Development of novel spme coatings and high-throughput automation of sample preparation for pharmaceutical and clinical samples

by

François Breton

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Increasing the efficiency of the methods used through the development and trial of novel pharmaceutical compounds is an important step to reduce the time required to develop new medical treatments. Before a drug can be used, multiple analyses are required to obtain their physical, chemical and biopharmaceutical properties. The aim of this thesis will be to show that SPME can be an advantageous technique in the field of pharmaceutical development due to its use both as a tool to determine the physical properties of drugs and to facilitate clinical development by easily and cheaply providing high-throughput analysis of compounds in biological fluids.

It will be demonstrated that a novel coating of triacontyl is capable of rapid equilibrium while providing enhanced sensitivity towards benzodiazepines when compared to shorter chain alkyl extraction phases. The same extraction phase will prove capable of providing a rapid determination of the hydrophobicity of structurally diverse β-blocker drugs while maintaining the use of solvents and analyte to a minimum. We will then show the possibility to produce large quantities of fibers using a robotic apparatus for high-throughput handling of samples. The 96 fiber plate produced will then be used to analyze the target drug loratadine in human plasma using the same apparatus.

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Table of Contents

List of Tables viii

List of Figures ix

List of Abbreviations xi

1 Introduction 1

1.1 SPME Background 2

1.1.1 Thermodynamic Theory 3

1.1.2 Kinetic Theory 5

1.2 SPME Coating Technologies 7

1.2.1 Coating Extraction Mechanisms 7

1.2.2 Coating Methodology 9

1.2.2.1 Polymeric Materials 9

1.2.2.2 Sol-gel 10

1.3 Current Methods for Drug Analysis 14

1.3.1 Classical Approaches 14

1.3.2 Automation Oriented Approaches 15

1.3.2.1 Solid Phase Extraction 15

1.3.2.2 In-tube Solid Phase Microextraction 16

1.3.2.3 Solid Phase Microextraction 18

1.3.3 Biocompatible Approaches 19
1.4 Octanol-Water Partition Coefficient 22
1.5 Thesis Objectives 24
2 Development of Silicate Triacontyl Coatings for Drug Analysis 25
2.1 Preamble 25
2.2 Introduction 25
2.3 Experimental 28
2.3.1 Chemicals and Materials 28
2.3.2 Coating Procedure 30
2.3.3 SPME Conditions 31
2.3.4 SEM and EDX Characterization 32
2.3.5 LC-MS Instrumentation 33
2.4 Results and Discussion 35
2.4.1 Development and Optimization of SPME Coating Procedure 35
2.4.2 Extraction and Desorption Properties 41
2.5 LogP Determination using C30 Fibers 45
2.6 Conclusions 50
3 High-throughput Automation for Drug Analysis 51
3.1 Introduction 51
3.2 Experimental 53

3.2.1 Chemicals and Materials 53
3.2.2 SEM Characterization 54
3.2.3 LC-MS/MS Instrumentation 55
3.2.4 Preparation of Stock Solutions and Samples 56
3.2.5 Preparation of Adhesive 56
3.2.6 Automated SPME Coating Procedure 57
3.3 Results and Discussion 59
3.3.1 Development and Optimization of SPME Coating Procedure 59
3.3.2 Optimization of the Automated Coating Procedure 62
3.3.3 Optimization of the SPME conditions 65
3.3.4 Validation of the Automated Coating Procedure 67
3.3.5 Application and Validation of Fibers in Human Plasma 69
3.4 Conclusions 74
4 Summary 75
Safety Considerations 76
References 77

List of Tables

Table 1 MRM transitions and MS parameters for β-blocker compounds..... 35

Table 2 Fiber-to-fiber and batch-to-batch reproducibility using C18 fibers. 39

Table 3 Calibration range and linearity of response using C18 and C30 fibers..... 45

Table 4 Carry-over of the β-blockers using C30 extraction phase..... 46

Table 5 Experimental and literature values used for LogP determination of β-blockers by SPME. 47

Table 6 Precision intra-assays of loratadine in spiked human plasma. 71

Table 7 Interday reproducibility of the fibers in human plasma. 72

List of Figures

Figure 1 Graphical representation of the boundary layer. 6

Figure 2 Schematic representation of the extraction process of liquid and porous solid materials..... 8

Figure 3 Formation of porous silica particles. 12

Figure 4 SiO_2 sol formed from silicic acid salt condensation. 13

Figure 5 Online sample clean-up using SPE column coupled to a traditional HPLC setup. 16

Figure 6 In-tube SPME apparatus coupled to LC-MSD 17

Figure 7 Surface derivatization followed by enzymatic cleavage of a pore at the surface of the RAM silica particles. 21

Figure 8 Structure of benzodiazepines. 28

Figure 9 Structure of β-blockers used in LogP correlation experiment 29

Figure 10 Scanning electron micrograph of an SPME fiber prepared by entrapping 5-µm porous silica particles in polymerized silicate. 37

Figure 11 Ruggedness of the coated fibers as represented by extraction efficiencies. . 40

Figure 12 Comparison of extraction efficiencies (% extracted) for C4, C8, C18 and C30-derivatized fibers using selected benzodiazepines. 42

Figure 13 Extraction time profiles for benzodiazepines using C18 fibers and C30 fibers. 44

Figure 14 Comparison of the β-blocker $LogK_{fs}$ values obtained experimentally using SPME, with the LogP values obtained from literature results. 48

Figure 15 Molecular structure of loratadine. 53
Figure 16 Schematic of the fiber holder apparatus with embedded samarium cobalt magnets. 58
Figure 17 SEM bondline of the adhesive without stationary phase and final product of adhesive and stationary phase after sonication step. 61
Figure 18 Modified 2 mL deep well plate used for automated coating procedure. 64
Figure 19 Desorption time profiles of loratadine using various solvents. 66
Figure 20 Determination of the effect of time on the epoxy preparation by observing capacity of fibers fabricated at different times. 68
Figure 21 Batch-to-batch reproducibility of the fiber preparation. 69
Figure 22 Specificity of the method for loratadine in plasma at LLOQ. 73

List of Abbreviations

ADME	Absorption, Desorption, Metabolism, Excretion
ADS	Alkyl Diol Silica
C4	Butyl
C8	Octyl
C18	Octadecyl
C30	Triacontyl
CAR	Carboxen
CDI	Carbonyldiimidazole
CEC	Capillary Electrochromatography
CW	Carbowax
DVB	Divinylbenzene
EDX	Energy Dispersive X-ray
ESI	Electrospray Ionization
GPP	Glycine-L-Phenylalanine-L-Phenylalanine
HPLC	High Performance Liquid Chromatography

ISRP	Internal Surface Reversed Phase
ISTD	Internal Standard
LLE	Liquid-Liquid Extraction
LLOQ	Lower Limit of Quantitation
MIP	Molecularly Imprinted Polymer
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometer
PA	Poly(acrylate)
PBS	Phosphate Buffered Saline
PDMS	Polydimethylsiloxane
RAM	Restricted Access Material
RSD	Relative Standard Deviation
SEM	Scanning Electron Microscope
SIM	Single Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
UV	Ultraviolet

XDS	Exchange Diol Silica

1 Introduction

Pharmaceutical innovation and development is directly linked to scientists' ability to monitor drugs as they are developed, including monitoring the drugs in biological fluids. The fast and efficient monitoring of drugs into our bodies is both a fascinating and a daunting task. As rugged methods are developed with higher efficiencies, new medications can be investigated more efficiently and therefore, increase the chances of new treatments reaching consumers faster. While many analytical methods deal with the analysis of compounds in air or water, pharmaceutics are normally concerned with living organisms; either animal or human. This involves the analysis of complex matrices such as hair, saliva, urine, blood and tissue homogenates to name a few. These samples contain other components, such as proteins, lipids, cells, in much larger quantities than the analyte itself. The way to properly analyze these matrices often means increased sample preparation. These extra steps tend to increase the workload for the analyst, increase the time necessary to get meaningful results and increase the likeliness of instrument failures or contamination. A constant concern is also the risk of infection the analyst faces when exposed to biological material when a lot of manipulation is involved. In this regard, more efficient sample preparation benefits the patients, the industry and the analyst.

Pharmaceutical compounds are administered in wide dosage concentrations and possess varied physical properties. Understanding their pharmacokinetic and physical properties is important to prevent unwanted side effects and toxicity. Development of

sample preparation methods that improve sample clean-up and preconcentration of analytes are therefore an important part of analytical research to help characterise these properties. The present work focuses on solid phase microextraction (SPME) as a means to efficiently quantify drugs due to the unique blend of qualities this method involves, as described later on.

1.1 SPME Background

Solid phase microextraction is a sorptive technique where selected coating chemistries are used to perform the extraction of analytes.[1] Developed in the early 1990's by Pawliszyn et al., the technique presents many advantages to the analytical chemist including:

-Ease of use

-Rapidity of analysis

-Preconcentration of analyte

-Small and practical format

-Amenable to automation

-Minimal use of toxic solvents

The SPME technique initially developed into a powerful tool for the analysis of environmental pollutants and also provided an opportunity for better *in situ* analysis in

this domain. Since then, SPME has gained popularity in a wide range of fields where the advantages mentioned above were needed such as food analysis, forensics and industrial applications to name a few. The physical design of the fiber itself is quite simple, generally consisting of a rod-like support usually made of fused silica or metal, and a stationary phase of controlled length and thickness. In order to understand how SPME is designed and how it is used, we must describe some of the underlying mechanisms of the technique. In the present thesis, only the theory pertaining to direct immersion of the stationary phase into the sample is relevant. The method of headspace extraction, although attractive in some applications because of minimal matrix effects, is not advantageous in our case due to the non-volatile nature of pharmaceutical compounds.

1.1.1 Thermodynamic Theory

The great qualities of SPME stem from the fact that the stationary phase can be chosen to match the type of analyte of interest. A proper choice of stationary phase can greatly help quantitate compounds, mainly by using a stationary phase with greater affinity to the analyte of interest, therefore increasing the sensitivity of the method. Analytes of low polarity will have greater affinity to a hydrophobic stationary phase whereas the opposite is true for highly polar compounds. As the analyte is partitioning into the stationary phase, an equilibrium is reached in time where the extracted amount becomes constant. The equilibrium conditions are defined as:

$$n = \frac{K_{fs} V_f V_s C_o}{K_{fs} V_f + V_s} \quad [1]$$

Where *n* is the amount extracted by the fiber, K_{fs} is the distribution constant between the coating and the sample, V_f is the fiber coating volume, V_s is the sample volume and C_o is the initial concentration of the analyte in the sample.[2] By allowing the microextraction to reach equilibrium, we are able to obtain reproducible results at the expense of some exposure time. It is clear from this equation that the amount of analyte extracted by the fibre is directly proportional to the initial concentration of our sample. In cases where the sample volume is negligible compared to the product of the distribution constant and the volume of the fiber, the equation takes the following simplified form:

$$n = V_s C_o \quad [2]$$

If the sample volume is much larger than the product of the distribution constant and fiber volume, the equation takes the following form:

$$n = K_{fs} V_f C_o \quad [3]$$

Generally, once a target analyte in a given sample is proposed, and a suitable stationary phase is found, we must look at kinetic parameters to improve the performance of the SPME method.

1.1.2 Kinetic Theory

The kinetic model proposed is a useful tool for the SPME user to increase productivity without sacrifices. Since SPME probes tend to be small compared to the bulk size of the sample, designing an SPME method also means agitating the sample in a way that will bring analytes efficiently to the surface of the stationary phase. The equation describing the time where equilibrium is reached is as follows:

$$t_e = t_{95\%} = 3\frac{\delta K_{fs} b}{D_s} \quad [4]$$

Where b is the fibre coating thickness, D_s is the diffusion coefficient of the analyte of interest in the given sample and δ is the boundary layer thickness (See Figure 1).

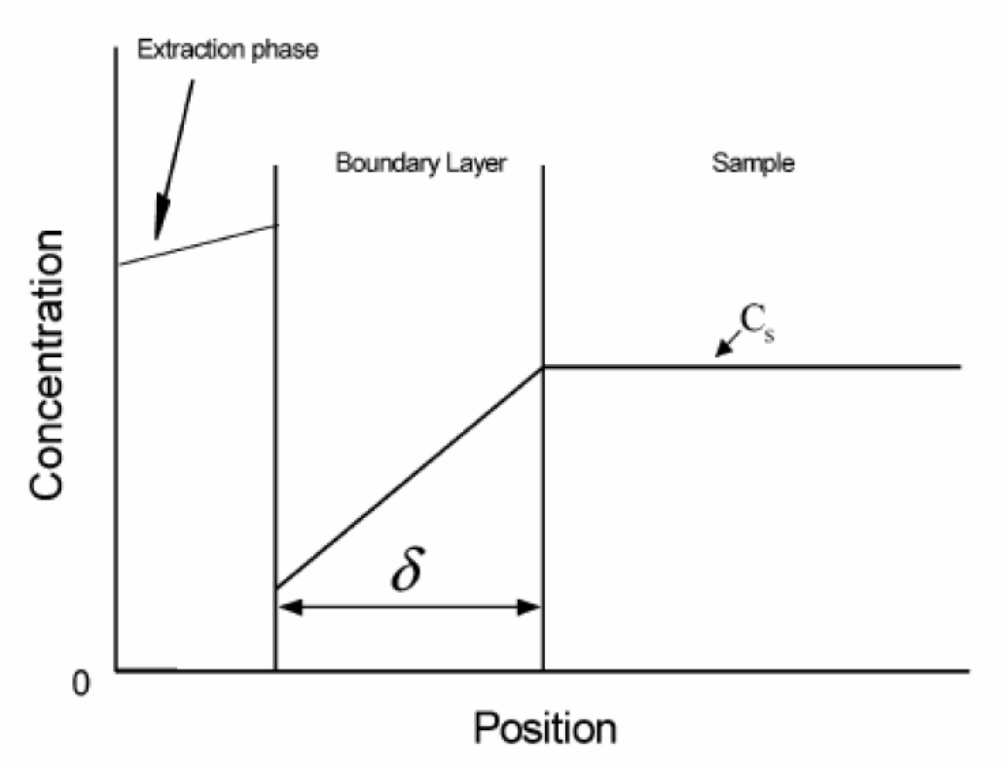


Figure 1 Graphical representation of the boundary layer.

The boundary layer is a static layer at the surface of the coating where no agitation occurs, acting like a stagnant liquid, which hinders the flow of analyte to the fibre considerably. In order to reduce the thickness of this boundary layer, increased agitation of the bulk sample must be performed and can be accompanied with an increase in sample temperature.

1.2 SPME Coating Technologies

At the basis, the qualities of SPME as a uniquely attractive extraction method can be attributed in large parts to the capacity of the fibres to have desired properties such as good analyte affinity, good capacity and even a desired selectivity (i.e. antibody coatings). It is by properly selecting or designing a fibre coating that the maximum potential of SPME can be achieved. In order to obtain a general idea of how SPME fibers are designed, it is necessary to discuss some background on the types of coatings involved in SPME. We will then discuss the technologies involved in applying these coatings successfully.

1.2.1 Coating Extraction Mechanisms

A multitude of high pressure liquid chromatography (HPLC) and gas chromatography (GC) columns are available on the market simply because there is no perfect column which can separate any compounds in any conditions. For the same reason, SPME technology relies heavily on the quality and choices of its coatings to be able to target as many analytes in as many conditions as possible. Although there is no perfect SPME fiber, some have the ability to extract a large library of compounds, such as polydimethylsiloxane (PDMS) based fibers. Others are intrinsically efficient at being very selective towards only a few select compounds, such as molecularly imprinted polymers (MIP)[3, 4] and antibodies[5].

There are 2 types of coatings available commercially: liquid coatings and solid coatings.[6] Liquid coatings, such as PDMS and poly(acrylate) (PA), extract the analyte through an absorption mechanism whereas the solid coatings, such as Carbowax (CW) based fibers, extract by adsorption (Figure 2).[6]

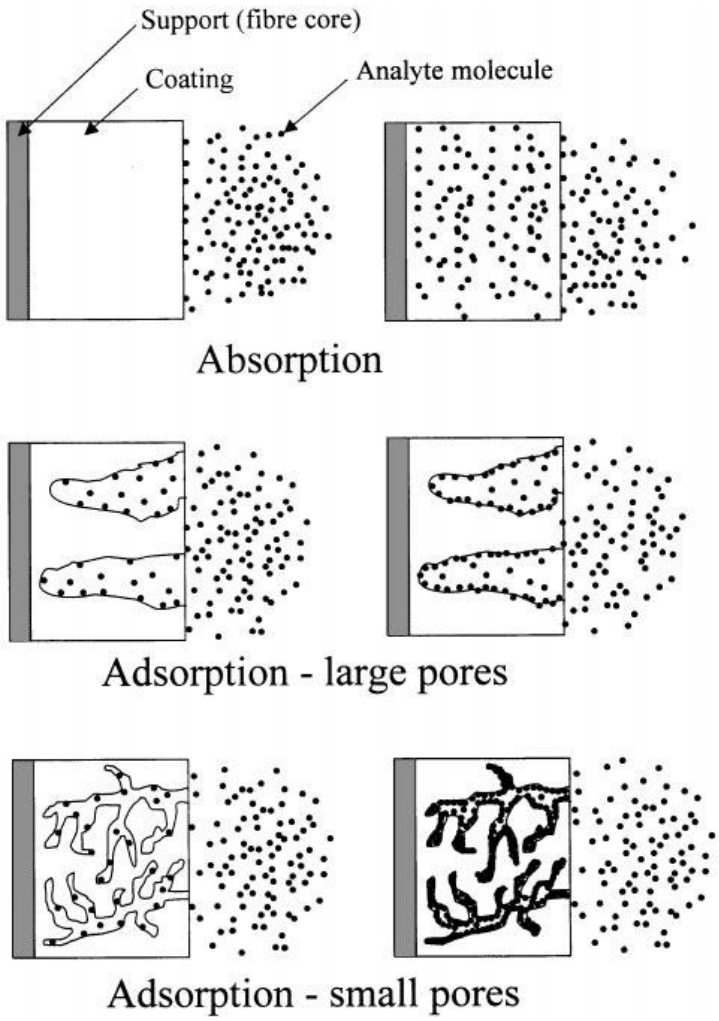


Figure 2 Schematic representation of the extraction process of liquid and porous solid materials.

Jiang et al. have classified coatings available as organic coatings and inorganic coatings.[7] Organic coatings are popular because they are usually easier to apply on the fiber and they can be more easily modified to have different functional groups. Coatings

of this class are PDMS and chemically modified PDMS. These coatings tend to be very versatile and are not affected by competition for adsorption sites on the fibers. The fibers discussed throughout this thesis will be organic coatings where absorption will be the main mechanism of extraction.

1.2.2 Coating Methodology

1.2.2.1 Polymeric Materials

By far, the most common SPME fibres consist of polymer based extraction phases. Amongst those, PDMS is most common, but other polymeric stationary phases are available, such as carbowax and divinylbenzene (DVB) based fibers. The coatings are assembled simply by depositing the coating to the surface of a fused silica fiber. Generally the coating is applied by running the fused silica rods through the solution followed by heat or ultraviolet (UV) curing, or they are simply applied by hand, followed by attachment to the fiber assembly. Further explanation on the process can be found elsewhere.[8] In 1997, polymer based adhesives were used to bind porous HPLC stationary phases on the surface of SPME fibers using Epotek epoxy adhesive.[9] This approach was impressive due to an 8 fold increase in extraction using a 30 µm thick C8 phase compared to a 100 µm thick PDMS phase. It was found that the fibers were rugged enough for GC injection, and they initiated interest due to their common extraction phase. This method of preparing the fibres would become popular for assembly of high capacity, HPLC compatible, fibers. Generally, these fibres are easy to

prepare but they do require high quality adhesives and proper curing treatment for optimal results.

1.2.2.2 Sol-gel

A popular approach to the development of a new fiber coating is the use of the sol-gel method. The sol-gel method generally involves the *in situ* generation of silica by base- or acid-catalyzed hydrolysis of tetramethoxy- or tetraethoxysilane in an alcohol-water mixture.[10] The sol-gel mixture consists of a "sol", which is the solvent in a liquid form, and the "gel", which is the non-soluble portion. Upon drying or catalysis, the product hardens and becomes glass-like. By exposing the resulting product to high temperatures, the polymerization of the silica is completed and residual solvents are removed.[10] In 1997, Chong et al.[11] were the first to propose the use of sol-gel technology to help improve SPME coating strategies.

Although SPME possesses many attractive advantages, some problems remain to be addressed regarding the coatings themselves. Most commercially available coatings have important drawbacks such as: (i) a relatively low recommended operating temperature (240-280 ^{o}C), (ii) instability and swelling in organic solvents (one of the biggest drawbacks to HPLC use), (iii) breakage of the fiber, (iv) stripping of the coating and (v) high cost.[12] The lack of proper chemical bonding between the stationary phase and the fiber surface, and the relatively high thickness of traditional SPME fibers seems responsible for some of the drawbacks, namely, the low operating temperature, the

solvent instability and the stripping of coatings.[12] The sol-gel coating technology has shown to be able to overcome these problems and create unbreakable fibers.[12, 13] The sol-gel possesses many qualities that make it interesting for SPME coating purposes. It is a relatively mild reaction and it is also controllable in order to obtain different physical properties, such as increased porosity, as well as the possibility to covalently incorporate desired chemical moieties into the coating while maintaining a rugged coating.

Although fiber coatings prepared using the sol-gel process have many advantages and improvements over traditional dip coatings, there remain some issues with the technique. The fabrication of a new coating using sol-gel necessitates a lot of optimization and the proper choices of precursor and catalyst. Much investigation is needed to determine the proper conditions such as temperature, concentration of reagents and proper curing. All the parameters can potentially have dramatic effects on the final product such as strength, uniformity and porosity of the coating. In order to simplify the procedure, we have made use of silicate chemistry to create a primary coating which would serve as a solid primer where various organosilane attachments could be performed. We use a mixture of pre-synthesized solid porous silica and liquid potassium silicate in order to achieve a rugged coating. The porous silica particles are synthesized primarily for use in HPLC columns due to their ruggedness, high porosity (about 200 m^2/g) and possibility to derivatize many stationary phases at their surface. They have been used extensively as a solid-phase extraction (SPE) cartridge material for similar reasons. The surface of these particles is made of reactive silanol groups (about 8 $\mu mol/m^2$), which can be used to functionalize the particles with a stationary

phase.[14] Silica is common in nature but is synthesized *de novo* in order to yield a pure particle (see Figure 3). The manufacturing of such particles is usually proprietary to companies producing these particles and a wide range of silica particles with many different physical properties are available.

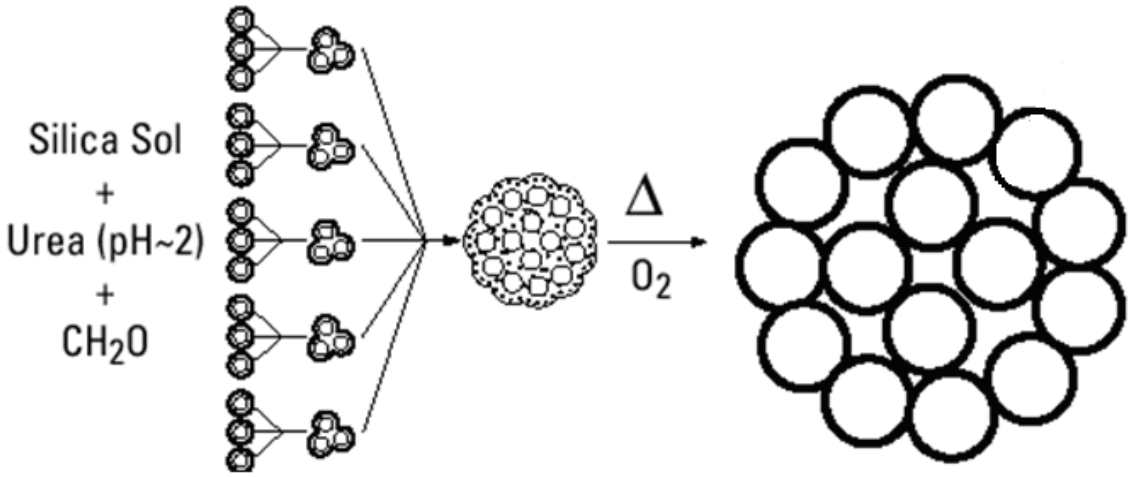


Figure 3 Formation of porous silica particles.

An important characteristic, as discussed before, is the porosity of the silica particles which, depending on the curing conditions determined by the manufacturer, can result in different pore volumes, different surface areas and different densities of the particles. In order to be able to provide a good bond between the silica backbone and the hydroxyl surface of stainless steel, we used potassium silicate in aqueous solution, which once cured, creates a tough and highly adhesive film with the proper surface. Upon curing, strong chains of inorganic molecules are formed (see Figure 4). Besides

being a strong adhesive towards metals and glasses/ceramics, potassium silicate is non-toxic, cheap, resistant to water and extremely resistant to heat (i.e. up to 1600^{o}C).

Figure 4 SiO_2 sol formed from silicic acid salt condensation.[15]

Potassium silicate is used in a wide array of applications. It is used as an adhesive for corrugated boards, foil-to-paper lamination, masonry coating, briquetting, pelletizing, agglomerating, binding ceramics or powdered metals for high temperature coating applications, welding rod coatings, paint, protecting stainless steel from abrasion, corrosion and cleaning agents, coating of bulk rail car interiors to prevent corrosion and water pipes treatment.[16] This rugged and versatile material is easy to work with and, coupled with the porous silica particles described previously, gave high surface area fibres which are highly rugged.

1.3 Current Methods for Drug Analysis

1.3.1 Classical Approaches

Nowadays, methods for drug analysis make use of breakthrough technologies developed with bioanalysis in mind (i.e. new HPLC columns or new instrument designs). Analysts are trying to look past the classical methods in order to avoid some of the problems associated with them, such as long analysis times. From an environmentally friendly point of view, liquid-liquid extraction (LLE) is an unfavourable method due to the use of toxic solvents in large quantities. LLE involves choosing a solvent in which the analyte of interest will partition, and mixing a considerable amount of the solvent with the sample. Since most drugs are non-polar, this often involves the use of ethers which are a health hazard both due to toxicity as well as extreme flammability. SPE, which uses a small cartridge to extract the analyte, can also necessitate a lot of solvent on the desorption step. The sample is usually fed through the cartridge by either pumps or vacuum. Once the analyte is retained on the cartridge, a large volume of desorption solvent, where the analyte prefers to partition, is run through the cartridge. This method is also a large consumer of toxic solvents. Furthermore, the cartridge is likely to get plugged since it is packed in a cartridge format. Hence, researchers are turning their attention towards more promising technologies, or trying to make older technologies more efficient (i.e. using automation).

1.3.2 Automation Oriented Approaches

1.3.2.1 Solid Phase Extraction

An example of an older technology made more efficient by automation is SPE. Normally carried out using a bulky vacuum apparatus and necessitating large volumes of solvent, SPE has been improved into an on-line method by incorporating it to HPLC. Unlike LLE, SPE is easier to bring to automation. This method usually consists of using 2 pumps, one with a loading solvent (pump1) which allows the sample to be run through the SPE cartridge and promote analyte partitioning to the SPE cartridge (represented as RAM – for Restricted Access Material – in Figure 5). RAM is a particular material used as SPE sorbent, but with desirable qualities for direct biological sample clean-up (described further). Once the analyte is extracted to the cartridge, the second pump backflushes the analyte using a desorption solvent where the analyte will partition. The backflush acts as an injection in itself and a clean-up of the cartridge is performed simultaneously. Everything eluted from the SPE cartridge then follows the path to the traditional chromatographic column for separation, and finally to the detector for quantitation. This automation procedure is an interesting approach, especially with novel SPE cartridges which allow direct biosample clean-up. Some drawbacks are obvious, the large cost of obtaining a second high-pressure pump for the apparatus and the high cost of the novel SPE cartridges capable of biosample clean-up.

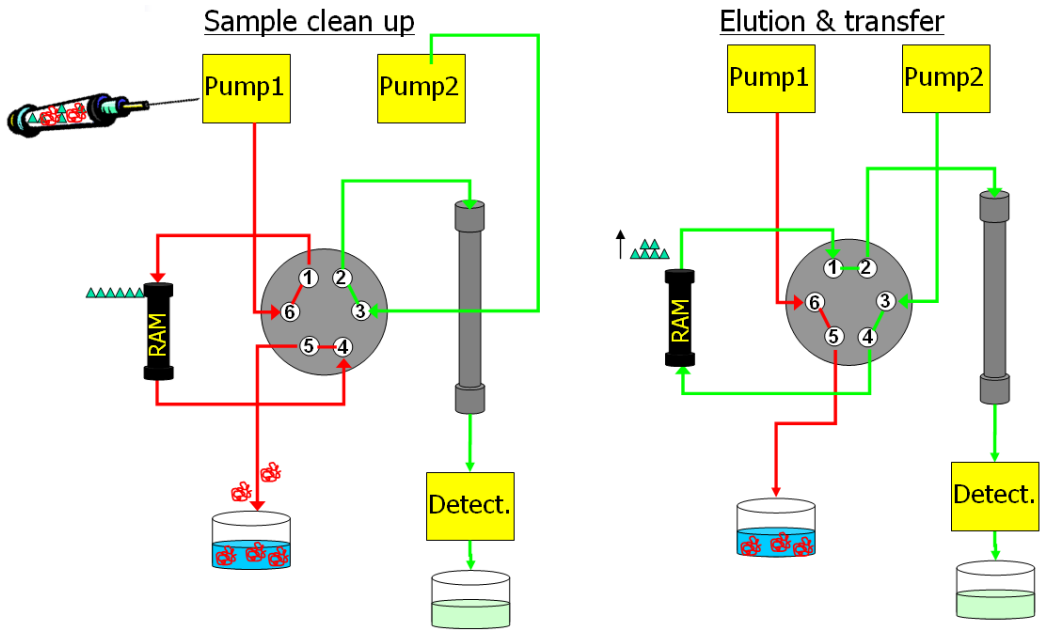


Figure 5 Online sample clean-up using SPE column coupled to a traditional HPLC setup.

1.3.2.2 In-tube Solid Phase Microextraction

Recent technologies which are gaining popularity in the field of bioanalysis is in-tube solid phase microextraction (in-tube SPME), which uses an open tubular capillary containing the desired extraction phase inside the tube. In-tube SPME has been used successfully in a variety of analyses such as the analysis of β-blockers in urine and serum samples[17], as well as amphetamines in urine[18], both with an omegawax stationary phase. Benzodiazepines were also analyzed successfully using a porous divinylbenzene stationary phase.[19] Immunoaffinity in-tube SPME, a method where receptors with very specific affinity are affixed to the surface of the tube, was also

applied to analysis of 7-aminoflunitrazepam in urine [20] and to the analysis of fluoxetine in serum.[21] In-tube SPME has the advantage of being easily automated, by inserting directly into the separation system, between the separation column and the injector (See Figure 6). This makes the method easy to optimize since autosampler programming allows us to determine optimal draw speed, draw cycles necessary and the volume of sample necessary, all this with minimal manual labour. This is ideal for quick method development while providing less chances of sample contamination or analyst contact with hazardous samples. Although chances of plugging the capillary itself are low, one must still be careful to prevent plugging of the injector system; therefore care should be taken to have a homogeneous sample.

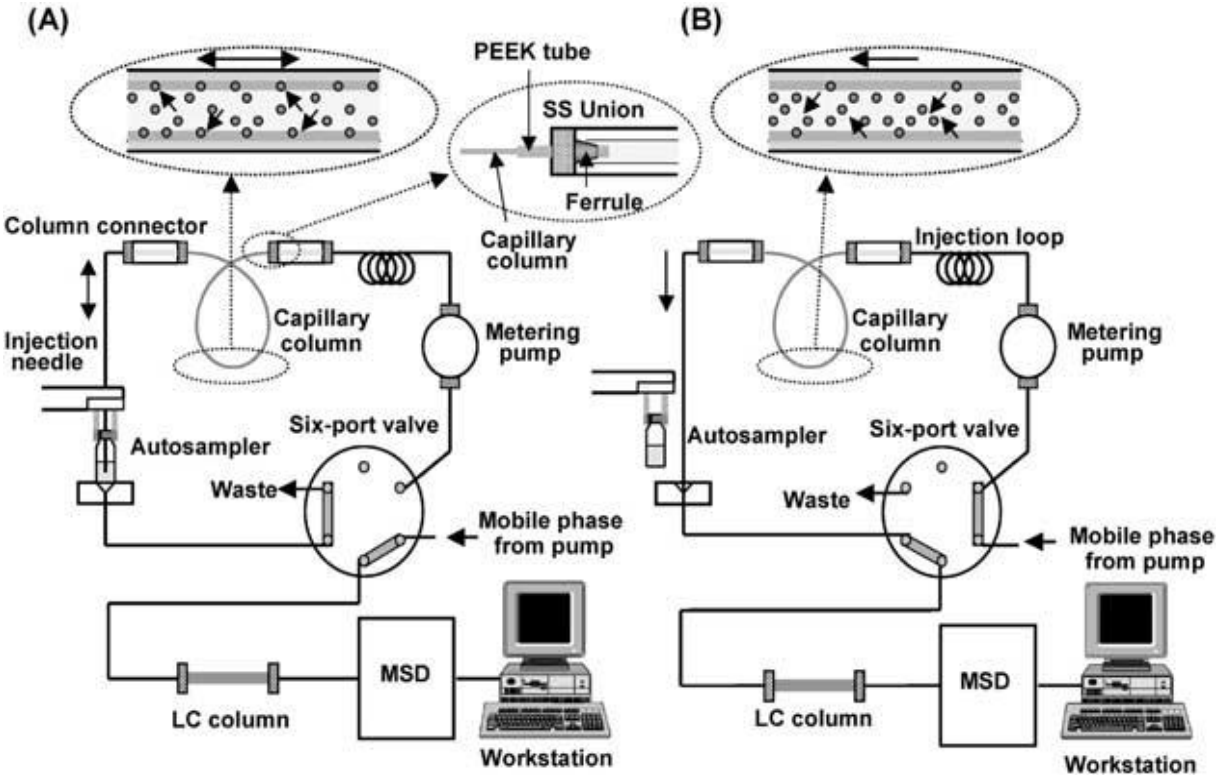


Figure 6 In-tube SPME apparatus coupled to LC-MSD (A: extraction position, B: injection position).[22]

1.3.2.3 Solid Phase Microextraction

Traditional SPME fibres have a design that is particularly attractive for bioanalysis because of the open-bed nature of the stationary phase, allowing easy access to the sample, with no risk of plugging the device. The potential for SPME as a technique which is ideally suited for automation was described in the early stages of the technique.[23] Automation of SPME is now well established and usually implemented by the use of a robotic arm such as the CombiPAL and its direct coupling to a chromatographic system. This system assures minimal direct human contact with the various processes of SPME such as extraction, agitation and desorption procedures. This in turn minimizes risks of contamination, accidents and reduces the analysis time. Nowadays, efforts are being focused on parallel analysis due to the development of 96 well plates, and more recently 384 and 1536 well plates. Since the extraction and desorption steps of the SPME process are the bottleneck of the technique, parallel sample preparation is therefore a very valuable high-throughput approach. Using this format, it is possible to considerably reduce extraction and desorption time and directly divide the time of the procedure by the number of samples run simultaneously. Automated SPME-LC is therefore a very valuable tool, especially since biological and environmental applications tend to generate numerous samples, in which case linear analysis may be impractical.[24] Multi-well plates coupled to various formats of multi-fiber holders have been investigated previously and demonstrate the benefits of such methods for automation.[25] The design has been since used for drug analysis and protein binding studies.[26] Further developments in the automation apparatus such as

the robotic arm and the fiber assembly will make this technique increasingly popular and easy to use while saving the analyst precious time.

1.3.3 Biocompatible Approaches

The more recent developments in stationary phases for added ruggedness in biological sample applications are worth mentioning. Porous silica particles are advantageous due to their high surface area and physical stability; however common stationary phases covalently bonded to their surface have limited use in dirty samples. The macromolecules and coagulation factors present in biological samples can irreversibly alter the surface of the stationary phase and hinder normal mass transfer. Materials capable of withstanding the fouling created by multiple immersions in biological samples are often referred to as biocompatible. Silica particles used for analyte extraction which have been designed for this purpose are referred to as Restricted Access Materials (RAM) due to their capacity at “restricting access” of the fouling agents to the extraction surface. Some description of special surfaces that have been developed and successfully applied to biological sample clean-up and can therefore be reused are described here.

The first RAM that was used for the clean-up of biological samples was named the Internal Surface Reversed Phase (ISRP) particles, which were introduced in 1985 by Hagestam and Pinkerton.[27] The particles worked by physically blocking access of the plasma proteins to the reversed phase embedded within the pores of silica

chromatography particles. These particles consisted of a tripeptide stationary phase made of Glycine-L-Phenylalanine-L-Phenylalanine (GPP). They initially derivatized their particles with a glycerylpropyl bonded phase (using silanization with γ-glycidoxypropyltrimethoxysilane) and subsequently covalently bound the GPP tripeptide with a carbonyldiimidazole (CDI) derivatization.[28] The hydrophobic amino acids are then cleaved from the outside of the particles with the help of the enzyme Carboxypeptidase A. This enzyme cleaves the phenylalanine moiety, while leaving the remaining glycine-diol on the surface to protect against protein adsorption and precipitation. It is important to note that since the size of the enzyme cleaving the outside stationary phase is smaller than most plasma proteins, it will have access more easily to the larger pores of the silica particle and therefore will prevent adsorption and precipitation of the protein by cleaving hydrophobic moieties of the large, potentially problematic pores. These researchers also demonstrated that smaller pore size diameter particles were resistant to a greater number of sample injections (240 injections for 80 +/- 30 Å) than their larger pore counterpart (about 50 injections for 123 +/- 30 Å).

A more recent material is Alkyl Diol Silica (ADS), developed in 1995 by Boos et al.[29] This material appeals particularly to analysts due to the common n-butyl (C4), n-octyl (C8) and n-octadecyl (C18) stationary phases used and can therefore have a predictable affinity towards the desired analyte. It is also possible to have ion-exchange properties inside the pores of these ADS particles, in which case they are referred to as exchange diol silica (XDS). The particles are first reacted with 3-glycidoxypropylmethyldimethoxysilane to obtain the glycerylpropyl coverage followed by butyroyl, capryloyl or stearoyl chloride to obtain the desired stationary phase. A

cleavage by lipases then effectively removes the hydrophobic surface reached by macromolecules and replaces them with a hydrophilic biocompatible surface where protein denaturation is prevented (see Figure 7).

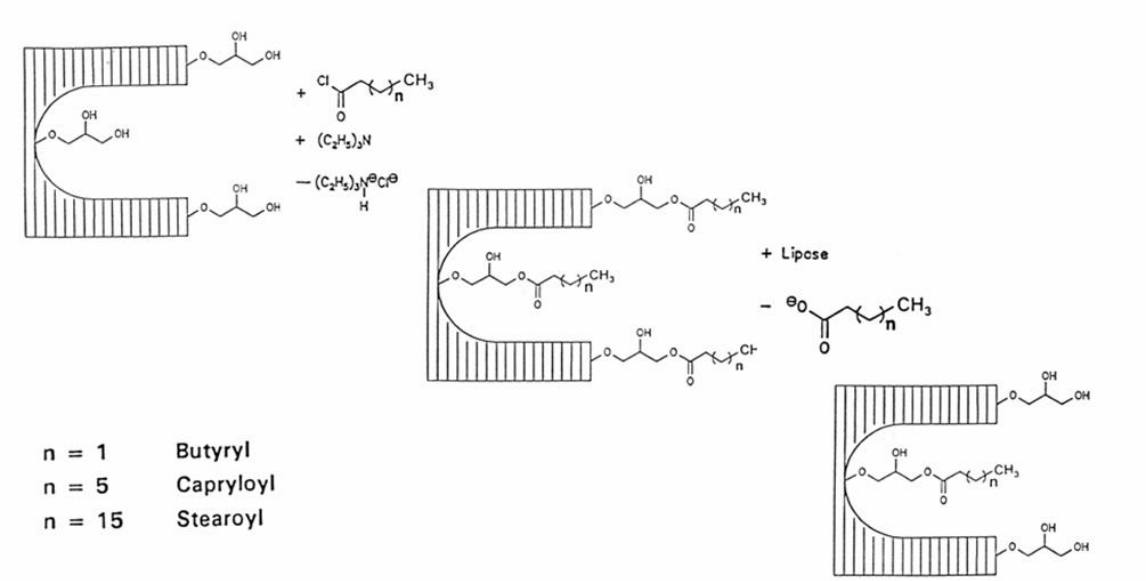


Figure 7 Surface derivatization followed by enzymatic cleavage of a pore at the surface of the RAM silica particles.

The biocompatible approaches described above have been used throughout the field of bioanalysis and serve as a good example of the fabrication approaches which can be used for bioanalysis using custom SPME fibers. Although these technologies are currently mainly used in SPE sample preparation, new SPME coatings have also been built using these materials.[30] These techniques and their applications show the importance of recent research to improve bioanalytical methods. It will be part of this thesis to demonstrate the capability of SPME as a bioanalysis tool to target pharmaceutical compounds in complex samples. We will achieve this using pre-

derivatized particles and adhesive to assemble a large number of probes, without emphasis on reusability.

1.4 Octanol-Water Partition Coefficient

Another important analysis during the development of potential pharmaceutical compounds is the logarithm of the octanol-water partition coefficient (LogP). The LogP is defined as:

$$LogP = Log \frac{[Analyte\ ]_{octanol}}{[Analyte\ ]_{water}} \quad [5]$$

It is important to understand that it is the logarithm of the unionized analyte. If the compound is ionized during the measurement a different equation applies and we would use LogD instead of LogP.

The LogP parameter is an important part of the pharmaceutical development process. It is used to screen compounds in the early stages of development and indicates the likelihood that the compound could progress and eventually be successful as a drug. Pharmaceutical compounds must possess the right set of physical properties to have proper ADME (Absorption, Distribution, Metabolism, Excretion) characteristics.[31] In order to know if a novel compound meets these characteristics, multiple tests are carried out in order to help predict the potential of the compound *in vivo*. LogP is generally one of the first parameters studied because if the values are outside

specifications, the compound is either too hydrophilic or too hydrophobic to have ideal ADME in the body, although some exceptions occur. A compound which is too hydrophilic might get excreted too fast to even have time to create a desired effect, whereas a compound with a high hydrophobicity might not make it into the body at all due to poor intestinal membrane absorption. Traditionally, LogP is obtained using the shake flask method, which consists of mixing a large amount of octanol and water into a flask and a considerable amount of drug into the mixture followed by shaking of the flask. The method would clearly benefit from reducing the amount of compound as well as solvents used, which is the reason we have used SPME to investigate an alternative which could help determine the hydrophobic character of a compound.

1.5 Thesis Objectives

The objective of this work will be to show the capabilities of SPME as a tool for high-throughput analysis of pharmaceutical compounds. A rapid and simple way to help determine the hydrophobicity of compounds using SPME will be described as well as a high-throughput multi-fiber method for the determination of a target compound in a complex matrix.

The first objective will demonstrate a new technique to assemble a porous silica coating which can be derivatized using organosilane chemistry, commonly used for HPLC column fabrication. This technique will give the analyst a tool to make custom stationary phases, which can provide a more specific coating for a given analyte or matrix. The fibers assembled in this work will be used to estimate the water/octanol partition coefficient of β-blockers in a buffered solution.

The second objective relates to the analysis of pharmaceutical compounds in complex matrices. It is a particularly challenging task even with all the sample preparation technologies currently available to the analytical chemist. A lot of time and effort is often required to obtain meaningful results from such analysis and therefore the area has seen a lot of efforts developed to help simplify methods. It is the goal of this work to demonstrate a way to prepare multiple fibres for high-throughput analysis of loratadine in biological samples.

2 Development of Silicate Triacontyl Coatings for Drug Analysis

2.1 Preamble

The following chapter is a modified version of a paper submitted for publication. The contribution of the co-author Maria Rowena M. Monton are both experimental help and manuscript revisions.

I, Maria Rowena M. Monton, authorize François Breton to use the material for his thesis.

Signature: 

2.2 Introduction

Coating procedures for the fibers have been investigated and many approaches have been developed to suit a wide range of analyte properties.[32-37] The multitude of coatings for SPME fibers is crucial since it will directly determine factors such as selectivity, reproducibility, speed and ruggedness of a method. The majority of commercially available SPME fibers consists of polymeric phases (e.g., PDMS, PA, CW, Carboxen (CAR), or composites) coated onto fused silica. However, recent interest in high-porosity fibers has picked up considerably because of their large surface areas and consequently, high extraction capacities resulting in significant sensitivity improvements. Unfortunately, fabrication protocols for highly porous coatings are often

based on adhesives, which have proprietary chemistries and render the process of coating a matter of trial and error. The adhesive must provide a rugged coating capable of resisting common solvents used in analyses.[38] At the same time, it should not impede mass transfer between the sample solution and the extraction phase during the extraction step, and between the extraction phase and the desorption solution during the desorption step. The method of attachment for the silica support is a parameter which cannot be overlooked.

The current chapter describes a new procedure for preparing silica-based coatings for SPME based on the entrapment of porous silica particles in a network of polymerized silicate, followed by *in situ* derivatization to attach the desired extraction phases. Such an entrapment strategy has been used previously to prepare monolithic, particle-loaded columns for use in capillary electrochromatography (CEC),[39, 40] and to coat the inner wall of separation capillary in capillary electrophoresis for electroosmotic flow control.[41] Soluble silicates dry to form tough, tightly adhering inorganic bonds or coatings[16], thereby rendering mechanically robust columns and highly stable coatings. We used this material to fabricate our SPME fibers, which provided us with a fiber capable of withstanding many extraction-desorption cycles without significant change in performance. As opposed to the column configuration in CEC, the open bed format of SPME fibers precludes direct inclusion of alkyl-modified phases in aqueous silicate; thus bare silica particles were used instead, and these were subsequently derivatized directly onto the fiber. As opposed to conventional coatings where the particles are separated by networks of adhesive that do not contribute to or hinder extraction, the porous silica particles and the inter-particle silica entrapment matrix provide a

continuous surface, which can be derivatized with the required chemical moieties for maximum coverage and ultimately higher extraction capacities.

Alkyl triacontyl (C30) phases are the longest chain of monomeric reversed phase-LC phases currently available[42]. They are considered as novel, although they have been used previously in the separation of cis-trans carotenoid isomers, due to their exceptional shape selectivity[43], and of fullerenes, in which the strong retention of the molecules was attributed to their effective interaction with the very long chains of the stationary phase[44]. More recently, in SPE, C30 was used as sorbent for extracting polyaromatic hydrocarbons in airborne particulate matter[45], and estrogens and their metabolites from water samples[46]. In both cases, C30 provided superior performance over C18.

The triacontyl fibers were selected to determine their viability as a tool to estimate the LogP value of structurally diverse β-blockers. Traditionally LogP values are determined using large quantities of drug and solvents (octanol/water) in order to experimentally determine the hydrophobic descriptor. Increasingly, the determination is performed using HPLC on a C18 reversed phase column and the retention time of the analyte is used to determine hydrophobicity.[47] However, it is still a solvent consuming technique requiring long columns and can be time consuming. This work exploits the high extraction efficiency of the developed extraction phases and the intrinsic advantages of the SPME technique, such as minimal solvent use, to demonstrate the usefulness of the fibers in determining the hydrophobic parameter of β-blocker drugs.

For this research, reversed phases (C4, C8, C18 and C30) for extraction were attached onto fibers following organosilane chemistry, thereby allowing optimization of extraction selectivity. The resulting SPME fibers were characterized by scanning electron microscopy (SEM) and evaluated for their ruggedness of preparation, endurance, and extraction-desorption properties using common benzodiazepines as model analytes.

2.3 Experimental

2.3.1 Chemicals and Materials

Drugs of the benzodiazepine class (see Figure 8) were selected to evaluate extraction qualities of the fibers prepared. Diazepam, oxazepam, nordiazepam and lorazepam were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg/mL certified standards.

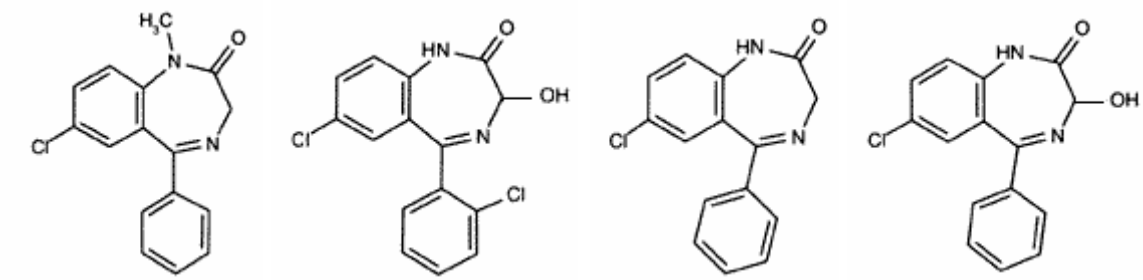


Figure 8 Structure of benzodiazepines (diazepam, oxazepam, nordiazepam, lorazepam), commonly used for their sedative and anti-anxiety properties.

Derivatization reagents consisted of n-butyldimethylchlorosilane for assembly of the n-butyl (C4) hydrophobic stationary phase, n-octyldimethylchlorosilane for n-octyl (C8) hydrophobic stationary phase, n-octadecyldimethylchlorosilane for n-octadecyl (C18) hydrophobic stationary phase and triacontyldimethylchlorosilane for the triacontyl (C30) hydrophobic stationary phase. These organosilane reagents were purchased from Gelest Inc. (Morrisville, PA, USA). Great care was taken to avoid exposure to humidity to preserve reagent quality.

Potassium silicate (Kasil 1) was donated by PQ Corporation (Valley Forge, PA, USA). Ascentis porous silica particles (underivatized), 5 µm in diameter, were provided by Supelco (Bellafonte, PA, USA). Anhydrous toluene and the β-blockers (see Figure 9) were purchased from Sigma Aldrich (Oakville, ON, Canada).

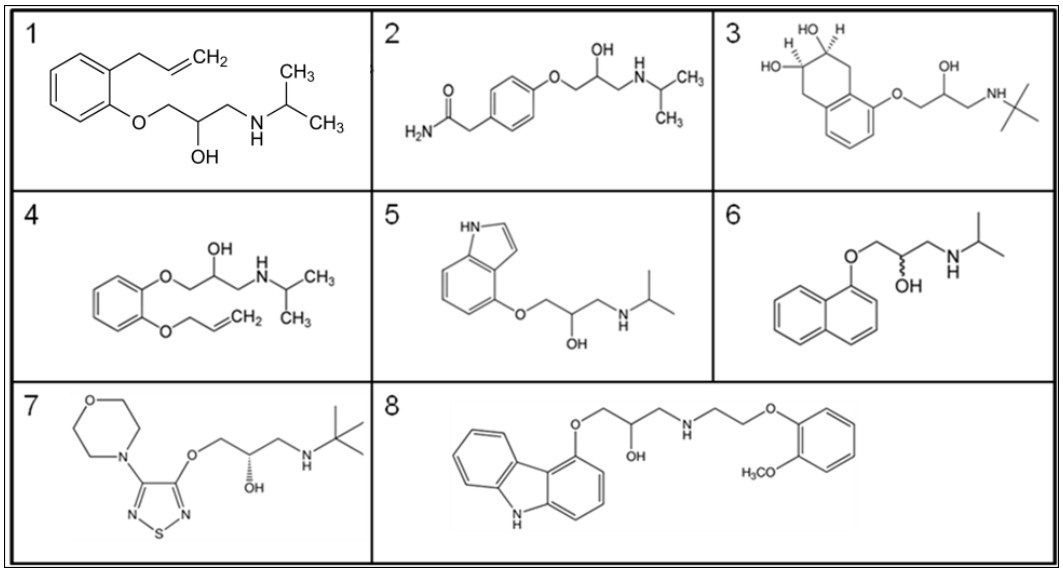


Figure 9 Structure of β-blockers used in LogP correlation experiment: 1. Alprenolol, 2. Atenolol, 3. Nadolol, 4. Oxprenolol, 5. Pindolol, 6. Propranolol, 7. Timolol, 8. Carvedilol.

The toluene was transferred from the original container through a septum, using a glass syringe, in order to avoid water contamination. Tetrahydrofuran, toluene, methanol and acetonitrile, HPLC grade, were purchased from Caledon (Georgetown, ON, Canada). Hydrochloric acid and nitric acid, ACS grade, were purchased from Fisher Scientific (Ottawa, ON, Canada). Water was purified and deionized using a Barnstead Nanopure water system (Dubuque, IA, USA). Stainless steel wires, grade 304, and a Xuron® music wire shear cutter were purchased from Smallparts (Miami Lakes, FL, USA).

2.3.2 Coating Procedure

The grade 304 stainless steel wires (0.061") were initially chemically etched for 30 minutes with hydrochloric acid to increase roughness of the surface and to activate the autoprotective hydroxyl layer of stainless steel. After a generous rinse with deionized water, the wires were dried in an oven at 130°C for 1 h. The dried and cooled wires were dipped in potassium silicate solution such that a length of 1.5 cm was covered, and then carefully rolled into 5-µm porous silica particles. The resulting silicate-silica coating was exposed to fumes of concentrated nitric acid for 10 s, which would set the coating together rapidly, and then was allowed to dry at ambient temperature for at least 12 h. To ensure thorough drying for maximum stability of the coating, as well as effective chemical bonding of the alkyl phases, heating in a programmed oven was performed. The coated fibers were initially exposed to 50°C,

then the temperature was gradually increased to 95°C at the rate of 1°C·min^{-1} and held for 15 minutes. The fibers were then brought from 95°C to 150°C at the rate of 1°C·min^{-1} and left to stand overnight. This slow optimized cure was necessary since a quicker cure resulted in blistering and bubbling of the coating.

The derivatization solutions consisted of 10% organosilane in anhydrous toluene (it was necessary for C30 to have some heating for complete dissolution). All steps were carried out using meticulously dry glassware and utensils. The coated wires were placed in vials containing 10 mL of the organosilane-toluene solution and purged with nitrogen before capping. Then, the vials were immersed in a silicone oil bath maintained at 70°C using a hot plate for the optimal time of 24 hours.

Following derivatization, the fibers were rinsed successively for 15-min periods with toluene, tetrahydrofuran, methanol, 50:50 (v:v) methanol:water and water. Finally, the fibers were allowed to dry overnight before initial use.

2.3.3 SPME Conditions

For the preliminary characterization work, extraction of the benzodiazepines was performed in 2-mL HPLC vials using 1.5 mL of phosphate buffered saline (PBS, pH 7.4) extraction solution. Prior to first extraction, fibers were conditioned for 30 min in 50:50 methanol:water and subjected to 5-min conditioning step between extraction-desorption cycles using the same conditioning solvent. Desorption was performed in 200 µL of 50:49:1 water:acetonitrile:acetic acid contained in 200-µL bottom spring inserts. Unless

otherwise noted, extraction and desorption times were 15 min and 5 min, respectively. In both steps, the samples were agitated at 2400 rpm using a vortex shaker DVX-2500 (VWR, West Chester, PA, USA).

For the LogP determination work, extraction of 16 ng/mL β-blockers was performed in 40 mL vials using 35 mL of sodium bicarbonate buffer (pH 10.5) as extraction solution. Extraction was performed for 1 hour. Stirring was performed using a magnetic stir bar at 500 rpm. Desorption and conditioning were performed as described above.

2.3.4 SEM and EDX Characterization

The fiber coatings were subjected to preliminary evaluation using an optical microscope (Reichert-Jung series 40, Heidelberg, Germany). High-magnification characterization of the fiber coatings was performed using a scanning electron microscope (SEM) LEO 1530 field emission SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany). Prior to analysis, the fibers were dried thoroughly, and then mounted using carbon conductive tape and specimen mounts (Ted Pella, Redding, CA, USA). Samples were sputtered with ~10 nm of gold and were analyzed using an acceleration voltage of 15 kV.

Energy Dispersive X-ray (EDX) analysis was used to perform a semi-quantitative evaluation of the derivatization efficiency onto the surface of the fibers. Using the same SEM apparatus, increases in elemental carbon onto the silica backbone of the coating were determined.

2.3.5 LC-MS Instrumentation

An Agilent 1100 LC-mass selective detection system (Hewlett-Packard, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a variable wavelength UV detector set at 230 nm, and a single quadrupole mass analyzer was used to perform characterization analysis. The model analytes were separated on an Ascentis C18 column (5 µm, 2.1 x 50 mm) (Supelco Inc. Bellafonte, PA, USA), preceded by a precolumn. Elution was carried out in isocratic mode using 50% 10:90:0.1 acetonitrile:water:acetic acid (solvent A) and 50% 90:10:0.1 acetonitrile:water:acetic acid (solvent B) at a flow rate of 0.5 $mL \cdot min^{-1}$, and a total runtime of 6 min. The injection volume was 20 µL. A 2-mm offset was used to prevent autosampler needle contact with the bottom of vial inserts.

LC was coupled to the mass spectrometer (MS) using an electrospray ionization (ESI) interface, operated in positive ionization mode. The MS parameters were: drying gas (N_2), 10 $mL \cdot min^{-1}$; drying gas temperature, 300°C; nebulizer pressure, 15 psi; capillary voltage, 4000 V; fragmentor voltage, 90 V; and quadrupole temperature, 100°C. The analytes were detected by selected ion monitoring (SIM) mode (nordiazepam, *m/z* = 271; diazepam, *m/z* = 285; oxazepam, *m/z* = 287; lorazepam (internal standard), *m/z* = 321) using isolation peak widths of 1 amu.

The LogP experiments were carried out on an Agilent 1200 LC system coupled to a Sciex API 3200 Q-trap mass spectrometer (Applied biosystems/MDS Sciex, Toronto, ON, Canada), using Multiple Reaction Monitoring (MRM) scanning (see Table

1). The runtime was 6 minutes and the injection volume was 10 µL with a 2-mm offset. The β-blockers were separated on a Zorbax XDB-C18 column (5 µm, 4.6 x 150 mm) (Agilent, Palo Alto, CA, USA). The elution was carried out using 99.9:0.1 water:acetic acid (solvent A) and 99.9:0.1 methanol:acetic acid (solvent B). The elution profile consisted of a gradual increase from 70% B to 90% B from 0 to 4 minutes, followed by a 30-second hold, a gradual decrease from 90% B to 70% from 4.5 to 5 minutes followed by a re-equilibration at 70% B for 1 minute. The flow rate was kept constant at 1 $mL.min^{-1}$ throughout the run. The mass spectrometry experiments were carried out using electrospray ionization (ESI) in positive mode. The curtain gas was maintained at 45 psi, nebulizer gas at 75 psi, turbo gas at 70 psi and collision gas at 7 psi. The ion spray voltage was set to 3500 V and the turbo gas temperature at 750°C. Data analysis was performed using the Analyst software.

Analyte	MRM transitions	declustering potential (V)	entrance potential (V)	collision cell entrance potential (V)	collision energy (eV)	collision cell exit potential (V)
Atenolol	267/145	46.00	4.50	17.63	30.00	3.00
Nadolol	310/254	61.00	6.00	17.15	21.00	4.00
Pindolol	249/116	56.00	10.00	21.55	37.00	4.00
Timolol	317/261	46.00	7.00	18.83	21.00	4.00
Oxprenolol	266/72	46.00	7.00	17.60	31.00	8.00
Alprenolol	250/91	46.00	6.00	17.15	23.00	4.00
Propranolol	260/116	46.00	6.00	17.43	23.00	4.00
Carvedilol	407/100	41.00	6.00	19.03	21.00	4.00

Table 1 MRM transitions and experimentally determined MS parameters for β-blocker compounds.

2.4 Results and Discussion

2.4.1 Development and Optimization of SPME Coating Procedure

In a study by Liu et al.,[9] a porous layer was shown to have 500 times greater surface area compared to a polymer coating in SPME, resulting in as much as 8 times increase in the amount of analyte adsorbed. In the present work, porous, 5-µm silica

beads were immobilized onto metal wires by entrapping them in networks of polymerized silicate. Aqueous potassium silicate was bonded by a combination of acid-curing and dehydration steps to render the bond insoluble, to reduce setting time, and to increase bonding strength[16]. Potassium silicate is commonly used in metal binding applications; hence, it can be expected to adhere strongly to the supporting wire. When applied, its tackiness held the silica beads in place. On exposure to acid fumes, the silicate ions polymerized readily, and the silanol groups on the surface reacted with the hydroxyl ions in the beads. In this manner, cross-linking siloxane clusters were formed, and led to a strong, coherent coating. To strengthen it further, it was subjected to a dehydration step, in which the oven temperature was ramped gradually to remove excess water slowly and to prevent blister and bubble formation. Exposure of the initial, unpolymerized coating to some acids at various concentrations were evaluated to determine how to obtain the most consistent coating. The acids investigated were trifluoroacetic acid, acetic acid, nitric acid, sulfuric acid and hydrochloric acid ranging in concentrations from 0.1% acid in deionized water to concentrated acid. Best results obtained from fumes of concentrated nitric acid occurred serendipitously and provided desired bonding of the coating while providing us with a rapid setting. Under SEM, the resulting silicate layer was found to be uniform with a thickness of ~ 3 μm. By rolling the wire dipped in silicate over the silica particles with a gentle, constant speed, a dense yet even loading of particles onto the surface could be achieved. As shown in the electron micrograph in Figure 10, the particles were only partially submerged in the entrapment matrix, forming a monolayer. This formation could maximize the surface area while keeping the thickness at a minimum.

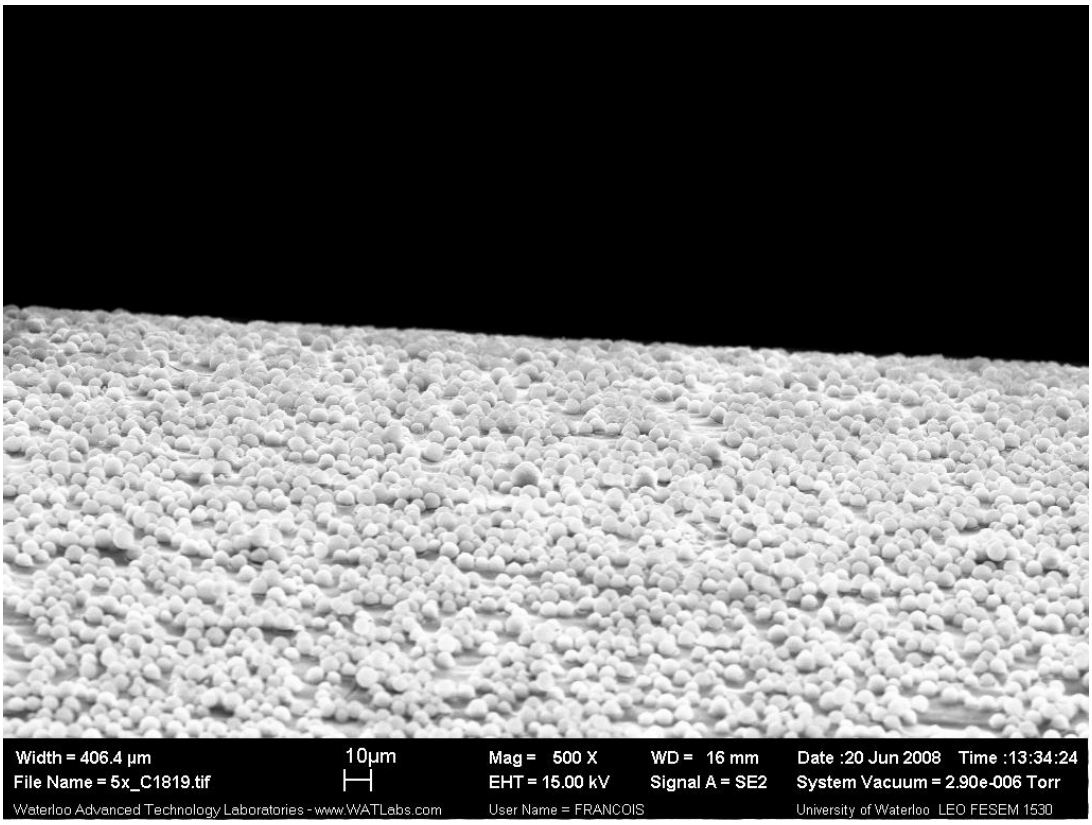


Figure 10 Scanning electron micrograph of an SPME fiber prepared by entrapping 5-µm porous silica particles in polymerized silicate. Derivatization with C18 performed *in situ*.

Preliminary evaluation of the ruggedness and robustness of the resulting coatings were done by a scratch test using a lint-free tissue paper, and 1-min sonication using 50:50 methanol:water. Fibers were considered suitable for subsequent organosilane reaction if no obvious changes in coating integrity could be observed under an optical microscope.

In contrast to the column configuration in CEC, the open bed format of SPME fibers does not allow direct inclusion of alkyl-modified phases in aqueous silicate; thus bare silica particles were used instead, and these were subsequently derivatized on-fiber. As opposed to conventional coatings where the particles are separated by networks of adhesive that do not contribute to, and may hinder extraction; the porous silica particles and the inter-particle silica entrapment matrix provide a continuous surface, which has the potential to be completely derivatized with the chosen chemical moieties. This approach could provide maximum coverage and therefore allow higher extraction capacities.

Alkyl derivatizations (using n-butyldimethylchlorosilane for C4, n-octyldimethylchlorosilane for C8, n-octadecyldimethylchlorosilane for C18, triacontyldimethylchlorosilane for C30) were performed according to the procedure described by Fields,[48] modified to fit the on-fiber format. Following derivatization, elemental analysis was performed using EDX to determine the increase in carbon content. An underivatized coated fiber and another that was derivatized with C18 phase were analyzed and compared. Results showed a 15% by weight increase in carbon content at the surface of the latter, indicating successful attachment of the alkyl phase.

To assess reproducibility, five independent batches of C18 fibers were prepared and the amount of benzodiazepines extracted and desorbed were determined by LC-MS following normalization using lorazepam as internal standard to correct for differences in injection volumes. The concentration of diazepam was maintained at 12.5 ng/mL while the others were maintained at 25 ng/mL. Results (Table 2) show that the fiber-to-fiber relative percent standard deviation (intra-batch) %RSD was within 18.6,

whereas the batch-to-batch (inter-batch) %RSD was within 20.2. These values attest to the ruggedness of the coating preparation protocol.

Batch number	Percent Extracted (n=6)					
	Nordiazepam		Diazepam		Oxazepam	
	Mean	% RSD	Mean	% RSD	Mean	% RSD
1	5.9	8.0	16.3	11.7	4.0	17.1
2	7.1	12.8	19.8	17.6	5.0	18.1
3	5.9	15.5	19.3	11.9	4.8	18.3
4	7.9	10.5	23.3	14.4	5.2	16.1
5	6.6	14.1	20.3	18.6	4.2	14.1
Interbatch (n=30)	6.7	15.7	19.8	20.2	4.6	19.8

Table 2 Fiber-to-fiber and batch-to-batch reproducibility using C18 fibers.

To determine endurance and reusability, a C18-coated fiber was subjected to a series of 100 successive conditioning (5 min)-extraction (2 min)-desorption (5 min) cycles, and the ratio of the amount of oxazepam was calculated for each cycle. The average response in injections 50-52 and 98-100 were found to be 96.6% and 88.9%, respectively, of the average response in injections 1-3 (Figure 11).

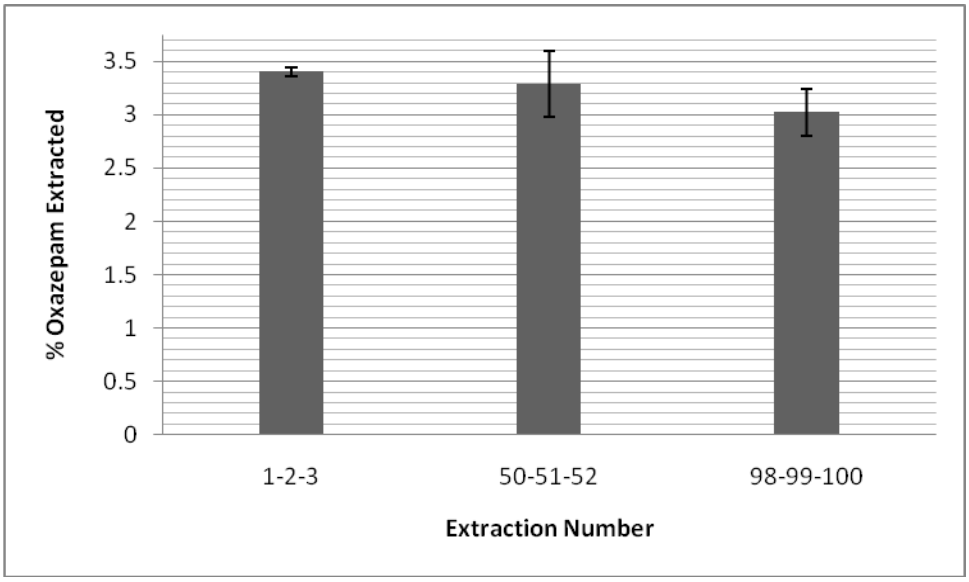


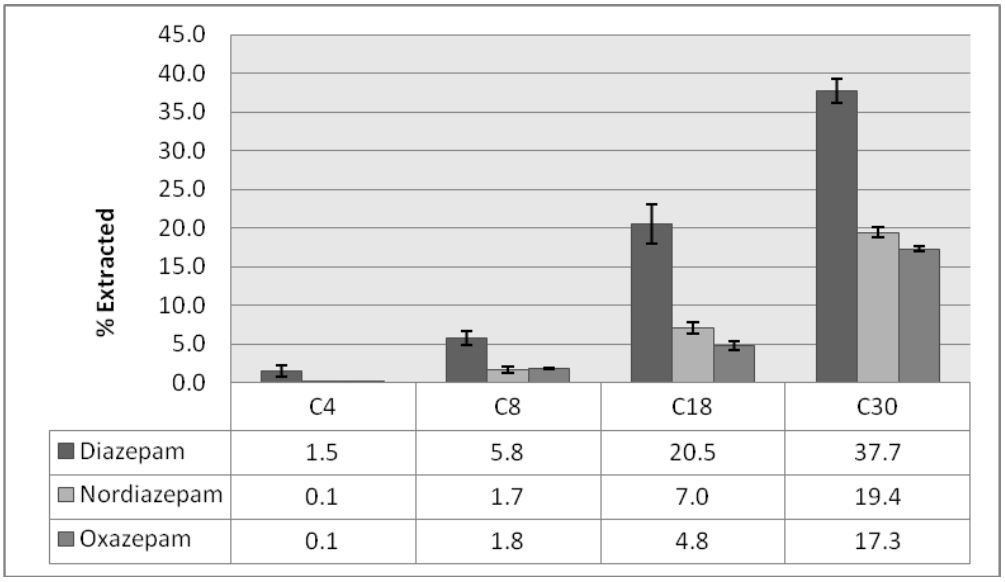
Figure 11 Ruggedness of the coated fibers as represented by extraction efficiencies.

The sturdiness of the coated fiber can be attributed to the interplay of four factors, namely good mechanical strength of stainless steel as supporting material, strong adhesion of silicate onto metal, effective immobilization of the silica beads in silicate, and robustness of the chemically bonded alkyl phase. Additionally, some fibers were kept continuously in 50:50 methanol:water for a period of one month, and no significant changes were observed upon visual inspection using an optical microscope.

2.4.2 Extraction and Desorption Properties

Fibers that were coated with porous silica particles entrapped in silicate, which were not derivatized, as well as fibers coated with silicate alone, but which were derivatized with C18 phase, did not extract any detectable amount of the test analytes. While the latter group contained silanol sites that could be alkylated, and therefore be subsequently used for extraction, the amount of analytes extracted were likely too low to be detected by the method. In contrast, fibers prepared by fixing porous silica particles with silicate, which have been subjected to derivatization, successfully extracted benzodiazepines.

In a study on the effect of the alkyl group (C1, C6, C8 and C18) bonded to the silica phase on the selectivity of extraction of polycylic aromatic hydrocarbons (PAHs) using SPME[49], it was demonstrated that small-chain alkyl-containing phases were better able to extract larger PAHs. This behaviour was attributed to the restriction imposed by longer chains on bulkier molecules, limiting their access to the full extent of the hydrophobic surface. On the other hand, in the present study, a comparison of the amount of benzodiazepines extracted using C4-, C8-, C18- and C30-coated fibers showed that the amount extracted increased with increasing alkyl chain length (Figure 12). The model analytes used here are small molecules belonging to the same compound class, and are therefore largely coherent structurally. Differences in extraction yield may be thought of as a function of hydrophobicity, with the more hydrophobic compound being extracted better, and in more hydrophobic extraction phase (i.e., greater hydrophobic space for enhanced interaction with the analyte).



	C4	C8	C18	C30
Diazepam	1.5	5.8	20.5	37.7
Nordiazepam	0.1	1.7	7.0	19.4
Oxazepam	0.1	1.8	4.8	17.3

Figure 12 Comparison of extraction efficiencies (% extracted) for C4, C8, C18 and C30-derivatized fibers using selected benzodiazepines. Conditions are as described in Experimental Section of this chapter.

Generally, SPME fibers prepared by gluing discrete particles together make use of the same sorbents utilized in solid phase extraction (SPE), or the same stationary phase particles used for packing LC columns, with C18 being the most common for reversed-phase (RP) systems. On the assumption that small, hydrophobic molecules could be better extracted the more hydrophobic the extraction phase is, we prepared C30-coated SPME fibers. As shown in Figure 4, C30 was able to extract benzodiazepines much better, even compared with the most commonly employed extraction phase in this series, i.e., C18. According to the solvophobic theory in LC, retention of analytes on the stationary phase is dependent on the molecular contact area between the solute and the stationary phase.[50] For a given solute, retention is expected to increase with the alkyl chain length of the bonded phase.

Extraction profiles using C18 (Figure 13A) and C30 (Figure 13B) fibers were prepared. For C18, equilibrium times as fast as 2 min could be obtained under the current conditions. Fast equilibration was rendered possible by the ultrathin layer (about 20 Å) of the extracting phase; thus, the influence of diffusion kinetics could be considered negligible [9]. The design of the fiber itself also allows for maximum rate of mass transfer to be achieved since the coating approach creates derivatized porous surfaces which are unhindered and situated at the surface of the coating, whereas approaches where particles are applied as slurry creates thicker coatings and the cured product might also block easy access to the derivatized surface. Due to this improvement in rapidity, it becomes advantageous to investigate C30 since the increase in equilibrium time (about twice longer than C18) is negligible compared to the gain in sensitivity.

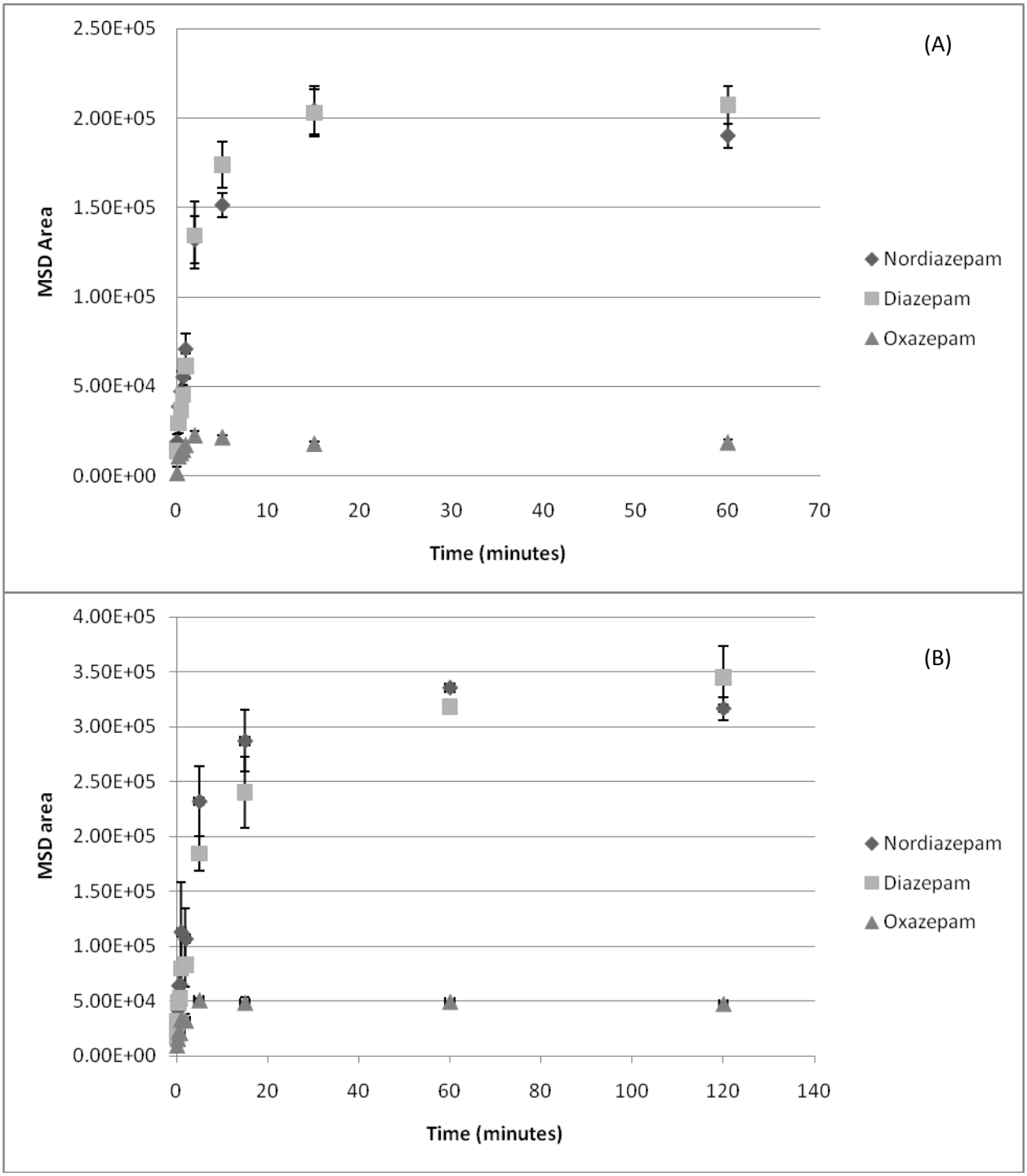


Figure 13 Extraction time profiles for benzodiazepines using C18 fibers (A) and C30 (B) fibers. Conditions are as described in Experimental Section.

Calibration lines were determined using C18 and C30 fibers (Table 3). In both cases, all three compounds showed a linear response over a concentration range of two orders of magnitude. The slopes of the calibration lines using C30 fibers were steeper than the corresponding lines using C18 fibers, further attesting to increased sensitivity of the method using the more hydrophobic C30 extraction phase for benzodiazepines.

	Concentration Range (ng/mL)	C18		C30	
		Slope of Calibration Line	R^2	Slope of Calibration Line	R^2
Nordiazepam	2 - 200	1015	0.9873	4987	0.9934
Diazepam	2 - 200	2622	0.9938	9733	0.9971
Oxazepam	2 - 200	309	0.9993	1659	0.9975

Table 3 Calibration range and linearity of response using C18 and C30 fibers.

2.5 LogP Determination using C30 Fibers

The interest with C30 stems from the increase in sensitivity shown towards more hydrophilic drugs. This particular quality would allow the fibers to have a wider dynamic range on the LogP scale to properly assess the hydrophobicity factor of the β-blockers. To determine if the SPME fibers developed could be used for LogP determination of pharmaceutical compounds we have assembled a list of 8 β-blockers and determined

their equilibrium profile. The volume of the sampling solution was increased to 35 mL in order to prevent depletion of the analyte in solution and provide a representative K value for the fibers. Carry-over of the fiber was determined to be at most 2.0% for the given analytes (See Table 4) and the highest amount extracted was 9% for carvedilol.

Analyte	Carry-over (%)
Alprenolol	1.2
Atenolol	*n/d*
Carvedilol	1.4
Nadolol	*n/d*
Oxprenolol	1.8
Pindolol	*n/d*
Propranolol	2.0
Timolol	*n/d*

Table 4 Carry-over of the β-blockers using C30 extraction phase.

Determination of the K value of SPME was done using the following equation[51]:

$$K_{fs} = \frac{nV_s}{V_f(V_sC_o - n)} \quad [6]$$

Results for the experimentally determined K_{fs} values can be found in Table 5 along with literature data for the LogP and pKa of the drugs of interest.

Analyte	MW ($g.mol^{-1}$)	$K_{fs_{experimental}}$	$LogP_{literature}$	$pKa_{literature}$
Atenolol	266.4	(2.8 ± 0.2) x 10^1	0.1[c], 0.22[e], 0.23[h], 0.026[i], 0.16[b], 0.25[g]	9.6[c], 9.54[e], 9.3[h], 9.17[i], 9.6[b], 8.07[g]
Nadolol	309.4	(1.5 ± 0.1) x 10^2	0.71[h], 1.17[i], 1.0[g]	9.7[h], 9.17[i], 9.4[b], 9.0[g]
Pindolol	248.3	(1.0 ± 0.1) x 10^2	1.83[e], 1.48[i], 1.75[b], 1.91[g]	9.54[e], 9.21[i], 9.7[b], 6.98[g]
Timolol	316.4	(3.8 ± 0.2) x 10^2	2.12[e], 1.91[f], 1.75[i], 1.91[b], 1.98[g]	9.53[e], 9.21[f], 8.86[i], 9.21[b], 9.19[g]
Oxprenolol	265.3	(6.6 ± 0.5) x 10^2	2.94[d], 2.51[e], 2.18[h], 1.83[i], 2.18[b], 2.3[g]	9.57[e], 9.6[h], 9.13[i], 9.5[b], 9.08[g]
Alprenolol	249.3	(1.1 ± 0.1) x 10^3	3.1[e], 3.1[f], 2.61[h], 2.81[i], 3.1[b], 3.15[g]	9.59[e], 9.65[f], 9.6[h], 9.19[i], 9.65[b], 9.34[g]
Propranolol	259.3	(1.6 ± 0.2) x 10^3	3.1[c], 3.48[e], 3.56[f], 3.65[h], 2.6[i], 3.56[b], 3.41[g]	9.53[e], 9.45[f], 9.5[h], 9.15[i], 9.5[b], 9.25[g]
Carvedilol	406.5	(1.6 ± 0.1) x 10^4	4.1[a], 4.23[d], 4.11[e]	7.8[a], 7.9[d], 7.97[e]

References: a [52], b[53], c[54], d[55], e[56], f[57], g[58], h[59], i[60]

Table 5 Experimental and literature values used for LogP determination of β-blockers by SPME.

A correlation was established to determine if the triacontyl fiber could be used to determine the hydrophobicity factor of the pharmaceuticals of interest. As can be seen

in Figure 14, a regression analysis showed clear correlation with a $R^2 = 0.9255$ between the values found in literature and the K_{fs} values obtained using the triacontyl fibers at equilibrium (t_{eq} = 1 hour). Given the variability between different methods to determine LogP, the method at hand is able to provide a rapid determination of this physical parameter without the need for large amounts of drug or solvents.

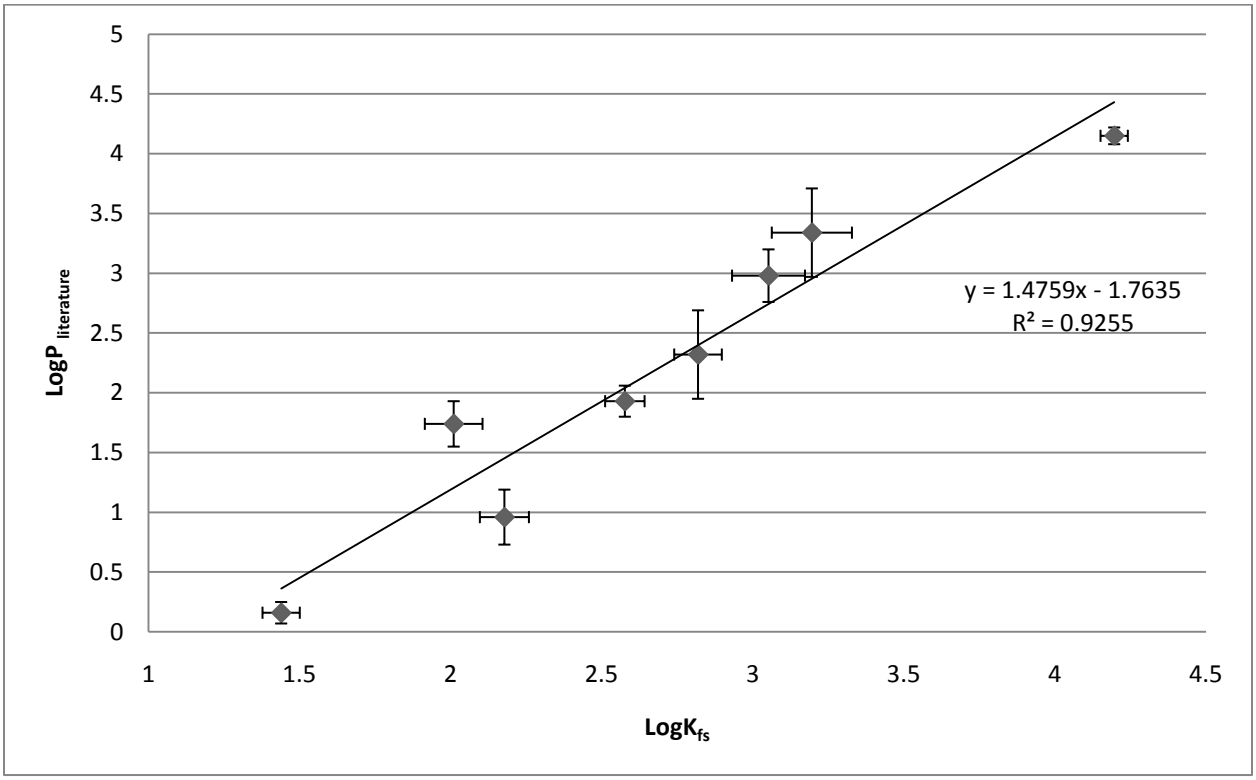


Figure 14 Comparison of the β-blocker $LogK_{fs}$ values obtained experimentally using SPME, with the LogP values obtained from literature results.

The design of the fiber itself also allows for maximum rate of mass transfer to be achieved since the coating approach creates derivatized porous surfaces which are unhindered and situated at the surface of the coating, whereas approaches where

particles are applied as slurry creates thicker coatings and the cured product might also block access to the derivatized surface. Due to this improvement in rate of mass transfer, it becomes advantageous to investigate C30 since the increase in equilibrium time (about twice longer than C18) is negligible compared to the gain in sensitivity.

2.6 Conclusions

The method described in this chapter provides a unique approach to the development of SPME fibers using well known technologies such as monolith casting and organosilane derivatization. Covalent immobilization of the various alkyl phases resulted in a robust and reproducible extraction coating that could be easily optimized for sample extraction selectivity and equilibrium time. This approach should permit the development of a wide range of coatings with tailored extraction properties to meet the needs of fast and efficient sample preparation in analytical chemistry. Covalent immobilization of the various alkyl phases resulted in a robust and reproducible extraction coating that could be easily optimized for sample extraction selectivity and equilibrium time. In the present study, it was demonstrated that C30 could be used to increase the sensitivity of benzodiazepine analysis over shorter alkyl chain extraction phases. It was also demonstrated that C30 can be used to estimate the LogP value of drugs and therefore provide a simple, rapid and reproducible way to estimate the octanol-water partition coefficient in early stages of pharmaceutical development. The present SPME fiber preparation, in combination with the well established organosilane surface derivatization, should help create a variety of surface chemistries and increase the applicability of SPME in more areas of analytical chemistry.

3 High-throughput Automation for Drug Analysis

3.1 Introduction

The various advantages of SPME, namely its simplicity, size and speed, make it an ideal technology for high-throughput sample analysis. The importance of SPME automation for routine analysis has been recognized early in environmental sample analysis.[23, 25] SPME is also a well established technique for drug analysis, as recently outlined in several reviews.[30, 61-67] Automation of SPME for bioanalysis has mainly focused on the area of in-tube SPME,[22, 68] however, this approach requires processing of samples in a serial fashion. Most recently, there have been efforts to demonstrate the suitability of SPME for 96 well-plate sample analysis of drugs in biofluids using commercially available fibers.[69] Although this work requires manual processing, this parallel and high-throughput analysis approach is necessary to meet the demands of drug analysis in biological samples.

Thus, the advancement of SPME as a technology used in large scale bioanalysis requires the development of robust, high-throughput and automated fiber fabrication protocols in order to obtain and process a large number of sampling devices, while maintaining rugged and uniform coatings for reproducibility. Furthermore, the amount of commercially available coatings on the market for high-throughput sampling in biological samples is limited and can necessitate fabrication *in situ*. This process is lengthy and requires a fair amount of optimization in order to obtain an acceptable sampling device.

We propose a high-throughput assembly procedure for a C18 SPME fiber using robotic assistance to help us achieve a uniform and rugged coating. The same robotic apparatus is then used for high-throughput sample preparation of loratadine in human plasma. Traditionally, fibers that are not commercially available to analyze a given compound are manually constructed by immersing a support material such as silica or stainless steel into the desired coating material. Silica bonded phases have shown desirable properties as coatings due to their large specific surface area. [9] Various coating procedures can be used to provide the desired parameters of the coating; however, the conditions under which the fiber preparation is performed can be tedious and hard to control (relies heavily on manual skills). To assemble the fibers using a simple and rugged method was one of the aims of our research to improve high-throughput. Our method therefore helped reduce manual labour induced errors in the fiber preparation step and provided many fibers at once, which in turn provided a fast and efficient way to perform high-throughput drug analysis.

The evaluation of our fiber preparation and extraction procedure was performed using loratadine, a long-acting tricyclic antihistamine with selective peripheral histamine H_1-receptor antagonistic activity, as a model analyte (Figure 15). Loratadine is a widely used allergy medication which has been analyzed in biological fluids by HPLC with mass spectrometry.[70-77] The majority of the methods used to determine loratadine in biological fluids relies on time consuming liquid-liquid extractions that are typically difficult to automate.

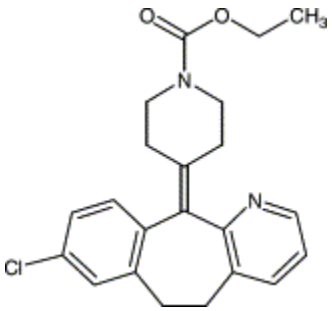


Figure 15 Molecular structure of loratadine (M.W. 382.89).

The objective of this work was to provide a fast, low cost, and reproducible method for SPME fiber preparation, capable of detecting loratadine within its therapeutic range. In our study, a custom designed robotic apparatus was used to automate the fibers preparation process in combination with the sample extraction procedure for the high-throughput analysis of loratadine human plasma.

3.2 Experimental

3.2.1 Chemicals and Materials

Loratadine [ethyl 4-(8-chloro-5,6-dihydro-11H-benzo[5,6]cyclohepta[1,2-b]pyridin-11-ylidine)-1-piperidinecarboxylate], and Loratadine-d4 were supplied by Merck Frosst and Co. (Kirkland, QC, Canada). Discovery C18 5μm silica particles were donated by Supelco (Bellefont, PA, USA). Ammonium acetate and acetic acid were purchased from Sigma-Aldrich (Oakville, ON, Canada). Acetonitrile, methanol, and isopropyl alcohol,

HPLC grade or better, were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Hydrochloric acid, ACS grade, was purchased from Fisher Scientific (Ottawa, ON, Canada), chloroform, ACS grade, was purchased from J.T. Baker (Phillipsburg, NJ, USA). Human Plasma EDTA was purchased from US Biologicals (Swampscott, MA, USA) and stored at -20°C. 353ND epoxy was purchased from Epoxy Technologies (Billerica, MA, USA). 96 deep well-plates (1 mL and 2 mL) were purchased from VWR (Mississauga, ON, Canada). Stainless steel wires grade 304 and a Xuron® music wire shear cutter were purchased from Smallparts (Miami Lakes, FL, USA). Samarium cobalt magnets (SmCo grade 18) were purchased from Master Magnetics (Castle Rock, CO, USA). Deionized water was obtained by a Barnstead Nanopure water system (Dubuque, IA, USA).

3.2.2 SEM Characterization

High magnification characterization of the fiber coatings were performed using a scanning electron microscope (SEM) LEO 1530 field emission SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany). Prior to analysis, the fibers were dried in an oven at 150°C for at least 2 hours in order to remove any moisture. Carbon conductive tape and specimen mounts (Ted Pella, Redding, CA, USA) were used to immobilize the samples for analysis. Sections of the fibers were cut into 7 mm lengths for horizontal views, or ~1 mm for a cross-sectional view. Prior to analysis, samples were sputtered with ~10 nm of gold and were analyzed using an acceleration voltage of 15 kV. Preliminary

characterizations were performed using an optical microscope (Reichert-Jung series 40, Heidelberg, Germany).

3.2.3 LC-MS/MS Instrumentation

A Varian (Palo Alto, CA, USA) 500-MS Ion Trap coupled to a 430 autosampler for 96 well plates and 212-LC pumps was used for this work. The chromatography was achieved using a Merck (Darmstadt, Germany) Chromolith Flash RP-18e column (4.6 x 25 mm) preceded by a Varian Pursuit C18 A3000MG2 pre-column. The mobile phase gradient consisted of 28% mobile phase A (90:10:0.1 (v/v) water:acetonitrile:acetic acid) changing to 90% mobile phase B (10:90:0.1 (v/v) water:acetonitrile:acetic acid) over 4.3 minutes at a flow rate of 1 mL/min, followed by re-equilibration for the column over 2.7 minutes. A Valco® t-splitter was used to obtain a 0.25 mL/min flow rate to the MS module. A needle wash solution of 50% methanol and 50% water was used with the autosampler. The injection volume was 20 µL.

The mass spectrometry experiments were performed using an electrospray ionization (ESI) interface in positive mode. The capillary voltage was maintained at +94V, Rf loading was set to 93%, and excitation amplitude was 0.86. The ESI housing was kept at 50°C, the drying gas was set to 400°C and 30 psi. The spray shield voltage was set to +600V and the needle voltage to +3600V. The mobile phase was diverted from the source except between t = 1.5 to 3.5 minutes to prevent contamination of the source. MRM mode was used for the quantitation of the analytes by LC-MS/MS. The precursor → product ion transitions were at *m/z* 383/337 for loratadine and *m/z* 387/341

for the d_4-loratadine. Data acquisition was done using a PC with the Varian MS Workstation software.

3.2.4 Preparation of Stock Solutions and Samples

A stock solution of loratadine and d_4-loratadine was prepared at 1 mg/mL in acetonitrile. The loratadine stock was further diluted in acetonitrile to obtain working solutions of 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL. A working standard of 200 ng/mL of the d_4-loratadine was prepared in acetonitrile. All working solutions were stored at 4°C and the stock solutions at -20°C.

After thawing the plasma samples, 37.5 µL of the working standards and internal standard was added to 675 µL of human plasma. Buffer samples were prepared in a similar fashion using 40 mM ammonium acetate adjusted to pH 6.8 using acetic acid. For the purposes of characterization and evaluation of the fibers for loratadine extraction, the internal standard was added after the extraction step in the final reconstitution solution.

3.2.5 Preparation of Adhesive

The Epotek 353ND adhesive was received as a 2 part kit. The ratio of part A:B was kept constant at 10:1 (w/w). Initially, 20 g of part A resin was weighed in a 40 mL screw cap vial and 16 mL of chloroform was added to the resin. The mixture was

magnetically stirred at 700 rpm for 30 minutes. Once homogenized, 2 g of part B hardening agent was added to the part A mixture and mixed well for 15 minutes under similar conditions. A quantity of 14 mL of the adhesive was required to fill the well of our coating apparatus.

3.2.6 Automated SPME Coating Procedure

A robotic arm (PAS Technology Deutschland GmbH, Magdala, Germany) designed for use as a multifunction autosampler was programmed using the Concept® Software to automatically coat 96 fibers with stationary phase using a novel custom built 96 fiber holder device (Figure 16). Grade 304 stainless steel wires (1.55 mm outer diameter) were cut into 5.5 cm fibers using an in-house modified Xuron® music wire shear. The resulting small wires were chemically etched in concentrated hydrochloric acid for 1 hour followed by a generous rinse with deionized water. The wires were dried at 150°C for at least 2 hours and allowed to cool to room temperature. Fibers were loaded onto the 96 fiber holder and retained by the individual magnets embedded in the holder (Figure 16). The 96 fiber holder was then properly coupled to the robotic arm and the software allowed the fibers to be coated row-by-row of 12 fibers into a modified 96 deep well plate with wells of 2 mL.

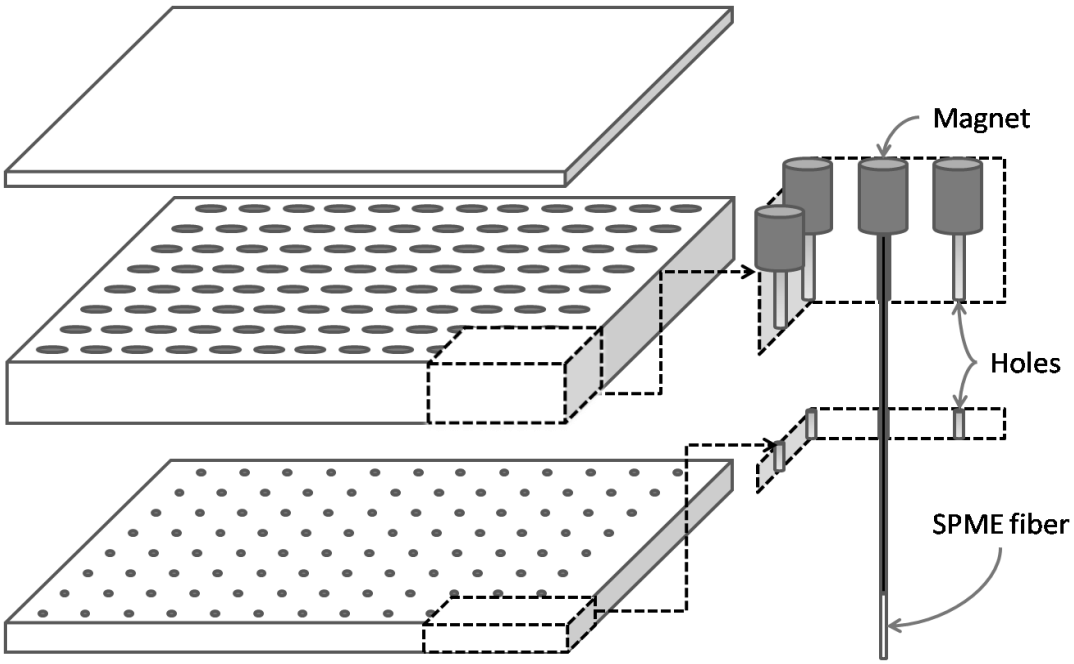


Figure 16 Schematic of the fiber holder apparatus with embedded samarium cobalt magnets.

The rows were sequentially dipped into the modified epoxy adhesive followed by blotting with an adsorbent material and finally into a section containing the Discovery C18 particles. The total program running time was under 6 minutes for the entire 96 fibres coating procedure. Once coated, fibres were removed from the robot and the 96 fibre holder was placed upside down for cure in a GC oven at 150°C for 1 hour, and then allowed to cool to room temperature. A secondary cure of 150°C for 1 hour was performed to insure maximum bonding of the particles to the wire. An aluminum rod specifically designed to suspend the 96 fibre holder in a sonication bath using a retort stand was used to sonicate the fibres in 10% acetonitrile, 90% water to remove any particles which had poor adhesion to the wire before they are used.

3.3 Results and Discussion

3.3.1 Development and Optimization of SPME Coating Procedure

Experiments were performed to determine the reproducibility of the robotically applied coatings and to utilize the SPME fibres for the accurate determination of loratadine in human plasma samples. The coating reproducibility was evaluated using loratadine spiked buffer samples. All experiments were carried at room temperature using a final concentration of 10% acetonitrile in the extraction solution due to loratadine's low solubility in water.

An octadecyl stationary phase was selected due to the hydrophobic nature of loratadine (LogP of 4.4 - 5.7). [78, 79] The particular homogeneity and low density of the Discovery C18 5 µm particles was crucial in obtaining a uniform coating. After investigating various adhesives, Epotek 353ND was determined to provide excellent adhesion of the stationary phase to the fiber support. Physical strength and robustness of the coating was determined to be of good quality if scratching the stationary phase with finger pressure using a kimwipe did not remove the particles as observed through an optical microscope at 30X. Furthermore, the integrity of the coating was also monitored via optical microscopy and maintained after exposure to sonication in various solvents. In order to obtain a thin, uniform but robust coating, the adhesive viscosity had to be modified by diluting Part A with chloroform. SEM was utilized to monitor the uniformity of the particles at the surface of the fiber and the thickness of the adhesive layer at various epoxy dilutions (See Figure 17). An adhesive thickness of 6-8 µm was

achieved at an optimum of 80% (v/w) chloroform in part A epoxy. This enabled a uniform monolayer of particle to be immobilized; improving the extraction kinetics of the fibers and yielding a more high-throughput application.

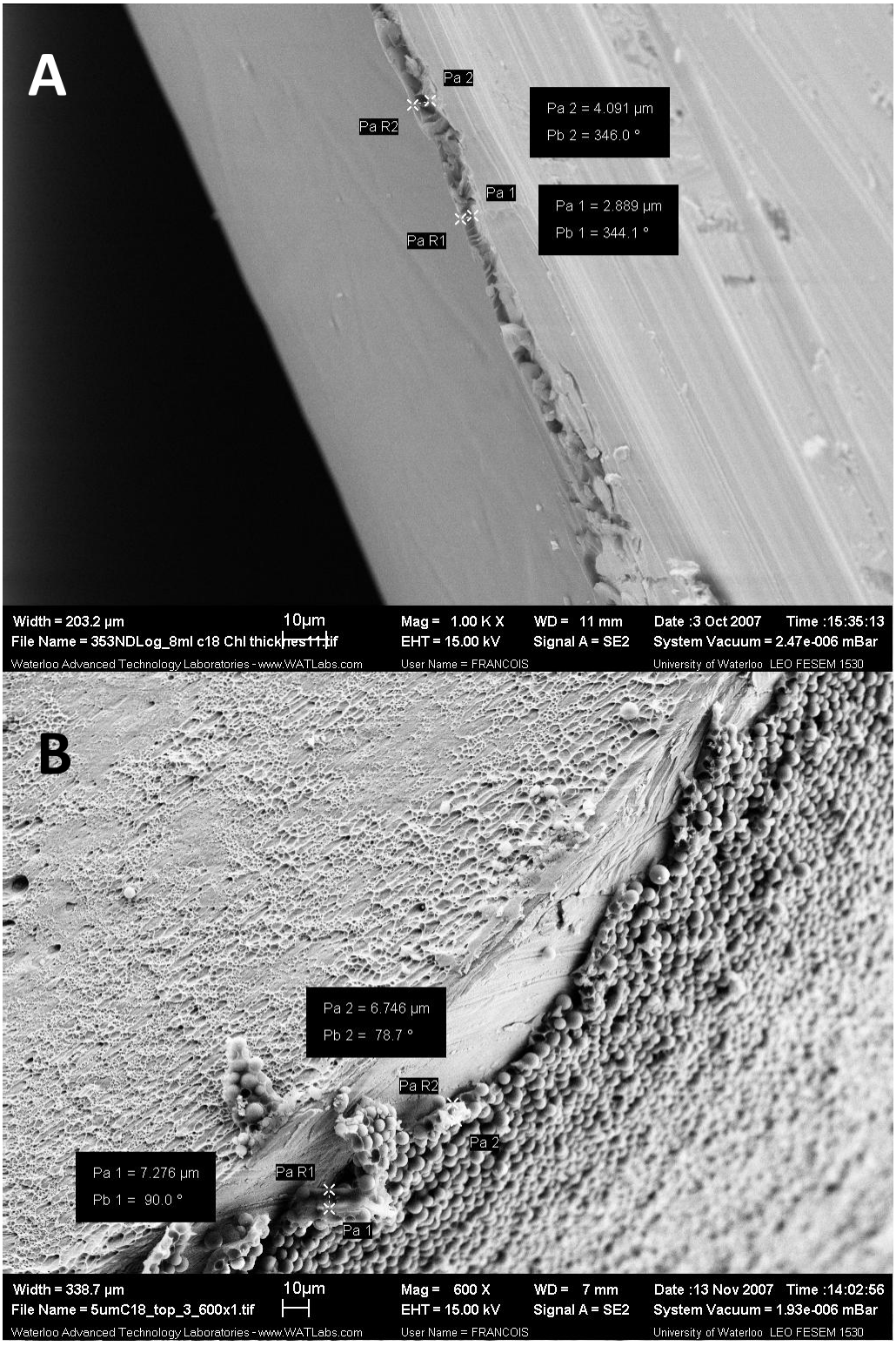


Figure 17 SEM bondline of the adhesive without stationary phase (A). Final product of adhesive + stationary phase after sonication step (B).

The choice of dilution using chloroform was determined to be the best approach since the coating procedure had to be easily amenable to automation. Alternatively, a 96 well plate warmer could also have been employed to slightly increase the temperature of the adhesive and decrease the viscosity of the adhesive. Manual approaches, such as brushing the adhesive on the wires, was considered too time consuming and irreproducible.

3.3.2 Optimization of the Automated Coating Procedure

In order to reproducibly coat each fibre on the fiber holder it was important to control tightly the adhesive application. The 96 fiber holder previously developed by Cudjoe et al.[80] was modified in order to provide us with a coating platform that would have a strong hold on individual fibers while being easy to use and, most importantly, capable of withstanding the high cure temperatures. The holder, as shown in Figure 16, consists of a plate (plate 1) containing 96 holes, to provide for lateral stability of the fibers during agitation. Evenly distributed spacers are then sandwiched between another, thicker plate (plate 2), with holes aligned with the previous plate to insert the upper part of the fiber, for rigidity. In this second plate we have larger holes above the holes where the fiber is inserted. These larger holes are about 6.4 mm in diameter and 6.4 mm deep and are filled with cylindrically shaped samarium cobalt magnets of similar dimensions. These rare earth magnets were selected for their strong magnetic energy and resistance to high temperatures. A last plate (plate 3) is finally applied over the magnets and screwed into place. This last plate's purpose consists of holding the

magnets into their seat and also has the proper coupling piece to be installed on the robot assembly. The design permitted a hands-off approach therefore eliminating possible manipulation errors of the uncured coatings resulting in discrepancies in fiber capacities. It also prevented the tedious need to remove every fiber from the holder to cure. This approach could also potentially allow fibers to be cured as part of the automation process if for example fibers were lowered into a heater at the proper cure temperature.

In order for every fiber to be coated to the same length, the support wires had to be of identical length. This was achieved by modifying a Xuron music wire shear, which provided a straight cut in itself, and to install a guide that would permit equal segment lengths of 5.5 cm.

The container holding the coating material consisted of a 2 mL deep well plate, modified to the specifications depicted in Figure 18. The wide sides of the plate had to be removed to prevent the fibers from touching the plate sides when lowered. The second modification consisted of creating a trench in row 1 where the adhesive is located. This step was performed to prevent small changes in the adhesive volume from influencing the coating length.

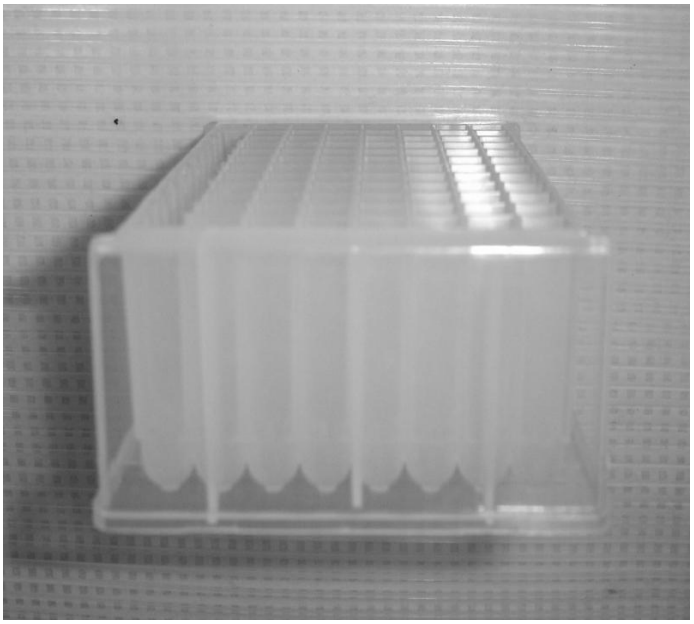


Figure 18 Modified 2 mL deep well plate used for automated coating procedure.

The total volume of adhesive used in this well was 14 mL, which was 18 mm from the top of the well. The low volume of adhesive required for the coatings allowed for a minimal change in volume of the adhesive well, however the well was adjusted to the fill line after each coating procedures. By having a single unseparated row the adhesive fill line would not be subjected to well-to-well irreproducibilities. The second row was loaded with individual cubic pieces of foam with sides of 70 mm which was coated with a light adhesive on one surface and a piece of kimwipe of similar dimensions was fixed on the upper surface by the light adhesive, deposited in each well using tweezers. This step was required to remove any droplet of adhesive which would accumulate at the bottom of the wire during the adhesive coating procedure. The third row was loaded with the particles up to within 3 mm from the top of the plate. The

parameters of the components in the 3 rows were adjusted to complement the Concept® software dipping procedure.

The dipping procedure, controlled from the Concept® software, was optimized to minimize any displacement of the adhesive and prevent uneven coatings. Initial work was carried by a simple dip at every step. This procedure yielded fibers with an even coating of adhesive, however poor coating of particles as observed by optical microscopy. It was observed that the quality of the coating was better with an agitation at ~250 rpm where C18 particles were thoroughly covering the adhesive. An 8 mm offset from the adhesive was required to prevent an uneven finish of the fiber coatings. Care was taken to ensure that the fiber holder was lowered without any inclination by using a digital level.

3.3.3 Optimization of the SPME conditions

Optimization of the SPME conditions was performed using hand fabricated fibers. The minimum time required to reach equilibrium and optimal sensitivity was determined to be 30 minutes using an agitation of 850 rpm. The experiment was performed using 750 µL of a 100 ng/mL loratadine solution consisting of 10% acetonitrile and 90% buffer. The desorption step was then optimized by monitoring the amount of analyte desorbed from the fibers and the residual carry-over. For high-throughput applications a removal of the analyte in a minimum time is required. We investigated the use of 3 desorption solvents to determine which of these would extract the analyte completely and rapidly.

Methanol and acetonitrile were found to achieve complete desorption faster than isopropyl alcohol (Figure 19).

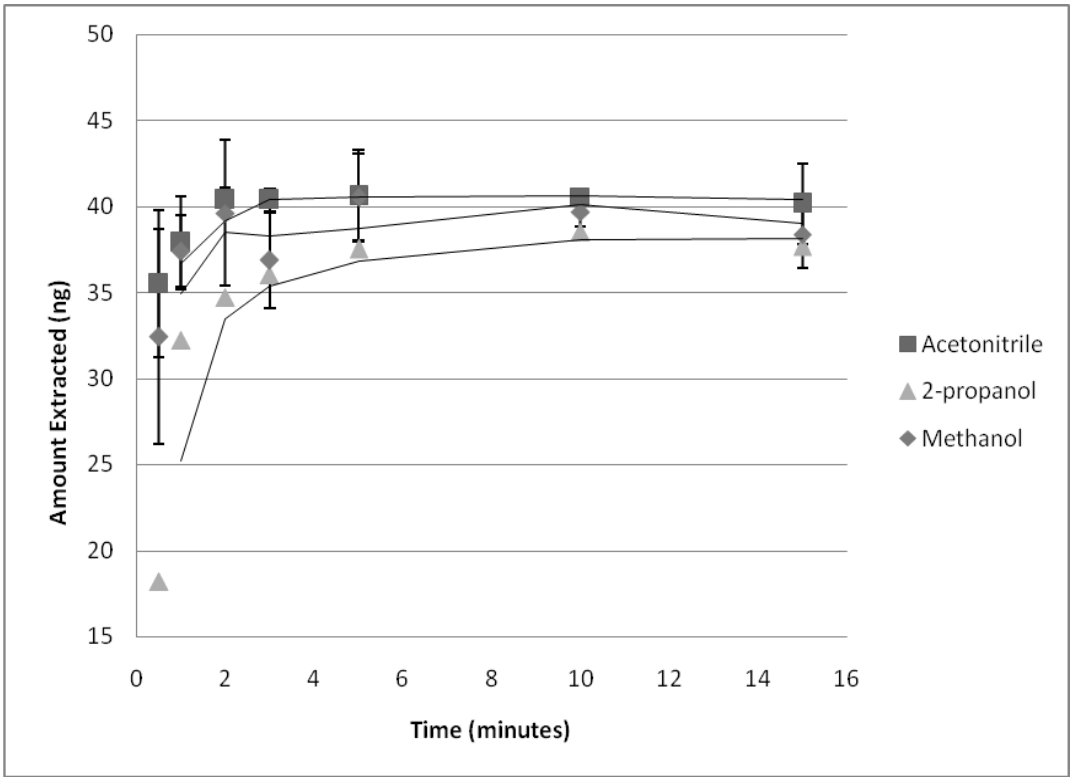


Figure 19 Desorption time profiles of loratadine using various solvents.

Acetonitrile was chosen and the use of 5 minute desorption was used since no more than 0.4% carry-over was detectable in a successive desorption after 5 minutes, as compared to isopropyl alcohol, which had consistently above 1.5% carry-over even after 5 minutes. Also, the short desorption times are desirable to avoid problems with evaporation of the desorption solvent. In our case, fiber carry-over was reduced to obtain good sensitivities, however fibers were used only once unless otherwise noted.

3.3.4 Validation of the Automated Coating Procedure

The 96 fiber coating procedure was validated by repeating the coating procedure and monitoring if a change in extraction capacity was observed. Initially, our focus was to determine if coating the fibers a certain time away from the initial mixing of both parts of the epoxy would modify the amount we extract. This measure of the amount extracted would then tell us if polymerization or changes in the adhesive properties would significantly modify the qualities of our coating. Using 7 fibers situated in approximately equidistant positions on the plate we obtained the amount extracted from our fibers which is directly related to the amount of stationary phase (coating) we have on our fibers. It was determined that for a 4 hour period, no significant change in the properties of the adhesive were found (< 12% RSD) to alter the coating integrity (Figure 20).

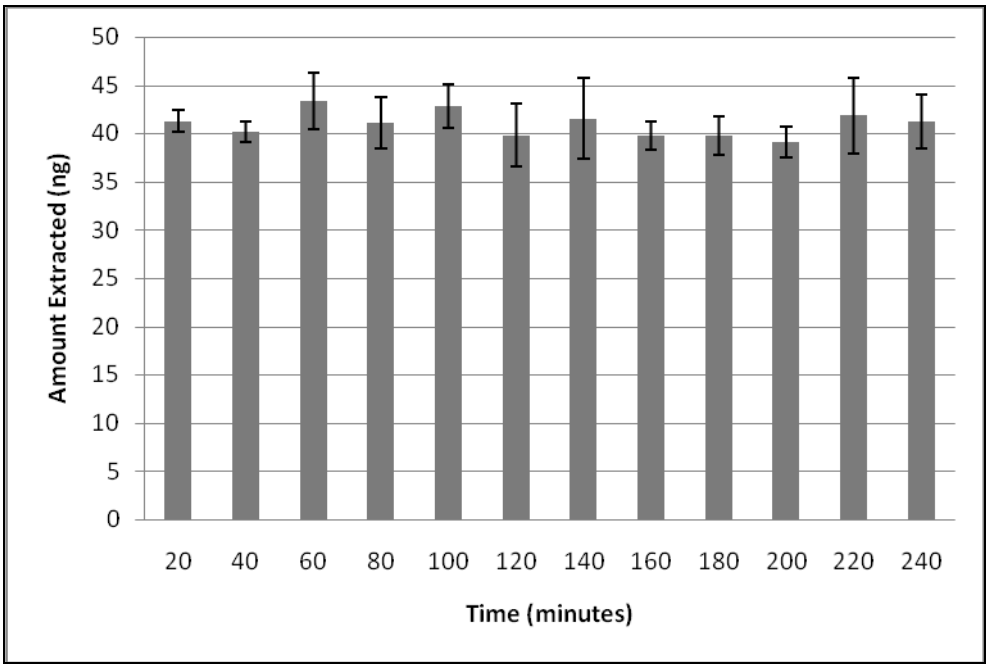


Figure 20 Determination of the effect of time on the epoxy preparation by observing capacity of fibers fabricated at different times. t=0 is considered to be the mixing of both components of the epoxy.

To determine if the coating procedure was reproducible, 5 batches of fibers (n=7) were prepared and evaluated for their extraction efficiency. Repeating the whole process produced fibers of similar capacity and sensitivities (Figure 21).

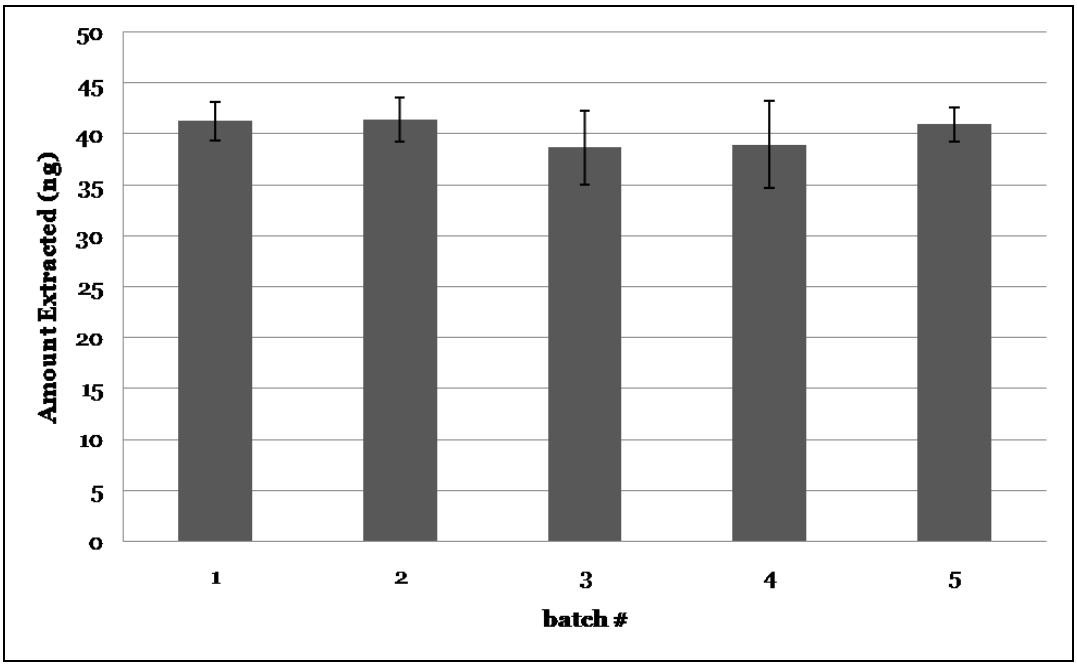


Figure 21 Batch-to-batch reproducibility of the fiber preparation.

The variation between fibers in each batch was also comparable as indicated by a 9.0% RSD for the evaluated fibers (n=35).

3.3.5 Application and Validation of Fibers in Human Plasma

The linearity of the SPME-LC-MS/MS method was determined using both buffer and human plasma samples as described previously. The calibrations gave linearity of $y = 0.126x – 0.019$ ($R^2 = 0.999$) and $y = 0.129x + 0.035$ ($R^2 = 0.999$) for buffer and human plasma respectively. The lower limit of quantitation (LLOQ) for the method in each matrix was determined to be the lowest sample concentration (n=3) which would give us a RSD (%) equal or less than 15%. The LLOQ in buffer was determined to be 0.5 ng/mL

with a RSD of 6.9%, and the precision at all other concentrations was determined to be equal to or lower than 5.3%. In human plasma the LLOQ was determined to be 1.0 ng/mL with a RSD of 6.0%, and the precision at all other concentrations was determined to be equal to or lower than 4.5%.

The accuracy of the method was determined by using replicate analysis (n=3) of the drug compound in both buffer and plasma. It was found that the precision of the method, with different concentrations of drug, was acceptable as indicated with maximum RSD of 6.0% (Table 6).

Concentration (ng/mL)	Ratio Std/ISTD	Accuracy (%)
1	0.21	135.7
	0.20	127.1
	0.19	117.2
Mean	0.20	
RSD (%)	6.0	
100	12.64	97.7
	11.97	92.5
	12.71	98.3
Mean	12.44	
RSD (%)	3.3	
250	33.21	102.9
	32.06	99.3
	32.17	99.6
Mean	32.48	
RSD (%)	2.0	

Table 6 Precision intra-assays of loratadine in spiked human plasma.

The stability of the stock of loratadine was investigated by preparing a fresh stock solution and determining the response factor of both standard solutions. The stock was stable for 10 days as the response was < 2.0% from the original solution (n=5) over this timeframe. In order to know if our assay was reproducible, we determined that intraday variation (n=5) was 3.0% in buffer solution and 3.2% in human plasma. The interday variation was determined to be 3.6% in human plasma samples. (Table 7)

Trial #	Day 1	Day 2
	Ratio Std/ISTD	
1	12.58	13.89
2	12.55	13.24
3	12.59	12.79
4	12.86	12.99
5	12.17	13.04
	Mean	12.87
	RSD (%)	3.63

Table 7 Interday reproducibility of the fibers in human plasma.

The specificity of the SPME LC-MS/MS method is demonstrated by a representative chromatogram of human plasma with and without loratadine (Figure 22). The sample clean-up provided by the C18 SPME coating and the selectivity of the LC-

MS/MS fragmentations transitions, effectively eliminated any background interferences as indicated by the absence of any peaks in the blank plasma baseline.

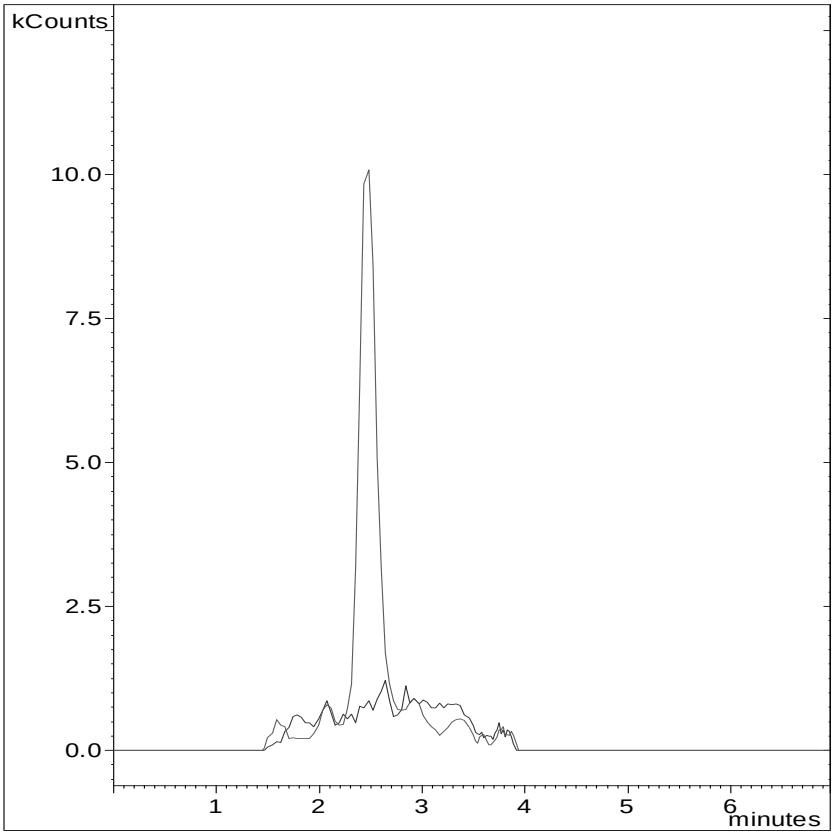


Figure 22 Specificity of the method for loratadine in plasma at LLOQ (1 ng/mL).

The ruggedness of our coatings was investigated by repeated use of 6 fibers over 20 extractions and no deterioration of the coating qualities were observed. Care was taken to prevent carry-over by performing a second desorption before a re-equilibration period of 15 minutes.

3.4 Conclusions

The highly uniform, and reproducible nature of the fibers prepared using a robotic apparatus, confirms the ability of SPME as a technology well adaptable to high-throughput oriented laboratories. The developed approach, in combination with the number of particulate extraction material that is commercially available, will allow for the simple manufacture of SPME fibers with a range of coating extraction chemistries, extending the versatility of the approach. Full automation of the fiber preparation, and sample extraction and desorption process also demonstrates the feasibility of high-throughput fiber fabrication for quantitative analysis of drugs in biological matrices. The developed method for the analysis of loratadine proved sensitive and reproducible for the determination of drug concentrations in human plasma samples. Above all, the automated and parallel approach will enable fast analysis for large amounts of samples.

4 Summary

The outcome of this work is the development of a novel fiber consisting of a triacontyl covalently bonded phase using a highly porous silica backbone. The fiber proved successful at extracting high amount of benzodiazepines as compared to shorter chain alkyl phases. It was also successful at establishing a relationship between the hydrophobic LogP value of β-blocker drugs in buffer and $LogK_{fs}$, thereby greatly reducing the amount of drug and solvent required to find this physical parameter. We have also shown the possibility to make 96 fibers with high uniformity which can be used to perform parallel analysis of multiple samples of biofluids using a robotic apparatus, thereby minimizing time of sample preparation as well as minimizing the need for analysts to come into contact with the biological material.

Through this work we hope to have demonstrated some possibilities and advantages of using SPME to simplify pharmaceutical development. The new coatings and methodology could greatly reduce the difficulty and tediousness of some current sample preparation protocols.

Safety Considerations

Care must be used when handling porous silica materials (derivatized or not) since their small size makes them easily inhalable. Concentrated acids and their fumes are hazardous and must be manipulated with necessary safety equipment in a fumehood. Human biological materials used in this work should be handled with care due to the possibility of harmful pathogens. Some of the adhesives used in this work are corrosive and toxic, proper safety equipment must be worn during handling. All chemicals, solvents, biofluids should be discarded according to University of Waterloo waste disposal guidelines.

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