BIOCOMPATIBLE SOLID PHASE MICROEXTRACTION

by

MIHAELA LACRAMIOARA MUSTEATA

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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.

I understand that my thesis may be made electronically available to the public.

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Abstract

Today's solid phase microextraction (SPME) is a well known technique that combines knowledge from different fields in an attractive, efficient, and economic way. The development of SPME has seen huge growth since its introduction as a new approach to sample preparation in the early 1990s. The applications of SPME are continuously expanding, and one of the most interesting current aspects consists of applying SPME for fast analysis of biological fluids, both in vitro and in vivo.

In spite of this great potential, development of new bio-applications is considerably hindered by the lack of suitable SPME products. The goal of this study is to find SPME coatings that can be utilized for in vivo and in vitro extractions, in direct contact with a biological matrix such as blood or tissue. This thesis presents several effective ways of preparing SPME coatings based on biocompatible polymers and silica-based extractive phases, focusing on their biocompatibility as a must. After fabrication, the proposed coatings are tested for biocompatibility and analytical utility.

Finally, some practical applications of the new coatings are presented, such as fast drug analysis and determination of drug plasma protein binding. Six test drugs with different physico-chemical properties are chosen for the investigation: diazepam, verapamil, lorazepam, warfarin, nordiazepam, and loperamide. It is shown that the application of these new SPME fibers for biological sample analysis greatly reduces the time required for sample preparation and limits the exposure of the analytical personnel to potentially infectious biofluids.

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ADS	alkyl-diol-silica
ASE	affinity sorbent extraction
C18/PEG	octadecyl - silica / poly(ethylene glycol)
CE	capillary electrophoresis
CW/DVB	carbowax / divinylbenzene
CW/TPR	carbowax / templated resin
DMF	dimethylformamide

ELISA	enzyme linked immunosorbent assay
ESCA	electron spectroscopy for chemical analysis
ESI	electrospray - ionization
ESI-MS	electrospray - ionization mass spectrometry
FDA	Food and Drug Administration
GC	gas chromatography
HPLC	high performance liquid chromatography
LC	liquid chromatography
LC-MS	liquid chromatography coupled with mass spectrometry
LLE	liquid - liquid extraction
MD	microdialysis
MIP	molecularly imprinted polymers
MSD	mass spectrometry detector
MW	molecular weight
ODS	octadecyl - silica
PAN / TPR	polyacrylonitrile / templated resin
PAN	polyacrylonitrile
PBS	phosphate buffer saline
PDMS	polydimethylsiloxane
PEG	poly(ethylene glycol)
P_{ow}	partition coefficient between n-octanol and water
PPB	plasma protein binding
PPY	polypyrrole

PU	polyurethane
RAM	restricted access materials
RSD	relative standard deviation
SFE	supercritical fluid extraction
SPE	solid phase extraction
SPME	solid phase microextraction
UV-VIS	ultraviolet - visible
XDS	ion exchange diol silica
XPS	X-ray photoelectron spectroscopy

Chapter 1 Introduction

The requirement to analyze drugs in biological samples and pharmaceutical products is becoming more and more frequent with the development of more selective and more effective drugs and with our need to understand more about their therapeutic and toxic effects. Knowledge of drug levels in body fluids, such as blood and urine, allows pharmacotherapy to be optimized and provides the basis for studies on patient compliance, bioavailability, pharmacokinetics and genetics, organ function, and the influences of co-medication.

Biological materials and pharmaceutical products are complex and often contain proteins, salts, acids, bases and organic compounds with properties similar to those of the analytes. In addition, the analytes often exist at low concentration in samples. Depending on the origins of samples and analytical objectives, drug analyses have been carried out using various analytical instruments in many circumstances such as clinical control for diagnosis and treatment of diseases, doping control, forensic analysis and toxicology. Despite the recent advances in the development of highly efficient analytical instruments for the determination of analytes in biological samples and pharmaceutical products, a sample preparation step is usually necessary in order to extract and isolate the analytes of interest from complex matrices.

Solid phase microextraction (SPME) is a sampling and sample preparation technique invented 16 years ago by Dr. Pawliszyn. SPME was developed to address the need for rapid sample preparation both in the laboratory and on-site (where the investigated system is

located).[1] Since its conception, the development of SPME has seen huge growth at both the fundamental and the application oriented levels.[2,3] In the few years of its practice, SPME has developed to a mature technique and a useful alternative to contemporary techniques in various scientific and research fields. Not surprisingly, SPME was one of the six "great ideas of the decade" as illustrated in a recent survey of Analytical Chemistry.[4]

1.1 Principles of SPME

In SPME, a small amount of extracting phase dispersed on a solid support is exposed to the sample for a well defined period of time.[2] Based on the total time of contact between sample and extraction phase, two methods are currently in use: (i) equilibrium extraction, when partition equilibrium is reached, and (ii) pre-equilibrium extraction, when the sample makes contact with the extraction phase for a short period of time. When partition equilibrium is reached, the convection conditions do not affect the amount of analyte extracted, that depends on the sample concentration and volume. On the other hand, in the case of preequilibrium extraction, quantification can be performed based on timed accumulation of analytes in the extraction phase. Several implementations of SPME exist based on the geometry of the extraction phase, such as coated fibers, vessels, stir bars, disks, and tubes coated on the inside. In terms of extraction from sample and subsequent delivery to an analytical instrument, the most convenient approach for manual operation and *in vivo* analysis is the fiber SPME design. The technique consists of exposing a small amount of extracting phase (coating) associated with a fiber to the sample for a predetermined amount of time. The transport of analytes from the matrix into the coating begins as soon as the coated fiber has been placed in contact with the sample (Figure 1-1).

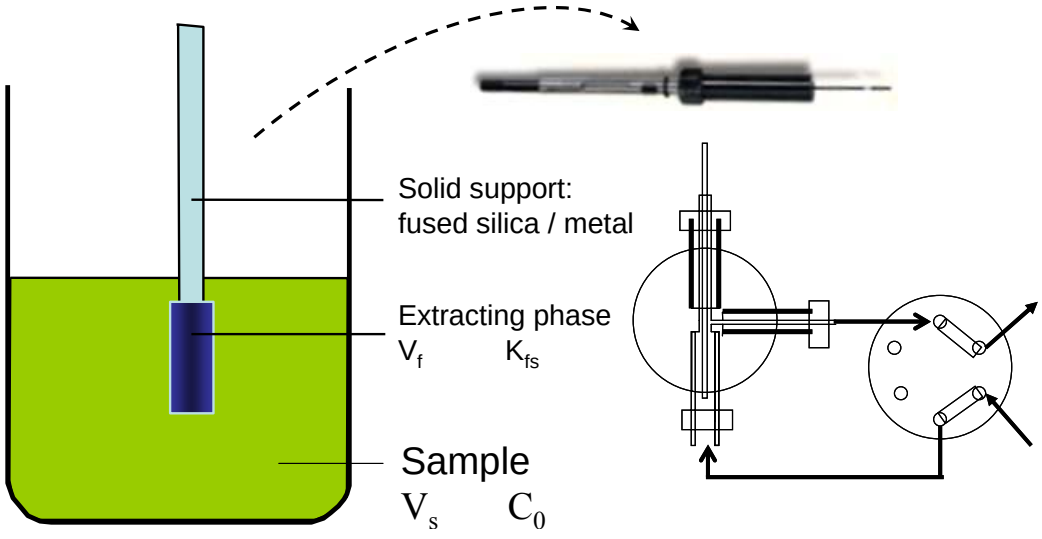


Figure 1-1. Sample preparation with SPME. V_f, volume of the fiber coating; K_{fs}, fiber/sample distribution coefficient; V_s, volume of sample; C_0, initial concentration of analyte in the sample.

If the extraction time is long enough, concentration equilibrium is established between the sample matrix and the extraction phase. When equilibrium conditions are reached, exposing the fiber for a longer time does not result in the accumulation of more analytes. Typically, the microextraction process is considered to be complete when the analyte concentration has reached distribution equilibrium between the sample matrix and the fiber coating. The equilibrium conditions can be described by equation 1.1, according to the law of mass conservation, if only two phases are considered (for example, the sample matrix and the fiber coating):[5]

$$C_0 \cdot V_s = C_s^{\infty} \cdot V_s + C_f^{\infty} \cdot V_f \qquad \textbf{(1.1)}$$

where C_0 is the initial concentration of a given analyte in the sample, V_s is the sample volume, V_f is the fiber coating volume, C_s^{∞} is the equilibrium concentration of analyte in the sample,

C_f^∞ is the equilibrium concentration of analyte in the fiber. The distribution coefficient K_{fs} of the analyte between the fiber coating and sample matrix is defined as:

$$K_{fs} = \frac{C_f^\infty}{C_s^\infty} \quad \textbf{(1.2)}$$

Equations 1.1 and 1.2 can be combined and rearranged into:

$$C_f^\infty = C_0 \cdot \frac{K_{fs} \cdot V_s}{K_{fs} \cdot V_f + V_s} \quad \textbf{(1.3)}$$

Finally, the number of moles of analyte n extracted by the coating can be calculated from equation 1.3:

$$n = C_f^\infty \cdot V_f = C_0 \cdot \frac{K_{fs} \cdot V_s \cdot V_f}{K_{fs} \cdot V_f + V_s} \quad \textbf{(1.4)}$$

Equation 1.4 indicates that the amount of analyte extracted onto the coating (n) is linearly proportional to the analyte concentration in the sample (C_0), which is the analytical basis for quantification using SPME.

Strictly speaking, the above discussion is practically limited to partitioning equilibrium involving liquid phases such as poly(dimethylsiloxane). The method of analysis for solid sorbent coatings is analogous for low analyte concentrations, since the total surface area available for adsorption is proportional to the coating volume, if constant porosity of the sorbent is assumed. For high analyte concentrations, saturation of the surface can occur, resulting in nonlinear isotherms. Similarly, high concentrations of a competitive interference compound can displace the target analyte from the surface of the sorbent.

Equation 1.4, which assumes that the sample matrix can be represented as a single homogeneous phase and that no headspace is present in the system, can be modified to

account for the existence of other components in the matrix, by considering the volumes of the individual phases and the appropriate distribution constants. The headspace is only important for volatile analytes.

In addition, when the sample volume is very large, i.e. $V_s >> K_{fs} \cdot V_f$, equation 1.4 can be simplified to:

$$n = K_{fs} \cdot V_f \cdot C_0 \qquad \textbf{(1.5)}$$

which points to the usefulness of the technique when the volume of the sample is unknown and large. In this equation, the amount of extracted analyte is independent of the volume of the sample. In practice, there is no need to collect a defined sample prior to analysis, as the fiber can be exposed directly to the flowing blood, ambient air, water, etc. The amount of extracted analyte will correspond directly to its concentration in the matrix, without depending on the sample volume. When the sampling step is eliminated, the whole analytical process can be accelerated, and errors associated with analyte loss through decomposition or adsorption on the sampling container walls will be prevented.

The time to reach the extraction equilibrium, ranging from minutes to hours, is dependent on the agitation conditions, the physicochemical properties of analytes and the fiber coating, and the physical dimensions of the sample matrix and the fiber coating. The amount of analyte extracted onto the fiber coating is at a maximum when the equilibrium is reached, thus achieving the highest sensitivity. If sensitivity is not a major concern of analysis, shortening the extraction time is desirable. In addition, the equilibrium extraction approach is not practical for solid porous coatings because of the displacement effect at high concentrations. For these circumstances, the extraction is stopped and the fiber is analyzed

before the equilibrium is reached. The kinetics of absorption of analytes onto a liquid fiber coating can be described as:[6]

$$n = \left(1 - e^{-a \cdot t}\right) \cdot C_0 \cdot \frac{K_{fs} \cdot V_s \cdot V_f}{K_{fs} \cdot V_f + V_s} = \left(1 - e^{-a \cdot t}\right) \cdot n_0 \qquad \textbf{(1.6)}$$

where t is the extraction time, n_0 is the amount of analyte extracted at equilibrium, and a is a time constant, representing how fast an equilibrium can be reached. When the extraction time is long, equation 1.6 becomes equation 1.4, characterizing equilibrium extraction. If the extraction equilibrium is not reached, equation 1.6 indicates that there is still a linear relationship between the amount (n) of analyte extracted onto the fiber and the analyte concentration (C_0) in the sample matrix, provided that the agitation, the extraction time, and the extraction temperature remain constant.

As equation 1.5 indicates, the efficiency of the extraction process is dependent on the distribution constant K_{fs}. This is a characteristic parameter that describes the properties of a coating and its selectivity toward the analyte versus other matrix components.

Because of its solvent-free nature and the small size of the fiber coating, SPME can be interfaced conveniently to analytical instruments of various types. Only extracted analytes are introduced into the instrument, since the extracting phase is non-volatile and insoluble in most organic solvents. Thus, there is no need for complex injectors designed to deal with large amounts of solvents, and these components can be simplified for use with SPME.[2,7] Depending on the method of subsequent analysis, the sensitivity of determinations using the SPME technique is very high, facilitating trace analysis.[8] Although in most cases the analytes are partly extracted from the sample, all extracted material is transferred to the analytical instrument, resulting in good performance. Carryover should be checked for each analyte and

the desorption conditions should be chosen so that the analyte remaining on the fiber is less than 1% of the initial amount. The solvent-free process results in narrow bands reaching the detector, giving taller, narrower peaks and better quantification.

SPME has been successfully coupled with gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) and has found many applications in different disciplines.[9] The approach has gained interest in the environmental, forensic, food, biological and pharmaceutical analysis fields.[3] There are many advantages of SPME over conventional sample preparation methods by integrating sample extraction, concentration, and introduction into the analytical system in a single device. This approach eliminates the excessive use of extraction solvents, and it is relatively simple.

Lately, this sample preparation method has begun to be involved in applications of non-volatile polar compounds from biological samples, and even for direct in-vivo extraction.[10] Usually, biological samples are complex heterogeneous mixtures. The heterogeneity of these samples frequently requires several sample preparation steps that are necessary to increase the analyte concentration and reduce the interference from other sample components.

Although SPME could reduce the solvent requirements for sample preparation, most of the commercially available fibers are not suitable for direct extraction from biofluids. Consequently, the applicability of SPME to biological samples is reduced. The preferred extraction mode for SPME in the case of biological samples is still headspace extraction. This procedure leads to minimal fiber fouling that usually results from protein adsorption during direct extraction. The extracts are clean and the fibers have a long lifespan. Unfortunately, most drug compounds are semi or non-volatile organic compounds. In these cases, headspace SPME is not suitable for drug extraction from body fluids.[3]

The most difficult and undesirable problem is the adsorption of proteins and other macromolecules on the surface of SPME fibers. These proteins and macromolecules constitute a diffusion barrier and decrease the extraction efficiency in subsequent extractions.

1.2 Current Methods for Separation of Drugs from Biological Samples

Drug monitoring is highly dependent on the development of new analytical instruments or techniques. The most widely used methods for separation of drugs from biological samples are: liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), ultrafiltration, dialysis, microdialysis (MD), supercritical fluid extraction (SFE), and affinity sorbent extraction (ASE).[11]

LLE is one of the first methods used for extraction. It depends on the partitioning of analytes between two immiscible liquids. The resulting extract may be directly analyzed or further purified and concentrated by subsequent LLE and evaporations. It is a simple, effective and efficient clean-up method, but it is labour intensive, time consuming, poses safety risks and it is difficult to automate.

SPE is a method for the isolation and concentration of selected analytes from a fluid sample by their transfer on a solid phase. The analytes are recovered by elution or thermal desorption. This method has high recovery, uses less organic solvent than LLE, does not produce foaming and it is easy to automate, but it is lengthy (it necessitates several stages: sorbent activation, removal of the activation solvent, application of sample, wash, desorption), expensive, and organic solvents are still required (for sorbent activation and desorption). LLE and SPE can be cumbersome and can cause harm to humans and environment.

SPME, a solvent free extraction method, consists of a single extraction step, but the experimental variables must be well controlled.

Ultrafiltration consists of filtering the sample through a special size-excluding filter, either by applying pressure (10 - 100 psi) or by centrifugation. The method is widely used, simple, efficient, but suffers from ligand binding to the filter and shift of equilibrium.

Dialysis and MD can be used to separate an analyte by diffusion through a semi-permeable membrane. They are efficient, widely used and have in vivo applications, but they are time consuming, face problems related to analyte binding to the membrane, can lead to sample dilution, and there is no analyte pre-concentration.

SFE combines liquid-like solvating capabilities with almost gas-like transport properties. The advantages of this method are a rapid extraction, selectivity, and an easy separation of analyte. However, there are some disadvantages: it needs high pressure and it is less effective for polar compounds.

The affinity sorbent may consist of an immobilized antibody or a molecularly imprinted polymer. This technique is highly specific and very sensitive, but the sorbent is difficult to prepare; it suffers from cross-reactivity and leaking of template.

1.3 Analysis of Biological Samples by SPME

Although SPME was initially applied only for the analysis of organic compounds from rather clean samples (air, water), it is now increasingly used in bioanalysis for determination of proteins, polar alkaloids, pharmaceuticals and surfactants, due to its successful coupling with LC (liquid chromatography) and CE (capillary electrophoresis). [12,13]

In vitro applications developed so far include analysis from serum, plasma, whole blood, milk, urine, saliva and hair, by headspace, direct immersion SPME and in-tube SPME. Headspace SPME consists of exposing the fiber to the headspace of gaseous, liquid or solid samples. The concentration of analyte in the headspace is generally increased by suitable sample pre-treatment steps. In direct immersion SPME, the fiber is directly immersed in liquid samples (Figure 1-2). pH adjustment is usually employed to improve partitioning of the drug between the sample and the fiber (the sample is acidified for acidic compounds and is made alkaline for basic analytes). In-tube SPME is an automated method; the analytes are directly extracted from the sample into the internally coated stationary phase of a capillary. All these applications make use of protein precipitation, pH adjustment, and salt addition as sample pre-treatment.[9,14] Unfortunately, none of these methods is applicable for direct in vivo extraction. The only SPME method applied so far for in vivo extractions consists of using polypyrrole for in vein extraction of benzodiazepines.[10]

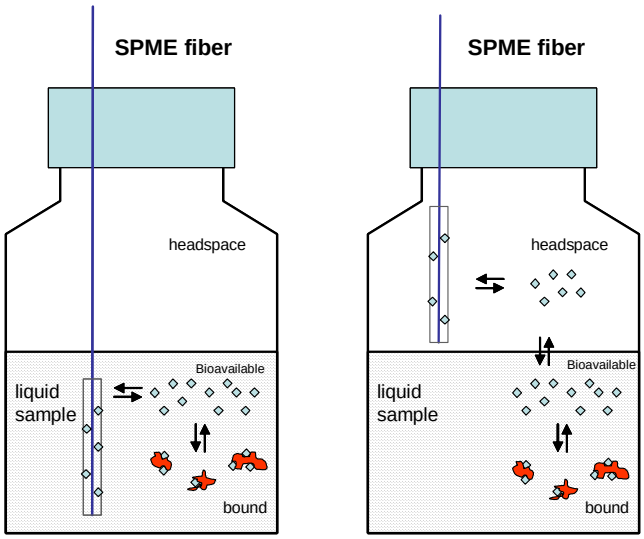


Figure 1-2. Schematic representation of the principle of direct immersion and headspace SPME.

Accordingly, in order to expand the applicability of SPME for in vivo and in vitro analysis, there is a critical need to develop new biocompatible SPME coatings.

1.4 Biocompatibility

Biocompatibility refers to non-rejection of biological tissue or artificial device, the compatibility of a donated organ or artificial device with the living tissue into which it is implanted or with which it is brought into contact. Incompatibility leads to toxic reactions or immunological rejection. Biocompatibility is a difficult concept to define in the absence of well-established clinical correlates. Besides anaphylactic reactions, which are reported more frequently, the idea of biocompatibility refers to any harmful effects induced by the contact of blood or tissue with xenobiotic materials.[15]

A material could be considered biocompatible if the sum of adverse humoral and cellular reactions occurring during exposure is lower than for a reference material. An extended definition of biocompatibility should also include factors related to the patient's clinical conditions, such as diabetes or systemic inflammatory disease, and to factors associated with the specific supportive technique. The creation of nonfouling surfaces is one of the major prerequisites for microdevices for biomedical and analytical applications.

As the concentration of therapeutic drugs is most often monitored in blood, the application of SPME for in vivo drug analysis requires good compatibility with blood. Hemocompatibility is an aspect of biocompatibility. In the absence of anticoagulants, all materials that come in contact with blood will induce the contact phase activation and subsequent reactions of blood clotting. The conversion of fibrinogen into insoluble fibrin is the

result of blood coagulation. The fibrin deposits on the surface of the exogenous material and forms a mesh of fibrils. In contact with fibrin, platelets are activated and aggregate, giving rise to a cooperative interaction that leads to blood clotting.

At the same time, blood cells are attracted by the thrombus, invade it and, through enzymatic release, contribute to further fibrin formation and platelet recruitment. These series of reactions and binding of clotting molecules onto the surface of the material contribute to the formation of a biological membrane that spreads over the material and is conventionally called "protein cake". This biological layer contains plasma proteins such as Factor XII, fibrinogen, high molecular weight kininogens and others whose activation results in further thrombogenesis, strong platelet aggregation and release of procoagulants like platelet Factor 4. Leukocytes are also involved in this process and by secreting proteases, lactoferrin and myeloperoxidase contribute to the proinflammatory effect. Hemocompatibility is also determined by the roughness of the polymer surface in direct contact with blood. It can be tested in vitro using different parameters related to the number of adhered platelets, time of the blood coagulation and haemolysis degree.

Biocompatibility is one general property that polymers that come in contact with living tissues and blood should possess. Polymers introduced into the living body should execute a definite function, not interfere (or even enhance) the functioning of a given organ, exerting neither local nor general toxicological action on the body.[16]

Another important property of biocompatible polymers is hemocompatibility. To create hemocompatible polymeric materials it is necessary to create a definite structure on the surface of the material, usually corresponding to the elements of the surface of the native

vessel. For example, introduction of a small amount of heparin leads to structural changes of polyurethane and promotes improvement of the parameters of the hemocompatibility.

It is of particular interest in the scientific world to develop and characterize membranes that can be well-controlled, stable and uniform. These membranes could help to separate viruses, proteins or peptides during biomolecular separation processes and the blocking of antibodies and complement molecules from encapsulated cells. In such applications if only one virus or antibody is able to penetrate the membrane, the whole system is compromised. Polymers such as polysulfones, polyacrylonitrile and polyamides are used to prepare membranes used for separation of sub-micron particles in biomedical applications. However, these membranes are not totally biocompatible. Some of the incompatibilities are

- Sensitization to sterilization
- Complement activation due to the reactivity of polymeric membranes
- Adhesion of various protein and immune components to membranes.

Several characteristics of polymeric membranes lead to thrombosis or systemic immunogenicity:

- Leaching of contaminants from polymers
- Interaction of blood proteins with reactive chemical groups on polymeric surfaces
- Mechanical instability of the membrane mediated primarily by shear stress.

In order to produce new biocompatible extraction systems, two main approaches can be used:

1. Preparation of biocompatible extraction materials
2. Protection of existing extraction phases, which may or may not be biocompatible, with a polymeric biocompatible membrane.

1.4.1 Biocompatible Extractive Materials

1.4.1.1 Restricted Access Materials

One class of promising biocompatible sample preparation material consists of restricted access materials (RAM), such as alkyl-diol-silica (ADS) and ion exchange diol silica (XDS). These materials consist of silica particles with a diameter of 5, 10 or 25 μm and with pores of about 3 nm in radius; the small pores yield a molecular mass cut-off of ~15 kDa that allows direct fractionation of a sample into the protein matrix and the analyte fraction. In addition to a defined pore size, the specific feature of diol silica particles is the topochemically bifunctional surface of the particles: the outer particle surface is modified with hydrophilic diol groups, whereas the inner pore surface is covered with hydrophobic alkyl chains and/or ion exchange groups.

RAMs have been applied mainly for the analysis of drugs in biological fluids. Examples include analysis of atenolol, propranolol, ibuprofen, naproxen, phenytoin, and carbamazepine by direct injection of plasma into an extraction column coupled to an HPLC system.[17,18] RAMs have also been successfully used as extraction phase in SPME for the determination of angiotensin 1 in whole blood and benzodiazepines in urine. [19,20]

A dynamic field of research is the development of synthetic receptor sites that are able to replace natural antibodies. In an ideal case, a synthetic receptor should have some qualities as: reusability, simplicity, affordability, high affinity and selectivity for the target and physical and chemical stability over a large range of experimental conditions.

These materials are useful in different areas, including stationary phase materials for HPLC, thin layer chromatography and capillary electrochromatography.[21] The fact that the

biocompatibility of MIP is not always optimal is the reason why they are sometimes used as the extraction phase of RAM.[3]

1.4.1.2 Ionic liquids

The term "ionic liquids" is used for a wide class of salts or salt mixtures which have appreciable liquid range and low melting temperature. They are composed entirely of ions and they melt below 100 ºC. These compounds are nonvolatile, and have no effective vapour pressure at room temperature.[22] Some of them (1-butyl-3-methylimidazolium hexafluorophosphate, 1-octyl-3-methylimidazolium hexafluorophosphate etc.) are compatible with HPLC, non-harmful to HPLC column, and they are suitable extraction solvents with adequate viscosity.[23]

They may be employed as very promising materials in creating new coatings, although there are not enough data about their toxicity, biocompatibility, biodegradability and other properties. They are known as green solvents but not all of them have this quality.[24] Their hydrophobicity varies significantly. Some scientists believe that the only common property of ionic liquids is that they melt below 100 ºC.[24]

1.4.1.3 Polydimethylsiloxane

Polydimethylsiloxane (PDMS) has been used in medicine for making devices such as implants, catheters, pacemaker encapsulants, and ocular lenses. More recently, the use of PDMS has been extended to the fabrication of microfluidic chips.

The application of PDMS has been driven by its good properties, which include flexibility and low toxicity. In spite of its multiple advantages, PDMS is a biomaterial that

presents serious surface instability characterized by hydrophobicity recovery.[25] Even when the surface is made hydrophilic, PDMS gradually reverts to the hydrophobic state due to surface rearrangements.

A lot of methods were used to improve the surface of PDMS. Modification procedures include exposure to energy sources such as plasma, corona discharge, and ultraviolet light, polyelectrolyte multilayers, radiation-induced graft polymerization, silanization, atom-transfer radical polymerization, chemical vapor deposition, cerium (IV) catalysis, phospholipids bilayer modification, and more recently, sol-gel modifications.

Most of these approaches do not solve the problem of hydrophobicity recovery via the silica-like layer or deformations since the hydrophilic groups are directly attached to the PDMS surface and thus exposed to the migrating groups from the bulk. A silica-like layer is produced on the PDMS surface during exposure to energy sources such as plasma or corona discharge; as a consequence, deformations could result because of the differences in elasticity between the brittle silica-like layer and the flexible PDMS bulk.

Cross-linking of polyelectrolyte multilayers by carbodiimide coupling and covalent attachment of poly (ethylene glycol) (PEG) chains to the polyelectrolyte produces stable, hydrophilic, protein-resistant coatings, which resist hydrophobicity recovery in air.[25]

1.4.1.4 Polypyrrole

Polypyrrole is a solid polymer with good adsorptive properties for moderately polar drugs. It is usually prepared by electrodeposition of pyrrole from aqueous solution. Polypyrrole is a well studied adsorbent, extensively used in biosensor technology. The

biocompatibility of the polymer is well known and this is one of the reasons for using it in many in vivo applications.[10]

1.4.1.5 Poly(ethylene glycol)

PEG is a linear or branched, neutral polyether available in a variety of molecular weights; it is a water soluble, nontoxic, and nonimmunogenic polymer with the unique ability of reducing nonspecific protein adsorption and cell adhesion and, therefore, is generally coupled with a wide variety of surfaces to improve their biocompatibility.

The performance of these modified surfaces for long-term biomedical applications largely depends on the stability of these PEG films. PEG is commonly used as a stationary phase in GC and LC. It is also used in some commercially available SPME coatings, CW/DVB for GC and CW/TPR for LC. Accordingly PEG can be used both as a membrane and an extractive phase.

PEG is one of the very few FDA approved polymers and hence of much interest in the biomedical communities due to its non-toxicity and ability to control biomolecular interactions with the surface of different devices. It has been used to prevent biofouling in bio-microsystems, tissue engineering applications, and drug delivery.

PEG is known to have unique properties in aqueous environments, acting much like a molecular windshield wiper. When a biomolecule such as a protein approaches the surface, the PEG chains which are in constant motion will wipe off the molecule from the surface, thus preventing biofouling.[26]

The protein-repelling behaviour of PEG is far from being simple to comprehend. It is a field in itself and an active area of research. Several theories have been proposed by physicists

and chemists, but none of them is adequate to explain its protein-resistant behaviour under all conditions. The unusual efficacy of PEG as an apparently biologically passivating surface film is linked both to the presumed biological inertness of the polymer backbone and also to its solvated configuration.

Despite many investigations, the mechanism of PEG's resistance to protein adsorption is still a mystery and will continue to attract the attention of researchers in the future. What is clear and well established is the proven ability of PEG to control biofouling.[27]

PEG – coupled surfaces can be created either by physical adsorption or by covalent immobilization such as grafting and chemical coupling. PEG thin films prepared by simple adsorption techniques are likely to elute off the surface due to weak forces of adhesion.

Theoretically, covalently coupled PEGs are considered to be more stable. Nevertheless, PEGs are susceptible to oxidative degradation and chain cleavage like other polyethers and many other hydrophilic polymers when exposed to aqueous environments. This may lead to loss of PEG film thickness and ultimately result in a loss of coating functionality.

Quite a few studies have addressed the stability of PEG in storage conditions as well as in extreme conditions of pH and temperature. Nevertheless, the issue of stability in in-vivo like environments remains rather unexplored.

As the maintenance of PEG film functionality is crucial for limiting biofouling, it is essential to develop insights into the causes and consequences of PEG film functionality over prolonged exposures to aqueous environments.[28]

1.4.2 Biocompatible Membranes

1.4.2.1 Polyacrylonitrile

Polyacrylonitrile (PAN) has been widely used as membrane material in the fields of dialysis and ultrafiltration. It has been found that its properties can be fine-tuned by using specific co-monomers; for example, PAN can be tailored with a reactive group for enzyme immobilization or with improved mechanical strength, solvent resistance and permeation flux, anti-fouling and biocompatibility, which give the PAN-based membranes great potential for the treatment of wastewater, the production of ultra-pure water, hemodialysis in artificial kidneys, and biocatalysis together with separation.[29]

PAN is one of the most important polymers used in the biomedical area because of its exceptional qualities, such as good thermal, chemical, and mechanical stability as well as biocompatibility. Membranes made of PAN are widely used as dialysers able to remove low to middle molecular weight proteins and as a high-flux dialysis therapy.[30] In terms of biocompatibility, PAN is one of the best polymers.[31]

1.4.2.2 Polyurethane

Some of the most biocompatible classes of polymers are polyurethanes (PU) and related compounds. Non-fouling PU polymers offer the advantage of being flexible because an appropriate choice of the isocyanate, diol and/or polyol monomers allows adjustment of the fine rheological properties of the final product. Polyurethanes may be the most prospective polymers in terms of hemocompatibility.

This characteristic can be further improved by chemically bonding heparin to the PU chain. As is well known, heparin is a highly active anticoagulant. Heparin was used to modify both the polymer surface and the bulk composition.

For this purpose, Lipatova et al. covalently bonded heparin to PU chains to realize a uniform distribution throughout the whole polymer volume or only at the surface. It was found that if one wants to produce polymers capable of biodegradation and replacement by the natural tissue, heparin should be bonded in bulk not only at the surface.[16]

Protein adhesion and cell attachment to PU may also be inhibited by partial esterification with phosphorylcholine; the resulting polymer mimics the thromboresistant surface of blood cell membranes and has low antigenic character.[32]

1.4.2.3 Chitosan

Chitosan is a weak cationic polysaccharide composed essentially of beta(1-4) linked glucosamine units together with some N-acetylglucosamine units. It is obtained by extensive deacetylation of chitin, a polysaccharide common in nature. Chitosan is a biocompatible, biodegradable, and nontoxic natural polymer that exhibits excellent film forming ability. As a result of its cationic character, chitosan is able to react with polyanions giving rise to polyelectrolyte complexes.

Therefore, because of these interesting properties, chitosan has become the subject of numerous scientific reports and patents on the preparation of membranes, microspheres, and microcapsules.[33]

1.4.2.4 Cellulose

A large variety of membrane materials are used at present and cellulose acetate is estimated to occupy about one-fourth of the total membrane market. Cellulose nitrate was invented in 1845 by Schönbein; in 1855, Fick made it into a membrane and used it in his study on dialysis. Cellulose nitrate solution, so called collodion, was used later for preparing membranes. Brown is the first one who prepared a membrane from cellulose acetate in 1910.

Cellulose acetate membranes have many advantages, including inertness to proteins (high recovery from a filtrate) and a high flux (fast diffusion of small molecules). According to Kesting cellulose acetate is a versatile membrane polymer. Its low cost, availability in a wide variety of viscosity grades, outstanding tractability, and reasonable resistance to oxidation, guarantee its continued utility for the foreseeable future. Cellulose acetate and regenerated cellulose have been successfully used as membranes for in-vivo microdialysis.[34]

Cellulose acetate is also important because of its capacity to adsorb specific leukocytes (granulocytes and monocytes) from blood, a quality that offers a promising remedy for autoimmune disease. Cellulosic materials can separate many enantiomeric pairs and this quality makes them appropriate to be applied not only to analysis but also to preparative separation. These materials have good durability and reasonable cost.[35]

1.4.3 Biocompatible Support Materials

SPME fibers for in-vivo applications should be robust, as thin as possible, unbreakable, highly flexible, and biocompatible. Few materials correspond to these stringent qualities; among them, stainless steel, Co-Cr alloys, and Ti-Al-V alloys are the most widely used. Ti and its alloys are among the most biocompatible metals but their wear resistance is relatively low.

Co-Cr alloys have better wear resistance but are less biocompatible. For the usual SPME applications, with in-vivo extraction times of maximum 30 min, all the above mentioned alloys are suitable. However, when highly flexible fiber cores are needed, Ti alloys should be used.

Body fluids contain approximately 1% NaCl that constitutes a corrosive environment for the metallic part of the SPME fibers. The interactions of the coatings with the body cells, the products of corrosion, and the metal ions released from the wear debris can have adverse effects on the body and on the coatings. These effects can include cellular damage, infections, blood coagulation (potentially leading to thrombosis) and failure of the probe.[36] Coating the fibers with biocompatible protective films, which can reduce corrosion and wear, may prevent or alleviate the problems described above.

1.5 Thesis Objective

This study has two main goals: to develop biocompatible (nonfouling) coatings for SPME, suitable for direct extraction from biological samples, and to use these new coatings for drug analysis and determination of drug plasma protein binding. Six test drugs with different physico-chemical properties are chosen for the investigation: diazepam, verapamil, lorazepam, warfarin, nordiazepam, and loperamide.

Analysis of biological samples with these fibers greatly reduces the amount of time required compared to using standard sample preparation techniques, and limits the exposure of the analytical personnel to infectious biofluids.

The current research presents the development of new biocompatible SPME coatings that are not fouled by protein adsorption during in vivo or in vitro extraction of biological samples. In the case of in vivo analysis, the new fibers eliminate the need to draw blood; for in vitro analysis, direct extraction of drugs from body fluids will be feasible, greatly reducing total analysis time. Furthermore, direct extraction from biological samples allows the investigation of supplementary parameters, such as binding to proteins.

Chapter 2 Development of Biocompatible SPME Fibers

A common problem of solid-phase microextraction coupled to liquid chromatography (SPME – LC) applications continues to be the unavailability of SPME coatings for polar and ionic analytes such as endogenous peptides, pharmaceutical drugs and their metabolites. Furthermore, there are few coatings that are suitable for direct in vivo extraction. In fact only coatings based on PPY were used so far for in vivo experiments.

2.1 Introduction

A useful strategy that can be used to overcome the problem of biofouling is to passivate conventional extraction phases by creating a thin biocompatible interface (or film) through the coupling of certain neutral and hydrophilic macromolecules, such as poly(hydroxyethyl methacrylate), polyacrylamide, poly(N, N-dimethyl acrylamide), dextran, polyacrylonitrile and poly(ethylene glycol). These protective layers repel proteins and allow extraction of small molecules of target analytes. The modified SPME fibers can be easily prepared by physical adsorption of the biocompatible polymer onto the surface of regular extraction phases. A different strategy consists of incorporating high capacity extraction phases into the bulk of a biocompatible polymer. These additional extraction phases may consist of C18-silica, CN-silica, HS-F5-silica, RP-amide-silica, or any other material used as stationary phase in HPLC.

This chapter presents the development of new biocompatible SPME coatings based on:

- Currently available coatings that are protected with thin biocompatible membranes (PAN)
- High capacity extraction phases incorporated in biocompatible polymers (PEG and PAN).

These new coatings are immobilized on biocompatible support materials such as stainless steel and titanium alloys. While stainless steel is suitable for most applications, titanium alloys offer advantages such as excellent biocompatibility, exquisite flexibility and shape memory. When non-biocompatible extraction phases are incorporated in a biocompatible one, care must be taken to completely cover the non-biocompatible material. When this is not feasible from a practical point of view, a solution is to cover the mixture with a new layer of biocompatible material. For example, when C18-silica particles are incorporated in PEG, some of them are partially exposed on the surface; the biocompatibility of such coatings can be improved by applying a new layer of PEG.

2.2 Materials and Reagents

Diazepam, nordiazepam, and lorazepam were purchased from Cerilliant. Polyacrylonitrile (PAN), verapamil, warfarin, loperamide, and phosphate buffer saline (pH = 7.4) were bought from Sigma (ON, Canada). Ammonium acetate was obtained from BDH Inc (Toronto, ON, Canada); N,N-Dimethylformamide (DMF) was supplied by Caledon Laboratories LTD (Georgetown, ON, Canada).

HPLC grade acetonitrile, acetone, and methanol were purchased from Fisher Scientific (Canada). Acetic acid and hydrochloric acid were bought from Fisher Scientific (Nepean, ON, Canada). Deionized water was obtained using a Barnstead/Thermodyne NANO-pure ultrapure water system (Dubuque, IA, USA). Human plasma (EDTA) was bought from Bioreclamation Inc (Hicksville, NY).

Stainless steel wires (127 μm and 254 μm diameter) were purchased from Small Parts Inc (Miami Lakes, FL); LiChrospher 60 XDS 25μm (SO_3/Diol) particles were supplied by

Merck KGaA (Darmstadt, Germany) as research samples. The C18-silica (5 μm), CN-silica, RP-amide-silica (5 μm), and HS-F5-silica (5 μm) particles were provided by Supelco (Bellefonte, PA, USA) as research samples. The PEG glue, synthesised according to a proprietary formula, was also supplied by Supelco.

2.3 SPME Fibers Based on PEG Glue

Biocompatible SPME fibers were prepared by coating stainless steel wires with PEG or a mixture of PEG and extractive particles (5 μm C18-silica or CN-silica). Stainless steel wires (0.005") are first etched by sanding the wires lengthwise in the area to be coated, with 400 grit silicon carbide sanding paper. Wires are then treated sequentially by:

- Sonication in acetone for 20 min
- Sonication in 6 M nitric acid for 20 min
- Sonication in water for 5 min
- Coating with a mixture of PEG and C18-silica, by passing the wires through a pipette tip containing the mixture
- Heating at 200 °C for 1 hour.

2.4 SPME Fibers Based on PAN

Compared to PEG, PAN has much better elasticity and mechanical stability. Accordingly, PAN can be used both for covering existing commercial fibers with a biocompatible layer, and for immobilizing extractive particles onto wires.

2.4.1 PAN as a Solid Matrix

An amount of 0.47 g particles (C18, RP-amide, HS-F5, used as HPLC stationary phases) were transformed into slurry with a 2 g solution (10%) of PAN in DMF. New SPME fibers were prepared by applying an uniform layer of slurry of PAN and different particles on the surface of stainless steel wires (254 μm or 127 μm diameter), allowed to dry under flowing nitrogen, and finally baked 1.5 min at 180 °C (in order to ensure better adherence of the PAN coating to the wire).

The wires were previously processed as follows: they were cut in 7.5 cm segments, washed (degreased) with acetone, etched for 1 min in concentrated hydrochloric acid, immediately washed with water, thoroughly cleaned by sonication (first in methanol and then in water), and finally dried at room temperature.

2.4.2 PAN as a Membrane

In order to obtain the coating, solutions of 2-10% PAN in dimethylformamide (DMF) were prepared. Existing fibers with conventional extraction phases were then coated with PAN by dipping them for 2 min in the solution of PAN. Consequently, the fibers were removed slowly from the solution, allowed to dry under flowing nitrogen, and finally cured by a short exposure (5 s) to a flow of nitrogen at 200 °C.

The thickness of the PAN protective layer was controlled by changing the concentration of the solution of PAN in DMF. The most stable coatings (for repeated use) were those obtained with a 10% solution of PAN in DMF.

2.5 Chemical Analysis

2.5.1 Standard Solutions and Sample Preparation

Stock solutions of the investigated drugs (diazepam, verapamil, warfarin, nordiazepam, loperamide, and lorazepam) with a concentration of 1 mM were prepared weekly in a water:methanol 1:1 mixture and kept refrigerated at 4°C.

Human plasma (with EDTA as anti-clotting agent) was stored at –20°C until analysis. For analysis, plasma was thawed at room temperature and aliquots of 1.5 mL plasma were transferred to clean vials. Appropriate amounts of spike were added to obtain final concentrations in the range 1 nM - 50 μM, followed by vortex mixing for 1 minute. Samples and standards in PBS (phosphate buffer saline) were prepared similarly, to a final concentration in the range 0.1 nM – 5 μM.

For extraction, the samples were placed on a digital vortex platform and the extracting phase of the SPME fiber was immersed in the sample for a precise period of time. The fiber was then briefly rinsed with water, and desorbed for analysis.

2.5.2 Apparatus and Analytical Conditions

2.5.2.1 LC/MS

LC-MS analyses were performed using an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum solvent degassing unit, a binary high pressure gradient pump, an autosampler, a column thermostat and a variable wavelength UV-VIS detector coupled on-line with an Agilent 1100 series MSD single

quadrupole instrument with atmospheric pressure electrospray-ionization. High purity nitrogen used as nebulizing and drying gas was obtained from an in-house generator.

Chromatographic separations were carried out on a Discovery® C18 column (5 cm x 2.1 mm, 5 μm particles, from Supelco), guarded by an on-line filter (0.2 μm). Data were collected and analyzed using the CHEMSTATION software from Agilent Technologies.

LC and ESI-MS conditions were as follows: column temperature 25 °C, mobile phase acetonitrile: 20 mM ammonium acetate pH = 7.0 with gradient programming (initial composition - 10:90, ramped to 80:20 over 6 min and maintained until the end of the run), flow rate 0.25 mL min^{-1}, nebulizer gas N_2 (35 psi), drying gas N_2 (13 L min^{-1}, 300 °C), capillary voltage 3500 V, fragmentor voltage 80 V, quadrupole temperature 100 °C, positive ionization mode. Total run time was 9 min.

For optimization experiments, scan mode in the range 100-1500 amu was used; for quantification experiments, selected ion monitoring is used, with a scan time of 0.42 s / cycle and a dwell time of 65 ms. The following positive ions were monitored: diazepam, m/z 285.1; verapamil, m/z 455.3; warfarin, m/z 309.1; nordiazepam, m/z 271.1; loperamide, m/z 477.3; lorazepam, m/z 321.0.

A sample chromatogram is presented in Figure 2-1. All other parameters of the mass-selective detector were automatically optimized using a calibration standard. Before each SPME experiment, the LC/MS system was calibrated with standard drug solutions. An example of such a calibration curve is given in Figure 2-2; for clarity, only diazepam, verapamil, warfarin and nordiazepam are shown.

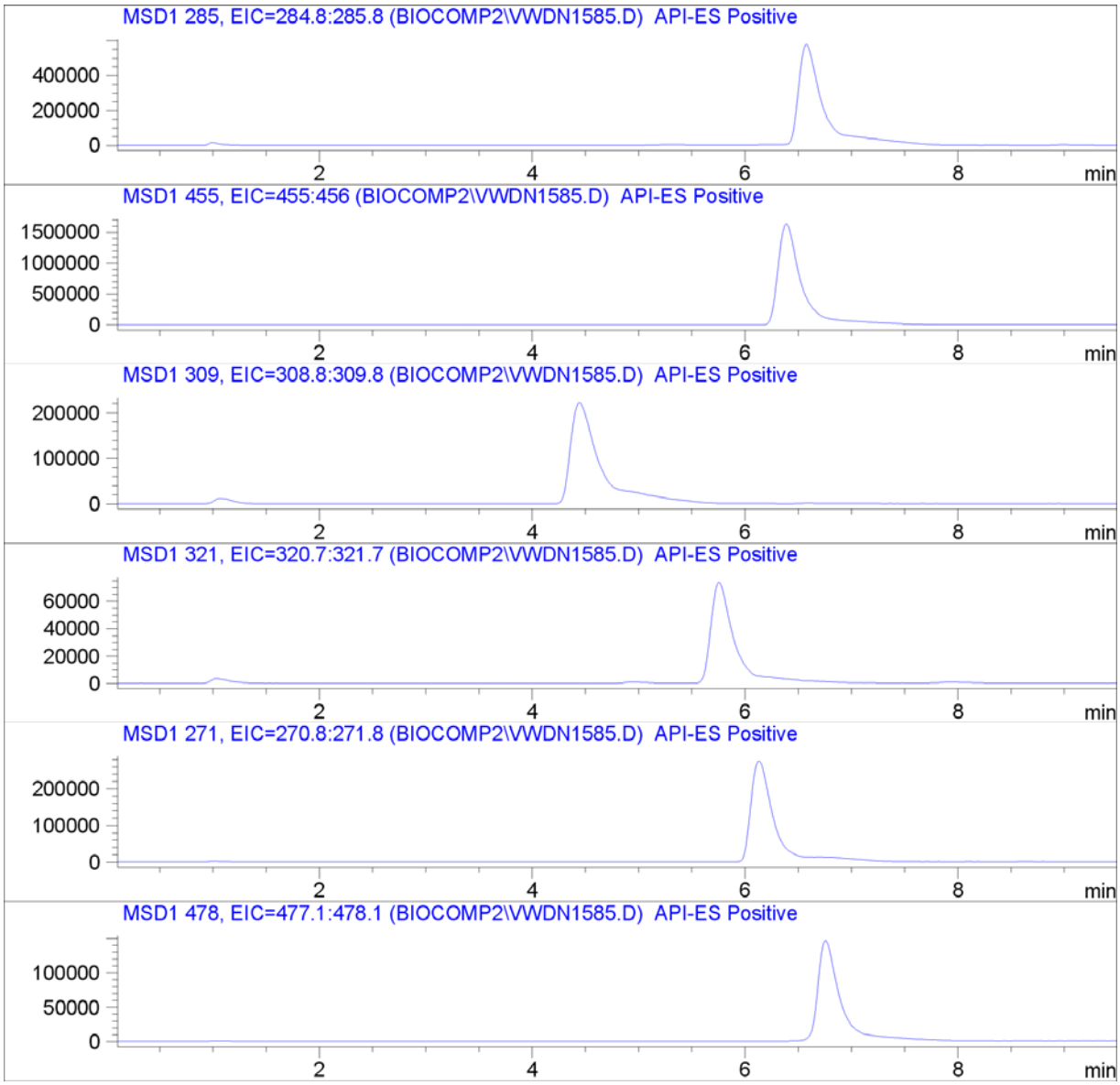


Figure 2-1. Sample chromatogram for diazepam, verapamil, warfarin, lorazepam, nordiazepam, and loperamide (this order, starting from the top).

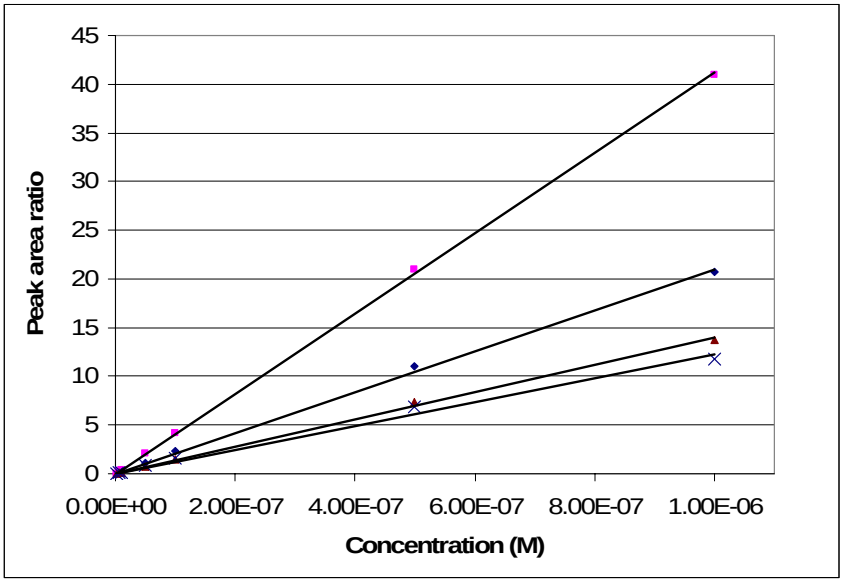


Figure 2-2. Instrument calibration curves for diazepam (◆, y=2.10E+07x, r^2=0.999), verapamil (■, y=4.12E+07x, r^2=0.999), warfarin (▲, y=1.39E+7x, r^2=0.999), and nordiazepam (x, y=1.22E+07x, r^2=0.993).

2.5.2.2 XPS

XPS analysis was performed by using a multi-technique ultra-high vacuum Imaging XPS Microprobe system (Thermo VG Scientific ESCALab 250) equipped with a hemispherical analyzer with a mean radius of 150 mm and a monochromatic Al-Kα (1486.60 eV) X-ray source. The spot size for the XPS analysis used for the present work was approximately 0.5 mm by 1.0 mm. The samples were mounted on a stainless steel sample holder with double-sided carbon tapes. The sample was stored in vacuum ($2x10^{-8}$ mbar) in the load-lock chamber of the Imaging XPS Microprobe system overnight to remove any remaining moisture before introduction into the analysis chamber maintained at $2x10^{-10}$ mbar. A combination of low energy electrons and ions was used for charge compensation on the non-conducting coating material during the analysis conducted at room temperature. Averages of five high resolution XPS scans were performed for each element (C, N, O, S). Curve fitting was performed using CasaXPS VAMAS Processing Software and the binding energies of

individual elements were identified with reference to the NIST X-Ray Photoelectron Spectroscopy Database.

All investigated fibers were exposed to undiluted human plasma at 37 ºC for 1h (this is considered a rigorous biocompatibility test).[25] They were then briefly washed with phosphate buffer and deionized water and dried in nitrogen before analysis. Survey scans and high resolution XPS scans were used to determine the atomic percentages of the surfaces before and after exposure to plasma. Figure 2-3 presents a survey spectra and a high resolution scan obtained for PAN/CW/TPR fibers after exposure to human plasma; for most coatings, sulphur was not detected.

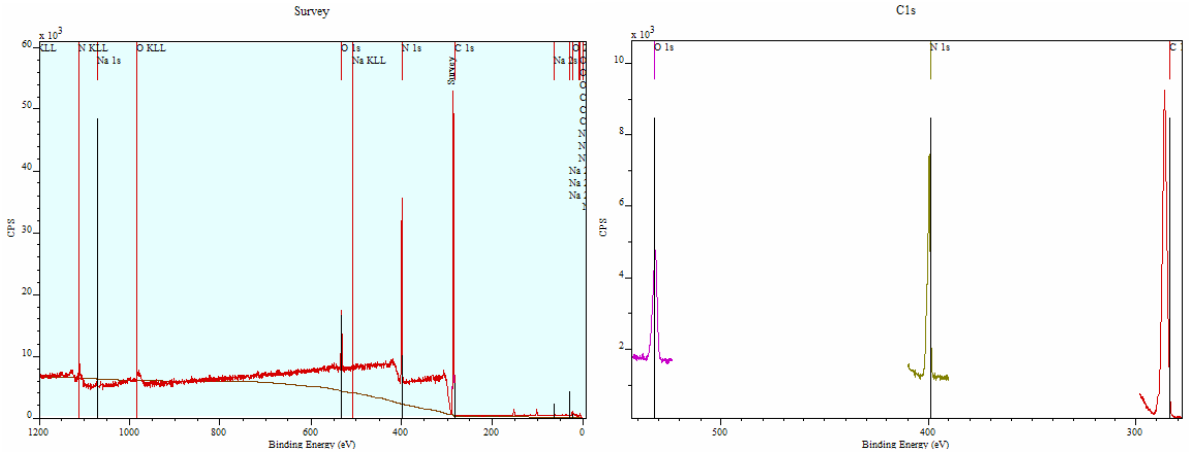


Figure 2-3. Survey spectra and high resolution (C1s, N1s, O1s) for PAN/CW/TPR fibers after exposure to undiluted human plasma.

2.6 Characterization of the New Coatings

Four absorptive phases, currently used for liquid chromatographic separations, were considered for the new SPME fibers: C18, CN, RP-amide and HS-F5. The platform of these particles consists of silica and they are available in different dimensions, pore sizes, and bonded phases.

Octadecyl-silica (C18) 5 μm particles contain octadecyl as the bonded phase. The bonded phase of CN-silica (CN) 5 μm particles is cyanopropyl. For RP-amide C16 5 μm, the bonded phase is palmitamido-propyl. All three types of particles (C18, CN, and RP-amide) are spherical, with 180 Å pore size, and 200 m^2/g surface area. HS-F5 5 μm particles contain pentafluorophenyl-propyl as bonded phase, the shape of the particles is spherical, the pore size is 120 Å, and the surface area is 300 m^2/g.

These particles were immobilized on the surface of stainless steel wires with either PEG glue or polyacrylonitrile. After polymerization, PEG turns into a very viscous liquid with rubber-like properties. On the other hand, because the PAN was prepared as a 10% solution in DMF, a highly porous solid PAN surface remained after solvent evaporation.

In order to verify the topography of the extractive particles within PEG or PAN at the surface of the fiber, optical microscope and scanning electron microscope (SEM) images were recorded. Selected SEM images of PAN/C18 coatings are shown in Figure 2-4, and optical microscope images of PEG and PEG/C18 coatings are presented in Figure 2-5.

For SEM imaging, the fibers were cut into 7 mm long pieces, coated with gold (~10 nm) and analyzed using a LEO 1530 Emission Scanning Electron Microscope at the Waterloo Watlab Facility.

SEM was also used to estimate the average thickness of each coating, which was found to be 16-30 μm for the 127 μm wires and 60-62 μm for the 254 μm wires. No swelling of the coating was observed during analysis time (extraction up to 2 h and desorption for 15 min).

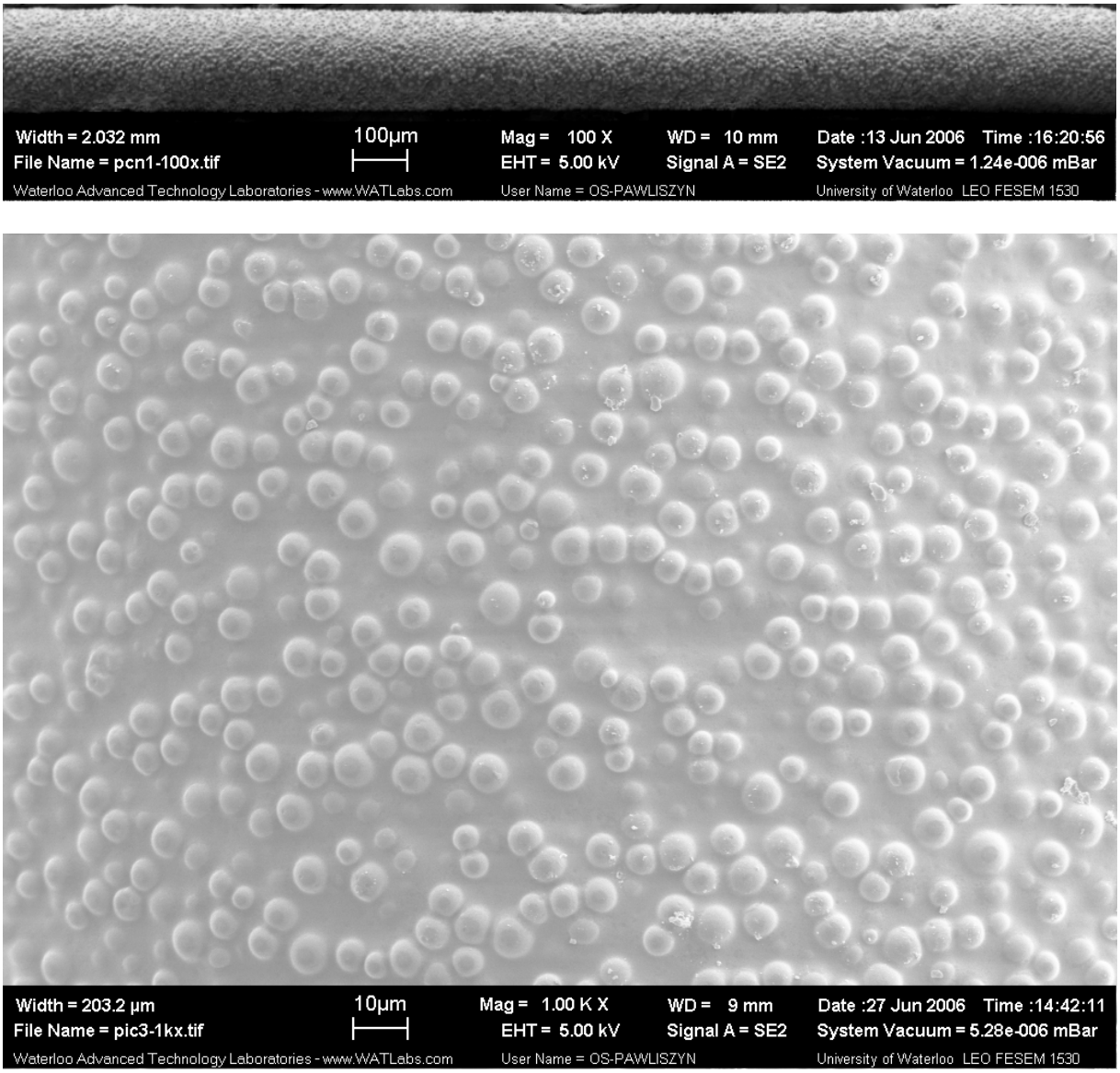


Figure 2-4. SEM images of PAN/C18 coatings, at 100x and 1000x magnification.

Biocompatible fibers were also prepared by protecting commercial CW/TPR fibers (Supelco, Bellefonte, PA) with PAN (Figure 2-6). The PAN/CW/TPR fibers have a stable-flex silica core and the coating has a thickness of 70 µm.

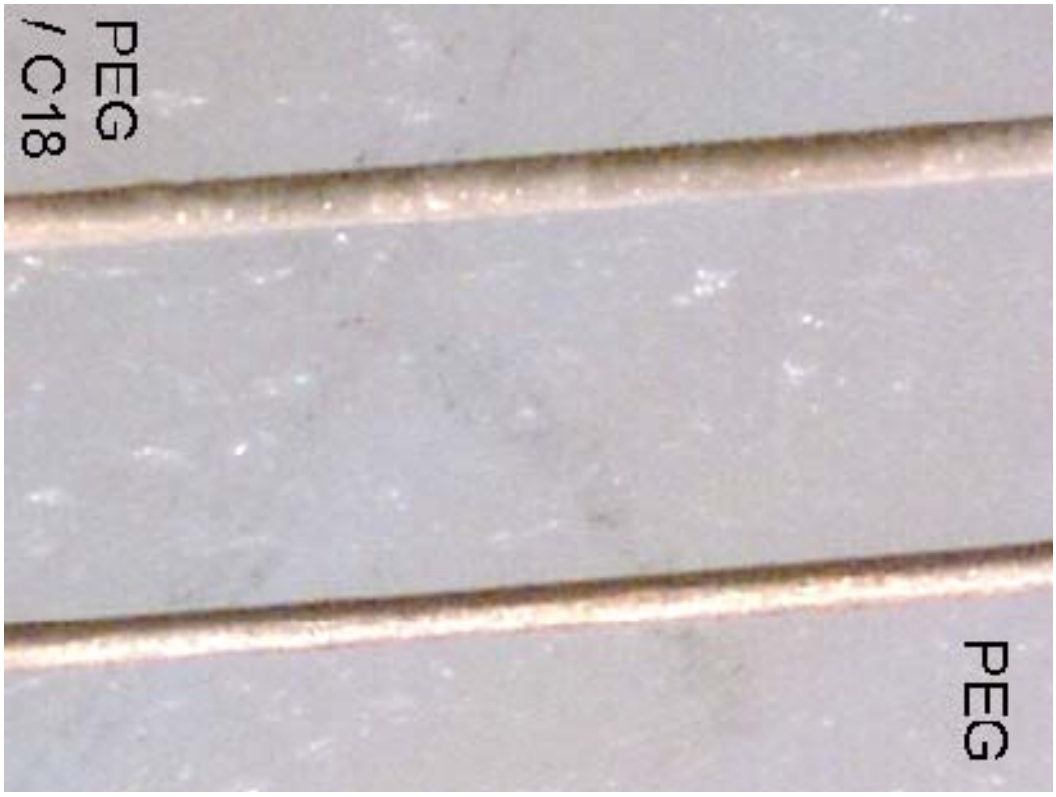


Figure 2-5. Optical microscope pictures of PEG/C18 and PEG fibers (60x magnification).

Figure 2-6. Fiber with PAN membrane (top) and original fiber before coating (bottom).

To characterize the newly developed coatings, their extraction performance toward the target analytes was investigated. The amount of analyte extracted with the new coatings was compared to that extracted with previously developed coatings (produced in-house or commercially available), under similar conditions. CW/TPR, PPY, PDMS, and RAM were chosen as reference materials in extraction efficiency and biocompatibility tests, because their biocompatibility is already accepted.[37,38,39]

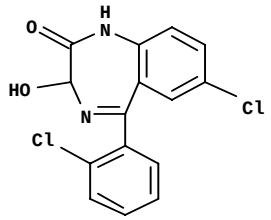
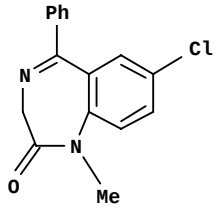
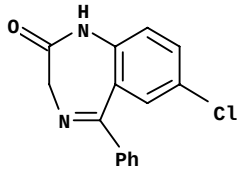
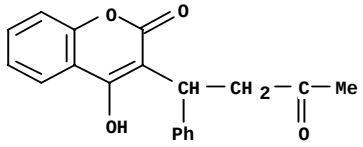
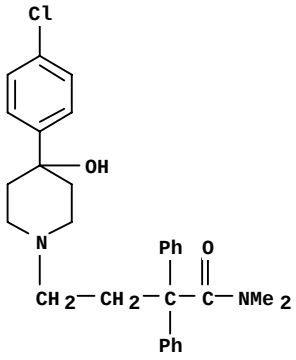
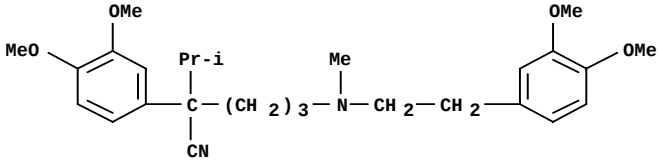
For the extraction efficiency test, the coatings based on C18, RP-amide, and HS-F5 showed on average a much higher extraction efficiency towards the test drugs: 90 times more than PPY, 50 times more than RAM or PDMS coatings, and 20 times more than commercially available CW/TPR. This result was indeed expected, as the particles that were used for the new coatings are widely known for their excellent properties as extraction phases in liquid chromatography.

2.6.1 Selection of Model Drugs

Six significantly different drugs (lorazepam, verapamil, diazepam, loperamide, nordiazepam, and warfarin - Table 2-1) were selected as target compounds for evaluating the performance of the newly developed coatings.

These drugs were chosen because their chemistry and pharmacological action are well known, they are widely used, and convenient to obtain. Lorazepam and diazepam were used alternatively as internal standard for compensation of variations in injection volume.

Table 2-1. Names, structures, molecular weights, and Log P_{ow} for target compounds.

Drug	Formula	Log P_{ow}
Lorazepam MW=321.1		2.5±0.5
Diazepam MW=284.7		3.0±0.5
Nordiazepam MW=270.7		3.2±0.4
Warfarin MW=308.3		3.5±0.5
Loperamide MW=477.0		4.3±0.5
Verapamil MW=454.6		4.9±0.4

As their Log P_{ow} suggests, these drugs have different polarities; the fact that they have significantly different polarities allows for systematic testing of the new coatings.

2.6.2 Optimum Desorption Time

Generally, the desorption procedure - removal of analytes from the SPME fiber for analysis - is very important and has to be investigated for every analyte, as one of the first steps in SPME method development. For the investigated drugs, several solvents and different stirring procedures were tested.

The sharpest chromatographic peaks and lowest carryover was obtained for a desorption time of 15 min, vortex stirring at 2400 rpm, and with a desorption solution prepared from acetonitrile:water:acetic acid (50:49:1). 60 μL desorption solution was used, containing lorazepam as internal standard (50 ng/mL), to compensate for variations in the autosampler injection volume.

Carryover, which is the ratio of the amount of analyte remaining on the fiber after the first desorption to the total amount of the analyte extracted, was examined and found to be well below 4% for all coatings based on PAN (with two exceptions, Table 2-2). PEG-based coatings generally exhibit lower carryover values (Table 2-3), but they also have lower extraction capacity.

The carryover was investigated by extraction from a volume of 1.5 mL PBS containing diazepam, verapamil, nordiazepam (all three at 5e-7M), warfarin (5e-6M) and loperamide (5e-8M). Much lower carryover values can be obtained by desorption in a larger volume of solvent.

For analysis, the desorption is usually followed by solvent evaporation and reconstitution in a lower volume of solvent suitable for direct HPLC analysis. Nevertheless, desorption in 60 μL solvent was found to be entirely suitable for the present study.

Table 2-2. Carryover for PAN-based coatings (after 15 min desorption).

Carryover %	*PAN/C18 0.005"*	*PAN/C18 0.01"*	*PAN/RP-amide*	*PAN/HS-F5*
Diazepam	0.7	1.4	0.9	1
Verapamil	1.3	2.2	2.9	9
Warfarin	0.2	0.6	0.7	0.7
Nordiazepam	0.5	1.1	0.8	1
Loperamide	2.4	3.9	2.9	13

Table 2-3. Carryover for PEG-based coatings (after 15 min desorption).

Carryover %	*PEG*	*PEG/CN*	*PEG/C18*	*PEG/C18/PEG*
Diazepam	nd	nd	nd	nd
Verapamil	0.7	0.4	0.4	4.5
Warfarin	nd	nd	0.9	1.4
Nordiazepam	nd	nd	-	-
Loperamide	4.8	3.2	-	-

nd = not detected

2.6.3 Equilibration Time

Whereas the concentration of the sample analyzed by SPME has no impact on the extraction time profile and equilibration time, the agitation conditions, coating thickness (especially for liquid coatings), distribution constant, and diffusion coefficient of the analyte play very important roles in determining the equilibration time.[40]

Sample temperature is important as well, since it has a great influence on the distribution constant and diffusion coefficient. The amount of analyte extracted in pre-equilibrium conditions is given by Equation 1.6.

The values of n_0 and a in Equation 1.6 can easily be determined from the experimental data (Figure 2-7 and Figure 2-8) by non-linear regression (or by linear regression, after taking the logarithm of both sides of the equation).

While the real equilibration time is infinite, the experimental value can be conveniently expressed as the time required to extract at least 95% of the maximum amount. In this case, the equilibration time can be calculated from Equation 1.6 as:

$$t_{95\%} = -\ln(0.05)/a \approx 3/a \qquad (1)$$

The time required to reach equilibrium at 2400 rpm vortex stirring was determined at room temperature for all target compounds by measuring the amount extracted at different time points and was found to be below 20 min for coatings deposited on 0.005" wires and between 4 and 133 min for coatings deposited on 0.01" wires (Table 2-4, Figure 2-7 and Figure 2-8).

To minimize the errors caused by different sampling times, the extraction time should be equal to or longer than the equilibration time. When the target drugs were analyzed in mixtures, an extraction time equal to the maximum value in the corresponding column of Table 2-4 was used.

Table 2-4. Equilibration times for all test compounds.

Equilibration time (min)	*PAN/C18 0.005"*	*PAN/C18 0.01"*	*PAN/RP-amide*	*PAN/HS-F5*
Diazepam	20	46	29	55
Verapamil	20	49	51	80
Warfarin	15	19	4	11
Nordiazepam	15	35	19	37
Loperamide	20	90	68	133

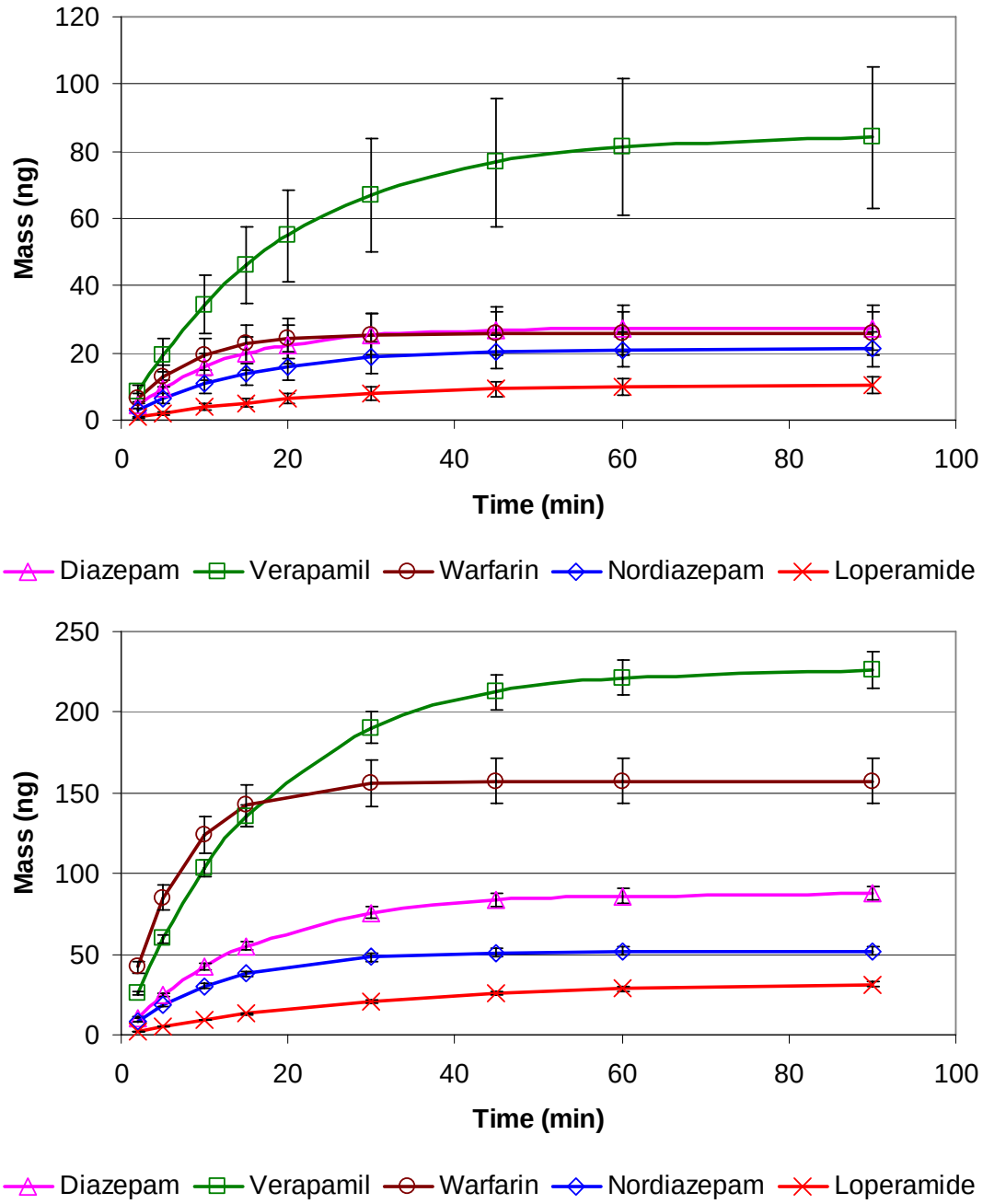


Figure 2-7. Extraction time profiles for diazepam, verapamil, warfarin, nordiazepam, and loperamide with PAN/C18 coatings on 0.005" (top) and 0.01" wires (bottom).

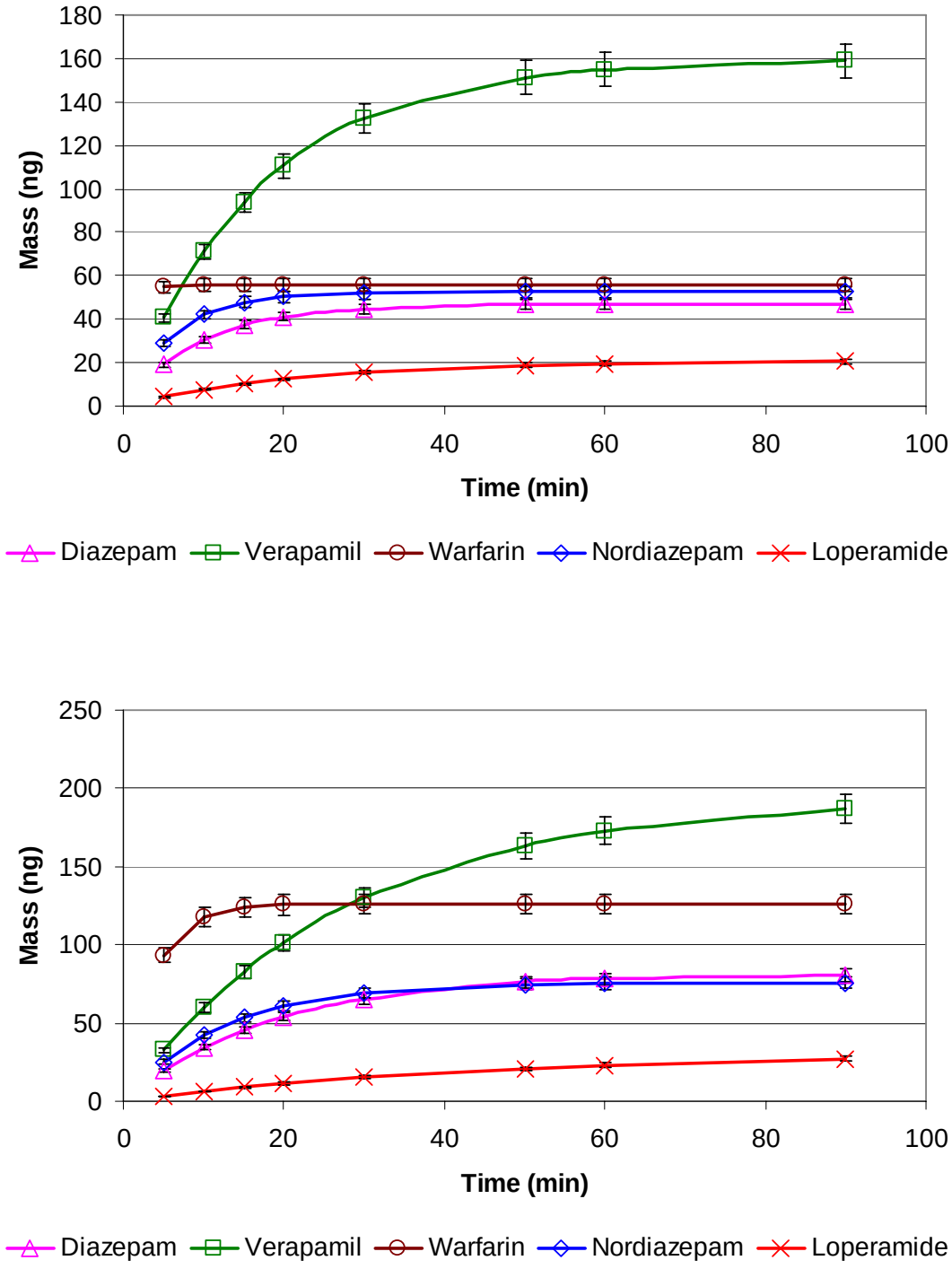


Figure 2-8. Extraction time profiles for diazepam, verapamil, warfarin, nordiazepam, and loperamide with PAN/RP-amide (top) and PAN/HS-F5 (bottom) coatings on 0.01" wires.

When existing commercial coatings are covered with a layer of PAN, the equilibration time remains essentially the same. Furthermore, the mechanic stability of the fibers coated with PAN is significantly improved: if original fibers can be used for 20 extractions before they break down, those coated with PAN last for more than 50 extractions. In addition to improved biocompatibility and durability, the resulting fibers offer almost the same extraction capacity as the initial ones (Figure 2-9).

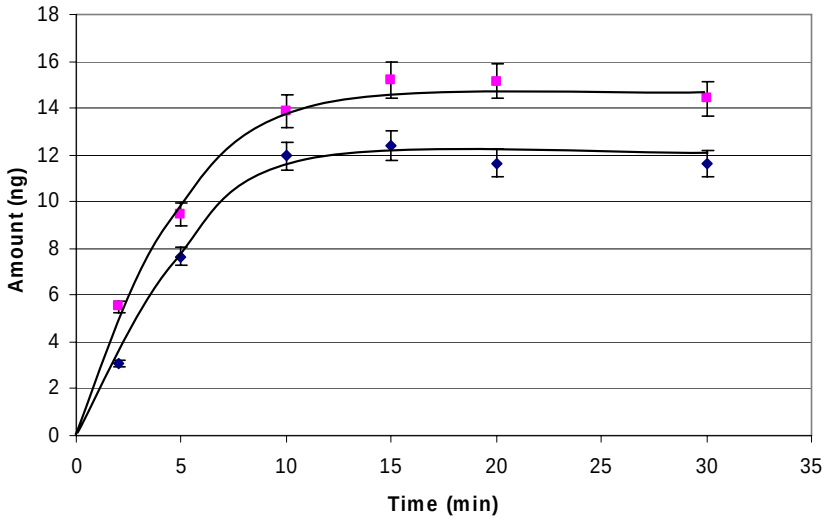


Figure 2-9. Comparative extraction time profile for verapamil, obtained with the commercial CW/TPR (■) fiber and PAN/CW/TPR (◆) fiber. Extractions were performed in phosphate buffer.

2.6.4 Reproducibility of Analytical Method

When a single fiber is repeatedly used for extraction, method precision can be expressed as "same fiber" reproducibility, which is important to determine especially for automated procedures, when a single fiber is used multiple times. On the other hand, when a new fiber is used for each determination, method precision is expressed as "fiber to fiber" reproducibility, which is important for on site sampling and in vivo determinations.

By using five different fibers of each kind for extraction and analysis in similar conditions, the fiber to fiber coating reproducibility was determined to be below 10% for coatings deposited on 0.01" wires and approximately 50% for 0.005" wires. Similarly, same fiber reproducibility was determined by using the same fiber for 5 times, and was found to be below 10% for coatings deposited on 0.01" wires (Table 2-5). The coatings deposited on thin wires had worse reproducibility because it was more difficult to produce a uniform coating.

It should be noted that in order to obtain good reproducibility for C18-based coatings, a conditioning step of at least 30 min in water:methanol 50:50 is required. Conditioning with water or higher proportion of methanol was found to lead to worse reproducibility. On the other hand, the other coatings in Table 2-5 require only a very brief conditioning step (less than 5 min), or even none at all.

Table 2-5. Reproducibility of SPME coating preparation, expressed as % RSD (range for the same fiber: intra-day and inter-day).

RSD % (n=5)	*PAN/C18 0.005"*		*PAN/C18 0.01"*		*PAN/RP-amide*		*PAN/HS-F5*	
	same fiber	fiber to fiber	same fiber	fiber to fiber	same fiber	fiber to fiber	same fiber	fiber to fiber
Diazepam	3-43	47	3-4	6	4-6	9	2-5	5
Verapamil	5-42	46	3-7	10	4-8	5	3-12	6
Warfarin	25-79	36	2-5	10	4-9	10	1-6	10
Nordiazepam	5-67	53	3-7	6	5-8	9	2-8	6
Loperamide	6-28	47	6-12	11	6-7	7	2-8	9

In the case of coatings based on PEG, the reproducibility is generally better than 10%, except for warfarin when it is below 20% (Table 2-6). It was noticed that the mixture of C18-silica and PEG produces a much more stable coating than PEG alone. The procedure is not limited to C18-silica: any particles that are useful as extraction phase in solid-phase extraction and solid-phase microextraction can be used.

Table 2-6. Reproducibility for the same fiber used several times and between different fibers (n = 7 for PEG/C18 and n = 5 for PEG, same day).

Drug / RSD%	Same fiber C18 (multiple use)	Same fiber PEG (multiple use)	Fiber to Fiber C18 (single use)	Fiber to Fiber PEG (single use)
Verapamil	3.4-7.5	7.7-12	3.9	5.5
Warfarin	9.1-16	3.1-10	20	11
Lorazepam	5.0-14	3.1-6.4	9.1	5.3

2.6.5 Calculation of the Distribution Constant

The target analyte's distribution constant (K_{fs}) defines the sensitivity of an analytical method based on SPME, gives more information about the experiment, and aids optimization. K_{fs} can be used to calculate the sample volume and coating thickness required to reach the desired sensitivity. For example, if the analytical instrument can detect only amounts larger than 3 ng, coatings based on PEG/C18 should be used (Figure 2-10). On the other hand, if sensitivity is not an issue, PEG coatings with shorter equilibration time should be used. The decision can easily be made based on Equation 1.4.

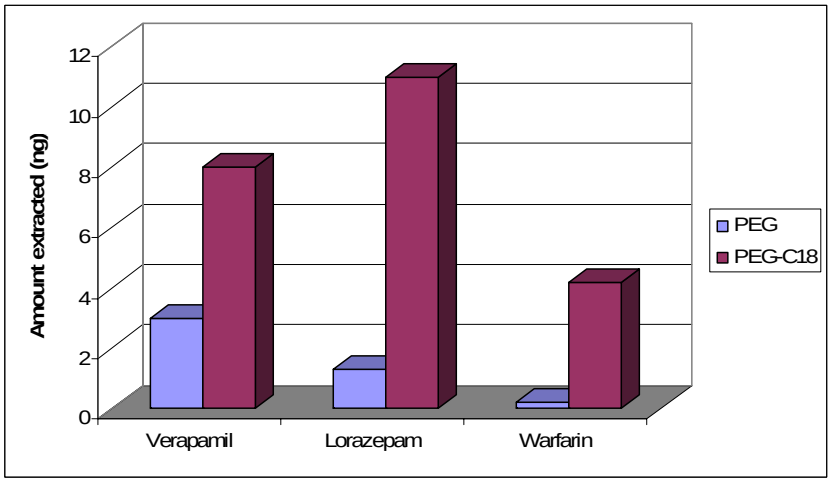


Figure 2-10. Comparison of amount of drug extracted by PEG and PEG/C18 fibers.

For the direct extraction mode, the distribution constant can be calculated from Equation 1.4:

$$K_{fs} = \frac{nV_s}{V_f(C_0V_s - n)} \quad \textbf{(2.1)}$$

For new coatings, the volume of the fiber can be determined from the coating length (b), coating thickness (d – obtained from SEM images), and radius of the support wire (r):

$$V_f = \pi b[(r+d)^2 - r^2] \quad \textbf{(2.2)}$$

Equations 2.1 and 2.2 were used to calculate the distribution constant for five target compounds in the case of PAN-based coatings (Table 2-7) and for three compounds in the case of PEG-based coatings (Table 2-8). Generally, PAN/C18 fibers have the highest distribution constant, but they need careful conditioning before extraction from samples. Even if the distribution constants for PAN/RP-amide and PAN/HS-F5 coatings are a little lower, they could be more convenient as they don't require activation prior to extraction. The less polar extraction phases showed a higher distribution constant for less polar analytes.

Table 2-7. Distribution constant values for coatings based on PAN (support wire diameter: 0.01", coating thickness: 60 µm).

K_{fs}	*PAN/C18 0.01", 60µm*	*PAN/RP-amide 0.01", 60µm*	*PAN/HS-F5 0.01", 60µm*
Diazepam	1.14E+03	4.57E+02	9.96E+02
Verapamil	3.25E+03	1.43E+03	2.13E+03
Warfarin	1.19E+02	4.02E+01	9.37E+01
Nordiazepam	5.60E+02	5.66E+02	9.70E+02
Loperamide	1.92E+04	2.34E+03	1.14E+04

Table 2-8. Distribution constant values for coatings based on PEG (support wire diameter: 0.005", coating thickness: 16 or 30 µm).

K_{fs}	*PEG 0.005", 16µm*	*PEG/C18 0.005", 30µm*	*PEG/CN 0.005", 30µm*
Verapamil	1.23E+2	1.62E+2	1.83E+2
Warfarin	1.20E+0	1.23E+1	1.02E+0
Lorazepam	7.55E+1	3.24E+2	3.67E+1

2.6.6 Reusability

Reusability is of little importance for in vivo applications when a single fiber is used for each determination. However, in the case of in vitro extractions, manually or automatically operated, reusability is very important in reducing the overall analysis cost; furthermore, fiber reusability may lead to better method reproducibility.

In order to investigate the reusability of the newly developed coatings, five new SPME fibers were used for repeated extractions from drug solutions in PBS (Figure 2-11) or human plasma (

Figure 2-12). Although sample-to-sample RSD was larger in the case of plasma samples, the biocompatible SPME coatings proved to be reusable for at least 10 times.

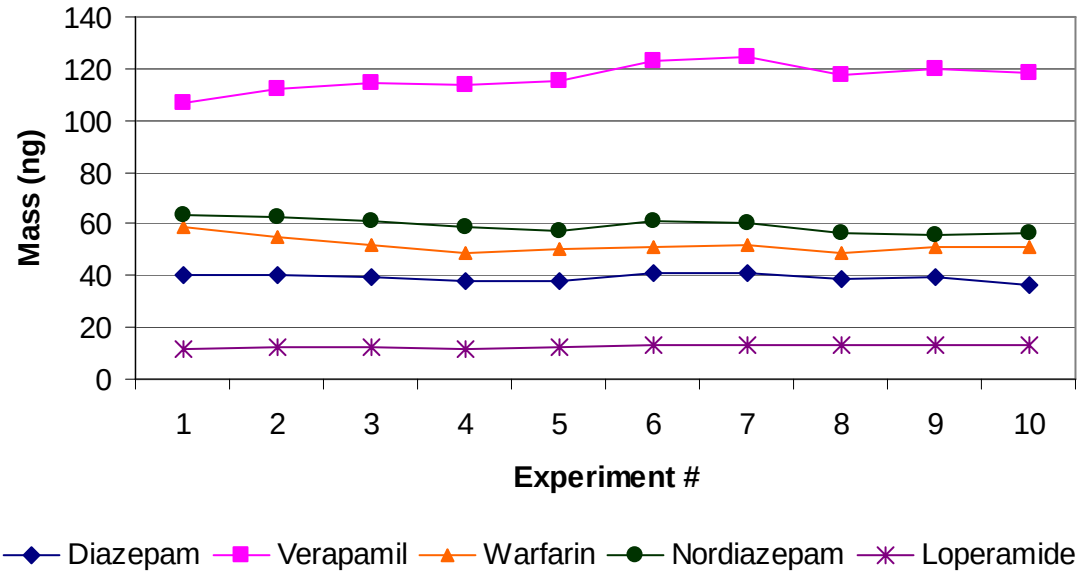


Figure 2-11. Reusability of PAN/RP-amide fibers for extractions from buffer solutions.

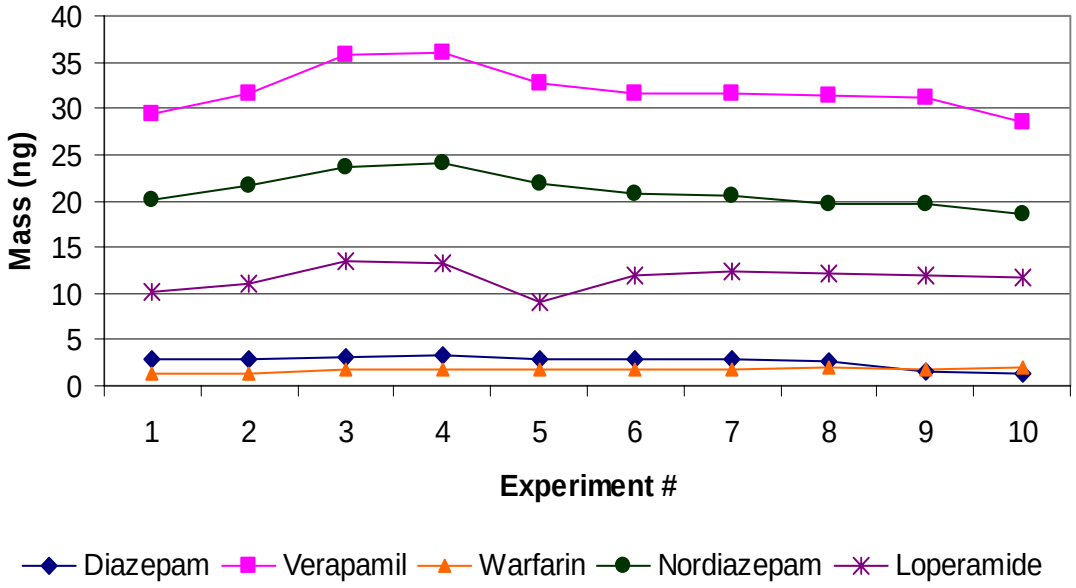


Figure 2-12. Reusability of PAN/RP-amide fibers for extractions from plasma samples.

2.6.7 Sterilization

Sterilization is necessary only if the new fibers are to be used for in vivo experiments. In this case, the coatings should be tested for endurance during sterilization. Current sterilization methods include heat, steam, chemical (ethylene oxide, alcohols, aldehydes), and radiation. The most preferable methods would be sterilization with steam or radiation. Radiation sterilization, although highly effective, poses a risk to human health and is not readily accessible. As a result, steam sterilization is most widely used. The new fibers were tested for extraction efficiency and biocompatibility before and after sterilization.

No change in extraction efficiency was observed upon sterilization with alcohols (methanol or ethanol), as this step is similar to the pre-conditioning step. In the case of

sterilization in an autoclave at 121°C for 30 minutes, most PEG-based fibers developed cracks and became mechanically unstable. However, the coatings based on PAN showed no sign of deterioration upon steam sterilization; this was expected, since PAN coatings are known to withstand GC-injector temperatures (> 250 °C). Although no signs of breakdown were observed, the extraction capacity decreased by 15% after sterilization, probably because of the combined effect of heat and water vapours on the fused silica particles.

2.6.8 Biocompatibility Test

Many methods have been applied for the study of biocompatibility, ranging from the simple visual inspection to the most sensitive atomic force microscopes. Nevertheless, only a few methods are widely used: X-ray photoelectron spectroscopy (XPS)[26], atomic force microscopy,[28,41] surface plasmon resonance,[42] and competitive ELISA.

XPS or electron spectroscopy for chemical analysis (ESCA) is one of the most common types of spectroscopic methods for analysis of surfaces. Briefly, the way in which XPS is used to examine a surface is the following: the surface of the solid sample is irradiated with a "primary beam", monochromatic X-radiation made up of X-Ray photons; the impact of this beam on the surface of the sample causes the formation of a "secondary beam" consisting of electrons. The emitted electrons produced by the incident beam are studied and give information about the surface of the sample. The spectrometric measurements consist of the determination of the power of the secondary beam as a function of the energy (or frequency) of the electrons.[43] XPS provides qualitative and quantitative information about the elemental composition of matter, particularly of solid surfaces, and also structural information. A survey

spectrum is a low-resolution, wide-scan XPS spectrum that serves as the basis for the determination of the elemental composition of samples. Subsequently, a high resolution spectra is recorded for the elements to be investigated.

In order to test the biocompatibility of the new coatings proposed by this research, X-Ray photoelectron spectroscopy was used as a powerful technique for determining the surface characteristics of those coatings. The sampling depth for this method is approximately 1-30 nm, which encompasses a surface region highly relevant for bio-interactions.[44]

Table 2-9. Atomic composition obtained by XPS for selected proteins and coatings (before and after exposure to human plasma); hydrogen is not considered, as it is not detectable by XPS.

Protein / Coating	C%	N%	O%	S%
Human serum albumin	*63.3*	*16.9*	*19.0*	*0.9*
Fibrinogen	*62.8*	*18.0*	*18.8*	*0.5*
PAN (bp*)	78.2	17.6	4.0	0.0
PAN (ap**)	73.5	15.1	11.3	0.0
PAN/C18 (bp*)	77.0	17.2	5.7	0.0
PAN/C18 (ap**)	73.6	13.9	12.5	0.04
PAN/RP-amide (bp*)	78.3	17.0	4.5	0.0
PAN/RP-amide (ap**)	68.6	15.5	15.8	0.0
PAN/HS-F5 (bp*)	79.3	20.1	0.4	0.0
PAN/HS-F5 (ap**)	70.9	15.2	13.7	0.0
PEG/C18/PEG (bp*)	43.0	0.15	56.8	0.0
PEG/C18/PEG (ap**)	54.9	6.1	39.0	0.0
PEG/CN (bp*)	40.0	0.6	59.3	0.0
PEG/CN (ap**)	53.5	6.4	40.1	0.0
PEG/C18/PAN (bp*)	78.5	18.3	3.2	0.0
PEG/C18/PAN (ap**)	73.3	14.8	11.8	0.0
PEG (bp*)	43.7	0.0	56.2	0.0
PEG (ap**)	60.5	7.0	32.5	0.0
PAN/RAM (bp*)	78.9	20.4	0.5	0.0
PAN/RAM (ap**)	72.6	16.3	10.4	0.5
PPY (bp*)	61.0	3.8	35.2	0.0
PPY (ap**)	69.7	12.4	17.6	0.3

* bp = before exposure to human plasma
** ap = after exposure to human plasma

After exposure of PAN-based coatings to plasma, the amount of nitrogen and carbon on the surface generally decreases, accompanied by an increase in the amount of oxygen (Table 2-9). These observations suggest that most of the molecules adsorbed from human plasma contain a high percent of oxygen, while their nitrogen content is lower than that of plasma proteins. Even more conclusive from a biocompatibility point of view is the amount of sulphur on the surface, since it is naturally present in proteins and absent from the investigated SPME coatings. When compared to RAM and PPY, materials that are regarded as highly biocompatible, [38,39] the new coatings based on PAN showed a much lower increase in sulphur.

In the case of PEG-based coatings, exposure to plasma leads to approximately 6% increase in surface nitrogen, an amount that is acceptable even for long-term implants. Furthermore, no sulphur was detected at the surface of these coatings, suggesting a good biocompatibility.

The biocompatibility test based on XPS suggests that the most biocompatible PAN-based coatings are PAN/RPamide and PAN/HSF5, followed closely by PAN/C18. Furthermore, the newly developed PAN-based coatings were inspected under the microscope after exposure to human plasma and whole mouse blood (without anti-clotting agents), and no clot formation was observed.

Chapter 3 Applications of Biocompatible SPME

3.1 Development of SPME methods for model drugs

The general steps in developing an SPME method are: selection of fiber coating, selection of sampling mode, selection of separation and/or detection method, determination of extraction time profile, selection of extraction time, optimization of matrix conditions, determination of desorption time, determination of the linear range, determination of method precision, selection of the calibration method, and determination of the limit of detection.

As the fibers to be developed should be biocompatible, coatings based on PEG and PAN were selected. The preferred sampling mode for in vivo and in vitro extractions of drugs is direct immersion. This extraction mode is suitable for compounds with high to medium polarity, which is the case for the drugs selected in this study.

Of all the separation methods currently used in conjunction with SPME, liquid chromatography is the method of choice for separation and analysis of verapamil, diazepam, warfarin, nordiazepam, loperamide, and lorazepam. These drugs are usually present in low concentrations in body fluids and therefore, a sensitive detection method is required, such as mass spectrometry. Accordingly, the analytes extracted with SPME fibers will be separated and quantified by liquid chromatography coupled to mass spectrometry (LC-MS).

Successful coupling of SPME with HPLC is dependent on the efficiency of the desorption step. Currently, there are two choices for desorption: on-line (manual introduction of the fiber into a desorption chamber) or off-line (in a vial or 96-well plate). Manual introduction usually gives good reproducibility and almost 100% injection of analyte, but is more difficult to use and automate, as a special interface is required. Off-line desorption offers more flexibility (choice of desorption solvent) and ease of automation, but offers less sensitivity, as not all the analyte is injected. Desorption solutions should be chosen so that a good compromise between desorption efficiency and peak shape is obtained. Good desorption efficiency is usually obtained with a solution that has high content of organic solvent, but it may lead to unacceptably broad peaks. As a compromise, desorption solutions with 50-75% methanol or acetonitrile are found to be optimal.

Desorption is influenced by the amount of time the fiber is in contact with the solvent; accordingly, the optimum time should be determined. The efficiency of the desorption is determined experimentally by measuring the carryover, and can be improved by adjusting the pH of the desorption solution towards higher values for acid analytes and towards lower ones for basic analytes. All these methods for improvement of desorption efficiency will be used when testing the coatings under development.

Once the SPME fiber is exposed to the sample, the analytes start to accumulate on it; the amount of analyte on the fiber increases until equilibrium is reached. Although there is no need to wait for the equilibrium to be reached, analyses performed at equilibrium are more reproducible and more sensitive.[1] Accordingly, the extraction time profile should be determined experimentally by measuring the amount of analyte extracted by a fiber as a

function of time. When equilibrium is reached, the total amount extracted remains constant and this point should be used as the extraction time for further experiments.

For in vivo experiments, this extraction time should be as short as possible, at least on the order of minutes. The time necessary to reach equilibrium can be decreased by using thin coatings or coatings with lower affinity for the analyte. Agitation conditions have a considerable influence on the equilibration time as well. For in vivo extraction from flowing blood, equilibration time will be related to the linear flow rate of the blood around the fiber.

In vitro experiments are currently performed in Dr. Pawliszyn's laboratory to determine the influence of blood flow on equilibration time and amount of analyte extracted at equilibrium. In the case of in vivo extraction from tissues the equilibration time will depend on the presence of cellular membranes and extra cellular fluids.

The next step in method development is the optimization of matrix conditions. For in vivo studies this step is not very flexible. The matrix conditions in blood cannot be modified without significantly changing the system, so the coating should be chosen in such a way that it provides good extraction in physiological conditions.

The linear range of newly developed coatings is determined by extraction from solutions with a wide range of concentrations. The limit of detection is usually determined by the sensitivity of the quantification instrument whereas the upper values are determined by both the instrument and the capacity of the fiber.

The linear range is usually larger for liquid-type coatings, smaller for solid coatings, and should cover the in vivo concentration range of the analyte. When the linear range for the assay of drugs in blood is determined, care must be taken to consider drug binding to plasma

proteins, because SPME detects only the free concentration; all the determinations should be performed with the matrix to be investigated.

The limit of detection is most often calculated from the signal to noise ratio for samples containing a concentration within a factor of ten of the estimated limit of detection. The concentration that generates a signal to noise ratio of three is considered to be the limit of detection.

Current calibration methods in SPME include: application of a known distribution constant, external calibration curve, standard addition, isotopic dilution, and the newly developed standard on the fiber approach.

The most suitable methods for in vivo experiments are the external calibration curve that is simple, fast and inexpensive, and the standard on the fiber method, when an isotopically labelled drug is loaded on the fiber before the in vivo extraction. In this case the fiber is exposed to the sample for a short period of time, without reaching equilibrium. The concentration of the sample is determined by measuring the amount of standard remaining on the fiber and the amount of analyte extracted.

Method precision is determined by successively analyzing identical samples during the same day and in different days. It is generally expressed as relative standard deviation (RSD). For the analysis of biological samples, that are very complex, RSDs are usually in the range 10 – 30%. Nevertheless, careful consideration of experimental parameters can help obtain RSDs as low as 15% for SPME methods.

3.2 Fast Drug Analysis – In Vivo and In Vitro

Although most biological samples are currently analyzed *in vitro*, many attempts are now directed towards *in vivo* analysis. It has been shown that the composition of the volatile extracts collected from detached or damaged plants can differ significantly from the mixture emitted by the live, undamaged specimen.[45] *In vivo* research is more suited to observe an overall effect than *in vitro* research, which is better suited to deduce molecular mechanisms of action. *In vitro* research aims to describe and understand the effect of an experimental variable on a subset of an organism's components. *In vitro* research has the advantage that there are fewer variables which can confound an experiment and the results are clearly visible. *In vivo* research has the advantage that the experimental system is a more complex biological system, and gives a better indication of what will happen in the real world.

3.2.1 In Vitro Drug Analysis

The advantages of the proposed SPME coatings were investigated by studying the extraction and separation from human plasma of several test analytes (described in section 2.6.1). Figure 2-1 demonstrates that this approach is feasible.

For in vitro sampling, drug analysis was performed as described in section 2.5.2.1. Calibration curves were constructed by spiking PBS and human plasma with drug concentrations in the range of 0.5 nM – 50 μM, which generally covers the therapeutic concentrations.

As shown in two examples in Figure 3-1 and Figure 3-2, a good linear relationship was obtained for this seven points calibration (n = 3).

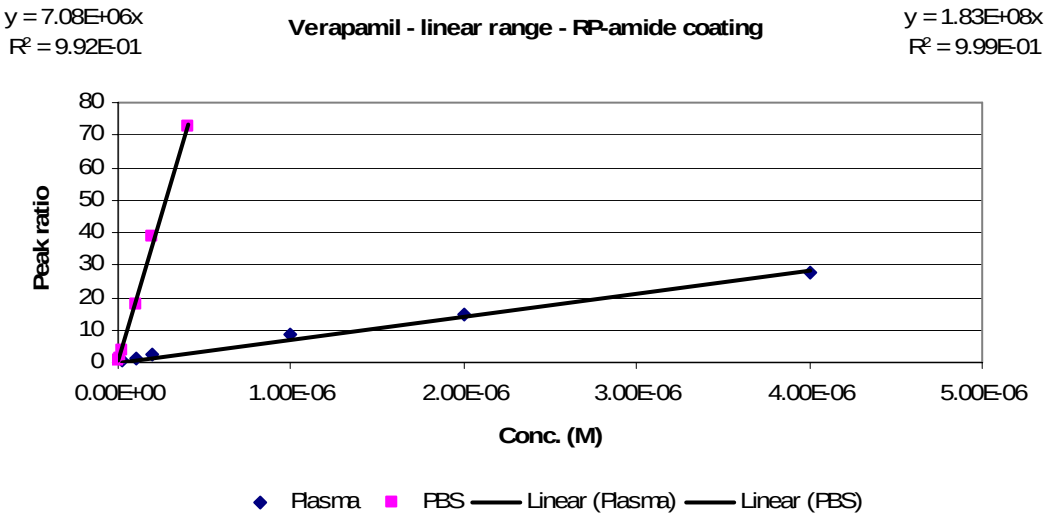


Figure 3-1. Calibration curve for verapamil in PBS and human plasma (with SPME).

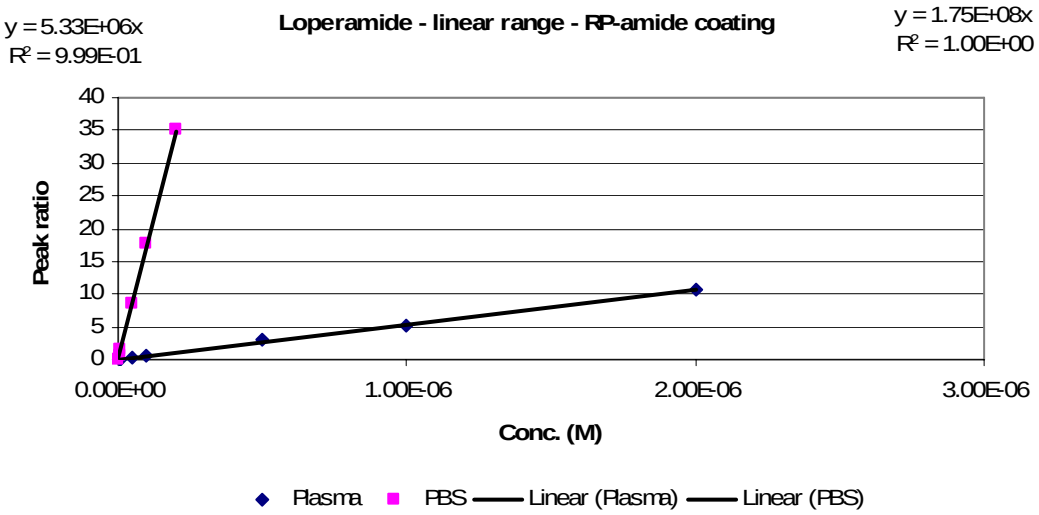


Figure 3-2. Calibration curve for loperamide in PBS and human plasma (with SPME).

The linear range covered more than three orders of magnitude for most drugs, with the exception of warfarin when the linear range spanned over two orders of magnitude. The full details are included in Table 3-1.

Table 3-1. Linear ranges for SPME-based analytical method.

Linear Range (moles/L)	*PAN/C18, 0.01"*		*PAN/RP-amide, 0.01"*	
	PBS	Plasma	PBS	Plasma
Diazepam	1e-9 –> 2e-6	1e-8 –> 1e-5	3e-9 –> 1e-6	5e-8 –> 1e-5
Verapamil	1e-9 –> 1e-6	5e-9 –> 5e-6	2e-9 –> 4e-7	2e-8 –> 4e-6
Warfarin	2e-8 –> 5e-6	2e-7 –> 5e-5	2e-8 –> 4e-6	1e-6 –> 4e-5
Nordiazepam	1e-8 –> 5e-6	1e-7 –> 2e-5	7e-9 –> 2e-6	2e-7 –> 2e-5
Loperamide	1e-9 –> 2e-7	5e-9 –> 2e-6	2e-9 –> 2e-7	2e-8 –> 2e-6

3.2.2 In Vivo Drug Analysis

In recent years there has been considerable interest in developing techniques to monitor levels of biologically active compounds in living systems in natural environments. *In vivo* sampling can eliminate errors and reduce the time associated with sample transport and storage, and can therefore result in collecting more accurate and precise analytical data.[46] An ideal *in vivo* sampling technique should be portable, solvent-free and offer integration of the sampling, sample preparation and analysis step. Reliable and accurate analytical methods are indispensable for *in vivo* research. On the other hand, the development of techniques appropriate for *in vivo* analysis poses significant difficulties, due to the low and unceasingly changing concentrations of target analytes in complex biological samples.

It is often impractical, frequently because of size, to remove suitable samples for study from the living system. In pharmacokinetic studies with rodents, the limited blood volume results in a large number of animals being used to generate profiles with sufficient numbers of data points. If blood were not removed for analysis, smaller numbers of animals would be required and the data generated would be improved by reduced interanimal variation. In any microextraction or membrane technique, compounds of interest are not exhaustively removed

from the investigated system. On the contrary, conditions can be conceived where only a small proportion of the total compound is removed, thus avoiding disturbing the normal balance of the chemical components.

In vivo analysis is a special application area where SPME is gaining ground because of its unique characteristics: on-site sampling, easy extraction, and analysis of the whole extracted amount. In any microextraction or membrane technique, compounds of interest are not exhaustively removed from the investigated system. On the contrary, conditions can be conceived where only a small proportion of the total compound is removed, thus avoiding disturbing the normal balance of the chemical components.

Early *in vivo* investigations with SPME focused on fragrances emitted by insects, fungi, and bacteria. These investigations were extended to biogenic volatile organic compounds emitted by animals and plants.[45] In a more recent application, the SPME technology was used for *in vivo* analysis of intravenous drug concentrations in a living animal. A novel SPME probe was developed and its effectiveness was demonstrated by acquiring the free and total concentration pharmacokinetic profile of diazepam, nordiazepam and oxazepam. The method was validated by comparison to conventional sampling methods.[38] For this initial study, most of the time points of a pharmacokinetic profile were acquired using PPY coatings. However, two of the time points were also obtained with PEG/C18 coatings, and good correlation to conventional sampling was achieved (Figure 3-3). While the linear range for PPY was from 5 to 750 ng/mL, the linear range for PEG/C18 coatings was from 1 to 2000 ng/mL. In this study, conventional analysis (based on centrifugation of blood cells followed by protein precipitation) took 90 min per sample, while the SPME approach required only 3 min. Moreover, no blood was removed for analysis in the case of SPME.

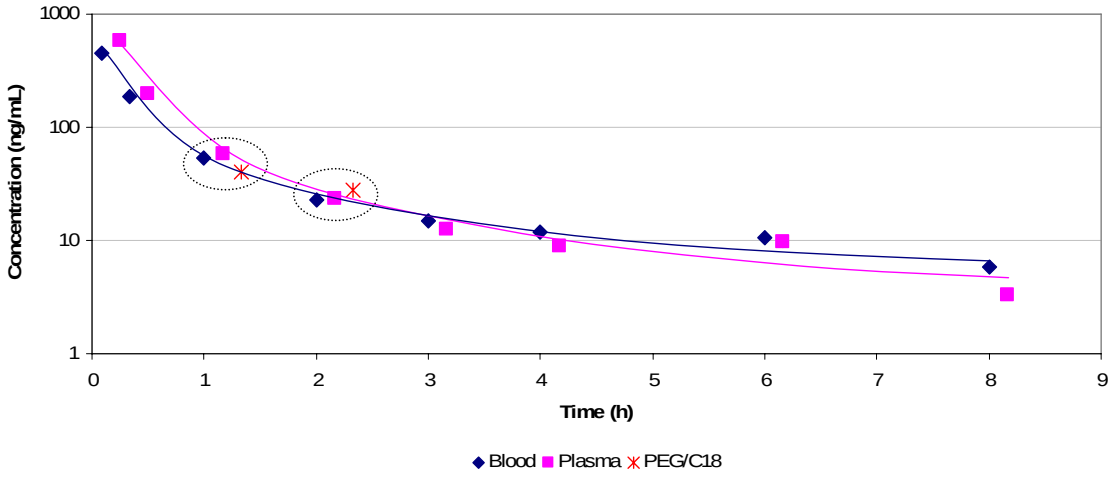


Figure 3-3. Diazepam pharmacokinetic profile, from three studies on three dogs (n = 9). "Blood": *in vivo* SPME with PPY probes; "Plasma": conventional analysis; "PEG/C18": *in vivo* SPME with PEG/C18 probes.

Furthermore, several colleagues in Dr. Pawliszyn's group have recently carried out a full pharmacokinetic study where all the time points were sampled with SPME fibers based on PEG/C18. Also, other colleagues in the group are using PAN/C18 and PAN/RP-amide fibers for in vivo studies in mice. A calibration curve for carbamazepine in whole mouse blood is presented in Figure 3-4 and shows very good linearity and wide linear range.

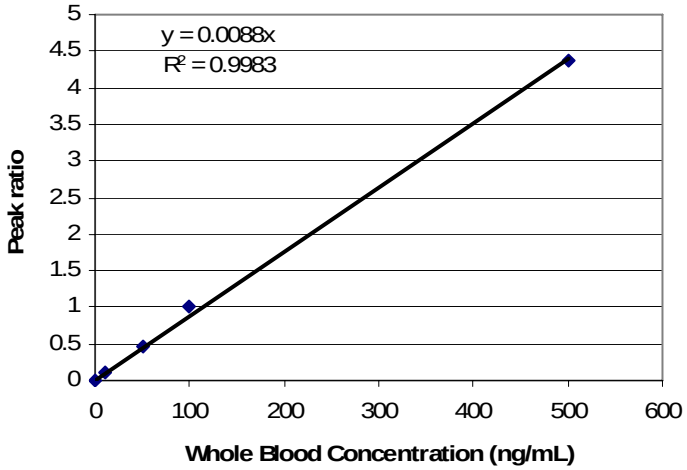


Figure 3-4. Calibration curve for carbamazepine in whole mouse blood (PAN/RP-amide coating).

3.3 Determination of Drug Plasma Protein Binding

Determining the amount of drug binding to plasma proteins is an essential step in both drug discovery and in clinical phases of drug development.[47-50] Plasma protein binding (PPB) affects the amount of drug available to diffuse into target tissues, e.g. brain,[51] the calculation of *in vivo* hepatic clearance,[52] and the interpretation of the drug's bioavailability.[48]

Due to the important clinical implications of plasma protein binding data and its role in characterizing a drug's behaviour and proper dosing, there is an increasing need to make this measurement as early as possible in the discovery process in order to understand drug disposition and to optimize individual drug therapy.

Although the main drug-binding proteins are albumin and alpha 1-acid glycoprotein, plasma contains many other proteins; consequently, there is a high probability that many small molecules will exhibit some levels of binding. To determine the extent of PPB, the molecule should be tested directly in a protein-binding assay using plasma or serum. This is a critical step in characterizing the distribution of a small molecule with respect to the plasma compartment.[53,54]

The determination of plasma protein binding by SPME is based on determining the free concentration of drug in the presence of plasma proteins, and has been comprehensively described before.[55] Briefly, the percentage of drug binding to plasma proteins (PPB) is calculated from the total and free concentration of drug:

$$\mathrm{PPB\%} = \frac{C_{\text{total plasma}} - C_{\text{free plasma}}}{C_{\text{total plasma}}} \cdot 100 = \left(1 - \frac{C_{\text{free plasma}}}{C_{\text{total plasma}}}\right) \cdot 100 \qquad \textbf{(3.1)}$$

Considering that the total drug concentration is directly proportional to the slope of the drug calibration curve in PBS and the free concentration is directly proportional to the slope of plasma calibration,[55] Equation 3.1 becomes:

$$PPB\% = 100 \cdot \left(1 - \frac{slope\ calibration\ \ plasma}{slope\ calibration\ PBS}\right) \quad \textbf{(3.2)}$$

Equation 3.2 was applied for the determination of drug plasma protein binding for the test drugs in section 2.6.1, and the results are presented in Table 3-2. Only the most reproducible coatings were used, and the results correlate very well with previously published values.

Table 3-2. Experimental and literature drug plasma protein binding values.

Plasma Protein Binding %	*PAN/C18 0.01", 60µm*	*PAN/RP-amide 0.01", 60µm*	*Literature Values (range)*[55,56]
Diazepam	98	99	96-98
Verapamil	96	96	88-98
Warfarin	99	99	98-100
Nordiazepam	98	98	97-98
Loperamide	96	97	95-97

Chapter 4 Conclusions

Successful preparation of biocompatible and hemocompatible SPME coatings represents an important step towards developing powerful biomedical, pharmaceutical and forensic applications, as the advantages of SPME would be directly useful for analysis of biological samples. One of the most important advantages provided by biocompatible SPME consists of in vivo blood and tissue analysis without the need to remove the sample from the biological system. The increased speed of sampling and analysis is an attractive issue of this research. One of the main deterrents of in vivo application of SPME has been the lack of suitable extractive phases. It is expected that the number of these applications will significantly increase with the introduction of new biocompatible coatings.

The present research towards development of biocompatible SPME fibers has focused on improving commercial CW/TPR fibers by protection with a layer of biocompatible PAN, and on the preparation of new coatings based on PEG glue and PAN mixed with silica-based extractive particles. The biocompatibility of these new coatings was assessed by XPS, and their performance was tested by developing an SPME/HPLC method for analysis of verapamil, lorazepam, loperamide, diazepam, nordiazepam, and warfarin from buffer solutions and from plasma.

The results are very encouraging: biocompatible coatings were successfully produced and used for in vitro analysis of drugs in human plasma and in vivo analysis in dogs. Two

strategies are used for developing new biocompatible coatings: protection of existing extraction phases with biocompatible polymeric membranes and preparation of biocompatible extraction phases.

Combining biocompatible SPME and LC/ESI-MS results in a very powerful analytical technique. This method for assay of several different drugs from plasma is fast, linear and selective. A single PAN-SPME fiber can be used at least 10 times for extractions from biological samples. An essential prerequisite for the determination of drug plasma protein binding by SPME is the utilization of non-fouling extraction phases. As expected, the new biocompatible coatings were very useful for measuring drug plasma protein binding (helpful for management of drug doses).

PEG-based coatings have a short equilibration time, but they need to be sterilized chemically or by radiation. PAN-based coatings are preferable when steam sterilization or when the best sensitivity is required.

Further developments in the area of biocompatible SPME could include analytical methods with lower limits of detection, applications for other drugs with a wider range of polarities, and automation of fiber preparation, extraction, and desorption.

Safety Hazards

Goggles, gloves and lab coats should be worn when working in the laboratory in order to avoid possible hazards. When handling solvents the best choice is to wear nitrile gloves because they are more resistant than ordinary ones made of latex.

Acetone is moderately toxic by various routes, a skin (defatting action) and eye irritant and narcotic in high concentration; it is responsible for headache from prolonged inhalation. It is dangerous because it is flammable and constitutes a fire and explosion hazard, being able to react vigorously with oxidizing materials. Ammonium acetate is poisonous by intravenous route and moderately toxic by other routes. Methanol is poisonous by ingestion, skin contact, intravenous, and intraperitoneal routes. It is moderately toxic by inhalation and other routes. It is known as being an eye and skin irritant and a systemic irritant by inhalation with narcotic effects. Its principal toxic effect is exerted upon the nervous system, particularly the optic nerves and possibly the retina and can progress to permanent blindness. Once it is absorbed it is very slowly eliminated. Coma resulting form massive exposures may last as long as 2-4 days. In the body, the products formed by its oxidation are formaldehyde and formic acid, both of which are toxic. Because of the slowness with which it is eliminated, methanol should be regarded as a cumulative poison. Though single exposure to fumes may cause no harmful effect, daily exposure may result in the accumulation of sufficient methanol in the body to lead to illness. Death from ingestion of less than 30 mL has been reported. It constitutes a fire hazard and moderate explosion hazard when exposed to heat, flame or oxidizers.

Safety measures should be taken when dealing with nitric acid, human plasma, mouse blood, and dog blood. Extra care is necessary when dealing with centrifuges and HPLC/MS. It is important to avoid any contact with the high voltage locations of the instruments. All protective barriers must remain in place during operation to prevent access to these areas. When preparing the mixture with extractive particles (C18, CN, RP-amide, and HS-F5), a mask must be worn because the particles are very fine and could be inhaled. Some operations should be executed in the fume hood (preparing of diluted nitric acid, sonication of the fibers as a prerequisite for developing the coatings etc.).

Attention should be paid when using acetonitrile because it is flammable and may be very harmful. Also care should be taken with any volatile solvent. Also, thick gloves should be worn when dealing with ovens and autoclaves.

All required safety and WHMIS training has been completed as required for conducting experiments at Waterloo.

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