpyrrolidone or hexafluordimethylcarbinol (or other strong hydrogen bond acidic monomers) as functional monomers that are copolymerized with acrylonitrile. Advantages of the phase inversion precipitation technique stem from the fact that polymerization occurs before the imprinting step. This temporal separation between polymerization and imprinting steps assures that the control and MIP polymers will be uniform. The presence of nitro-containing groups in the template will have no bearing on the preformed polymer. Also, the method is extremely mild since the template is not exposed to harsh thermal or irradiation (ultraviolet or gamma initiation) conditions associated with initiation, or to chemically reactive species during polymerization. This curtails problems with template chemical transformation that are often countered when using traditional imprinting techniques. This attribute will allow imprinting of additional labile compounds such as nerve agents.

Intended Use:

The intended use for these high affinity materials will be either as selective sampling sorbents or as selective coatings for sensors. Both approaches have numerous applications that are summarized below.

If implemented as a selective sampling sorbent, the MIP can be used for direct air sampling or, alternatively, as a secondary sorbent to be situated immediately behind a more traditional nonselective air sampling sorbent (such as XAD-2 resin) [21]. In the latter case, the trace explosive, along with the organic matrix interferences from the air sample, are transferred to the MIP using an organic solvent or a supercritical fluid. In this case, selective capture of the target explosive occurs on the secondary MIP stage. In either arrangement, the matrix interferences are not retained on the MIP and can be diverted to waste. The end result is that enormous matrix discrimination takes place on the MIP resulting in a highly purified sample. The high purity of the sample allows simplification of subsequent analytical instrumentation required to perform high integrity analyses at extremely low detection limits. The enormous matrix discrimination, obtained up-front during sampling or immediately thereafter, makes possible the construction of compact field-portable instrumentation.

Once selective capture has been accomplished, the explosive is released from the MIP sorbent and either directly detected or processed first through a single-stage chromatographic column, if required, to separate out minor impurities before being detected. Suitable detectors will have a selectivity that is orthogonal to the MIP, have a rapid response time, and be highly sensitive toward the explosive. Possible detectors include ion mobility spectrometry, electron capture, mass spectrometry, electrochemical, or thermal energy analysis detection.

The phase inversion precipitation typically results in a polymer membrane deposited on a glass surface. Thickness of the film varies depending on conditions but is on the order of roughly 50 μm. This format is ideal for preparing certain sensor surfaces including SAWs [22]. Modification of methods cited in the literature can be expected to provide sub-micron

layer deposition for use with microcantilevers [23]. Both these sensor devices may be most effective when the MIP portion is operated as part of a sensor array that contains other coatings having differing selectivity toward the target analyte [24]. Additional sensor approaches can be envisioned for selective capture of the nitro-explosive followed by optical or photoacoustic detection of the explosive directly on the MIP polymer. For chromatographic studies, the polymer material can be removed from the glass surface and processed into a sorbent. Alternatively, precipitation could be performed directly on glass beads and the coated beads used as a chromatographic packing material.

This invention addresses numerous applications that are critical for national and homeland security. One of the high priority applications is to exploit the high selectivity of these materials for developing bomb detection instrumentation. This instrumentation could be used for detecting explosives in luggage, cargo holds, and shipping containers. Additional applications involve detection of unexploded ordnance in mine fields and artillery ranges.

Environmental applications involve analysis of vegetation and crops that have been exposed to munitions [25-27]. Plant exposure occurs through growth in munitions-tainted soil or by irrigation with munitions-contaminated aquifer water. Plant uptake can result in bioaccumulation of the parent explosive and/or possible formation of toxic metabolites. Detection of trace level munitions in plants requires selective separations due to the extreme complexity of the biological matrix. MIPs should be particularly suited for isolating explosives from complex organic plant extracts due to their high selectivity. For example, one would expect to obtain selective retention of explosives even in extremely strong mobile phases that will not allow retention of most organic compounds. This provides an effective strategy to clean up highly complex samples that contain explosives; one simply needs to inject the sample and collect the retained fraction that will contain all the explosive and very little matrix background interferences. Explosive-specific MIPs would also be useful for the monitoring pollution plumes of explosives in aquifers at ultratrace concentrations [28]. This analysis could proceed using a novel analytical approach that permits general MIP analysis of aqueous samples [29]. This development is significant since water is typically considered an incompatible matrix for most MIP sorbents.

Current State-of-the-art and Approach:

Very little research has focused on preparing MIPs that are specific toward explosives. Most work related to this topic has originated from George Murray's group at Johns Hopkins University [5,6]. This group created imprinted sensor coatings specific for certain explosives. These coatings incorporated both molecular recognition capabilities and a transduction mechanism to serve as a basis for detection. Murray's group has proposed two approaches. The first approach is described in the peer-reviewed literature and is based on formation of a charge-transfer complex between a tertiary amine functional monomer unit and a nitroaromatic explosive [5]. The second approach is only described in the patent literature and involves interaction of a nitroaromatic explosive with a porphyrin functional monomer [6]. Both techniques detect the explosive based on an absorption wavelength shift that occurs upon interaction with the explosive. These approaches are very limited in scope since they apply only to nitroaromatic compounds. Nitroaromatics comprise only a small proportion of the explosives class. It is important to note that although these concepts have been developed by Murray and co-workers, neither their literature publication nor patent applications present experimental data indicating success with these techniques.

Our approach will not be as restrictive as Murray's due largely to our chromatographic emphasis that allows for downstream detection. In other words, our polymers do not need to incorporate a concurrent transduction mechanism. Therefore, we have more flexibility in the design of the imprinted polymers. A significant advantage over Murray's work is that our polymers are designed to generally recognize nitro-containing explosives (*i.e.,* aliphatic and aromatic nitramines, nitroaromatics, aliphatic nitro compounds including polynitro cyclical cage explosives, and organic nitrates). As previously mentioned, this is a much broader group than the narrowly focused nitroaromatic explosives that Murray's work addresses.

Polymers we propose to prepare are based on Porapak R or strong hydrogen bond acidic polymers. Extensive work has been done in both these areas [28,30-33]; however, imprinted variants of these polymers have not been described. Research in our group has demonstrated that Porapak R has selectivity toward nitro groups [28,34], as well as nitroso

and nitrosamine functionalities [35,36]. It has also been hypothesized that this recognition mechanism would be active for organic nitrate and nitrite ester compounds [37]. It is expected that many of these compounds could be imprinted with the techniques described in this invention report by using vinylpyrrolidone as the functional monomer. Another approach would be to utilize strong hydrogen bond acidic monomers, such as styryl hexafluorodimethylcarbinaol, that are capable of selectively interacting with analytes that have a basic character [28,30-33,38]. This compound category includes organic compounds that contain nitro, nitrate ester, phosphoral, or ketone groups. It is anticipated that compounds that contain these functionalities can be imprinted using the techniques described herein. The phosphoral group is of particular importance to national security as this encompasses the nerve agent and insecticide classes of compounds. It is anticipated that other strong hydrogen bond acidic functional monomer units, besides styryl hexafluorodimethylcarbinol, would be useful for preparing imprinted polymers toward basic analytes [28,30-33,38]. Since template molecules are not subject to a harsh chemical reaction environment, the preformed polymer imprinting approach would be advantageous for imprinting nerve agents and other hydrolytically labile and otherwise unstable compounds.

Funding Support and Division of Intellectual Property:

This intellectual property (IP) was developed entirely through DOE funding. Specific project support for this IP was partly through the Imprinted Media project (F52301) funded by internal laboratory-level Laboratory Directed Research and Development funds (50%) as well as through the Ultraselective Sorbents (F45028) NA-22 project (50%). Scott D. Harvey is the Principal Investigator of both these projects. IP contributions to the concept described in this invention report are equally divided as follows: Scott D. Harvey, PNNL (50%) and Mingdi Yan, PSU (50%).

Concept Origin Information:

Concept origin can be traced to an NA-22 project review of the Ultraselective Sorbent project that was held at PNNL on May 25, 2004. Dr. Scott Harvey, the PI for the MIP half of the project, summarized work conducted on this project toward preparing explosive-specific MIPs. Progress toward this goal is also documented in a previous

PNNL invention report (#143797-E). Mingdi Yan was serving as an independent academic reviewer at this NA-22 review. Dr. Yan suggested that approaches similar to those described by Kobayashi and co-workers might avoid some of the polymer consistency problems that were encountered. This suggestion led to the preparation of this joint invention report.

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APPENDIX III

Manuscript Accepted for Publication

Simplified Synthesis and Purification of the G-Series Nerve Agent Hydrolysis Products: Cyclohexyl Methylphosphonic Acid and Isopropyl Methylphosphonic Acid

Manuscript by Gary M. Mong, Scott D. Harvey, and James A. Campbell, accepted for publication in *Phosphorus, Sulfur, and Silicon*

REVISED

Synthesis of Alkyl Methylphosphonic Acid Esters

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Simplified Synthesis and Purification of the G-Series Nerve Agent Hydrolysis Products: Cyclohexyl Methylphosphonic Acid and Isopropyl Methylphosphonic Acid.

Keywords: Cyclohexyl methylphosphonic acid; Isopropyl methyphosphonic acid; Methylphosphonic dichloride

Abstract

A synthesis and isolation scheme is described for producing mono alkyl esters of methylphosphonic acid based upon stoichiometric addition of alcohol to methylphosphonic dichloride. Solvent extraction applied to the reaction mixture to separates the mono alkyl esters in reasonable yield from dialkyl methylphosphonate and unsubstituted methylphosphonic acid. The singly alkylated materials are important hydrolysis products of the G-series nerve agents, and are not generally available from chemical suppliers.

Introduction

The development of analytical techniques for the detection and analysis of G-series nerve agent hydrolysis products (alkyl methylphosphonic acid esters) is hampered by the lack of available reference materials. With the exception of pinocolyl methylphosphonic acid ester, these materials are not commercially available (1). The rapid environmental hydrolysis of the G-series nerve agents produces a relatively persistent alkyl methylphosphonic acid ester as the primary product (2). Further hydrolysis affords methylphosphonic acid. The goal of this work is to devise a simple synthesis and separation for G-series nerve agent hydrolysis products that can be performed in most research laboratories, which results in material suitable for use in chemical warfare analysis methodologies.

Several published reports describe the synthesis of alkyl methylphosphonic acid esters. Chittenden *et al*. described a synthetic path to these compounds through methyl phosponothioic acids (3). This procedure is complex and requires the synthesis of starting materials. Timperly et. al. detailed their production through an Arbuzov synthesis (4). A direct route from partial hydrolysis of methylphosphonic dichloride has been successfully employed (5,6), with overall yields in the 12% to 47% range (5). This latter procedure requires the reaction of methylphosphonic dichloride with water resulting in a hydrolytically unstable, glassy anhydride that is used as a starting material for esterification. This method, as well as that of Timperly *et al*., apparently requires vacuum distillation to obtain pure product.

The synthesis and extraction techniques developed in this study can be applied using commonly available equipment and materials to rapidly produce high purity (98+%) alkyl methylphosphonic acid esters. An additional advantage of the method described here is that vacuum distillation is not required to obtain pure product.

Our approach is based on the direct reaction of alcohols (isopropanol and cyclohexanol) with methylphosphonic dichloride (Figure 1) in toluene solvent, followed by hydrolysis of the mono-chloride to produce the desired alkyl methylphosphonic acids. The desired product is isolated from other products by solvent extraction. The overall synthetic approach, combined with solvent extraction-based purification, results in gram quantities (typical yield of >30%) of high-purity (98+%) product. Inherent to the reaction is the production of transient pyrophosphonate species (Figure 2) which are easily hydrolyzed to the suite of idealized products shown in Figure 1.

Since the alkyl methylphosphonic acids are not amenable to direct gas-chromatographic (GC) analysis, reaction aliquots and samples from extractions were treated with ethereal diazomethane to create methyl derivatives (7) prior to analysis by gas chromatography-mass spectrometry (GC-MS). This technique has been applied to acidic phosphate-related components in nuclear defense wastes (8). Many of the alkyl organophosphous compounds have relatively straightforward mass spectral fragmentation patterns (8). As analytical methylation and silylation are likely to be used by other

researchers for analysis of monoalkyl methylphosphonic acid esters, GC and MS data for the compounds synthesized in this work is provided in Tables 1 and 2.

Figure 1 at bottom of text

Figure1. Reaction between methylphosphonic dichloride and alcohol resulting in a statistical production of products.

Figure 2 at bottom of text

Figure 2. Pyrophosphonate artifacts.

Results and Discussion

Attempts to form mono-alkyl phosphonic esters from direct reaction of alcohols with methylphosphonic dichloride creates a mixture of products. Upon hydrolysis of these mixtures, depending upon conditions, a statistical mixture of methylphosphonic acid, dialkyl methylphosphonates, and the desired mono alkyl methylphosphonic ester results (Figure 1). Linear alkyl pyrophosphonates of the type shown in Figure 2 were found when hydrolysis was done at ambient temperature. The linear pyrophosphonates either form during hydrolysis of the phosphoryl chlorides or result from *in situ* side reactions involving the alcohols which generate water. Pienaar (5) and Petrov (6) report that the reaction of water with methylphosphonic dichloride initially results in a cyclic dimer. In our approach, the alkyl ester is formed first, allowing alkylated linear species to form upon hydrolysis. The pyrophosphonate dimers are readily hydrolyzed by quenching the reaction with water at toluene reflux temperature.

The mixture of products (Figure 1) is purified by first partitioning the highly acidic aqueous phase from the toluene. The dialkyl methylphosphonate ester, as well as

most of the unreacted alcohol, is extracted into the toluene layer. The desired product and methylphosphonic acid form a separate layer.

An approach fashioned after the work of Hardy and Seargill (9) was used to remove methylphosphonic acid,. These authors found that dibutyl phosphate could be isolated from technical butyl phosphate mixtures by extracting with carbon tetrachloride. We have found that the difference in polarity between methylphosphonic acid and mono-alkyl methylphosphonic acid esters can be exploited in a similar fashion. Extracting the aqueous mixture with chloroform, and subjecting it to successive partitions with water affords a clean separation of the monoalkylester. The extracted mono-alkylated materials were nearly colorless and displayed GC traces in which >98% of the peak area was product, suggesting that further purification of these materials for use as analytical or experimental standards was not necessary. The overall levels of byproducts found in the extracts were comparable to that found in samples of previously available(1) commercial isopropyl methyphosphonic acid (<2%). Examination of the GC-MS trace for the cyclohexyl analogue afforded similar impurity levels; however no standard material was available for comparison. Finally, distillation under vacuum afforded only one fraction, with very little residue left in the distillation pot. GC-MS traces of this distillate were not markedly different from the extracts.

The solvent extraction efficiency was investigated by diazomethane derivatization and GC-MS analysis of each step. Initial removal of dialkyl methylphosphonates by toluene was found to be very efficient. Nearly all of the methylphosphonic acid remains in the aqueous phase upon partitioning with chloroform; however, the last traces of methylphosphonic acid were removed by back-extracting the chloroform with water. Isopropyl methylphosphonate has a moderate extraction distribution from chloroform to water, so two only two back extractions with water were done. The cyclohexyl compound did not exhibit significant loss during this step and three back extractions were done to remove methylphosphonic acid from the chloroform. In both cases, the end products were found to be 98+% mono-alkyl methylphosphonates in terms of the total peak area in the GC-MS traces; it is recognized, however, that ion production mechanisms in the mass spectrometer are very different for the components;

therefore TIC areas cannot be used for precise quantitation. The isopropyl methylphosphonate obtained from this procedure, when derivatized, exhibited a GC trace virtually identical to that obtained from the commercial sample.

Table 1 exhibits the GC, electron ionization (70 eV) mass spectrometry (EI-MS) data, and chemical ionization mass spectrometry (CI-MS) data for the methyl and trimethylsilyl derivatives of isopropyl and cyclohexyl methylphosphonates. Table 2 addresses the EI-MS and CI-MS data obtained for selected pyrophosphonates. The MS ions are listed in relative abundance to the base ion. Authentic isopropyl methylphosphonate (1), was used for GC-MS comparisons. We noted a discrepancy between previously reported data (5) for isopropyl methyl methylphosphonate and that which we obtained using diazomethane for derivatizing our isopropyl methylphosphonate product. In light of our experience in interpreting mass spectra of these types of compounds, the presence of readily interpretable fragment ions, and the verification of molecular weight by CI-MS, we believe the data reported in Table 1 and Table 2 to be correct.

Table 1. GC and GC-MS Data for Derivatives of Cyclohexyl and Isopropyl Methylphosphonic Acids.

Compound $(R_1O)(R_2O)P(O)CH_3$	GC Index	EI-MS m/z (Relative Intensity)	MW (CI)
R1 = CH_3 , R2 = isopropyl	982	93(100), 111(97), 137(38), 79(21)	152
R1 = TMS, R2 = isopropyl	1107	153(100), 169(20), 75(18), 195(8)	
R1 = CH_3, R2 = cyclohexyl	1383	111(100), 93(27), 79(9)	192
R1 = TMS, R2 = cyclohexyl	1492	153(100), 169(43),75(11)	

Table 2. GC and GC-MS Data for Pyrophosphonate Methyl Derivatives.

Pyrophosphonate Compounds $(R_1O)P(O)(CH_3)OP(O)(CH_3)(OR_2)$	EI-MS m/z (Relative Intensity)	MW (CI)
R1=R2= CH_3	157(100), 93(52), 172(22), 89(11), 202(9), 187(8)	202
R1= CH3, R2= isopropyl	171(100), 189(84), 111(88), 93(80),157(44), 79(30)	230
R1=R2=isopropyl	175(100), 157(95), 97(44), 79(33), 143(22), 201(16)	258
R1= CH3, R2=cyclohexyl	189(100), 111(80), 93(31), 157(28),171(16), 211(8)	

Based on our studies of isopropyl methylphosphonic acid and cyclohexyl methylphosphonic acids, we believe this synthesis would be generally applicable for producing alkyl methylphosphonic acid esters from other unhindered alcohols in the C_4-C_7 range.

Experimental

Alkyl methylphosphonic acid esters were prepared as follows. Alcohol [16.5 mmol of either cylcohexanol (1.64 g) or isopropanol (0.992 g)] was dissolved in 2.0 ml of dry toluene and placed in an addition funnel that had been swept with dry nitrogen. Methylphosphonic dichloride (2.18 g, 16.5 mmol), dissolved in 2.0 mL of dry toluene,was placed into a dried 100-mL round-bottomed flask maintained under dry nitrogen and equipped with a reflux condenser. Alcohol was added dropwise to the round-bottomed flask at ambient temperature. The reaction was stirred for 15 minutes before bringing to reflux at 105°C for 2 hours.

The reaction mixture was quenched while still warm (*ca.* 80°C) with two equivalents of water (0.60 mL), and then refluxed for an additional 10 minutes. The reaction was cooled and the organic phase (approximately 4.0 mL of yellow liquid) was separated from the denser aqueous layer.

Methylphosphonic acid was removed by adding 5.0-mL chloroform to the acidic extract and back-extracting the chloroform two times (isopropyl synthesis) or three times

(cyclohexyl synthesis) with 3.0-mL portions of water. The chloroform extracts were dried over sodium sulfate followed by quantitative removal of the chloroform under vacuum. Overall yields of purified extracted product were 38% for isopropyl methylphosphonic acid and 31% for cyclohexyl methylphosphonic acid (based on the weight of purified product remaining after removal of chloroform).

The temperature programmed GC retention indices for methyl derivatives and trimethylsilyl (TMS) derivatives were determined by a linear interpolation between bracketing *n*-alkanes on a capillary column coated with a 1.0 μm film of poly(5% diphenyl/95% dimethylsiloxane). Trimethylsilyl derivatives were created using bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (10). GC-EI-MS data (70 eV) was collected using a Hewlett-Packard Model 5890 GC (Agilent, Palo Alto, CA, USA) interfaced with a Hewlett-Packard 5972 mass spectrometer through a heated transfer line (280°C). Splitless sample introduction (injection port at 250°C) was followed by separation on a Restek (Bellefonte, PA, USA) Rtx-5 capillary column [30-m x 0.25-mm i.d., d_f = 0.25 m film of bonded poly(5% diphenyl/95% dimethylsiloxane) stationary phase]. After an initial hold at 45°C for 2 min, the column was programmed to 250°C at 8°C/minute and held at this final temperature for 5 min. This program was adequate to elute all expected products and dimers from the chromatographic system. GC-CI-MS data was determined using a Hewlett-Packard Model 5890 GC interfaced with a JEOL SX102/SX102 mass spectrometer (Peabody, MA, USA) operated in the CI mode (source temperature 190°C, source pressure 2 x 10^{-4} torr) using isobutane reagent gas to observe M+1 ions. The JEOL instrument used a column identical to that used to collect EI-MS data.

Acknowledgements

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Un-embedded figures

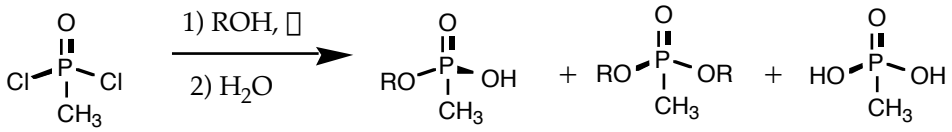

Figure 1 - Caption in text

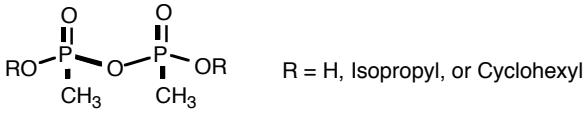

Figure 2 – Caption in text

C. 12

APPENDIX IV

PNNL Invention Disclosure #14442-E

Novel Trace Enrichment Instrument Based on Molecularly Imprinted Polymers (MIPs) for Analysis of G-series Nerve Agent Hydrolysis Compounds (and Related Phosphoric Acid Half-acid Esters) in Aqueous Samples

Inventor: Scott D. Harvey, Witnesses: Bob W. Wright and Michael A. Lind, Submitted on 6-30-2004.

D. 2

Novel Trace Enrichment Instrument Based on Molecularly Imprinted Polymers (MIPs) for Analysis of G-series Nerve Agent Hydrolysis Compounds (and Related Phosphoric Acid Half-acid Esters) in Aqueous Samples

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Invention Summary:

A variety of important applications depend on the selective concentration and detection of half-acid esters of alkylphosphonic or phosphoric acids. The G-series nerve agents hydrolyze in the environment rapidly to form the relatively stable half-acid esters of methylphosphonic acid. Detection of these compounds provides indirect evidence for nerve agent use and is often preferred over analysis of the parent nerve agent due to the higher stability of the half-acid ester in the environment. Other compounds of interest to national security fall into this general category of compounds. For example, bis(2-ethylhexyl) hydrogenphosphate is a half-acid ester of phosphoric acid that is a nuclear signature compound associated with uranium reprocessing.

This invention report describes a novel on-line analysis technique that will concentrate and selectively analyze target half-acid esters of methylphosphonic and phosphoric half acid esters from aqueous media. In this approach, the target compound is first concentrated from water on a traditional nonselective sorbent (reversed-phase or possibly ion-exchange supports). Water is then removed from the sorbent by a stream of dry gas before the sorbent is eluted with an organic solvent. The eluted fraction is directed to a molecularly imprinted polymer (MIP) sorbent where the target compound is very selectively sorbed while the interfering compounds pass through the sorbent to waste. The final sep in analysis is to elute the target compound from the MIP using a water-containing (or possibly an alcohol-containing) mobile phase followed by detection

of the half-acid ester. A variety of detection devices can be used including conductivity, potentiometric, mass spectrometric, or electrochemical.

Because of the moderate degree of selectivity obtained on the first capture stage combined with highly selective capture of the target by the MIP phase, this analysis can be expected to display extreme selectivity. Because of this exceptional selectivity, requirements for detection can be relaxed which allows development of simplified lightweight field-portable instrumentation. In common with all on-line instruments, sample utilization is highly efficient. This feature, when combined with the aqueous trace enrichment abilities and extreme selectivity, allows for ultra trace analysis.

Intended Use:

This invention can be used in a variety of ways. Instrumentation is compatible with collection of field samples on C-18 cartridges that can then be transported to the laboratory for analysis. The C-18 cartridge fits into simple instrumentation that can conveniently be placed at a variety of locations (*e.g.,* permanent or transient laboratories, hotels, embassies, and covert analysis locations). Alternatively, the on-line instrument can be assembled in a field-portable format that will allow continuous analysis of aqueous streams. This instrument can be used for the analysis of the aqueous microsurface layer that is reported to concentrate some organic compounds over the bulk solution concentration [1,2]. In this format, the instrument starts with a water fraction that already has an enhanced organic content for further concentration of the C-18 cartridge. Selective capture of the target from this complex mixture on the MIP will allow for very low detection limits. These capabilities are of high interest to intelligence and armed forces communities. In addition, this instrument imparts novel environmental monitoring capabilities for early threat detection related to national and homeland security.

Current State-of-the-art and Approach:

It is known that the half-acid esters can be preconcentrated on reversed-phase and ion-exchange chromatographic sorbents. The experimental work described below will utilize

trace enrichment on a reversed-phase sorbent. MIPs specific toward the methylphosphonic half-acid esters have been described in the literature [3]; however, these polymers have not been prepared using advanced suspension polymerization techniques described below [4]. The preparation techniques described here offer the significant advantages of producing monodisperse spherical macroporous particles that have more desirable chromatographic properties compared to the irregular bulk cast polymer materials that are usually used.

MIPs typically operate at peak efficiency in organic solvents, not in aqueous solutions. This invention incorporates MIPs in the analysis train by combining an initial nonselective trace enrichment from water using a C-18 sorbent followed by a solvent exchange to an organic solvent with subsequent transfer and selective capture of the target on the MIP phase. In order to effectively accomplish this analysis, all water must be removed from the nonselective C-18 phase with a stream of dry gas before the target is transferred to the MIP. Acetonitrile is the mobile phase used for transferring components from the nonselective phase to the MIP in the proof-of principle experiments presented below. MIPs are capable of very selectively retaining the target species from acetonitrile while the rest of the matrix components are diverted to waste. To detect the target compound, an aqueous- or alcohol-containing mobile phase is used to elute the half-acid ester from the MIP and transport the analyte to a detector. Due to the high selectivity of the MIP, it is anticipated the compound will be pure enough to be taken directly to a detector for quantification of the compound of interest. Detection can be based on a variety of methods that include, but are not limited to, conductivity, potentiometry, or electrochemical detection. In the case of electrochemical detection, the ester bond must be cleaved by a phosphonate or phosphate ester hydrolase with subsequent electrochemical detection of the resulting alcohol.

Synthesis of Polymers:

For each MIP that was prepared, a control polymer was made in parallel at the same time using the same reagents. The difference between the control and the MIP was that the control reaction mixture lacked the template and, therefore, the resulting control polymer cannot be imprinted. Since both the control and the MIP contain the same polymer matrix, any differences in analyte interaction should be due to the presence of the imprint on the MIP.

Reagents:

Most of the reagents required for syntheses were obtained from Aldrich: 1) trimethylolpropane trimethacrylate, 2) pinacolyl methylphosphonate (PMP), 3) chloroform, 4) 2,2'-azobisisobutrylnitrile (AIBN), 4) basic alumina, Brockmann activity I, 150 mesh, 5) perfluoro(methylcyclohexane) (PMC), and 6) methacrylic acid 99%. Two specialty chemicals were required and were procured as follows: 1) polyfluoro-acrylate (PFAC-1), #007207 from Oakwood Products, West Columbia, SC, USA, and 2) poly(ethyleneglycol 1000 monomethyl ether monomethacrylate, #16666 from Polysciences Inc., Warrington, PA, USA.

This study required the use of compounds that were produced by custom organic synthesis at PNNL. The G-series half-acid esters [isopropyl methylphosphonate (IMP) and cyclohexyl methylphosphonate (CMP)] were produced as described by Mong et al. [5]. For MIP synthesis, a special surfactant was synthesized that, when added to the reaction mixture, allowed control of the sorbent particle diameter. Surfactant synthesis was accomplished by a modification of the method of Mayes and Mosbach [4] that reflects use of products that are readily available in the United States. Towards this end, 10.0 ml chloroform, 0.38 g acroyl PEG 1000 MME, 24 mg AIBN, 4 g acroyl PFA-1, were placed in a tube, purged with nitrogen, and sealed. The reaction was heated at 60°C for 48 hrs, the solvent removed by a stream of dry nitrogen, and the product placed under house vacuum (100 mtorr) for 1 week.

MIP and Contol Synthesis:

Polymers were synthesized according to methods described by Mayes and Mosbach [4]. Inhibitors were removed from all reagents immediately before use. Ethanol stabilizer and monomethyl ether hydroquinone inhibitor were removed from chloroform and trimethylolpropane trimethacrylate, respectively, by passing over basic alumina. Hydroquinone inhibitor was removed from methacrylic acid by vacuum distillation. The control reaction mixture consisted of 11.4 mg surfactant, 0.400 g of methacrylic acid (4.65 mmoles), 1.57 g of trimethylolpropane trimethacrylate (14.7 mmoles), 20 mg of 2,2'-azobisisobutyronitrile (0.122 mmoles), 4.6 g of chloroform, and 20.0 ml of PMC. The MIP reaction mixtures contained 1.16 mmoles of the template molecule [IMP, PMP, CMP, or the bis(2-ethylhexyl) hydrogenphosphate] in addition to the control polymer starting materials listed above. Control sorbent preparation was performed at the same time, with the same reagents, and under the same conditions as the

MIP polymers. Independent experiments determined that 11.4 mg of surfactant added to the above reaction mixtures resulted in particles that were approximately 12 μm in diameter.

The polymerization reaction involved initial stirring at 2000 RPM for 5 min to form a stable emulsion followed by purging with nitrogen for 5 min. Polymerization then occurred by irradiating with UV light while stirring at 500 RPM for 3 hrs. The reaction was stopped at this point, the beads were isolated by filtration and washed extensively with acetone.

HPLC columns:

Sorbent material was sent to Higgins Analytical (Mountain View, CA, USA) for packing into 37 x 3.0 mm stainless steel 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. For capturing the target organics from aqueous solution, commercially available cartridges (37 x 3.0 mm) packed with reversed-phase (d_f =10 μm) C-18 packing were used.

Initial Studies:

For initial studies, PMP was analyzed by HPLC. A PMP standard (23.4 μl of a 5mg/ml solution of PMP in acetonitrile) was introduced through a Rheodyne (Model 7125, Alltech, Deerfield, IL) injection valve to the MIP or the corresponding control column. Acetonitrile (100%) was delivered at 1.0 ml/min as the mobile phase using an Intelligent pump (Model 301-S, Japan). Elution of compounds for the MIP column was monitored by a BioRad Refractive Index Monitor (Model 1755, Foster City, CA, USA). Fractions corresponding to 4.0 ml of acetonitrile were collected, evaporated, derivatized with diazomethane, and analyzed by capillary GC on a Hewlett-Packard 5890 Series II gas chromatograph after diluting to a total volume of 10.0 ml with pentane. For these GC studies, a valve fitted with a 20-μl sample loop delivered sample injections to a 15 m x 250 μm i.d. non-polar deactivated retention gap (Supelco, Bellefante, PA, USA) that was connected to a 15 m x 250 μm i.d. XTI-5 separation capillary column (Supelco, d_f = 1.0 μm) using press-fit column connector. Helium head pressure was adjusted to achieve a linear velocity of 30 cm/sec. Injections were accomplished by switching the helium

carrier gas through the sample loop for 75 seconds before returning to the loop by-pass position. The temperature program was initiated upon return of the recorder pen to the on-scale position after elution of the solvent peak. After an initial 2 min hold at 40°C, the column was programmed at a linear 6°C/min linear ramp to a final temperature of 225°C. The final temperature was held for 15 min before the column was cycled to its original temperature.

Results show that relative to a 100% PMP control sample, initial 4 min HPLC fractions from the control sorbent and the PMP-specific MIP contained 98.0% and 27.5% of the target compound, respectively. Therefore, selective retention on the MIP is clearly achieved with practically all the PMP that was introduced to the system (72.5%) being selectively retained on the PMP-specific MIP column.

Additional HPLC experiments were conducted with bis(2-ethylhexyl) hydrogenphosphate. Comparison between the bis(2-ethylhexyl) hydrogenphosphate-specific MIP and control show that this compound is selectively retained on this compound-specific MIP over the control sorbent. These studies were based solely on refractive index detection and were not verified by capillary GC as was done for the PMP studies described above.

Further experiments utilized the PMP-specific MIP sorbent incorporated within the on-line instrumental format. These studies are further described below.

Instrument design and evaluation:

Figure 1 shows the instrument diagram. For analysis, valve 1 can deliver either injections (for standards) or aqueous solution to the reversed phase C-18 cartridge at a flow of about 1.0 ml/min. The reversed-phase cartridge will enrich hydrophobic components from injections or aqueous streams, including the half-acid alkyl esters of methylphosphonic and phosphoric acids. Once the organics from the sample have been captured (anywhere from 10 to 100 ml), valve 1 is switched to allow a flow of dry gas (at 20 psi) to pass through the C-18 cartridge for 5 min. Valve 2 is then switched to back flush the captured organic components off the dry C-18 cartridge with acetonitrile and transport to the MIP. The target component is selectively captured by the MIP from the acetonitrile mobile phase while the rest of the organics originally captured by the C-18 cartridge pass through the MIP cartridge and are diverted to waste. Four min is allowed for the transfer of the target compound to the MIP.

This amount of time allows complete wetting of the dry C-18 phase, removal of bubbles from the system, and adequate time for the matrix interferences to pass through the MIP. Finally valve 3 is switched to elute the half-acid ester from the MIP and transport to the conductivity detector. A valve timing sequence is included in Table 1 for operation of the instrument as described above. The experimental parameters have not been optimized (sorbent bed size, mobile phase flow rates, times at each valve position) and it is possible that different conditions would lead to enhanced performance.

A series of chromatograms are included to show proof-of-principle operation of this instrument. Figure 2 represents a water injection blank. The large peak that occurs at about 8.0 min is due to bubbles arriving at the conductivity detector from introduction of acetonitrile into the dry C-18 cartridge. This peak appears in all chromatograms regardless of whether the target analyte is present or not. The rise in baseline at 13 min is due to arrival of the aqueous MIP elution mobile phase at the detector. The baseline offset is due to the difference in background conductivity between acetonitrile and water.

Figures 3 through 5 present chromatograms of PMP standards analyzed with the PMP-specific MIP installed on the system. These chromatograms correspond to injections of 234 μg, 117 μg, and 23.4 μg of PMP for Figures 2, 3, and 4, respectively. On-line experiments were not conducted with the bis(2-ethylhexyl) hydrogenphosphate-specific MIP but it is assumed similar results would be obtained.

Extensions of Invention:

This invention describes a general approach for exploiting the enormous selectivity of MIP sorbents for analyzing organic species in aqueous samples. All that is needed to address different compounds is to insert the appropriate MIP sorbent into the system. One could exploit the ability of MIPs to recognize chemical structures similar to the template. This cross reactivity is advantageous when trying to capture a class of structurally related compounds, or when performing ultra trace analysis where residual template bleed from the MIP may be problematic. To capture a number of different target signatures, a mixed sorbent bed containing all the relevant MIP phases could be used. The advantage of the described instrument over more traditional methods is directly related to the high MIP selectivity that allows extremely large analysis volumes which, in turn, makes possible achievement of very low detection limits.

Studies presented here focus on nonselective concentration using C-18 or the related restricted-access material (RAM) phases. Other sorbents may be used for nonselective capture; however, compatibility must be maintained between the nonselective stage, the MIP stage, and the detector. Selective capture on the MIP is most conveniently accomplished from an organic solvent. In the instrument described in this invention, compatibility is maintained by removing water from the C-18 before transferring analyte to the MIP with acetonitrile. Although ion-exchange would be useful for initially capturing half-acid esters, the salt or acid mobile phases used to elute these resins would not promoting selective capture on the MIP.

Funding Support and Division of Intellectual Property:

These intellectual property (IP) concepts were developed entirely through DOE funding. Specific project support for this IP was partly through the Imprinted Media project (F52301) funded by internal laboratory-level Laboratory Directed Research and Development funds (50%) as well as through the Ultraselective Sorbents (F45028) NA-22 project (50%). Scott D. Harvey is the Principal Investigator of both these projects and is the sole contributor to the development of this IP.

Concept Origin Information:

Concept origin can be traced to demonstration portion for the Homeland Security Initiative. A portion of this demonstration features the analysis of aqueous solutions for the hydrolysis products of the G-series nerve agents. The specific instrument concept for achieving this analysis, along with the initial experimental results included in this invention report, were first detailed in an e-mail and FAX to Bob W. Wright on June 16, 2004. On June 23, the same FAX was sent to Michael Lind and an additional PowerPoint file was forwarded to Bob Wright and Michael Lind. The same data related in these memos and PowerPoint files are presented in the Tables and Figures contained in this invention report.

References:

1. J. Chi, G.-L. Huang, X. Lu, D.-G. Ma, and Y. Wang, DEHP enrichment in the surface microlayer of a small eutrophic lake, *Water Research*, **37**:4657-4662 (2003).

2. C. Guitart, N. Garcia-Flor, J. Dachs, J.M. Bayona, and J. Albaiges, Evaluation of sampling devices for the determination of polycyclic aromatic hydrocarbons in surface microlayer coastal waters, *Marine Pollution Bulletin*, **48**:961-968 (2004).

3. M. Zi-Hui and L. Quin, Determination of degradation products of nerve agents in human serum by solid phase extraction using molecularly imprinted polymer, *Anal. Chim. Acta*, **435**, 121-127 (2001).

4. A.G. Mayes and K. Mosbach, Molecularly imprinted polymer beads: Suspension polymerization using a liquid perfluorocarbon as the dispersing phase, *Anal. Chem.,* **68**:3769-3774 (1996).

5. G.M. Mong, S.D. Harvey, and J.A. Campbell, Simplified Synthesis and Purification of the G-Series Nerve Agent Hydrolysis Products: Cyclohexyl Methylphosphonic Acid and Isopropyl Methylphosphonic Acid, *Phosphorus, Sulfur and Silicon*, submitted (May 2004).

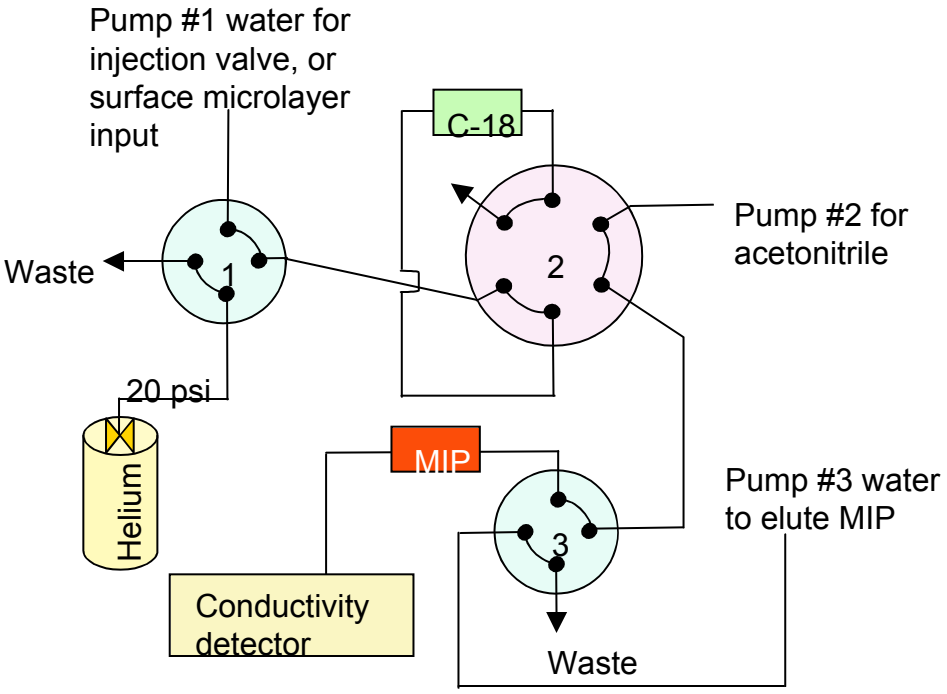

Figure 1. Instrument diagram detail showing the specific valving arrangement.

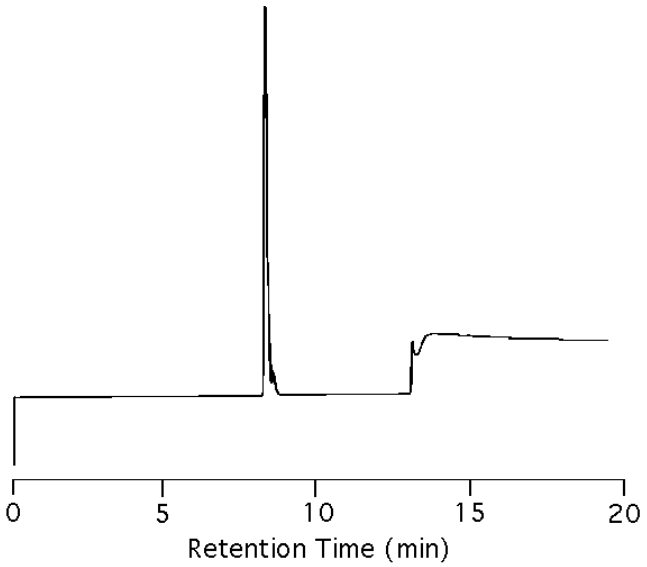

Figure 2. Chromatogram resulting from the injection of a water blank. The peak at 8 min is due to bubbles arriving at the detector from elution of the dry C-18 sorbent. The baseline offset at 13 min corresponds to the difference in background conductivity between acetonitrile and water.

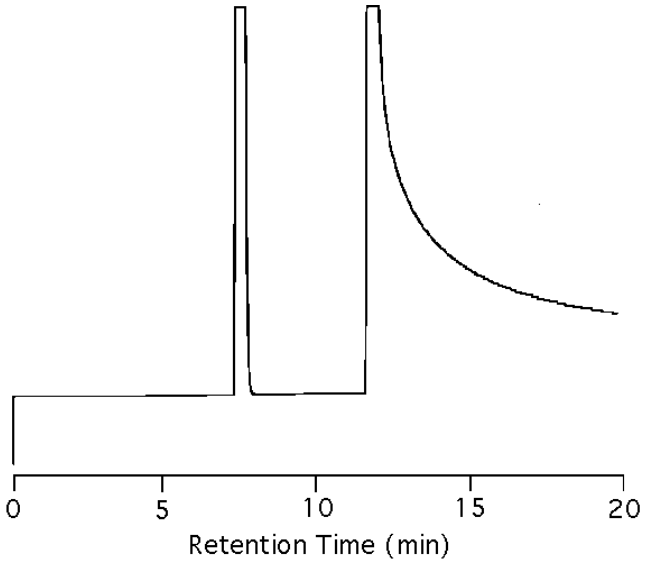

Figure 3. Chromatogram resulting from an injection of 234 μg PMP.

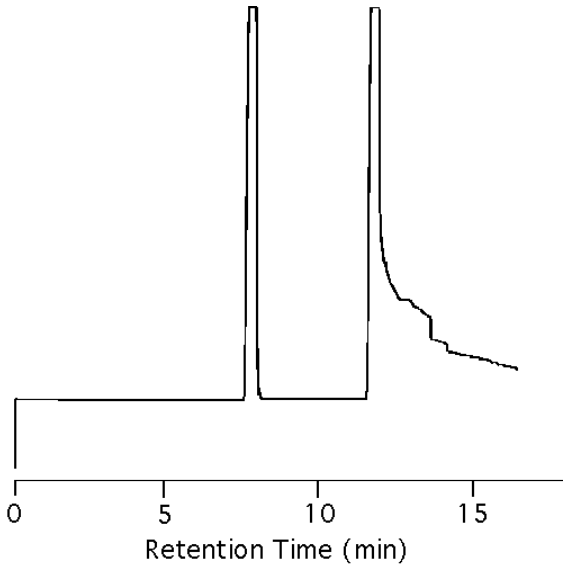

Figure 4. Chromatogram resulting from an injection of 117 μg PMP.

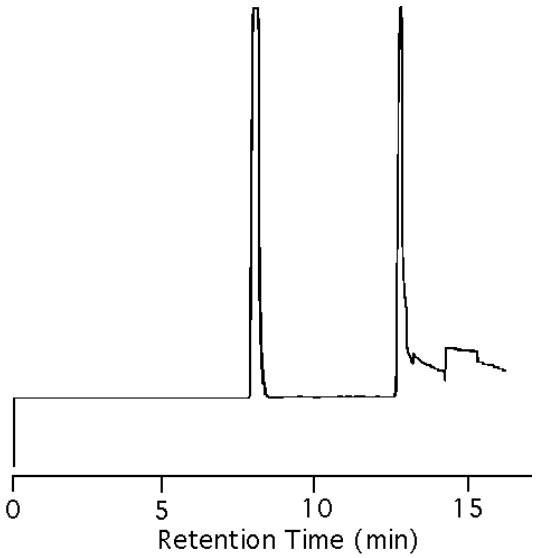

Figure 5. Chromatogram resulting from an injection of 23.4 μg PMP.

Table 1. Valve position and timing sequence to accomplish principal steps in the analysis.

Valve Number/ Operation	Valve 1	Valve 2	Valve 3
Load water sample (2 min)	Position A	Position B	Positions B
He purge to dry C-18 (5min)	Position B	Position B	Position B
ACN back flush of C-18 and transfer to MIP (4 min)	Position B	Position A	Position B
Elution of half-acid ester from MIP and transfer to detector	Position B	Position A	Position A