Molecularly Imprinted Solid-Phase Extraction and Liquid Chromatography/Mass Spectrometry for Biological Samples

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Till Malte och Janne

Table of contents

Preface

I was introduced to the fascinating field of molecularly imprinted polymers (MIPs) during my undergraduate work at AstraZeneca, Södertälje. The combination of sample clean-up with solid-phase extraction and the chemistry behind the recognition mechanism for MIPs really interested me. So, I became enthusiastic when I was given the opportunity to continue working with MIPs as a PhD project.

At the beginning of the PhD project I focused on the applications of MIPs, and challenges associated with their application, for analysing a specific group of analytes, organophosphate flame retardants, rather than investigating the material itself, which many groups already appeared to be doing. My first paper focused on the preparation of a MIP with high selectivity for a hydrolysis product, or possible metabolite, of the flame retardant triphenyl phosphate (Paper I). Triphenyl phosphate had been found in relatively high concentrations in the air in occupational environments and at μg/g concentrations in blood donor plasma. The objective was to develop methods that can detect the corresponding diphenylphosphate ester as a metabolite in human urine, by clean-up using SPE with a tailored MIP sorbent (molecularly imprinted solid phase extraction, or MISPE). Another reason for choosing the target analyte was that it possesses a functionality that I thought could be suitable for MIP preparation. The goal to produce a MIP with high selectivity towards the target analyte was successfully accomplished, as demonstrated in the first paper.

I was very optimistic after the first successful attempt to extract the analyte from aqueous standards using the synthesized MIP. I had read a lot about the problems associated with "complex samples" arising from biological fluids, and thought that MIPs could be very useful for resolving them. However, when the MISPE method was applied to the extraction of human urine rather than standards, I had real experience of these problems, which I had previously only read about. Nothing worked as before and I didn't know were to start seeking the reasons for the failure. Hence, from this point onwards the work took another

direction. I started to investigate the MIP material more thoroughly to develop a better understanding of the mechanisms responsible for the retention of analytes from aqueous samples, the effects of the sample matrix on the selectivity and, not least, ways to suppress these effects (Paper II-V).

From the experience gained during this work I think that it is highly important to do more than merely apply a method to an analytical problem. The investigator needs to understand the chemistry associated with the problem and know the analytical system well in order to use it efficiently. I am also convinced that the importance of efficient sample pretreatment should not be underestimated. Once I was asked during a presentation at a conference I attended if it is really necessary to include a selective clean-up step when you use a selective detector? I thought about this for a while, but I don't remember what I said in reply. However, I am now sure that for most analytical applications it is of great importance to do so.

Abstract

This thesis focuses on the use of molecularly imprinted polymers as selective sorbents for solid-phase extraction (MISPE). The MISPE methods developed were mainly intended for use with biological samples, such as human urine and blood plasma. These body fluids are complex samples, which often need an effective clean-up step before analysis to reduce the levels of possible interfering substances from the matrix, especially if the analytes are present in trace amounts. Solid-phase extraction (SPE) is a well-established and routinely used method for clean-up and preconcentration of samples from diverse matrices. However, conventional SPE sorbents often lack selectivity, leading to co-extraction of interferences, which negatively affects the following detection method. One of the advantages of MISPE is the built-in selectivity for a target analyte, or class of structurally related analytes, enabling the efficient clean-up that is often required for biological samples. The built-in selectivity of MISPE originates from the preparation of a highly crosslinked copolymer network in the presence of an imprint molecule, i.e. the template. Subsequent removal of this template molecule leads to the creation of defined recognition sites, complementary to the shape and functionality of the template.

In this work, molecularly imprinted polymers were synthesized for the first time for several types of target analytes, including diphosphate esters (Papers I-III) and a protein adduct (Paper IV) and evaluated as sorbents for solid-phase extraction. A MISPE method for extracting local anaesthetic drugs from human plasma was also evaluated (Paper V). The development of appropriate methods for using the prepared polymers to extract target analytes directly from body fluids, and the elucidation of factors that influence their performance, were major foci of all the work underlying this thesis. These are not straightforward tasks, since the recognition mechanism of the material is often based on polar interactions, which are not favoured in aqueous environments. In such cases, non-selective adsorption of the analyte(s) to the polymer surface often occurs. In order to use the MIP sorbent most effectively it is important to suppress this non-selective adsorption, without disrupting the selective adsorption of the target analyte(s) to the imprints. Generally in these studies, this strong

analyte-polymer surface interaction could be repressed, and selective adsorption enhanced, by carefully optimising the conditions for washing the sorbent, in terms of organic solvent volumes, solvent polarity and the addition of an ionic modifier. The sample matrix, mainly urine, was found to strongly decrease the capacity of the MIP. Hence, this effect was further investigated. It was found that the presence of NaCl in the sample negatively affected the recovery and repeatability of the method. Furthermore, these parameters could be improved by adjusting the sample pH. It was important to control the pH of the sample, in order both to achieve selective extraction and to increase the extraction recoveries. The selectivity of MISPE for the extraction of diphosphate esters from human urine was demonstrated by comparing its performance with that of a conventional SPE sorbent, a mixed-mode-anion exchanger (MAX). Due to its efficient clean-up, MISPE generated extracts that yielded less complex ion chromatograms in subsequent LC/ESI-MS analysis than extracts from the MAX cartridge. Signal suppression from the interfering co-eluting compounds was detected when the MAX extracts were analysed, which was not the case for the MISPE extracts. These findings show the importance of efficient and selective sample preparation, even if a selective detector is used.

Development of LC/ESI-MS methods was also an extensive component of this work (Papers I-IV). Different chromatographic conditions have been evaluated for the optimal separation and detection of the investigated compounds. Use of ion-pairing agents and suitable HPLC columns (Hypercarb and C18 Aquasil) for the acidic, polar analytes, was found to give better retention and separation than use of conventional reversed-phase columns. To improve the selectivity and detectability further, selected ion monitoring (SIM) and selected reaction monitoring (SRM) acquisition modes were used for quantification of the investigated compounds.

In summary, the aim of this work was to contribute to the knowledge of the recognition mechanisms of molecularly imprinted polymers in aqueous matrices, which is important for extending the use of MISPE for several types of bioanalytical applications.

List of papers

This thesis is based upon the following papers, which are referred to in the text by the corresponding Roman numerals:

I. Synthesis and evaluation of molecularly imprinted polymers for extracting hydrolysis products of organophosphate flame retardants.
Kristina Möller, Ulrika Nilsson, Carlo Crescenzi
Journal of Chromatography A 938 (2001) 121-130.

The author was responsible for all the experimental work (except for packing the polymers into the stainless steel columns and the NMR analysis and evaluation). The author was also responsible for writing the major part of the paper.

II. Determination of a flame retardant hydrolysis product in human urine by SPE and LC-MS. Comparison of molecularly imprinted solid-phase extraction with a mixed-mode anion exchanger.
Kristina Möller, Carlo Crescenzi, Ulrika Nilsson
Analytical and Bioanalytical Chemistry 378 (2004) 197-204.

The author was responsible for all the experimental work and for writing the major part of the paper.

III. Investigation of matrix effects of urine on a molecularly imprinted solid-phase extraction.
Kristina Möller, Ulrika Nilsson, Carlo Crescenzi
Journal of Chromatography B 811 (2004) 171-176.

The author was responsible for all the experimental work and for writing the major part of the paper.

IV. Evaluation of a molecularly imprinted solid-phase extraction method for a 1,2:3,4-diepoxybutane adduct to valine in hemoglobin.
Kristina Möller, Ronnie Davies, Charlotta Fred, Ulrika Nilsson, Margareta Törnqvist
Submitted to Journal of Chromatography B.

The author was responsible for the synthesis of the MIP, evaluation of the MISPE method and, in collaboration with Charlotta Fred, for the development of the LC/MS method. The author was also responsible for writing a substantial part of the paper.

V. Development of a molecularly imprinted polymer based solid-phase extraction of local anaesthetics from human plasma.
Lars I. Andersson, Emilia Hardenborg, Maria Sandberg-Ställ, Kristina Möller, Johan Henriksson, Inger Bramsby-Sjöström, Lars-Inge Olsson, Mohamed Abdel-Rehim
Analytica Chimica Acta 526 (2004) 147-154.

The author's main contributions to the experimental work for this paper were in the initial optimization of the solid-phase extraction protocol.

Introduction

Chemical analysis generally consists of a chain of procedures, illustrated in Figure 1, to quantify and/or identify one or several components in a sample of matter. The needs for improved analytical methods are increasing, especially for compounds with known or possible effects on human health, due to the increasing number of environmental pollutants, drugs and their metabolites, and additives used in the food industry. These needs place high demands on the analytical methods employed, which must be efficient, accurate and predominantly automated. Recent advances in instrumentation and the range of detectors available enable analytical scientists to measure and identify target analytes at lower and lower concentrations. However, despite the improvement in instrumentation, the major rate-limiting step in the overall analytical procedure is generally the sample preparation. Nevertheless, much research still seems to focus on the final separation and detection steps, while less attention is paid to the development of faster, more selective clean-up methods.

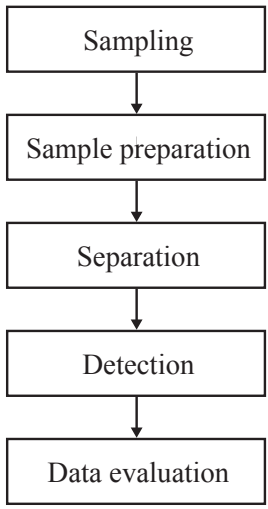


Figure 1. The analytical chain of procedures.

Aims

The work this thesis is based upon focused on the sample preparation step in the overall chain of analytical procedures. The objectives were to investigate and evaluate the use of molecularly imprinted polymers (MIPs) as sorbents for solid phase extraction of polar compounds in aqueous matrices, and to explore the effects of the sample matrix on the adsorption mechanism, in terms of selectivity. This subject is important both for the development of generic extraction protocols and to improve knowledge of the retention mechanisms in these types of matrices. A general aim was to contribute to the development of new sample preparation techniques for biological samples, such as urine and plasma, and to highlight the importance of efficient clean-up for these complex types of samples.

Sample preparation of biological samples

The aim of sample preparation is to enable instrumental analysis or improve the instrumental analyte signals in comparison to those obtained from non-treated samples. The sample preparation steps may consist of extraction of the analyte from the sample matrix, a clean-up step and/or a preconcentration step. Sometimes the analytes are chemically modified, derivatised, to give them more suitable properties for the following separation and/or detection. The sample preparation is often laborious and can be, as previously mentioned, the major source of error in the overall analytical process. For these reasons this part of the analytical chain should ideally be minimized (or avoided if possible). However, in many cases extensive sample pretreatment is necessary to obtain acceptable analytical results. This is often the case for bioanalytical methods where biological samples are processed.

Biological samples, such as urine, blood serum or blood plasma, contain large amounts (and numbers) of endogenous components and are generally referred to as complex matrices. The matrix components often

interfere and may adversely affect the following separation and detection if they are not efficiently removed. This is especially important if very low amounts of the analytes are present in the samples. Extracting hydrophilic compounds from these aqueous matrices is an analytical challenge. The sample types analysed in this work were human blood plasma and urine. Blood contains a mixture of many components, including a variety of proteins, fats, salts and suspended cells. The red blood cells can be removed from the clear fluid, the plasma, by centrifugation after addition of an anti-coagulating agent. Urine consists of both organic and inorganic components and is usually relatively free of proteins. The simplest form of sample preparation for these kinds of samples, involves dilution, centrifugation, filtration and/or evaporation. Some commonly used techniques for sample preparation, especially to clean-up biological fluids, are briefly described below.

Techniques for sample preparation

In order to determine compounds such as drugs or drug metabolites in biological fluids, the proteins generally have to be removed prior to the final analysis. Proteins may denature in the LC solvents or at the high temperatures used for GC and cause clogging in the analytical system. Some common methods for removing proteins are:

- *Protein precipitation*
  This technique removes proteins from the samples by first denaturating them directly in the initial sample, commonly by the addition of a water miscible organic solvent (e.g. methanol, ethanol, acetonitrile or acetone) or a strong acid, such as trichloroacetic acid (TCA). The denatured proteins are then removed from the sample by centrifugation.

- *Dialysis*
  In dialysis the proteins are separated from the sample analytes by exploiting their slower migration rates through a semi-permeable membrane than those of low molecular weight compounds, including the target analyte(s).

The analyte molecules are transferred, by diffusion, to a dilute solution. Thus the volume of the dialysate often needs to be reduced to increase the concentration of the analyte.

- *Ultrafiltration*
  This technique is similar to dialysis, in that a membrane filter is used to separate the proteins. However, a centrifugal force is applied to accelerate the diffusion and, thus, the entire process. Low molecular-weight compounds are able to pass through the filter, which has pores of a certain size range, while the proteins are retained on the filter.

Protein removal is not a very efficient clean-up technique, since the analyte(s) may be adsorbed on the proteins, and many interferences from the sample may still remain. Some techniques that offer an extraction step and more efficient clean-up are:

- *Liquid-liquid extraction (LLE)*
  LLE is a classical technique involving the partitioning of solutes between two immiscible liquids. It is important to select appropriate solvents for this purpose; the solvent should match the analytes polarity while still being immiscible with water, and it should preferably be compatible with the following detection method. A larger volume of the extraction solvent, compared to the sample, favours partitioning and the enrichment of the analytes is often inefficient. However, the sample extract can easily be evaporated if a volatile solvent is used to increase the analyte concentration. Other factors, such as pH and ionic additives may greatly affect the extraction efficiency.

- *Solid-phase extraction (SPE)*
  SPE is a very common type of clean-up technique for bioanalytical purposes, due to its simplicity and versatility. Many different types of SPE sorbents are commercially available, for diverse applications. SPE with tailored MIP sorbents (MISPE) is currently a rapidly growing field. This type of technique was evaluated in this thesis.

Other examples of extraction techniques are solid-phase microextraction (SPME), supercritical fluid extraction (SFE), membrane extraction and affinity sorbent extraction. SPE and affinity sorbents are further described in the following chapters. General considerations for the extraction of biological samples and a more detailed description of the abovementioned sample preparation techniques are presented in the chapters by Kataoka et al. [1] and Stevenson et al. [2].

Solid-phase extraction (SPE)

SPE is well established and widely used in many different areas of chemistry. Development of SPE methods and their use in analytical chemistry greatly increased during the late 1980s and the 1990s, although their history dates back to at least the early 1970s [3]. SPE involves passing a liquid sample through a solid sorbent bed, usually consisting of modified silica particles. The aim is to retain the analytes in the sorbent bed, wash away interferences and finally elute the analytes as a clean extract in a small volume. The collected extract can then be analyzed by a suitable method, for instance LC/MS. A wide range of different formats and sorbents for SPE applications is available, and the technique can be used either off-line or on-line. The principle is very similar to that of LLE, except that the solutes are not partitioned between two immiscible liquid phases but between a solid phase (sorbent) and a liquid phase (sample matrix). SPE was initially developed as a technique to complement or replace LLE. One advantage of SPE over LLE is that it requires lower amounts of organic solvents for the extraction, which is important for environmental and health reasons. SPE also often offers higher selectivity for the target compound(s) due to the wide range of sorbent types available. Other advantages of SPE are its higher concentration factors and the fact that the extraction steps can readily be automated.

Since the area of SPE is very large this subject is not fully covered in this thesis. The following sections are focused on SPE for organic compounds and liquid samples. For further reading the book by James S. Fritz [3], and the cited book sections [4, 5] and review papers [6-9] are recommended.

Theory of SPE

The theoretical basis of separation by SPE sorbents is the same as that of HPLC separation/retention mechanisms. In SPE the sorbent acts as the stationary phase and the sample as the liquid phase. Distribution of a solute between these two phases follows the laws of chemical equilibrium. The partition coefficient, K, of a compound can be given by:

$K = C_S/C_M$

where C_S is the concentration of the compound in the stationary phase and C_M its concentration in the mobile phase, at equilibrium. The molar ratio between the two phases is related to K by:

$$\frac{N_S}{N_M} = K \times \frac{V_S}{V_M}$$

where V_S is the volume of the stationary phase and V_M is the volume of the mobile phase. For efficient extraction in SPE the partitioning of an analyte between the mobile phase and the stationary phase should strongly favour the latter. This means that K should be as large as possible. In column chromatography the N_S/N_M ratio is called the capacity factor or the retention factor and is represented by, k´. For HPLC, where the aim is to separate compounds from each other, k´, should be in the range of 1-10. For SPE the capacity factors should be very high (100, 1000 or more) in the loading solvent. There is normally no separation between individual analytes in SPE and they are retained as a sharp band on the sorbent. On the other hand, for elution of the analytes the k´ value should be as low as possible to elute the group of analytes in a narrow band.

As well as the capacity factor in the loading solvent, the efficiency of extraction will depend on the number of separate equilibrations with the adsorption sites in the column. In column chromatography the number of equilibrations is called the theoretical plate number, represented by N. For HPLC the efficiency of the column is usually very high, up to 25000 in plate numbers. For SPE N is usually low, below 100. In SPE the particle size is larger than in HPLC, at least 40-50 µm, compared to 3 or 5 µm in HPLC. The particles are also often irregular in shape and the sorbent bed

is not packed as carefully in SPE, so N is smaller. However, since SPE is not intended to separate compounds, the N value is normally sufficient. In HPLC the analyte(s) ideally emerge(s) as a peak with the shape of a normal distribution curve. The shape of the chromatographic peak depends on the plate number; a narrow peak indicates high efficiency. In SPE the sample is continuously flowing through the sorbent and no sharp concentration maximum is reached. The elution profile could instead be described by a frontal or breakthrough curve, as shown in Figure 2. Such a curve gives the breakthrough volume, V_B, which is defined as the point at which the sample volume exceeds the capacity of the sorbent [5].

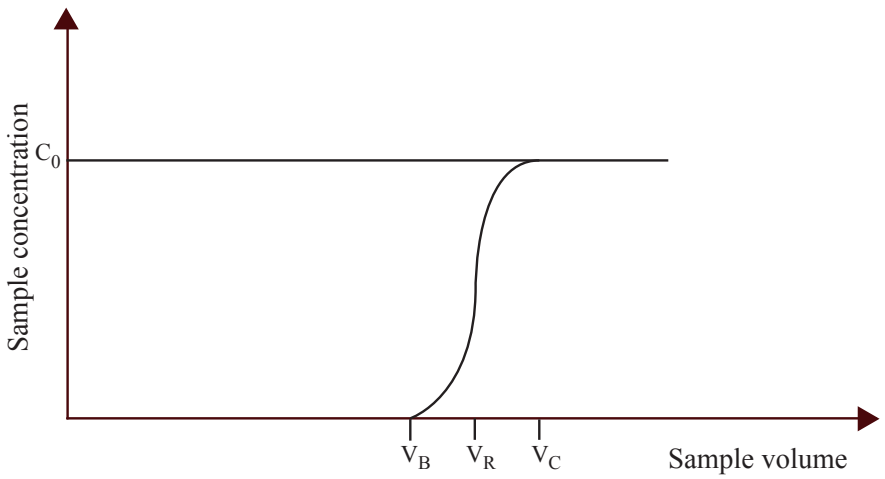


Figure 2. A theoretical representation of a typical breakthrough curve. V_B is the breakthrough volume, V_R is the chromatographic elution volume and V_C corresponds to the sample volume at which the capacity of the sorbent is saturated. At V_C the concentration of the analyte entering the sampling device is the same as the concentration of the analyte exiting the device, C_0.

A desirable property for SPE particles is a large surface area. The equilibrium between the sample and the sorbent is shifted to the sorbent when the area is larger. Common SPE sorbents have surface areas between 200 and 800 m^2/g. Since the surface area decreases as the average pore size increases, small pores are to be preferred. A typical nominal pore size is 6 nm.

Principles of SPE

The analyte can be retained by the sorbent surface by a range of different types of interaction, such as hydrogen bonds, dipole-dipole or dipole-induced dipole mechanisms, dispersive, charge transfer or ionic interactions. Ionic interactions involve higher energies (50-200 kcal/mole) than both hydrogen bonds (5-10 kcal/mole) and the dipole types of interactions (1-10 kcal/mole) [4]. The three main modes of SPE for organic compounds utilizing one or several of these interactions are: reversed-phase, normal-phase, and ion-exchange systems. In reversed-phase SPE the aim is to extract nonpolar analytes from a polar sample using a sorbent of nonpolar character. For normal-phase mode an opposite approach is used; the sorbent consists of polar particles while the sample matrix is nonpolar and polar analytes are extracted. Ionic interaction occurs between an analyte carrying a charge (positive or negative) and a sorbent carrying the opposite charge.

The SPE process usually consists of four distinct steps: (1) conditioning/equilibration, (2) adsorption/sample application, (3) washing and (4) elution, as shown in Figure 3.

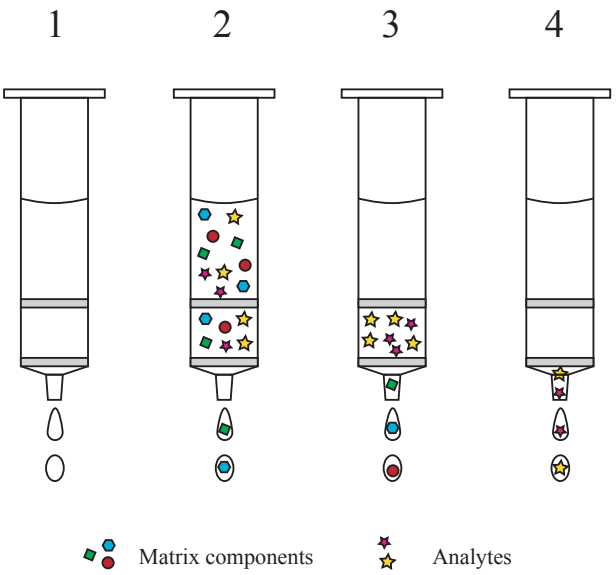


Figure 3. The four steps of the SPE process, conditioning (1), sample application (2), washing (3) and elution (4).

(1) Conditioning of the sorbent is important to promote good surface contact between the phases. This is usually done by wetting the sorbent with an organic solvent of appropriate polarity. This step also includes the equilibration of the sorbent with a solution as similar to the sample as possible, in terms of polarity, ionic strength and pH.
(2) In this step the sample is percolated through the sorbent bed, simply by gravity, or by the application of a gentle vacuum or pressure. It is important to control the flow rate in this step to promote efficient mass transfer of the analytes to the sorbent. A suitable flow rate depends on the particle size distribution, the column dimensions and the packing characteristics of the sorbent.
(3) Here the aim is to wash off retained interferences from the sample matrix without eluting the analytes.
(4) The elution step involves disrupting the retention of the analyte on the sorbent. A solvent offering a combination of the possible interactions is often most effective. It is advantageous if the elution solvent volume is small enough for the analyte to be preconcentrated. Furthermore, the solvent should ideally be compatible with the final analysis.

An alternative approach for the SPE process is to retain the matrix components, while the analytes of interest pass through the sorbent bed.

SPE formats

The most common format for SPE materials is the cartridge, or SPE column, which consists of a reservoir, most often of polyethylene, with the sorbent bed fitted between two frits, as shown in Figure 3. Such columns are available with a wide range of different sorbents, sorbent sizes and reservoir volumes. Typical amounts of sorbents are between 25-500 mg with reservoirs volumes of 1-10 ml. This format is used for off-line applications, while for on-line use the sorbent is packed in so-called precolumns. Typical amounts of sorbents in a precolumn are 20-100 mg. The on-line SPE process is similar to the off-line protocol, including conditioning, washing and application of the sample to the precolumn. The main difference is in the elution step, since the retained analytes are eluted directly onto the analytical column. The elution solution then acts as the mobile phase and has to be compatible with

the following separation and detection method. The most common on-line coupling of SPE when analyzing biological samples is with liquid chromatography [7]. This is explained by the good compatibility of the LC aqueous mobile phases with the SPE of biological samples, which are mainly aqueous. On-line coupling with gas chromatography is not as straightforward as SPE/LC for biological samples. Since GC is not normally compatible with water a drying step is usually required before elution with a solvent suitable for GC injection. On-line coupling of SPE to GC for the analysis of biological samples has recently been reviewed [10].

Another SPE format is in disks, in which the sorbent is supported on a membrane. Such disks have a wide, thin bed, and contain smaller particles than those used for the columns. Advantages of disks, compared to the cartridges, are that higher flow rates can be applied due to the lower backpressure generated, and that the risk of channelling in the sorbent bed is reduced, since the sorbent particles are held closely together between the membrane filters. The disk technology is well described in reference [3] for further reading.

SPE sorbents are also available in 96-well plates, which were developed in the early 1990s for high throughput and small-volume SPE in bioanalysis. These SPE blocks are now available for several types of sorbents and different sample volumes. Most plates are used in particle bed format, but they are also available as disk-based devices [11].

The development of these diverse formats for SPE has facilitated automated sample processing. Several systems are available for automated SPE, both off-line and on-line. A review of the automation trend, the advantages, the equipment and some generic approaches was published some years ago [12].

Different types of SPE sorbents

The most common types of SPE sorbents are bonded-phase silica particles. These particles consist of siloxanes chemically modified by molecules with appropriate functionalities for the intended application, e.g. hydrophobic carbon chains for reversed phase SPE, as illustrated

in Figure 4. A list of common bonded phase silica sorbents supplied by several commercial manufacturers is presented in Table 1, of which C18 (octadecyl modified silica) is by far the most widely used phase. A disadvantage with silica-based sorbents is that they are not very stable outside the pH range 2-8. Another problem with silica sorbents is that the residues of free acidic silanol groups can give undesirable secondary interactions, for example hydrogen bonds with alcohols and amines. To avoid the problems associated with free silanol groups a process called end-capping is often used, which blocks the free silanol groups, commonly with methyl groups.

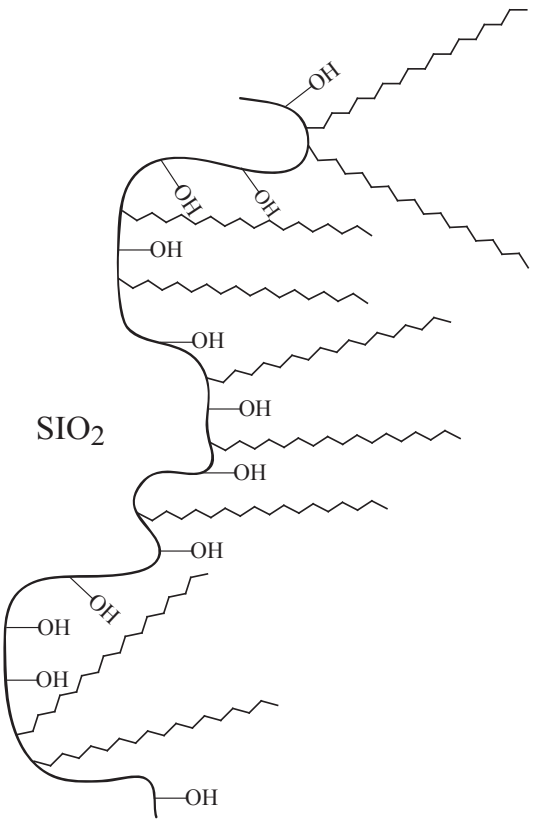
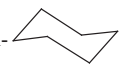


Figure 4. Illustration of the surface of a bonded-phase silica particle.

Table 1. Common silica based sorbents for SPE.

Phase	Designation	Secondary interaction	Structure
Reversed phase sorbents (Primary interactions are non-polar)			
Octadecylsilane	ODS, C18		-Si-$C_{18}H_{37}$
Octyl	C8		-Si-C_8H_{17}
Butyl	C4		-Si-C_4H_9
Ethyl	C2		-Si-C_2H_5
Phenyl	PH		-Si- 
Cyclohexyl	CH		-Si- 
Normal phase sorbents (Primary interactions are polar)			
Cyanopropyl	CN		-Si-$(CH_2)_3CN$
Silica	SI	Weak anion exchanger	-Si-OH
2,3-Dihydroxypropoxypropyl	DIOL		-Si-$(CH_2)_3$-OCH_2CH(OH)-CH_2(OH)
Aminopropyl	NH2	Weak anion exchanger	-Si-$(CH_2)_3NH_2$
Ion exchange sorbents (Primary interactions are ionic)			
Anion exchangers			
Primary secondary amine*	PSA	Weak non-polar	-Si-$(CH_2)_3NH(CH_2)_2NH_2$
Quarternary amine**	SAX	Weak non-polar	-Si-$(CH_2)_3N^+(CH_3)_3Cl^-$
Cation exchangers			
Propylcarboxylic acid*	CBA	Weak non-polar	-Si-$(CH_2)_3COOH$
Benzenesulphonic acid**	SCX	Strong non-polar	-Si-  -$SO_3^-H^+$
Propylsulphonic acid**	PRS	Weak non-polar	-Si-$(CH_2)_3SO_3^-H^+$

* Generally referred to as weak ion exchanger
** Generally referred to as strong ion exchanger

Other common types of SPE phases are organic polymer-based sorbents, the most widely used being porous polystyrene-divinylbenzene (PS-DVB). These sorbents have the advantages over silica-based sorbents that they are stable over the entire pH range and often have higher surface areas due to high levels of crosslinkers. Both PS-DVB and hydrocarbon-

modified silica sorbents have hydrophobic surfaces, which must be conditioned and activated to be compatible with aqueous samples. To improve their compatibility with aqueous samples and increase the retention of polar compounds more hydrophilic polymer sorbents have also been developed. These sorbents can be obtained by the introduction of a functional group to the existing co-polymer [13, 14]. One example is the ENV+ sorbent from IST, which consists of a PS-DVB backbone chemically modified by the incorporation of hydroxyl groups. Another hydrophilic sorbent is the Oasis HLB (hydrophilic lipophilic balance) from Waters (a mechanistic study of the sorption properties of Oasis HLB has been presented by Dias et al. [15]). This sorbent incorporates a hydrophilic monomer to increase its water compatibility. Oasis HLB is also available as a mixed-mode sorbent, providing scope for two retention mechanisms to be exploited in the same phase. This sorbent contains ion exchange groups, either for cation exchange or anion exchange, which allows additional ion exchange interactions as well as the reversed phase interactions. The structure of the HLB polymer backbone with anion exchange groups is illustrated in Figure 5. The advantage of a mixed-mode sorbent is its ability to extract compounds of different polarities simultaneously, due to the dual retention mechanisms.

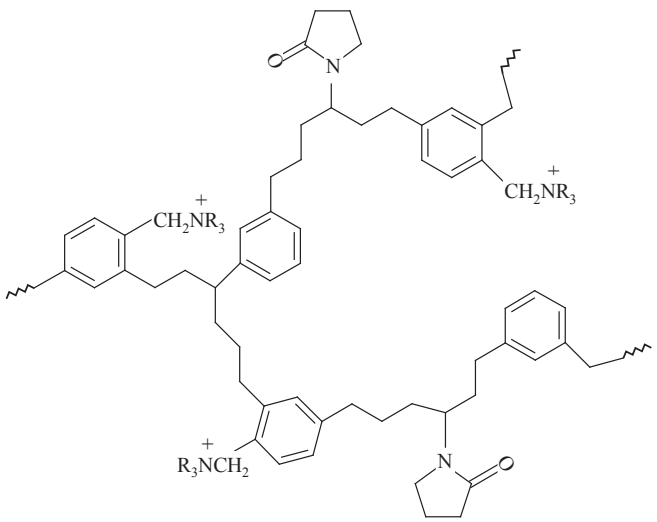


Figure 5. Chemical structure of the Oasis MAX sorbent, a mixed-mode anion exchanger.

Another type of mixed-mode sorbent is the restricted-access material (RAM), which is a combination of a size-exclusion and a reversed-phase sorbent. This material is used in precolumns for the direct injection of biofluids in on-line systems. The sorbent prevents access of proteins either by a physical barrier, such as the pore size, or by a chemical modification of the particle surface to avoid irreversible adsorption of proteins. The internal surface areas of the particles contain the hydrophobic moieties (C_4, C_8, or C_{18} hydrocarbon chains) where the smaller analytes are retained [7].

Other examples of SPE sorbents include various forms of carbon, such as graphitized carbon blacks (GCBs) and porous graphitic carbon (PGC). These sorbents are capable of trapping very polar analytes. However, the retention of some compounds can be excessive or irreversible, making their elution problematical. PGC is a macroporous material, unlike GBC which retains compounds on the external sorbent surface, with high mechanical stability and is stable over the whole pH range. The surface of a PGC particle consists of a two-dimensional graphite structure, composed of layers of hexagonally arranged carbon atoms with delocalized electrons, as illustrated in Figure 6. Its retention mechanism is different from that of silica and polymer-based sorbents. The analytes are retained by a combination of dispersive and stronger electrostatic interactions. The latter interactions are due to the high polarizability of the surface [16].

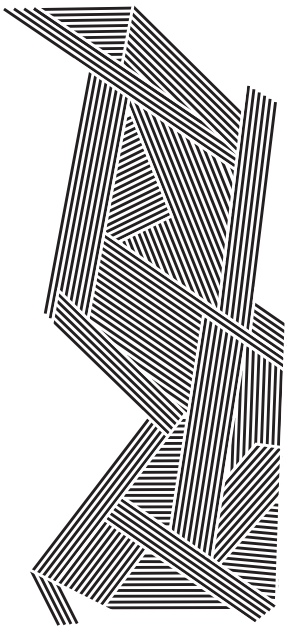


Figure 6. Illustration of a porous graphitic carbon (PGC) surface.

Although, there is a wide range of different SPE sorbents commercially available they often lack in selectivity. This is a problem especially

when the analytes occur in trace amounts and co-elute with interferences from the sample matrix. For biological samples this may necessitate an additional sample clean-up prior to final analysis. For this reason more selective sorbents, such as immunoaffinity sorbents and MIPs are often desirable.

Immunoaffinity extraction

This technique is based on the ability of antibodies to recognise and bind specifically the target analytes. Antibodies are produced by the mammalian immune system in response to the presence of foreign substances (antigens). Antibodies can be very selective and antibodies against large biomolecules, such as hormones, enzymes, proteins, viruses and peptides are easily obtained. However, compounds of low molecular mass (<1000) are unable to evoke this immune response and have to be modified before immunization. The modification consists of binding the antigen to a larger carrier molecule, usually a protein. This complex is called a hapten and its design is very important since the antibody should be able to recognise the target analyte, rather than the whole complex.

When using immunoassays, the sample is incubated with the antibodies until equilibrium is established between the analyte and the antibody binding sites. The immunoassay can be either competitive, where the analyte competes with a labelled form of the analyte for a limited number of antibody binding sites, or non-competitive where the antibody binding sites are non-limiting. An example of a non-competitive immunoassay is the enzyme-linked immunosorbent assay (ELISA). Radioimmunoassay (RIA) is an example of a competitive assay, where the label consists of a radiolabelled form of the analyte. The advantages of these techniques are their, high throughput capacities, sensitivity and selectivity. A characteristic for many immunoassays is their ability to detect the analyte in complex samples, such as whole blood, plasma, serum or urine, without prior extensive sample preparation. A drawback with the technique is the possible cross-reactivity of the antibodies; an antibody is not always able to distinguish between structural analogues, for instance between the analyte and its metabolites [17].

In immunoaffinity extraction (IAE) antibodies are covalently bound onto an appropriate sorbent and packed into a solid-phase extraction column or precolumn. The principle is then the same as for conventional SPE. Hence, the immunoextraction protocol consists of the same four steps; conditioning the sorbent, percolation of the sample, washing away interferences, and elution of the target analytes. If possible, the sorbent is regenerated. The mechanisms of adsorption and desorption are different from those involved in ordinary SPE and IAE, so each of the steps differ too. After the extraction a separation step, chromatographic or electrophoretic, is often performed. This may solve the problems associated with the crossreactivity of the antibodies.

Immunoaffinity extraction provides unique and powerful techniques, due to the high selectivity of the antibody-antigen interaction, which enable selective extraction and concentration of individual compounds or classes of compounds. Wide ranges of applications of solid-phase immunoaffinity extraction have been developed, both off-line e.g. for a human glycoprotein hormone from urine followed by LC/ESI-MS-MS [18], for steroid estrogens in wastewater coupled with LC/ESI-MS [19] for morphine and its glucoronides from human blood followed by LC/fluorescence detection [20] and on-line e.g. for Flunitrazepam, a potent hypnotic drug, and its major metabolites from human serum followed by LC/UV [21]. A on-line method have also been developed for extracting a novel retinobenzoic acid from human plasma by directly loading the sample onto a pre-column, followed by a column-switch for separation by HPLC. The immunoreactivity was then detected by a competitive radioimmunoassay [22]. IAE has been thoroughly reviewed by several authors [23-26].

The drawbacks of immunosorbents include their cost and time-consuming development. The design and production of antibodies may take several months or even years and it can be difficult to obtain specific antibodies for low molecular weight molecules. Another drawback is the sensitivity of the antibodies to pH changes, elevated temperatures and organic solvents. These drawbacks have led to the development of synthetic antibody mimics, so-called molecularly imprinted polymers.

Molecularly imprinted polymers (MIPs)

The technique of molecularly imprinted polymers is based upon molecular recognition and is often referred to as synthetic antibodies. The principle is that a polymer network is created around a template, i.e. the imprint molecule. Subsequent removal of the template leaves a cavity, or imprint, with both chemical and steric affinity for the template molecule. Since the material exhibits a predetermined selectivity for a target molecule, or class of structurally related molecules, this is of great interest for several analytical techniques and applications. The interest in the technique is demonstrated by the almost exponential increase in the number of publications on it during the last decade. In 1995 there were approximately 40 publications concerning the concept compared to over 250 in 2005. However, even if interest and research in the area are relatively new, the advent of molecular imprinting dates back as far as to the beginning of the 19th century and the pioneering work of a Soviet chemist, M. Polyakov. In 1931 Polyakov published an investigation on the effects of drying silica in the presence of different additives on its pore structure [27]. Polyakov reported a correlation between the structure of the additives used during the drying process and the extent of their rebinding to the silica. Another important advance towards current MIP technology was the polymerisation of sodium silicate using a dye as template, presented by Dickey in 1949 [28]. This method was described as a preparation of adsorbents with specific affinity for predetermined substances. In this method the dye, template, was added prior to the polymerisation, which is the way that polymers are usually prepared today, and the adsorption power was compared with that of control gels, prepared in the absence of the template. The first works on imprinted organic polymers came from two different groups, independently, in 1972. One of these studies, presented by the Klotz group [29] involved the synthesis of binding sites from methyl orange in a polyethyleneimine-crosslinked network. The other study, presented by Wulff et al. [30] involved an imprint that showed enantiomeric affinity for the D-form of glycerolic acid. Both of the cited studies involved a covalent linkage of the template molecule to the monomers before polymerisation. The most common approach today is to use non-covalent linkage of the monomers

to the template molecule. This non-covalent approach for producing organic imprinted polymers was introduced in the early 1980s by the group of Mosbach [31], who called the non-covalent approach "host-guest polymerization".

Fundamentals

A polymer is defined as a large molecule that consists of smaller repeating units. A monomer is the building block of the polymer and combines with other monomer molecules to form a polymer. The monomers can be of the same type, in which case the polymer is called a homopolymer. If the polymer is composed of two or more different monomers, it is referred to as a copolymer. The reaction between the monomers is called polymerisation. Different types of polymerisation reactions can be used, depending on the types of monomers involved [32]. The most widely used process for molecularly imprinted polymers is free-radical polymerisation.

Free-radical polymerisation is a chain-growth reaction, which means that the growth of the polymer is caused by a kinetic chain of reactions. The mechanisms and rates of the reactions that initiate, continue and terminate the polymer growth vary. The first step is the creation of a free-radical to start the reaction. This is most commonly accomplished by a photochemical or thermal decomposition of a compound, called an initiator (I), which forms two free-radicals ($R^{\bullet}$), as shown in Reaction (1).

$$I \rightarrow 2R^{\bullet} \qquad (1)$$

The next step is the initiation of the polymerisation reaction where the formed radical adds to a monomer (M) and creates a new monomer-ended radical ($M_1^{\bullet}$), as in Reaction (2).

$$R^{\bullet} + M \rightarrow M_1^{\bullet} \qquad (2)$$

The reaction in which the polymers are formed is the propagation reaction and involves the addition of a monomer to the monomer-

ended radical (Reaction 3). The propagation reaction continues until the free radical reacts to form an inactive covalent bond. This is the last step in the free-radical polymerisation process, and is called the termination. Termination can occur in many ways. The most important ways are by two different mechanisms where two radicals combine (Reaction 4) or where a disproportionation reaction (Reaction 5) occurs, forming a paired electron bond [32].

$$M_i^{\bullet} + M \rightarrow M_{i+1}^{\bullet} \quad (3)$$

$$M_n^{\bullet} + M_m^{\bullet} \rightarrow M_{n+m} \quad (4)$$

$$M_n^{\bullet} + M_m^{\bullet} \rightarrow M_n + M_m \quad (5)$$

Some advantages with free-radical reactions are their relatively low sensitivity to impurities and their ability to occur in a range of different solvents with varying polarity. These properties are especially suitable for MIP synthesis, since they facilitate the use of a variety of different combinations of monomers and solvents.

Approaches of molecular imprinting

In molecular imprinting most of the polymers are copolymers, i.e. two or more different monomers are used to build up the polymer. Various approaches can be used to synthesize the copolymers (covalent, metal-ion coordinated, semicovalent and noncovalent) of which the last is by far the most commonly used due to its simplicity and versatility. The differences between these approaches lie mainly in the prearrangement of the template molecule and the monomers before initiation of the polymerisation. In covalent imprinting the template molecule is covalently linked to a monomer before polymerisation. The advantage with this approach is that a strong interaction is preserved during polymerisation, leading to well-defined imprint sites, allowing an exact stoichiometric ratio of the template molecule and the functional monomer. The drawbacks are that the covalent binding must be chemically cleaved in order to extract the template after polymerisation, and the approach can only be used for a

limited number of compound classes. The metal coordination approach is based on the formation of a complex between the monomer and a metal ion, which in turn coordinates to the template molecule before the initiation of the polymerisation reaction. In the semi-covalent approach the template is covalently linked before polymerisation, as in the covalent approach, but the rebinding of the compound of interest is based on non-covalent interactions to the imprints.

Since the non-covalent approach was used in the work underlying this thesis, the focus here will be on this technique. The other approaches are well described in the cited book sections [33, 34] and the review by Mayes and Whitcombe [35], which are recommended for further reading.

Technology for non-covalent molecular imprinting

The principle of molecular imprinting involves three main steps, as demonstrated in Figure 7:

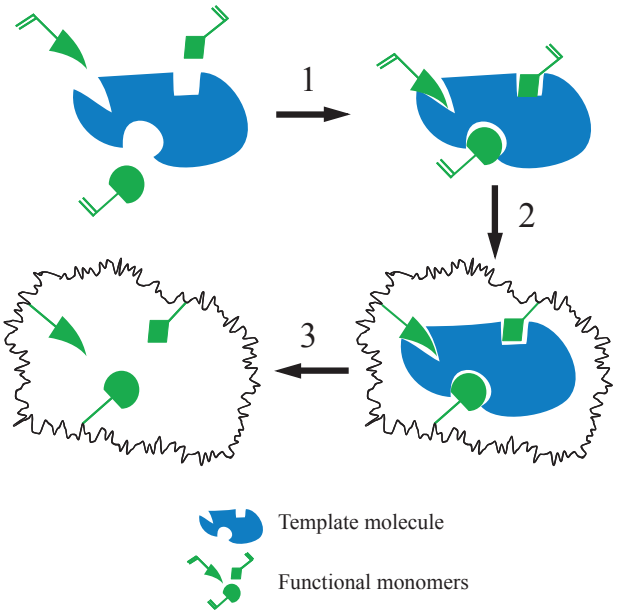
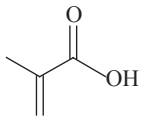


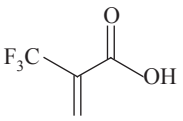
Figure 7. Schematic illustration of the principle of molecular imprinting.

1. The formation of a prepolymerisation complex between the template molecule and the functional monomers,
2. Polymerisation of the formed template-monomer complex in the presence of a cross-linker,
3. Extraction of the template molecule from the polymer matrix.

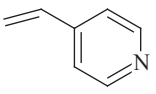
The monomers used to create the prepolymerisation complex should form non-covalent interactions by self-assembly with the template molecule. This means that the monomers should contain functionalities that can interact with those of the template, for instance the monomer may be basic for an acidic template. Typical interactions involved are ionic interactions and hydrogen bonds. Other types of interactions, such as dipole-dipole, dipole-induced dipole and charge-transfer mechanisms, may also be involved during the formation of the prepolymerisation complex [36]. A stable prepolymerisation complex is important for the molecular recognition properties of the material. The stability of this complex is due to the strength of the interactions involved; preferably the template used should have several functionalities that can interact with the monomers. Steric factors are also important to consider, since the interaction strength is dependent on the distance and orientation between the template and the monomers. Typical functional monomers used in molecular imprinting are shown in Figure 8. By far the most commonly used of these is methacrylic acid, MAA. MAA was used as the monomer in the studies described in Papers IV and V. In the studies reported in Papers I, II and III, 2-vinylpyridine (2-VPY) was used as the functional monomer. The functional monomers are usually added in excess relative to the amount of template used, to favour the complex formation. The most commonly used monomer:template ratio is 4:1, but higher proportions of functional monomers have also been used in some cases. A larger monomer:template ratio has been shown to increase the number of selective sites [37].



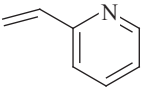
Methacrylic acid (MAA)



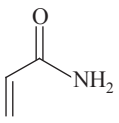
Trifluoromethyl acrylic acid (TFMAA)



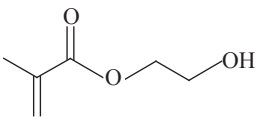
4-vinylpyridine (4-VPY)



2-vinylpyridine (2-VPY)



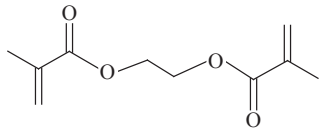
Acrylamide (AAM)



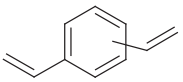
2-hydroxyethylmethacrylate (HEMA)

Figure 8. Structures of monomers used for molecular imprinting.

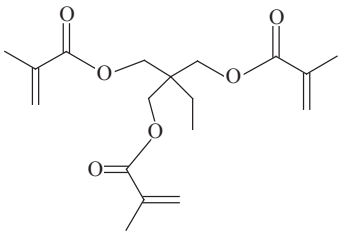
To preserve the three-dimensional arrangement of the monomer-template complex, a cross-linker is added in large amounts, typically over 80% of the total monomer amount. It has been observed that levels over 50% yield selective imprints and that the selectivity increases with the crosslinker concentration [38]. A high level of cross-linker also gives the material a macroporous structure and high mechanical stability. Ethylene glycol dimethacrylate, EDMA, is the most common cross-linker for MIP applications. In Figure 9 the structure of EDMA is presented, together with that of some other cross-linkers which have been used for MIP preparations. EDMA was used as cross-linker in all the papers included in this thesis.



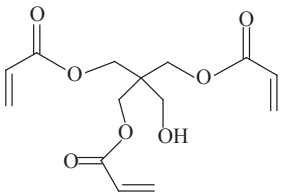
Ethyleneglycoldimethacrylate (EDMA)



Divinylbenzene (DVB)



Trimethylopropane trimethylacrylate (TRIM)



Pentaerythrotil triacrylate (PETRA)

Figure 9. Structures of crosslinkers used for molecular imprinting.

The solvent used as the diluent for the reagents, often referred to as the "porogen", in the polymer production, affects the morphology of the material. The noncovalent approach generally generates a heterogeneous material with a range of pore sizes and types of binding sites. The morphology of the polymers was not considered in the studies this thesis is based upon, and this subject is more thoroughly described in the review by Cormack et al [39] and the book section by Sellergren [40]. The choice of diluent also affects the stability of the prepolymerization complex. Since the interactions involved in the prepolymerisation complex often are of polar type e.g. hydrogen bonds, the diluent should preferably be nonpolar and aprotic to generate a polymer with high selectivity [41]. Commonly used solvents include toluene (Paper V), chloroform (Papers I, II and III), benzene, dichloromethane and acetonitrile (Paper IV).

The polymerisation reaction is generally initiated by either a thermal or photochemical decomposition of a free radical initiator. The most commonly used initiators are the azobisnitriles, such as 2,2-azobis(2-methylpropionitrile) (AIBN) and 2,2-azobis(2,4-dimethylvaleronitrile) (ABDV). Photochemical initiation of AIBN at a low temperature has

been shown to promote higher selectivity in the resulting MIP than thermal initiation [41, 42].

Extraction of the template is a critical step for the recovery of accessible binding sites. Since the template molecule can be deeply embedded in the polymer matrix extensive washing is often required. The solvents chosen for the wash protocol should be able to disrupt the interactions created during polymerisation. Thus, solvents of a polar nature, with the addition of a base or acid, are often chosen. However, even if careful washing is performed there is a risk that the template will bleed, which raises problems for quantitation. This issue is further discussed in the section about *MISPE*.

Polymerization procedures

Bulk procedures are usually used to prepare MIPs. A typical protocol for this technique is exemplified by a scheme for the preparation of the ditolyl MIP used in the studies described in Papers I-III and illustrated in Figure 10. The reagents for the MIP synthesis, i.e. the functional monomers, the cross-linker and the template, are dissolved in the diluent together with the radical initiator. The reagent solution is then degassed to remove oxygen and placed under UV-light to photochemically decompose the initiator into free radicals. The polymerisation reaction is then allowed to proceed for 24 h. The resulting hard block polymer is then crushed, milled and sieved to desirable particle sizes. The polymer particles are then washed to remove the template and unreacted reagents. Throughout this work, the particles were packed in empty SPE cartridges for subsequent evaluation. This polymerization procedure is quite simple and straightforward, and does not require special equipment. However, the method is relatively laborious and the MIP production takes a couple of days. The resulting particles are also irregular in shape, which limits their chromatographic performance. For those reasons other methods for the preparation of MIPs have been developed.

Several techniques have been presented for making imprinted polymer beads. Suspension polymerisation techniques have been developed using liquid perfluorocarbon [43] and mineral oil [44] as the continuous

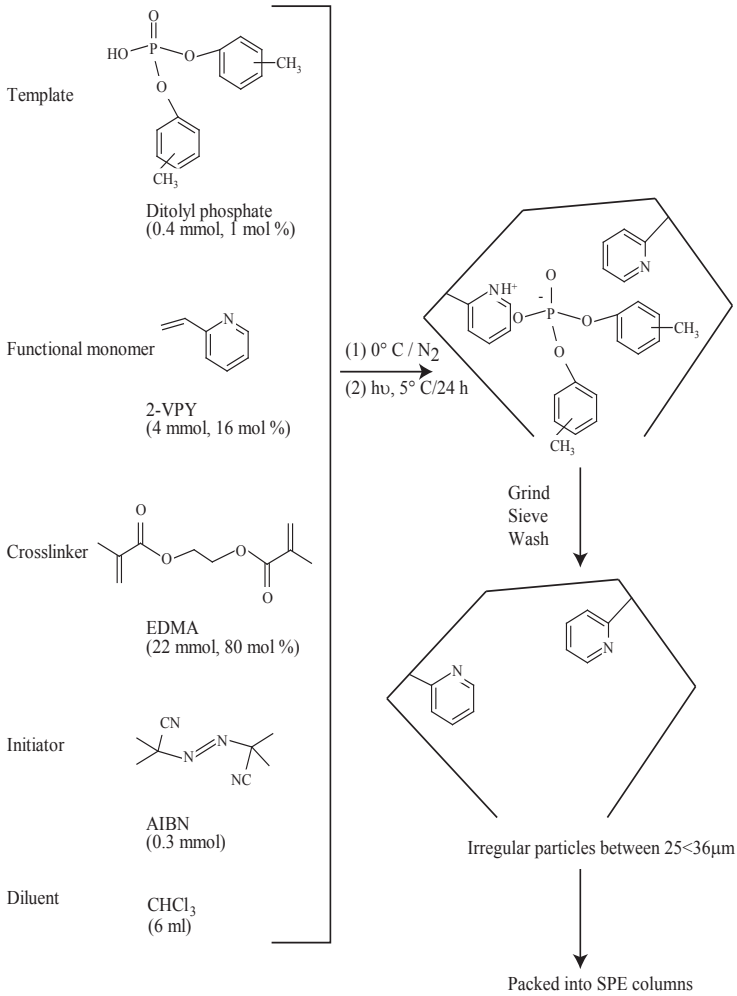


Figure 10. Schematic picture of the imprinting procedure for the MIP used in Paper I-III.

phases for the production of molecularly imprinted beads. Conventional suspension polymerisation has also been used, with water as the continuous phase [45, 46]. Another polymerisation technique that has been evaluated for MIP production is dispersion or precipitation polymerisation [47, 48]. Some surface modification techniques have also been developed, including grafting of molecularly imprinted polymer films on silica particles [49] and the preparation of surface-modified

imprinted polymers by a multi-step swelling and polymerization method on seed particles [50].

When developing a new MIP for a certain compound, the selection and proportions of functional monomer, crosslinker, initiator and solvent are often based on previously developed MIP protocols for other templates, or trial-and-error. However, for some compounds these general protocols might not work or have to be improved since the resulting protocols give poor selectivity, capacity or chromatographic performance. Since the number of variables affecting the imprint process are very large, approaches for more effective and rational design to obtain an optimal MIP for certain templates have recently been developed, based on combinatorial and computational techniques [51-53].

Molecularly imprinted solid-phase extraction (MISPE)

The first application of MISPE was presented by Sellergen in 1994 [54]. The cited study presented a selective extraction of pentamidine, a drug used for the treatment of AIDS-related pneumonia, from human urine. Since then this has proved to be one of the most promising applications of MIPs, as described in several reviews [55-60] and book chapters [61]. MIPs potentially offer a higher degree of selectivity than conventional SPE sorbents. This feature is highly attractive for the clean-up and enrichment of complex samples, which are common in bioanalytical and environmental applications. Hence, several MISPE applications concern the clean-up of biofluids and aqueous samples, as exemplified in Table 2. These applications have been developed for single target analytes, for the simultaneous extraction of classes of structurally related compounds, and for both off-line and on-line protocols.

Principles of MISPE

The concept of MISPE is based on the same main four steps as conventional SPE, i.e. conditioning of the sorbent, percolation of the sample, washing away interferences and elution of the target analytes. However, the principle of adsorption is based on a different mechanism, so a different method development strategy is required. Since the advantage of MISPE is its selectivity, it is important to optimize the selective retention of the

Table 2. Examples of studies where MISPE has been applied to biological and environmental samples.

Target analyte or analytes	Template	Sample matrix	Protocol	Reference
Biological and pharmaceutical samples				
Tamoxifen (antioestrogenic drug for treatment of breast cancer)	Tamoxifen	Human plasma and urine	Off-line	[62]
Sameridine (local anaesthetic and analgesic drug)	Structural analogue	Human plasma	Off-line	[63]
Bupivacaine (local anaesthetic drug)	Pentycaine	Human plasma	Off-line	[64]
Phenytoin (anticonvulsant drug for treating epilepsy)	Phenytoin	Human plasma	Off-line	[65]
Clenbuterol (β_2-agonist used for growth promotion)	Brombuterol	Calf urine	Off-line	[66]
Scopolamine (anticholinergic, anti-infective and analgesic pharmaceutical)	Hyoscyamine	Human serum and urine, calf serum and urine	Off-line	[67]
Class of β blockers	Propanolol	Rat plasma	On-line, precolumn packed with RAM-MIP particles (hydrophilically surface-modified MIP particles)	[68]
Verapamil and metabolites (antihypertensive and antiarrythmic drug)	Verapamil	Rat plasma and cell culture, human urine	On-line, coupled to a RAM precolumn	[69]
Tetracycline and oxytetracycline (antibiotic drugs)	Oxytetracycline	Pig kidney tissue extract	Off-line	[70]

Environmental samples				
Class of triazines (pesticides)	Simazine	Apple extract, urine, humic acid	On-line coupled to a C18 precolumn	[71]
4-Nitrophenol	4-Nitrophenol	River water	On-line	[72]
Class selective for triazines (pesticides)	Terbuthylazine	River water	On-line coupled to a RAM precolumn	[73]
Primicarb (pesticide)	Primicarb	Tap water, river water, sea water	On-line	[74]
Class of eight naphthalene sulfonates (environmental pollutants from the chemical industry)	1-naphthalene sulfonic acid	River water	Off-line	[75]
Class of four organophosphorus (pesticides)	Monocrotophos	Surface water (river), tap water, soil	Off-line	[76]

target analyte(s) to the imprints and to suppress non-selective interactions to the polymer surface. Two different approaches can be used to obtain this selectivity; selective adsorption or non-selective adsorption followed by a selective desorption step. These two approaches are illustrated in Figure 11.

- *Selective adsorption*
  Most MIPs are prepared using an organic solvent as the diluent or porogen during the polymerisation reaction. As already mentioned, the interactions involved in the establishment of the prepolymerisation complex are often of polar nature and hence stabilized in a nonpolar solvent. Thus, to achieve selective adsorption the sample needs to be loaded on the MISPE column in an organic solvent of low to medium-polarity. It has been shown that the ideal solvent for selective rebinding to the imprints is often the same as the porogen [77, 78]. A selective adsorption approach to MISPE often requires extraction in an organic solvent prior to loading the sample [77, 79].

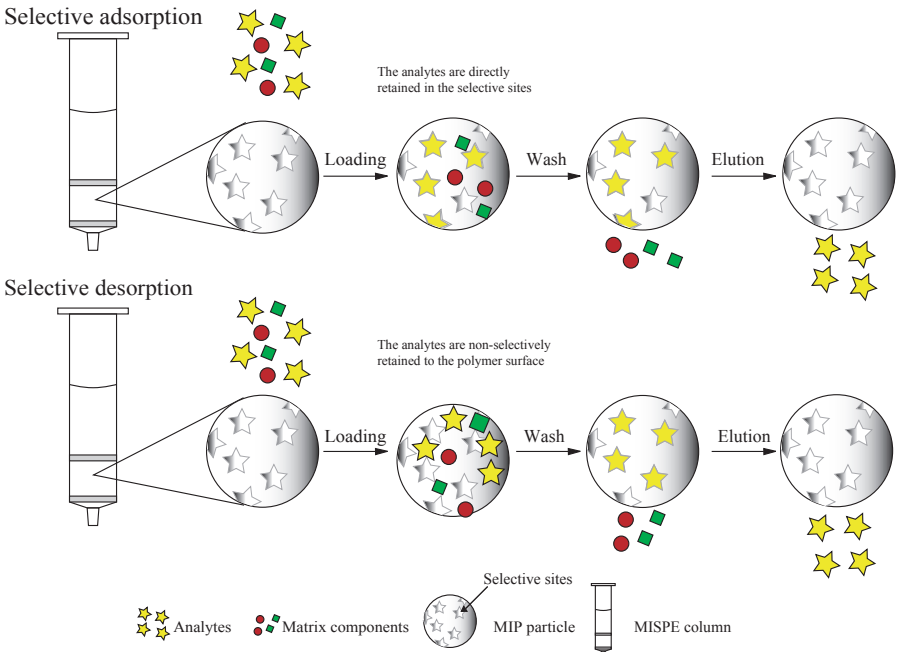


Figure 11. Illustration of the two different approaches used for MISPE.

- *Selective desorption*
  Samples that are suitable for MISPE are often of aqueous nature, such as biological fluids and environmental water samples. However, when an aqueous sample is directly applied to the MIP sorbent, extensive non-selective adsorption to the polymer surface generally occurs. The polar interactions are weak in aqueous media and the hydrophobic forces stronger. However, for relatively non-polar compounds or compounds that can be retained by an ion-exchange mechanism this non-selective adsorption is often strong and can be utilised for adsorption. The MIP sorbent then works as a conventional reversed phase or an ion-exchange SPE sorbent during the loading step. Furthermore, the non-selective adsorption can be converted to selective adsorption to the imprints by washing the cartridge with carefully chosen solutions, for instance the analytes may bind to the imprints in conditions that favour the interactions established during creation of the MIP. The porogen [80] or organic

solvents with low polarity [65, 81] are often used in a "selective" wash step. This wash step should also remove the non-selective bound interferences from the sample matrix. If the non-selective adsorption also involves ionic interactions, a weak acidic or basic modifier could be added to the wash solvent in order to disrupt them [66].

Other approaches to improve the water compatibility and reduce non-selective adsorption to the MIP have also been presented. For example, Dirion et al. [82] increased the water compatibility by incorporating a hydrophilic co-monomer in an existing MIP protocol. Another approach is to use more polar solvents, such as mixtures of alcohols and water [75, 83] as diluents for the MIP synthesis. Adding a detergent to the aqueous sample has also been shown to reduce the non-specific binding [84, 85]; this approach was used in the study described in Paper V. The hydrophobic parts of the detergent are adsorbed on the polymer surface as protecting layers, while the hydrophilic heads are in contact with the water. This layer hinders the interactions of the analyte with the polymer surface.

Template bleeding

An important aspect to consider when using MISPE for quantitative measurements is the potential risk of template bleeding. During the production of the MIP a high concentration of template is present, and leakage of this molecule during extraction can lead to erroneous results in the subsequent quantitative analysis. Complete removal of this template compound is necessary if the method is to be used for samples with the analyte in trace amounts. However, it has been shown that complete removal is difficult even if an extensive washing protocol is applied [62-64]. For this reason an approach to circumvent this problem has been developed by Andersson et al. [63], based on the use of a structural analogue to the analyte as the template compound. This approach was used in all the studies underlying this thesis; the templates used could be separated from the analytes by liquid chromatography (LC, Papers I-IV) or gas chromatography (GC, Paper V).

Other techniques utilizing MIPs

Besides MISPE the attractive features of MIPs (including their unique selectivity, high chemical resistance and mechanical stability) make them very useful for a wide range of other applications. A few of the main types of applications are described below, but for more complete lists and discussion a recent review by Alexander et al. [86] and the book by Sellergren [87] are highly recommended.

- *Sensors*
  MIPs have proved to be very promising recognition media for highly selective analytical sensors used in various transduction, detection, systems [88-90]. Some applications include: a selective luminescent sensor for measuring a hydrolysis product of a chemical warfare nerve agent in water [91], detection of polycyclic aromatic hydrocarbons in water with fluorescence and mass sensitive measurements [92], a MIP-coated quartz crystal microbalance for the selective rebinding of the anabolic steroid nandrolone [93], a voltametric sensor for the detection of vanillyl mandelic acid [88] and homovanillic acid [94], a quartz-crystal microbalance sensor prepared by surface-imprinting for detecting a plant virus in aqueous media [95] and the production of surface-plasmon resonance sensor chips, using MIPs embedded with gold nanoparticles [96].

- *Membranes*
  Incorporation of MIPs in membranes provides further applications, in which they introduce selectivity into the transport through the membrane barrier [97, 98]. MIPs have been grafted onto the surface of a microfiltration membrane to introduce specific binding sites in the porous matrix [99, 100]. These MIP membranes were used in solid-phase extractions of triazine herbicides from water. Another application, involving surface-grafted membranes, is a screening method for identifying optimal conditions to generate a MIP protocol for a given template [101]. MIP membranes have also been prepared by the incorporation of synthesized MIP particles as solid adsorption phases in a polyvinyl chloride/dibutyl phthalate membrane matrix [102]. The resulting affinity membranes were used for extracting antibiotic drugs from water.

- *Catalysis*
  Since MIP materials contain spatially and functionally selective cavities for the template they can be applied in catalytic processes [103, 104]. Catalytically active imprinted polymers have been prepared using transition-state analogues as templates for ester hydrolysis [105-107], ester hydrolysis including a metal-ion coordination [108] and for a Diels-Alder cycloaddition reaction [109].

- *Chromatography*
  The use of MIPs as stationary phases for chromatography is by far the most intensively studied application of imprinted polymers, especially for liquid chromatography [110, 111]. The pioneering work by the Mosbach group on non-covalent imprinted polymers included separation of amino acid derivatives by LC using an MIP as the stationary phase [112, 113]. The material was found to have enantio-selective properties, retaining the enantiomer used as template more strongly than the other enantiomer. Since then, several chiral compounds have been separated by MIP-based LC , including peptides [114] and commercial drugs [115-117].

  MIP-based LC has also been used extensively for evaluating the binding characteristics or to confirm that an imprint has been successfully established. This is commonly done by comparing the retention behaviour, such as the capacity factor (k´), of the MIP LC column with that of a reference column, usually one packed with the corresponding non-imprinted polymer (NIP). The selectivity is then often expressed as the imprinting factor, IF. The IF is the k´ ratio between the MIP column and the NIP column. The selectivity for compounds other than the template or analyte is also commonly investigated by this approach. This type of evaluation was applied to the MIP prepared for diphenyl phosphate, with ditolyl phosphate as template (Paper I).

  MIPs have also been used in capillary electrochromatography (CEC), primarily for enantiomeric separations [118-120]. Several different capillary formats have been presented, such as monolithic imprinted

polymers inside a fused-silica capillary [121], imprinted polymer coatings for open-tubular CEC [122], the use of silica particles grafted with imprinted polymer films for packed silica capillaries [123] and partial filling of the capillary with MIP microparticles [124].

Problems related to the heterogeneity of the binding sites and their variability in accessibility in the material impose limitations on the use of MIP-based chromatography. These problems lead to differences in the mass transfer rates for a given compound and, thus, poor chromatographic performance, manifested in broad and tailing peaks.

- *Binding assays*
  Due to the similar binding characteristics as antibodies, MIPs have been used as alternatives or substitutes to antibodies in sorbent assays [125-127]. The principle of this technique was described above in the *Immunoaffinity extraction* section. The advantage of this alternative approach is that the chemical and mechanical stability of MIPs allows them to be used in conditions that are not suitable for antibodies. The production of MIPs is also generally cheaper and faster. Binding assays based on the rebinding of radio-labelled ligands have been developed for the enantioselective rebinding of (S)-propanolol [128], the selective rebinding of a herbicide (2,4-dichlorophenoxyacetic acid) [129] and the screening of a propanolol-imprinted library [44]. Assay-analogues to enzyme-linked immunosorbent assays (ELISAs) have also been developed with MIPs used as antibody substitutes [130, 131].

  Binding assays are also used to evaluate the binding characteristics of the produced MIP. In these cases a fixed amount of polymer is incubated with the analyte until equilibrium is reached, and the free amount of analyte in the supernatant is then measured. This approach was used in the study reported in Paper V, in which a radio-labelled form of the analyte was incubated with the MIP and the corresponding NIP to investigate the MIP's imprinting efficiency. The PC_{50} values (the polymer concentration required for binding

50% of the added radio-ligand) were calculated and compared for the different polymers. A low value for the MIP, in combination with a high value for the corresponding NIP, indicates that the former has a high density of high-affinity sites.

Liquid chromatography/mass spectrometry (LC/MS)

Liquid chromatography/mass spectrometry is now a robust and routinely used technique for bioanalysis. The technique can be coupled to a versatile chromatographic system and offers, selective and sensitive detection. The development of atmospheric pressure ionization (API) techniques has enabled LC to be easily and reliably interfaced with MS. The API techniques, which include atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and the recently introduced atmospheric pressure photoionization (APPI) are generally referred to as soft ionization techniques, since they usually cause little or no fragmentation of the analyte ions. Several recent reviews are recommended for further reading regarding the applications of these techniques [132-134].

Electrospray mass spectrometry

ESI-MS is a commonly used technique in bioanalysis. It was originally developed in 1984 by John Fenn and M. Yamashita [135, 136], based on the pioneering work of Malcolm Dole [137]. In ESI, the liquid sample enters the atmospheric pressure chamber, the ion source, through a metal or silica capillary, to which a high voltage, several kV, is applied. The ESI interface transforms the ions in the liquid phase into ions in gas phase. To enable ion evaporation ions must be present in the mobile phase before the sample is introduced. This is usually accomplished by using aqueous mobile phases containing a small amount of acid or base. Often the analytes generate either protonated, $[M+H]^+$or deprotonated, $[M-H]^-$, quasi-molecular ions, depending on the electric field polarity. ESI is not strictly an ionization method, but rather a combination of ionization and desorption of

already formed ions. Besides protonation or deprotonation, ions are often formed by adduct formation with buffer ions and with alkali-metal ions, the latter commonly originating from the glassware, such as the bottles containing the mobile phase. Among the common MS techniques, ESI is unique in that multiply-charged gas phase ions may be formed. Multiple charging in ESI enables the analysis of very large compounds such as peptides and proteins.

In the ESI source (or interface) a fine spray of charged droplets is produced, from which the solvent is evaporated to give the gas phase ions. During evaporation, which is usually facilitated by a stream of warm nitrogen, the electrical charge density at the droplet surface increases until a critical point, known as the Rayleigh instability limit, is reached. At this point the charge repulsion exceeds the surface tension and the droplets divide into smaller droplets. This process is repeated until smaller and smaller droplets are formed and finally the sample ions are ejected into the gas phase, as illustrated in Figure 12. Two different theories have been proposed to explain the ejection into the gas phase: the ion evaporation mechanism (IEM), and the charge residue model (CRM). According to CRM, the detected ions are the

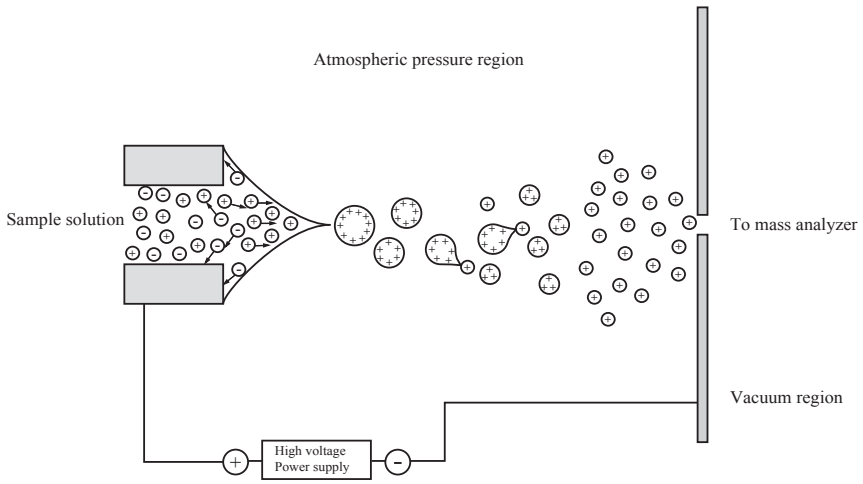


Figure 12. Schematic representation of the ESI process, in this case in positive ion mode.

charged species left when all of the solvent has evaporated from the droplet, while according to IEM direct emission due to field desorption of solvated ions from the droplet occurs when the droplet size reaches a certain radius (10-20 nm). Neither of these mechanisms has gained universal acceptance. However, a review discussing these mechanisms [138] was published some years ago and its author concluded that IEM is most likely to occur for small ions and CRM for larger protein ions. For further reading regarding ESI-MS the books edited by Cole [139] and Pramanik et al. [140] are recommended.

LC coupled to electrospray mass spectrometry

Many parameters affect the performance of the ESI process. To maximise sensitivity, the pH, solvent, solvent additives, and flow rates for the LC effluent must all be considered. Generally in ESI ion formation is most efficient at low flow rates, optimally around 5-10 µl/min [141]. However, if the solvent evaporation is pneumatically assisted, often referred to as ion spray, the flow rates can be increased up to 1 ml/min, with an optimum flow rate of around 200 µl/min. Various LC/ESI devices and LC instruments have been developed to meet the low flow rate criterion, and a practical consideration of the different devices and miniaturization is described by Abian, et al. [142]. Suitable solvents for the LC mobile phase must permit formation of ions in solution. The additives used for the mobile phase should be volatile to avoid contamination or plugging of the sample orifice. Additives in the mobile phase are used to control the pH; a high pH should be used for negative-ion detection, and for analytes with acidic sites, while a low pH is normally used for positive ion-detection and analytes with basic sites. The additives may also act as ion-pairing agents for the LC separation, since ionic and polar compounds often have poor retention in the commonly used reversed phase chromatography. The ion-pairing agent should not be strong enough to neutralize the analyte and it should be added in a low concentration to avoid ion-suppression. Other issues to consider when combining LC with ESI are described more thoroughly in reference [141].

A number of aspects related to the retention of polar compounds by HPLC systems for subsequent ESI-MS analysis must also be considered.

The composition of the mobile phase must be compatible with the ESI-MS detection system and allow chromatographic separation. Sometimes a compromise between the sensitivity and the retention might be necessary. The separation efficiency of the column is important, even if the MS detector is selective and can separate compounds by their mass-to-charge ratios (m/z). Co-eluting peaks may give rise to severe suppression of the analyte response. A retention time long enough to separate the analyte from interferences, especially early eluting polar matrix interferences, is often advantageous.

Acquisition techniques

The generated ions are separated according to their m/z values in an analyser, for instance an ion-trap or quadropole, before detection. Depending on the application different acquisition modes can be utilized for the analyser: full scan, selected-ion monitoring (SIM) or selected-reaction monitoring (SRM), which is also sometimes called multiple-reaction monitoring (MRM). In the full scan mode the complete spectrum is measured between two m/z values, and the sum of the intensities of all the detected ions can be shown in a total ion current chromatogram (TIC). In the SIM mode ions of one or a number of m/z values are selected and all the other ions are ejected from the mass analyser. SIM is often advantageous for quantification, since the time length of the signal integration is greater for the selected m/z values, compared to full scans, which significantly reduces the noise levels, and thus the detection limit. SIM was used for quantification of diphenyl phosphate in human urine in the studies described in Papers II and III. SRM is a two stage MS/MS technique, in which a selected ion, the "precursor" ion, of a certain m/z value is first isolated in the analyser. In the next stage, the precursor ion is dissociated into fragment ions, product ions, by collision with a background gas in the analyser, usually referred to as collision-induced dissociation (CID). The product ions are then scanned to produce an SRM mass spectrum and an SRM TIC. This two-stage technique is even more selective than SIM, and often yields lower detection limits. SRM also gives more definitive identification of the analyte(s) than SIM, due to the specific fragmentation reaction.

Matrix effects on the MS detection

Matrix effects are problems associated with LC/ESI-MS, and have recently been reviewed [143]. Even if a selective scan method is used, such as SIM or SRM, which avoids the detection of most interfering compounds, such compounds may still affect the ionization efficiency. ESI has proved to be generally more susceptible to matrix affects than APCI [143, 144]. The matrix effect is detected as a suppression (most commonly) or enhancement of the analyte signal response arising from the co-elution of matrix components. The exact mechanism is still unclear, but probably involves competition between the analyte and the co-eluting components from the sample matrix for access to the droplet surface in the electrospray [143]. A mechanistic investigation of electrospray ionization was presented by King et al [145], who suggested that the ion suppression is probably not caused by reactions in the gas phase, but rather to arise from a high concentration of non-volatile materials present in the ESI spray together with the analytes, inhibiting their transfer to the gas phase. Matrix effects on the ionization can be assessed by an addition of the analyte after extraction of the sample and to compare the resulting signals with those obtained from a standard solution. Different approaches for compensating for or reduce these problems have been presented, such as reducing the flow [146] and using 2D-LC [147], where the primary problems associated with co-eluting matrix components was attributed to column overload. The use of an internal standard may compensate for matrix effects, but this may also contribute to ion suppression. Liang et al. have presented a study showing that the use of co-eluting isotope-labelled internal standards suppressed the analyte signals [148]. Another study also reported problems with matrix effects when using a stable isotope analogue as IS [149]; here the ion suppression was greater for the IS than for the analyte, causing variations in the response ratios. The matrix effects can also be compensated for by using standards prepared in a blank matrix to create the calibration curve for quantification. However, such measures cannot avoid the loss of sensitivity due to ion suppression. The only solution for avoiding ion suppression effects is to improve the sample clean-up to remove co-eluting components.

Results and discussion

Applications in this thesis

For the majority of this work (Papers I-III), the target analytes were organic diphosphate esters, see Figure 13. The organophosphate triesters are widely used as flame retarding additives and/or plasticizers in diverse materials, for instance electronic equipment, lubricants, textiles and plastics. Since the compounds are used as additives and are not covalently incorporated in the material, they have a tendency to diffuse from the products, for instance into the surrounding air. Several of the compounds have also been identified in indoor environments [150]. One of these agents, triphenyl phosphate, has been shown to be emitted from computer video display units [151], and has been identified in human donor plasma [152]. Several toxicological effects of exposure to these compounds have been reported. Triphenyl phosphate exhibits contact allergenic properties [153], and has been shown to be a potent inhibitor of human blood monocyte carboxylesterase [154]. Some of the arylated triphosphate esters, such as tritolyl phosphate, also have neurotoxical effects [155].

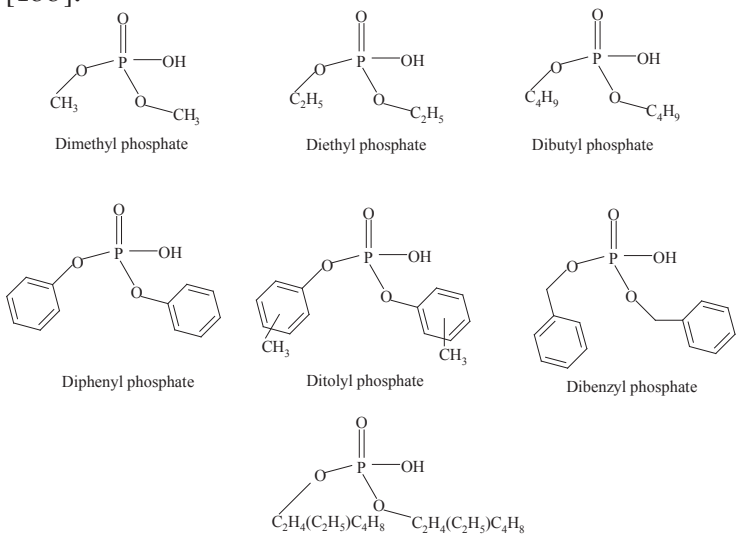


Figure 13. Structures of the organic diphosphate esters used in this work.

The corresponding diphosphate esters are degradation products or metabolites of the triphosphate esters. Little has so far been described in the literature on the metabolism of organophosphate esters and the occurrence of these metabolites in exposed animals, or humans. However, some of the diesters have been identified as metabolites from the corresponding flame retardant in rat urine, such as bis(2-chloroethyl) phosphate [156] and di-p-tolyl phosphate [157]. Bis(2-chloroethyl) phosphate ester has also been identified as a metabolite from the metabolism of tris(2-chlorethyl) phosphate by human and rat liver preparations [158]. Diphenyl phosphate has been identified as a major degradation product from triphenyl phosphate in both water and sediments and from 2-ethylhexyl diphenyl phosphate in fish tissue [159].

The dialkyl phosphate esters, such as dimethyl and diethyl phosphate, have been measured as human urinary metabolites as markers for exposure to organophosphate pesticides [160-162].

In order to evaluate risks of exposure to widespread compounds over time, such as the organophosphate flame-retardants and organophosphorus pesticides, analytical techniques are required that are sufficiently sensitive, accurate and efficient. Therefore it is of great interest to develop techniques and to improve existing ones for measuring these types of compounds. Most of these compounds are also very polar, with low pKa values, which make them particularly difficult to extract from biological matrices, such as urine, using conventional techniques.

In the study described in Paper IV a MIP was synthesized for use with a hemoglobin adduct from the reactive metabolite of 1,3-butadiene, 1,2:3,4-diepoxybutane (DEB). This metabolite has been shown to form a specific ring-closed adduct in the reaction with N-terminal valine in hemoglobin [163]. In this study, the MIP was designed for the hydrazide of this ring-closed adduct, Pyr-Val hydrazide. The structure of Pyr-Val-hydrazide is shown in Figure 14.

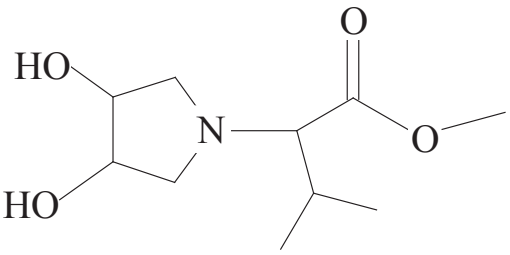


Figure 14. Structure of Pyr-Val hydrazide, (N,N-(2,3-dihydroxybuta-1,4-diyl) valine hydrazide).

DEB has high mutagenic potency compared to mono-functional epoxides, and has been assumed to play an important role in the carcinogenicity of 1,3-butadiene [164, 165]. Measurements of *in vivo* levels of reactive metabolites are essential in cancer risk assessments, however this is not generally possible. The formation of stable reaction products (adducts) with nucleophilic sites in biomacromolecules in the body, e.g. proteins or DNA, offers possibilities to measure these short-lived compounds *in vivo*. The approach of determining adducts of reactive compounds to blood proteins has been applied for various purposes, for example monitoring of occupational and environmental exposures in humans [166].

In Paper V a MISPE method for extracting the local anaesthetic drugs ropivacaine and bupivacaine from human plasma was evaluated. The chemical structure of the drugs is shown in Figure 15.

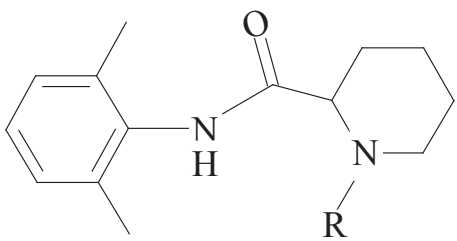


Figure 15. Structure of the local anaesthetics studied. Bupivacaine: R = n-butyl, ropivacaine: R = n-propyl.

MISPE

In the work underlying this thesis, several different MIPs were prepared and evaluated as selective sorbents for SPE. As already mentioned, the main goal was to use these sorbents for extracting target analytes from biological samples. A MIP was synthesized for the first time (to my knowledge) and evaluated with respect to its selectivity for diphosphate esters (Paper I). This MIP was further investigated for the extraction from aqueous standards and human urine (Papers II-III). The developed MISPE method was also compared with the use of commercially available SPE sorbents (Paper II). The MIP prepared for the extraction of Pyr-Val hydrazide (Paper IV) was also synthesized for the first time (to my knowledge) and was then evaluated primarily for aqueous standards. The MIP made for the local anaesthetics (Paper V) was used both in aqueous buffer-based experiments and for the extraction of human plasma samples.

Evaluation of the selectivity of the prepared MIPs

The selectivity of the MIP, or confirmation of the establishment of imprints, is often evaluated by comparing its retention parameters with those of a non-imprinted form of the polymer, NIP, synthesized in the same way, and often in parallel to the MIP, except for the addition of the template molecule. The MIP is often evaluated prior to its use in MISPE by a chromatographic evaluation (as in Paper I) or by equilibration batch rebinding experiments (as in Paper V). However, for the majority of this work (Papers I-IV), the selectivity and optimization of the extraction protocol were evaluated by comparing the recovery and breakthrough parameters of the produced MIP and the corresponding NIP, by using them directly as SPE sorbents. Fractions from both columns were collected and further analyzed by LC/ESI-MS. The analyte recovery in each fraction was quantified by comparing the analyte/internal standard response ratio with that in an external standard.

For the new MIP synthesized and evaluated for the selective extraction of diphenylphosphate in Paper I, selective adsorption was achieved when the analyte was extracted from methanol. Although this solvent is polar

a selective interaction was established. The selectivity was manifested by a large breakthrough for NISPE when the analyte was applied in methanol, compared to a complete retention for MISPE. In Paper II the selectivity of this MIP was evaluated for several diphosphate esters and structurally related compounds, by comparing the recoveries obtained from the MIP and the corresponding NISPE. The results showed that the imprints possessed cross-reactivity for several of the tested compounds, demonstrated by a stronger retention on the MIP compared to the NIP. The compounds that are most structurally similar to the template molecule were most strongly retained, indicating that both their shape and functionality influence the recognition mechanism.

Direct extraction from aqueous standards

Since the prepared MIPs were intended to be used for extracting analytes from human body fluids, their selectivity and recognition mechanisms were further evaluated under aqueous conditions. As already mentioned, several problems are related to this issue. Since the polymer backbone is of hydrophobic nature there is a problem with wettability of the surface and transport to the imprints in these conditions. Another problem related to these conditions is that the polar interactions, e.g. hydrogen bonds, formed in the pre-polymerisation complex are weakened by the presence of the polar and aqueous solvent. Extracting aqueous samples often leads to most of the adsorption to the polymer surface being of non-selective nature. However, this non-selective adsorption can be disrupted and transformed to selective adsorption to the imprints by carefully choosing the washing protocol. This effect was further investigated in the studies described in Paper II when extracting diphenyl phosphate from an aqueous standard. The analyte was completely retained on both the MIP and the NIP columns under these conditions. However, when the columns were washed with methanol the non-selective adsorption was interrupted, resulting in desorption from the NIP column, while the analyte was still retained on the MIP sorbent. Methanol then acted as a selective washing agent, allowing analyte diffusion and adsorption of the analyte to the imprints. In Paper III this wash step was further investigated in terms of the effects of the amount of organic modifier on the non-selective adsorption. It was found that levels of 50% or

more were needed to disrupt this interaction, as illustrated in Figure 16. The results from the wash step investigation provide information about the mechanism of retention to the imprints, which is important for the development of the MISPE method. Diphenyl phosphate has a strongly acidic functionality, with a pK_a of 0.3 [167], and relatively non-polar substituents, the phenyl groups. A strong ionic interaction between the acid functionality and the basic functional monomers (2-Vpy) is probably the major mechanism of retention to the imprints, since the retention to the imprints was preserved in the presence of the polar and protic solvent. Since increasing the methanol content interrupted the non-selective adsorption to the surface adsorption, this interaction was mainly of hydrophobic character.

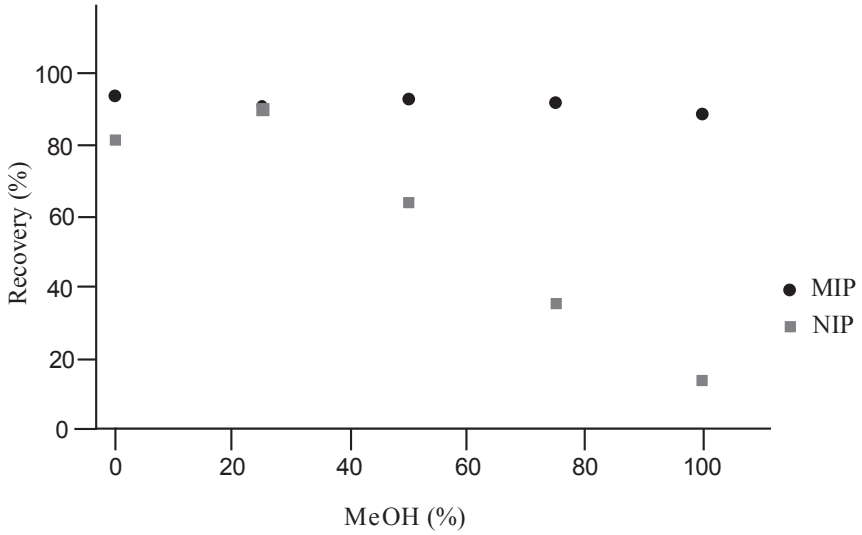


Figure 16. Recoveries of diphenyl phosphate extracted by MIP and NIP columns from 1 ml of water. The columns were washed with 5 mM NH3 containing different percentages of methanol. Each value represents an average of duplicate samples.

Extraction of the analyte Pyr-Val-hydrazide (hemoglobin adduct) from an aqueous solution containing 10% acetonitrile, was also evaluated (Paper IV). In this case, selectivity was achieved in aqueous conditions. When loading the analyte onto the MIP and NIP columns, respectively, approximately 10% breakthrough was detected from NISPE.

However, when washing with water this breakthrough increased for the NIP while the analyte was still retained on the MIP, as illustrated in Figure 17. Hence, in this case water was able to transport and facilitate retention of the analyte to the imprints. Since water was able to disrupt the non-selective adsorbance to the polymer surface this adsorption was not mainly of hydrophobic type. The non-selective interaction proved to be relatively strong in acetonitrile, which was the diluent used for the polymer synthesis. This solvent is aprotic and allows hydrogen bonding, which most likely is the predominant mechanism of the non-specific binding of analyte to the polymer. The analyte contains several functional groups that may form hydrogen bonds with the functional monomer used, MAA, and the crosslinker, EDMA. Since hydrogen bonding is weak in aqueous environment these non-selective interactions were interrupted when the columns were washed with water. However, the retention to the imprints was still strong under these conditions, probably due to a combination of several hydrogen bonds, steric factors and ionic interactions between the basic functionality of the analyte and the acidic monomer.

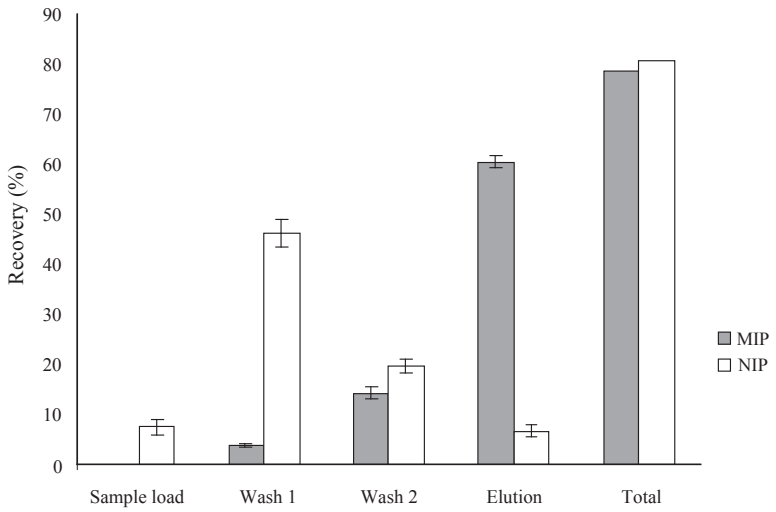


Figure 17. Recovery of Pyr-Val-hydrazide in each fraction using MISPE and NISPE, respectively, loaded in 0.5 ml 10% acetonitrile. Wash 1 = 1 ml water, Wash 2 = 0.5 ml acetonitrile, Eluent = 2 ml ethanol. Columns represent the average of three replicates and the error bars show the standard deviation.

As reported in Paper V, extraction of the local anaesthetic, Bupivacaine, from an aqueous solution resulted in strong non-selective adsorption, demonstrated by comparing the binding isotherms, obtained from radioligand binding experiments, of the MIP with the corresponding NIP. It was found that addition of an organic modifier, 5% ethanol, suppressed the non-specific interaction. The organic modifier facilitates the wettability and the diffusion of the analyte to the imprints. As mentioned before, the addition of a detergent could also be used to suppress this type of adsorption to the polymer surface. This was demonstrated in this study by the addition of Tween 20 to the incubation solution. The pH also proved to be an important parameter to control to reduce the non-selective adsorption. The effect of pH is further discussed in the next section.

Another important parameter to optimize to achieve high recoveries and, in some cases, selective extraction is the elution step. After the selective wash the analyte can be deeply embedded in the polymer matrix and strongly bound to the imprints. To interrupt these interactions a polar solvent is commonly used with the addition of a strong base or acid. In the studies described in Paper I a strong acid, trifluoroacetic acid (TFA), was added to the elution solvent, methanol, to displace the analyte. It was found that TFA concentrations as high as 4-6 % were needed for efficient desorption of the analyte. However, in Paper II it was found that a strong base, triethyl amine (TEA) more efficiently disrupts the interactions with the imprints. TEA was also used for desorption in the studies described in Paper V. In this work TEA gave more selective extraction than the addition of an acid, as manifested by a cleaner GC chromatogram.

Extraction from human body fluids and matrix effects

Biological samples are challenging to extract since they contain many compounds that can be coextracted simultaneously by the sorbent. As described above, the adsorption to the MIP in aqueous environment is often due to a non-selective interaction, which may be disrupted by a selective wash step. In the case of extraction from body fluids the sample matrix may also affect the selective rebinding to the imprints. It is

therefore of great importance to investigate these effects in order to use the MIP most efficiently.

In the investigations reported in Paper II MISPE of diphenyl phosphate from human urine was tested. When transferring the developed extraction protocol from aqueous standards, including the selective wash with methanol, to human urine samples no recovery at all was obtained. It was found that substantial breakthrough occurred during the sample-loading step, when urine was applied directly to the column. The urine matrix decreased the capacity of the polymer, suppressing both the selective and the non-selective adsorption. To investigate the effects of pH the sample was diluted with a buffer. At low pH, 3.0, the adsorption was strengthened and the recovery increased to over 80%, when extracting from urine. However, the non-selective interaction also increased and was difficult to disrupt. At low pH the functional monomer (2-VPY, pK_a 5.9) is completely charged, leading to a stronger ionic interaction, both to the imprints and to ionic non-selective sites in the polymer. These non-selective sits are due to randomly incorporated pyridine residues, since the functional monomer was added in excess to the template molecule during the MIP preparation. In extractions from aqueous standards at higher pH the non-selective adsorption was mainly due to hydrophobic interactions, and thus easily suppressed with methanol. At low pH the selective wash step was modified with a basic additive in order to suppress the stronger non-selective adsorption to the surface, which was probably based on both hydrophobic and ionic interactions. In Paper III the effect of pH was further assessed and it was shown that pH was an important parameter to control for both the recovery and the selectivity, as shown in Figure 18. This effect of pH was also demonstrated in the studies described in Paper V, in which an acidic monomer was used (MAA, pK_a 4.3) and the interactions were stronger at higher pH. Both the selective interactions with the imprints and the non-selective interactions with the surface increased in strength with increasing pH. However, the maximal difference between the binding to the MIP and the corresponding NIP was found to be at pH 5.0. Thus, fine-tuning of the pH was shown to be important to achieve a selective rebinding to the imprints and high recovery, although this may require a compromise between these parameters.

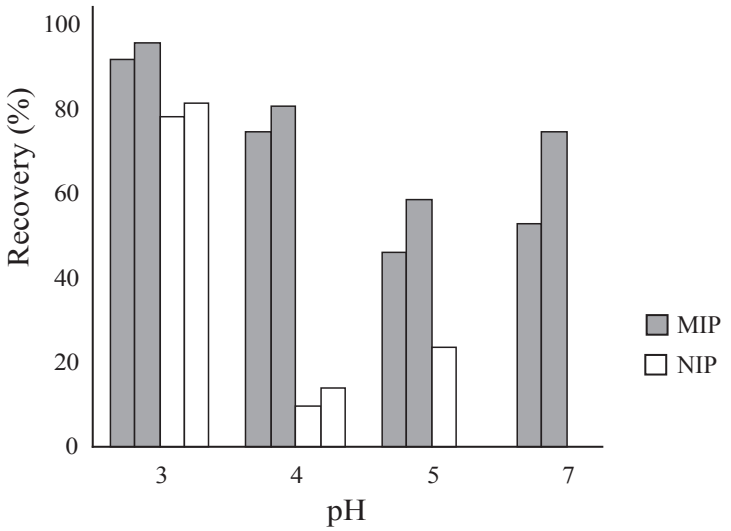


Figure 18. Recoveries of diphenyl phosphate extracted by MISPE and NISPE from 1 ml of a salt standard (urine mimic). The salt standard contained both NaCl and urea and was diluted with a buffer at several different pH prior to extraction. Columns represent duplicate samples at each pH.

The effects of the urine matrix were further investigated in order to optimise the recovery and selectivity (Paper III). In an attempt to mimic the urine matrix, a number of naturally occurring urine components (urea, NaCl, creatinine and p-aminohippuric acid) at naturally occurring levels were added to an aqueous standard solution containing the analyte. These solutions were then extracted using the MIP and the NIP sorbents to investigate the effects of these components on the recovery. It was found that NaCl negatively affected both the recovery and repeatability of the MISPE, while urea, creatinine and p-aminohipuric acid had little or no effect, even when they were added in large excess to the analyte. The Na^+ ions were supposed to interact with the acidic functionality of the analyte, thereby hindering an ionic interaction between the analyte and the basic sites in the MIP. The effect of cations has also been described in a study describing the extraction of triazines from mineral water [168]. In the cited study, Ca^{2+} ions were found to adversely affect the extraction recoveries and this was postulated to be due to ion-exchange between the cation and the acidic functional monomer, MAA. Interestingly, the

addition of p-aminohippuric acid, added at levels up to 1000 times that of the analyte, did not affect the recovery of diphenyl phosphate. This compound contains an acidic functionality, which might interact with the basic monomer and has a more hydrophobic structure than urea and creatinine. The effect of NaCl was suppressed by simply diluting the solution with a buffer at pH 4.0, as demonstrated in Figure 18. The improvement in recovery at lower pH is most likely due to increases in the density of positively charged sites in the polymer, which suppress the complex formation with Na^+.

Comparison with conventional SPE sorbents

To investigate the suitability of MISPE for extracting diphenyl phosphate, the method was compared with SPE using several commercially available SPE sorbents (Paper II). Since the analyte has a strongly acidic functionality, but also hydrophobic moieties (the phenyl rings) SPE sorbents with both hydrophobic and ion exchange functions were tested. Three different anion exchangers were tested - a strong anion exchanger (SAX), a weak anion exchanger, an aminopropyl modified silica column (NH2) and a mixed mode anion exchanger (MAX) – and a commonly used reversed-phase (C18) sorbent. Diphenyl phosphate was loaded onto the different columns in an aqueous solution. On the reversed-phase sorbent the analyte was retained in the loading step, but substantial breakthrough was detected when the cartridge was washed with water. The analyte was not retained on the weak anion exchanger (NH2), and was detected as a large breakthrough in the loading fraction. On the other anion exchangers the analyte was retained, but its retention on the SAX cartridge was extremely strong and no suitable elution conditions were found, even when a higher than recommended concentration of acidic modifier was used. For the MAX column it was possible to elute the analyte using up to 6 ml of methanol containing 2% of a strong acidic modifier, trifluoroacetic acid (TFA).

The MAX sorbent was further tested for the extraction of diphenyl phosphate from urine and high recovery was achieved. The MAX sorbent consists of a HLB copolymer backbone with incorporated cationic groups, for the structure see Figure 5. These functionalities appear to be

suitable for interactions with diphenyl phosphate. However, although the recoveries were high (102%) the sorbent provided less selectivity than the developed MISPE, resulting in high levels of matrix components in the sample, which negatively affected the following LC/ESI-MS detection, as further discussed in the *ESI-MS and matrix effects* section. Another disadvantage with this type of sorbent was that a large volume of solvent was needed for elution of the strongly retained analyte.

The results demonstrate that it may be difficult to identify appropriate SPE sorbents for certain analytes, especially for the extraction of ionic or polar analytes from aqueous matrices. In such cases MIPs may provide an alternative or complements to conventional/commercial sorbents.

LC/MS

LC/MS methods were developed for the majority of the techniques presented in Papers I-IV. Due to its strengths in both sensitivity and identification, MS is often preferable to other detection methods. However, to my knowledge, these were the first uses of LC/MS for detecting the investigated phosphate diesters. In previous studies including this class of compounds, especially the dialkylated phosphate esters, dimethyl and diethyl phosphate, the samples have been derivatised then detected by GC/MS [160, 161]. However, ESI-MS offers a means for rapid and less laborious analysis that allows the ionic diphosphate esters to be directly quantified in their native form.

Separation of polar and ionic compounds by LC

Separation of ionic compounds on a reversed-phase column usually requires ion-pairing agents to be added to the mobile phase. In the studies described in Papers I and III a reversed-phase column, C18, was used to separate the strongly acidic diphosphate esters. In the study described in Paper I triethylamine (TEA) was used as an ion-pairing agent and a baseline separation of the polar compounds was achieved, while in Paper III ammonia was used as additive to the mobile phase instead

of TEA, since the latter resulted in reductions in sensitivity after long-time usage, probably due to contamination of the sample orifice. The basic modifiers may also promote the ion formation of the acidic esters, although since the diesters are very acidic, with pK_a values below 1, they should already be completely dissociated when the pH is higher than 3. The modifiers were therefore added mainly to improve their retention and separation.

In Paper II the suitability of a Hypercarb column for separating the diphosphate esters was tested. Hypercarb consists of porous graphitic carbon particles, which were briefly described in the *Different types of sorbents* section. The advantage of this column for LC/ESI-MS applications is that it strongly retains polar compounds and can be used with a high content of organic modifier in the mobile phase. The retention involves a combination of two mechanisms: dispersive interactions between the analyte-mobile phase and the graphite surface and charge-induced interactions of a polar analyte with the polarizable graphite surface. The first mechanism is the same as in conventional reversed-phase LC, while the second requires a different method development strategy [169]. The diesters were strongly retained by the Hypercarb column; it was not possible to elute them using a gradient up to 100% of acetonitrile, including ammonia. However, when tetrahydrofuran (THF) was used instead as organic modifier the diesters were eluted with a gradient of up to 40% of THF, including 10 mM NH_3.

An LC/MS method was also developed for the hemoglobin adduct, Pyr-Val-hydrazide, the deuterated internal standard and the template, Pyr-Leu-hydrazide (Paper IV). These compounds are very polar and were difficult to retain on a conventional C18 column, eluting very early, almost with the void even though a water-rich gradient was used. By using a polar endcapped C18 Aquasil column, the retention could be increased and a separation of the hemoglobin adduct from the template was achieved. The Aquasil column is designed to cope with water-rich gradients and to retain polar analytes, due to the hydrophilic endcapping groups incorporated between the C18 alkyl chains [170].

ESI-MS and matrix effects

As reported in Paper I, an SRM method was developed for the studied diesters, diphenyl phosphate, ditolyl phosphate, dibutyl phosphate and bis(2-ethylhexyl) phosphate. The precursor ions formed under negative-ion conditions were the deprotonated quasi-molecular ions, $[M-H]^-$. For all the investigated compounds, the corresponding monoesters were formed as stable product ions by CID. For each of the aromatic diesters the major fragment obtained was the aromatic moiety including one oxygen, as shown in Figure 19. An MS/MS method was also developed for the protein adduct, Pyr-Val-hydrazide (Paper IV). The adduct, template and internal standard were determined in positive mode under acidic conditions,

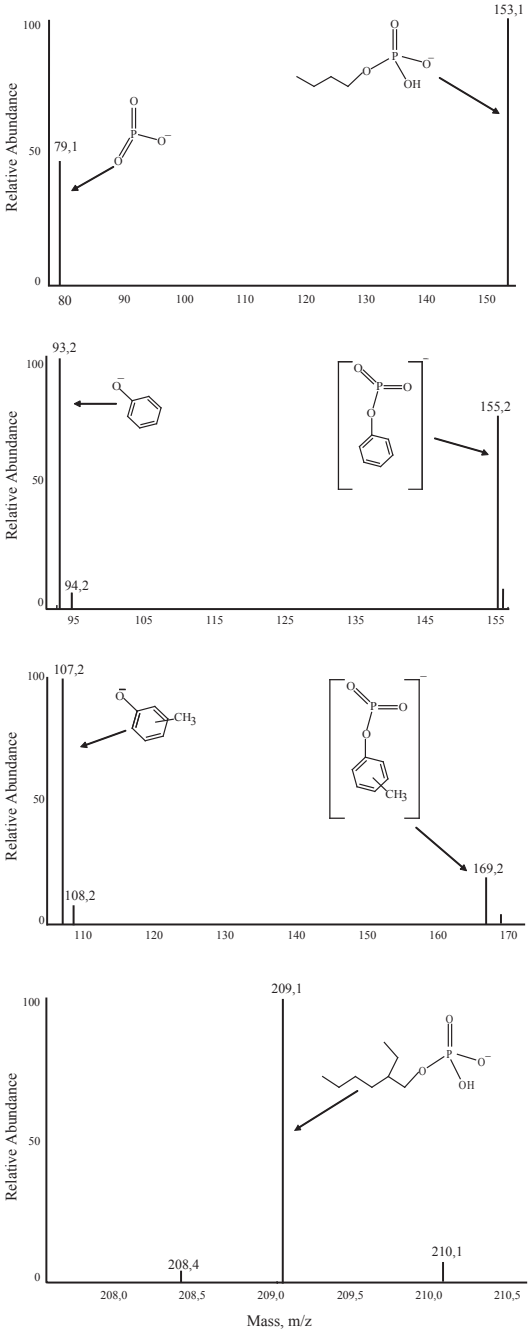


Figure 19. Mass spectrum of the investigated diphosphate esters after collision induced dissociation. Proposed structures of the ions are shown in the spectrum.

which generated protonated quasi-molecular ions $[M+H]^+$. This protein adduct has been shown in earlier LC/ESI-MS studies of DEB-modified peptides to generate a major fragment at m/z 158, corresponding to the pyrrolidinium ion [171]. In our study, this ion was also generated by CID from the analyte, as shown in Figure 20, and a similar one for the internal standard at m/z 172.

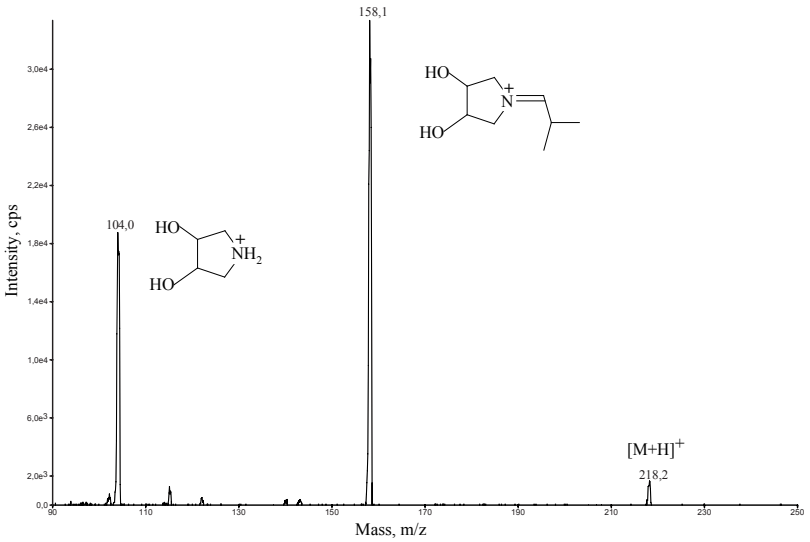


Figure 20. Mass spectrum of Pyr-Val-hydrazide after collision-induced dissociation. Proposed structures of the fragment ions at m/z 104 and m/z 158 are shown in the spectrum.

In Paper II the effects of a more selective clean-up step on the matrix effects in ESI is presented. In this study a commercial mixed-mode anion exchanger was compared with the developed MISPE sorbent for extraction of diphenyl phosphate from urine. The urine extract from the MAX column still contained substantial amounts of matrix components, manifested as interfering peaks in the ion chromatograms, which were more complex than those obtained when the MISPE cartridge was used, as shown in Figure 21, even though selective-ion monitoring was used. These interferences also caused ion suppression, which was observed by comparing the signal intensity of the volumetric internal standard, with the signal intensity of an external standard. The matrix also affected

the repeatability of the MAX method (RSD 17%, n=4) compared to the MISPE method (RSD 11%, n=4).

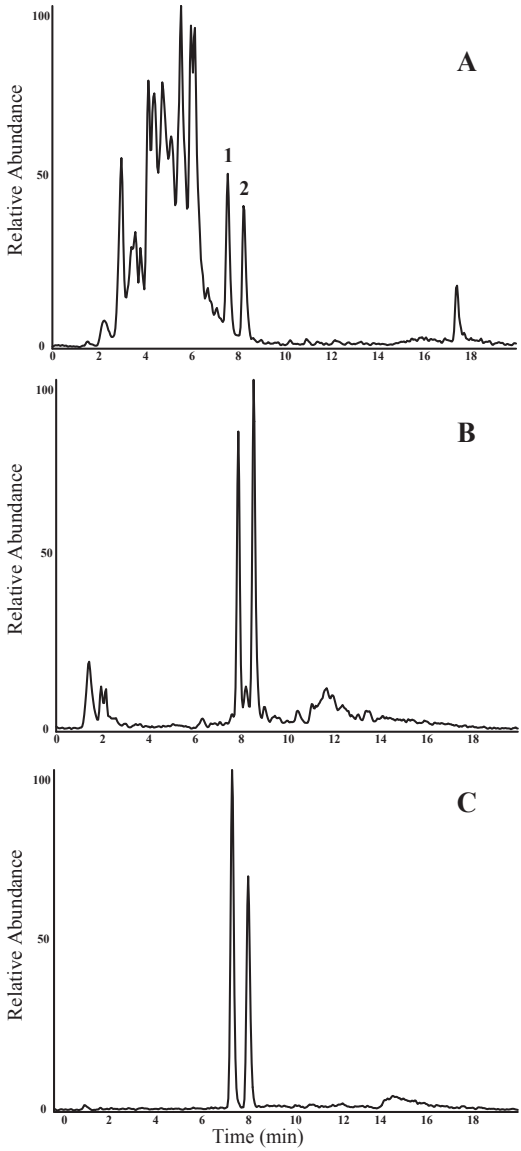


Figure 21. Total ion chromatograms obtained from an extract of a 1ml urine sample spiked with 260 ng diphenylphosphate. (A) MAX extract, (B) MIP extract and (C) external standard. Peak 1 corresponds to diphenyl phosphate and peak 2 to dibenzyl phosphate (internal standard).

Signal suppression in ESI may also arise from additives to the mobile phase, such as ion-pairing agents [172]. The ion-pairing agents, TEA and ammonium acetate, which were tested for their effects on the retention of the highly acidic analyte, diphenyl phosphate (Paper I) both suppressed the analyte response in the tested range, 0.1–10 mM, causing sensitivity to decrease as the concentration of the agents increased. TEA at 0.5 mM affected the sensitivity to a lesser extent than ammonium acetate and was hence chosen to improve the chromatography. The results demonstrate the importance of minimizing additives to the mobile phase, even if they are added at relatively low concentrations.

Conclusions and future perspectives

The following questions were initially addressed in the work underlying this thesis. Is it possible to use molecularly imprinted polymers in selective sample preparation techniques to measure levels of highly polar and acidic environmental pollutants in human body fluids, and what advantages could they offer compared to well-established commercially available techniques? Although we did not fully answer these questions, we went a long way towards doing so, arriving at several conclusions and raising several new questions to resolve.

From the results presented in the papers included in this thesis, it could be concluded that it is possible to produce MIPs that are selective for metabolites of environmental pollutants. The developed methods allowed efficient and selective clean-up from human biofluids. When evaluating the produced MIP as an SPE sorbent several parameters that affect the extraction recovery, reproducibility and selectivity were identified. The most important parameters to optimize were the solvent used for washing the sorbent and the sample pH. It was found that a substantial amount of the adsorption to the MIP when extracting aqueous matrices was non-selective adsorption to the polymer surface, resulting in poor reproducibility. However, by carefully choosing the solvent composition for the wash step this interaction could be suppressed and the selective sites in the MIP could be exploited. It was also found that is important to identify the effects of the sample matrix, in order to suppress them and to optimise the developed MISPE method. Identifying the sample matrix effects and the conditions needed to achieve selective extraction improved our understanding of the recognition mechanism of MIPs in aqueous matrices. This knowledge should facilitate the development of general extraction protocols such as those that have been established for conventional SPE.

It was demonstrated that the unique selectivity of the developed MISPE method has several advantages over the tested commercially available SPE sorbents. The developed MISPE method provided more efficient clean-up, which proved to be important even when a detection system

as selective as ESI-MS was used. Direct extraction of polar and water-soluble compounds from an aqueous matrix was difficult to achieve with the conventional SPE sorbents tested. These findings indicate that MISPE may provide an important alternative to conventional SPE sorbents for the extraction of polar or ionic metabolites from biofluids.

The method developed for the investigated phosphate esters should be further evaluated for its applicability to structurally similar and environmentally relevant compounds from human urine. The capacity of the polymer also needs to be further investigated, in terms of how much the sample volume can be increased. To make the MIP preparation easier, the polymerization could be performed by suspension polymerization. Since the retention mechanism seems to mainly depend on ionic and hydrophobic interactions a conventional suspension polymerization with water as the continuous phase might work. This would speed up the MIP preparation and also generate a higher amount of particles in the useful size range directly from the prepared batch. Finally, the method should be applied in exposure studies of organophosphorus environmental pollutants, for example in indoor environments with high levels of flame retardants. In the case of the MISPE method developed for the hemoglobin-adduct, further studies must investigate how the degraded protein matrix affects the MIP, in terms of capacity and selectivity. Hopefully, this method should prove to be useful for measuring the protein adduct as a marker for exposure to butadiene.

In conclusion I think that MISPE should provide a viable solution to several analytical problems in the future, and that the application and scope of this technique will expand. I hope that this thesis, and the studies it is based upon, will facilitate these developments, and help promote the use of MISPE as a routine analytical extraction method.

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Abbreviations

AAM	Acrylamide
ABDV	2,2´-azobis(2,4-dimethylvaleronitrile)
AIBN	2,2´-azobisisobutyronitrile
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
CEC	Capillary electrochromatography
CID	Collision induced dissociation
CRM	Charged residue model
DEB	Diepoxybutane
DVB	Divinylbenzene
EDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionization
GC	Gas chromatography
GCB	Graphitized carbon black
HEMA	2-hydroxyethylmethacrylate
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
IAE	Immunoaffinity extraction
IEM	Ion evaporation mechanism
IF	Imprinting factor
IST	International sorbent technology
LC/MS	Liquid chromatography mass spectrometry
LLE	Liquid-liquid extraction
MAA	Methacrylic acid
MAX	Mixed-mode anion-exchange
MeOH	Methanol
MIP	Molecularly imprinted polymer
MISPE	Molecularly imprinted solid-phase extraction
MRM	Multiple-reaction monitoring
NIP	Non imprinted polymer
NISPE	Non imprinted solid-phase extraction
NMR	Nuclear magnetic resonance

PETRA	Pentaerythritol triacrylate
PGC	Porous graphitic carbon
PS-DVB	Polystyrene-divinylbenzene
RAM	Restricted-access material
RSD	Relative standard deviation
SAX	Strong anion exchanger
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SRM	Selected reaction monitoring
TCA	Trichloroacetic acid
TEA	Triethyl amine
TFA	Trifluoroacetic acid
TFMAA	Trifluoromethyl acrylic acid
THF	Tetrahydrofurane
TIC	Total ion current
TRIM	Trimethylopropane trimethylacrylate
2-VPY	2-vinylpyridine
UV	Ultraviolet

Populärvetenskaplig sammanfattning

Inom analytisk kemi vill man mäta om ett ämne finns i ett visst prov och hur mycket av ämnet som finns i provet. Proverna kan till exempel vara blod, urin eller vatten där man vill mäta halten av en läkemedelsubstans eller se om det finns miljöföroreningar i vattnet. I många av dessa prover är halten av ämnet man vill mäta väldigt låg medan halten av andra ämnen som naturligt finns i proverna är mycket högre. För att kunna mäta de här låga halterna behöver man fånga ämnet och rena bort de andra ämnena ur provet, vilka annars stör analysen.

I den här avhandlingen har en teknik för att fånga och rena upp ämnen väldigt selektivt undersökts. Tekniken kan jämföras med kroppens immunförsvar: vid en attack mot kroppen av främmande ämnen bildar immunförsvaret antikroppar som binder det främmande ämnet. Om ämnet återigen attackerar kroppen kommer antikropparna ihåg ämnet och fångar det igen. Speciella antikroppar bildas för varje ämne och de fångar bara det ämne de har bildats för. I tekniken den här avhandlingen handlar om har en antikropps egenskap försökts härmas, dvs. att fånga ett ämne väldigt selektivt, genom att tillverka en syntetisk antikropp i plast. Den här syntetiska antikroppen kallas molekylavtryck och tillverkas genom att man i plast gjuter ett avtryck av en molekyl. När molekylen sedan tvättas ur plastmallen kan avtrycket användas för att fånga ämnet, eller ämnen som är väldigt lika avtrycksmolekylen.

I arbetet bakom den här avhandlingen har olika molekylavtryck tillverkats för att rena upp och fånga ämnen från urinprover och blodprover. Ämnena har varit läkemedel och nedbrytningsprodukter från miljöfarliga ämnen. Molekylavtrycken har packats i plaströr och proverna har hällts ner i dessa rör. När provet vandrar genom röret fastnar ämnena i avtrycken och resten av provet rinner igenom. Genom att sedan hälla på olika kemiska lösningar kan man tvätta bort andra ämnen från provet som kan ha fastnat på plastens yta. Efter tvätten tillsätter man en lösning som gör att ämnet man vill mäta släpper från avtrycken. Den lösning man då får fram är väldigt ren och tillåter att en mycket låg halt kan mätas.

För att kunna använda molekylavtrycken på ett bra sätt är det viktigt att försöka förstå hur de kemiskt fungerar när de binder ämnen i ett prov och hur provets egna ämnen påverkar inbindningen. Detta har undersökts genom att testa olika kemiska lösningar för att späda ut provet, för att tvätta plasten med och för det sista steget då ämnet släpper från avtrycken. För att kunna verifiera att ett avtryck har bildats och att det är selektivt för det avtryckta ämnet har plasten jämförts med en referensplast. Referensplasten har tillverkats på samma sätt förutom att avtrycksmolekylen aldrig tillsatts, den saknar alltså avtrycket.

Resultaten från undersökningarna visade att vi lyckats tillverka avtrycksplaster och att vi kunde använda dem för att fånga ämnena från urinprover och blodprover. Plasten som utvecklats för urinprover visade sig påverkas negativt av saltet som finns naturligt i urin. Genom att späda ut urinprovet och korrigera pH-värdet kunde den effekten hävas. När samma plast jämfördes med en vanligt förekommande metod, visade det sig att den gav en bättre upprening på grund av sin förmåga att selektivt fånga ämnet. Det visade sig också vara viktigt att noga välja lösning för att tvätta plasten. När provet tillsattes fastnade nämligen det undersökta ämnet även utanför avtrycket, på ytan av plasten. Rätt vald tvättlösning kunde då istället tillåta att ämnet transporterades till avtrycken och fastnade där.

Sammanfattningsvis hoppas jag att den här avhandlingen har bidragit till kännedomen om hur den kemiska inbindningen sker för molekylavtryck, framförallt för biologiska prover, såsom urin- och blodprover. Den kunskapen är viktig för att den här tekniken ska kunna utvecklas för fler ämnesklasser och provtyper samt få utökad användning inom analytisk kemi.