High-throughput analysis of biological fluids using 96-blade (thin-film) solid phase microextraction system

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Chemistry

Waterloo, Ontario, Canada, 2012

AUTHOR'S DECLARATION

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

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Abstract

Sample preparation is considered to be the most time-consuming step for a quantitative analytical process. Many analytical laboratories deal with several hundreds of samples on a daily basis; therefore, the manual analysis of the samples in a linear sequence is demanding and impractical. During the last decade, automated and high-throughput sample preparation strategies, such as automated multi-well plate format systems, have gained popularity in order to address this issue.

Solid phase microextraction (SPME) is a non-exhaustive sample preparation technique in which the SPME device is directly exposed to the sample. Selective extraction of compounds takes place based on the degree of distribution of the analyte between the SPME coating and the sample matrix. Since the extraction and desorption steps are the most time-consuming steps of the SPME method, the parallel extraction and desorption of multiple samples on a multi-well plate format would contribute to significant time efficiency and throughput.

This thesis involves the utilization of an automated 96-well format SPME system coupled with LC–MS/MS. Therefore, the system is able to obtain improved reproducibility and sample throughput through the automation and simultaneous analysis of up to 96 samples, respectively.

Solid phase microextraction is an equilibrium-based sample preparation method and employs a small volume of an extractive phase that may provide limited recovery for the analysis of trace amount of compounds. To address the issue of sensitivity, the studies

reported in this thesis utilized thin-film geometry for increasing the volume of extractive phase, and consequently improving the sensitivity and efficiency of the system.

Most available commercialized SPME coatings are mainly designed for gas chromatography applications and are limited in addressing the needs for the efficient extraction of those polarity ranges of compounds, which are analyzed in liquid chromatography systems. In addition, these coatings usually suffer from drawbacks such as physical instability, swelling in organic solvent, lack of inter-lot reproducibility, and high cost.

The initial research of this thesis involves the evaluation of different strategies for the development of highly stable coatings for the automated 96-blade (thin-film) SPME system coupled with liquid chromatography–tandem mass spectrometry (LC–MS/MS). Sol-gel technology was used for the preparation of octadecyl (C18)-silica gel thin-film 96-blade SPME system. Various factors (e.g., sol-gel composition, aging time, coating preparation speed, coating thickness, and drying conditions), affecting the quality of the C18-silica gel thin-film coating, were studied and optimized. The results show that the stability and durability of the silica gel coating are functions of the coating's thickness and drying conditions. The evaluation of the C18-silica gel SPME extractive phase resulted in stable physical and chemical characteristics and long-term reusability with a high degree of reproducibility.

The adhesion of macromolecules, such as particulates and proteins, to the coating surface can significantly influence the kinetics of the extraction and the amount of analyte extracted by the coating. Therefore, (as described in Chapter 3 of this thesis) biocompatible polyacrylonitrile (PAN) polymer was used for the preparation of particle-based extractive phases in order to improve the biocompatible characteristics of SPME coatings for the

extraction from biological samples. As the main goal of this thesis consists of developing highly stable coatings for the automated 96-blade SPME system, different immobilization strategies (dipping, brush painting, and spraying) were evaluated. The spraying was found to be the optimal method in terms of stability and reusability for long-term use.

The increased volume of the extraction phase in the thin-film C18-PAN coating resulted in significant enhancement in the extraction recovery when compared to that of conventional rod fiber geometry. Furthermore, the optimized C18-PAN coating demonstrated improved biocompatibility, stability, and reusability for the extraction of benzodiazepines from human plasma in comparison with those of C18-silica gel coating.

Due to irreversible attachments of blood cells on the coating surface, it is challenging to reuse the typical biocompatible SPME coatings for direct immersion extraction from whole blood over the long-term. To improve the biocompatible properties of the C18-PAN SPME coating for long-term direct analysis from whole blood, different modification strategies were studied and evaluated. The modification of the coating with an extra layer of biocompatible polyacrylonitrile polymer along with the application of a new washing strategy resulted in significant improvement in the blood compatibility of the original coating in long-term use. The PAN-over C18-PAN coating provided at least 30 reproducible extractions from whole blood (relative standard deviation (RSD) = 12% for n = 6) without loss of efficiency and without blood cell attachment on the coating surface.

‘Extracted blood spot’ (EBS) sampling was introduced as a novel approach to overcome the limitations of dried blood spot (DBS) sampling. EBS includes the application of a biocompatible SPME coating (instead of filter paper/card in DBS) for spot sampling of blood

or other biofluids. In EBS, four steps of sampling, analytes' isolation and pre-concentration, and sample clean-up are combined in one single step.

The compatibility of EBS sampling with different analytical methods was demonstrated via LC–MS/MS and direct analysis in real time (DART) – MS/MS systems. The utilization of EBS as a fast sampling and sample preparation method resulted in a significant reduction of matrix effects (ion suppression) through the isolation of analytes and a cleaner sample that is less susceptible to interference caused by complex matrix components.

Many studies (e.g., metabolomics) necessitate the analysis of compounds with a wide range of polarity and from different classes. The lack of proper stationary phases for the simultaneous analysis of both polar and non-polar compounds necessitates the repetition of the analytical process with different SPME coatings for each group of analytes. This is not only time-consuming but is also limited by the available volume of the sample and the stability of the analytes of interest. In this thesis, this issue was addressed through the development of modified polystyrene-divinylbenzene (PS-DVB)-PAN and phenylboronic acid (PBA)-PAN 96-blade SPME coatings. The coatings were evaluated for the extraction of analytes in a wide range of polarity (log P = 2.8 to –3.7) and demonstrated efficient extraction recovery for both polar and non-polar groups of compounds. The PS-DVB-PAN and PBA-PAN coatings presented chemical and mechanical stabilities and reproducible extraction efficiencies for more than 100 usages in phosphate-buffered saline (PBS) and human plasma.

The traditional sample preparation techniques for food analysis are time-consuming and lack efficiency. In addition, the lack of proper sample clean-up in these techniques may result in co-elution of matrix interferences with the analyte of interest, and consequently suppression/enhancement of the analyte's signal in LC–MS analysis that may result in

significant errors in quantitative data. As a result, this thesis reports, for the first time, on the application of an automated SPME-LC–MS/MS system for food analysis. The PS-DVB-PAN 96-blade SPME system was optimized for the extraction of the phenolic compounds from grape, berry, and wine samples. The developed method was evaluated in terms of recovery, reproducibility and reliability, and the results of the SPME-LC–MS/MS analysis were compared with the solvent extraction technique coupled with LC–MS/MS. The developed 96-blade SPME method achieved excellent recovery and reproducibility for the extraction of grape, berry, and wine samples, and a significant reduction of ion suppression/enhancement of analytes was obtained through efficient clean-up of the sample matrix from interfering components. In addition, the SPME method provided valuable insights on the existence of binding/adsorption interactions of the phenolic compounds with the binding matrix in the food samples under study.

Acknowledgements

I express sincere gratitude to my supervisor, Professor Janusz Pawliszyn, for this research opportunity and his continuous support, guidance, and encouragement during my studies. I thank my committee members, Professor Wojciech Gabryelski, Mr. Leonard Sidisky and Professor Mario Gauthier for their time and helpful advice during my studies and throughout the preparation of this thesis. I am also very grateful to my external examiner, Professor Daniel Armstrong, and my internal examiner, Professor Praveen Nekkar, for their invaluable time on evaluation my thesis.

I would like to appreciate my advisory and examiner committees for their effort in providing the feedback about the quality and structure of my thesis. Thanks for indicating my research achievements valuable to the field of analytical chemistry and thanks for nominating my thesis for the Pearson Medal award.

I would like to express my thanks to all my collaborators, co-workers, and colleagues for their collaboration, support, and friendship during the years of this journey, with a special acknowledgement to Mr. Dietmar Hein, Dr. Barbara Bojko, Ms. Fatemeh Mousavi, and Mr. Krzysztof Gorynski.

I sincerely thank my parents Zahra Mohamammad Beygi and Ebrabim Mirnaghi, my sister and brothers Maryam, Mehdi, and Hadi Mirnaghi and my close friends Shabnam Azimi and Elnaz Aalam for their ongoing love and support, even from thousands of miles away.

Finally and most importantly, I owe my deepest gratitude to my husband, Amir Ghods, for his endless support, motivation, and positive energy. This thesis would never have been possible without his love, care, understanding, and encouragement.

Dedication

I lovingly dedicate this thesis to my wonderful husband and my adoring parents … your unconditional love, support, and encouragement inspires my life. Everything I do, I do it for you!

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List of Abbreviations and Symbols

a	Time constant
A	Surface area
b	Thickness of the coating
C18	Octadecyl
C18-TEOS	Octadecyl triethoxysilane
C_f^{∞}	Equilibrium concentration of analyte on the coating
$C_{f\,\max}$	Maximum concentration of active sites on the coating
CE	Collision energy
C_s^0	Initial analyte concentration
Cs	Analyte concentration in the bulk of the sample matrix
CHD	Coronary heart disease
CXP	Collision cell exit potential
CW	Carbowax
CW-TPR	Carbowax-templated resin
d	Depth of the blade substrate
δ	The thickness of boundary layer
D_s	Diffusion coefficient of the analyte in the sample matrix
DART	Direct analysis in real time
DBS	Dried blood spot
DMF	N, N-Dimethylformamide
DVB	Divinylbenzene
DP	Declustering potential
η	Viscosity of the solution
EBS	Extracted blood spot
EP	Entrance potential
ESI	Electrospray ionization
FP	Focusing potential
GC	Gas chromatography

HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
HS	Headspace
K_{fs}	Distribution constant of the analyte between SPME coating and sample at equilibrium
K_{Afs}	Adsorption constant of the analyte at equilibrium
l	Length of the coating
LDL	Low-density lipoprotein
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
Log P	Log of octanol/water partition coefficient
LOQ	Limit of quantitation
LPME	Liquid-phase microextraction
MEPS	Microextraction in a packed syringe
MIP	Molecularly imprinted polymer
MPC	2-Methacryloyloxy ethyl phosphorylcholine
MRM	Multiple reaction monitoring
MS	Mass spectrometer/spectrometry
MS/MS	Tandem mass spectrometry
n_e	Amount of analyte extracted by SPME fiber at equilibrium
NA	Not applicable
ND	Not detected
ORMOSIL	Organically modified silica
PAN	Polyacrylonitrile
PBA	Phenylboronic acid
PBS	Phosphate buffered saline
PDMS	Polydimethylsiloxane
ppb	Parts per billion
PPB	Plasma protein binding
ppm	Parts per million

PPT	Protein precipitation
PS-DVB	Modified polystyrene-divinylbenzene copolymer with a weak anion exchanger
QC	Quality control
r	Radius of the support wire
R^2	Linear regression coefficient
RAM	Restricted access material
rpm	Revolutions per minute
RSD	Relative standard deviation
SBSE	Stir bar sorptive extraction
SE	Solvent extraction
SEM	Scanning electron microscopy
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SPME	Solid-phase microextraction
SVP	Standardized voltage and pressure
t	Sampling time
TEOS	Tetraethyl orthosilicate
UV	Ultraviolet
v	Withdrawal speed
V_f	Volume of SPME coating
V_s	Volume of sample matrix
w	Width of the coating substrate
XIC	Extracted ion chromatogram

Chapter 1

Introduction

1.1 The importance of sample preparation for biofluid analysis

Biofluid analysis consists of quantitative methods for the determination of low and high molecular weight species in a given biological system (e.g., living cells, plants, animals, and human bodies). The quantification of analytes such as drugs, metabolites, hormones, toxins, and nutrition ingredients in biofluids is necessary in the fields of drug discovery and development, medicine, therapeutic drug monitoring, toxicology, and food analysis.[1-4] Depending on the type of application, the analysis of complex biological fluids can be very challenging: this might be due to low concentrations of the analyte of interest in the sample (e.g., sub or low part per billion) that can contain thousands of other components including salts, acids, bases, peptides, proteins, drugs, endogenous metabolites, and numerous other organic components.[5] When dealing with instrumental methods with low analytical selectivity and sensitivity, the abundant components present in the sample, which have chemical properties similar to those of the analytes, can potentially interfere with the analysis of the analyte of interest, and consequently result in an unsuccessful analysis and/or unreliable analytical results.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has gained popularity over the last few decades due to its high sensitivity and selectivity; it has become the method of choice for bioanalytical applications.[2,3] Liquid chromatography provides

chromatographic separation of the analyte from other sample components, and tandem mass spectrometry presents selective quantitation of analytes by multi-step mass spectrometry based on their mass-to-charge ratio. However, in most cases there are limitations for direct injection of a biological sample into an LC–MS/MS system, including: (i) clogging of the instrument compartment due to the presence of large particulates in untreated biological samples or the precipitation of some sample components in contact with the LC mobile phase, (ii) deterioration of the performance of the chromatographic column due to the presence of contaminants, and (iii) incorrect analytical results due to ionization suppression/enhancement (called the matrix effect) in the presence of co-extracted components from the sample.[1, 6]

Different mechanisms have been proposed to explain the matrix effect, including: (i) a decrease in the evaporation efficiency of the spray droplet and reduction in the capability of the analytes to reach the gas phase, (ii) competition between the analyte and the interfering compounds for ionization, and (iii) neutralization processes.[7] It is important to verify and minimize the possibility of the matrix effect during method development and validation of LC–MS methods, since it can significantly influence the precision, accuracy, and reliability of the method. Selection and optimization of a proper sample preparation method, optimization of LC conditions, and application of an appropriate analytical calibration technique are the main strategies to reduce the possibility of the matrix effect in electrospray MS.[6, 8]

In fact, the sample preparation of the biological samples is completed with three major goals: (i) cleaning up the sample by removing unwanted matrix components that can interfere with the analysis of the analyte of interest (improving method specificity and

reliability), (ii) concentrating the analyte to improve the limit of detection of the analytical method (improving method sensitivity), and (iii) exchanging the analyte from the sample into an organic solvent compatible with the chromatographic system. In addition, as a result of sample clean-up, sample preparation presents better chromatographic column and mass spectrometer performance and life time in LC–MS applications.

1.2 Sample preparation methods for biofluid analysis

During the last several decades, many different sample preparation approaches have been developed for biofluid analysis. Ultrafiltration, dialysis, and protein precipitation (PPT) are sample preparation methods mainly used to remove proteins from biological samples.[9, 10] In contrast, other sample preparation techniques such as liquid-liquid extraction (LLE),[11] liquid-phase microextraction (LPME),[12-14] solid phase extraction (SPE),[15,16] solid phase microextraction (SPME),[13, 17, 18] stir bar sorptive extraction (SBSE),[19, 20] and microextraction in a packed syringe (MEPS)[21] are much more efficient techniques in terms of producing cleaner extracts for analysis. In addition, immunoaffinity extraction,[22, 23] molecularly imprinted polymer (MIP)-based extraction,[24, 25] and membrane-based extraction[26, 27] are also efficient sample preparation techniques with high specificity and selectivity. These techniques function on the basis of selective isolation of the analyte of interest from the complex biological matrix. Among all the above-mentioned sample preparation techniques PPT, LLE, and SPE are currently the most predominate sample preparation techniques for biological samples prior to LC–MS analysis, and are described in summary in this chapter.[1, 5, 6, 11]

The principle of PPT is based on the addition of an organic solvent or inorganic acid to the biological samples and the removal of proteins.[28] This method is fast and easy to develop, but it suffers from poor sample clean-up and a high possibility of matrix effects.[8] The LLE method is based on the addition of an appropriate water-immiscible organic solvent to the biological sample, and the extraction is performed by partitioning of the analyte into the organic layer (with higher solubility toward the analyte) and leaving the interferences, such as salts and proteins, in the aqueous layer. The LLE suffers from consumption of large volumes of organic solvent and the formation of emulsions, which result in the loss of analyte.[11] In SPE, a solid extractive phase contained in a cartridge or disk is used to extract the analyte of interest from the biological sample. After introducing the sample to the SPE cartridge, any loosely adsorbed matrix interferences are washed by a weak solvent and the elution of the analyte of interest is obtained using a small amount of an appropriate solvent. The SPE method suffers from the possibility of clogging the packed bed sorbent with any large biomolecules and particulates present in the sample; therefore, additional steps of pre-treatment (e.g., centrifugation, filtration, etc.) are required prior to the analysis of a complex sample.[29]

1.3 Solid-phase microextraction (SPME)

Solid-phase microextraction (SPME) is an equilibrium-based sample preparation method that permits integration of sampling, sample clean-up, and pre-concentration in a single step. The method was developed to address the need for rapid and solvent-free sample preparation for both laboratory and on-site analysis. SPME was originally designed for the extraction of organic compounds in combination with GC analysis.[30, 31] However, over the

past two decades this technique has gained popularity in both GC and LC applications for the extraction of different classes of compounds from various matrixes in diverse scientific fields (e.g., environmental, food, pharmaceutical, forensic, and clinical areas of study).[32-34] In this technique, a small volume of extractive phase (which is immobilized on a solid support) is exposed to the sample for a well-defined period of time. In SPME, the extraction is performed by the isolation of analytes which have a high affinity toward the coating. The extraction is based on the diffusion of the analytes from the sample matrix to the extraction phase through a boundary layer with the ultimate goal of reaching equilibrium between phases.[31] After extraction, the extracted analytes on the SPME coating are desorbed using thermal desorption by direct insertion of fiber into a chromatographic injector (i.e., GC applications) or solvent desorption (e.g., LC applications). The extraction process can be carried out via headspace (HS) extraction through exposing the SPME coating to the vapor phase above the sample matrix (extraction of volatile and semi-volatile compounds) or by direct immersion of the SPME extractive phase with the sample matrix (extraction of non-volatile or low-volatile analytes).

Over the years, several implementations of SPME such as fiber, in-tube, stir bar, vessel walls, disk/membrane, and suspended particles have been introduced (Figure 1.1). However, fiber coated configuration, as the original design of SPME, is most often used for SPME applications.[35]

1.3.1 Advantages of SPME for biofluid analysis

Solid phase microextraction presents remarkable benefits over traditional sample preparation methods for the analysis of biological samples, which are discussed in detail in this section.

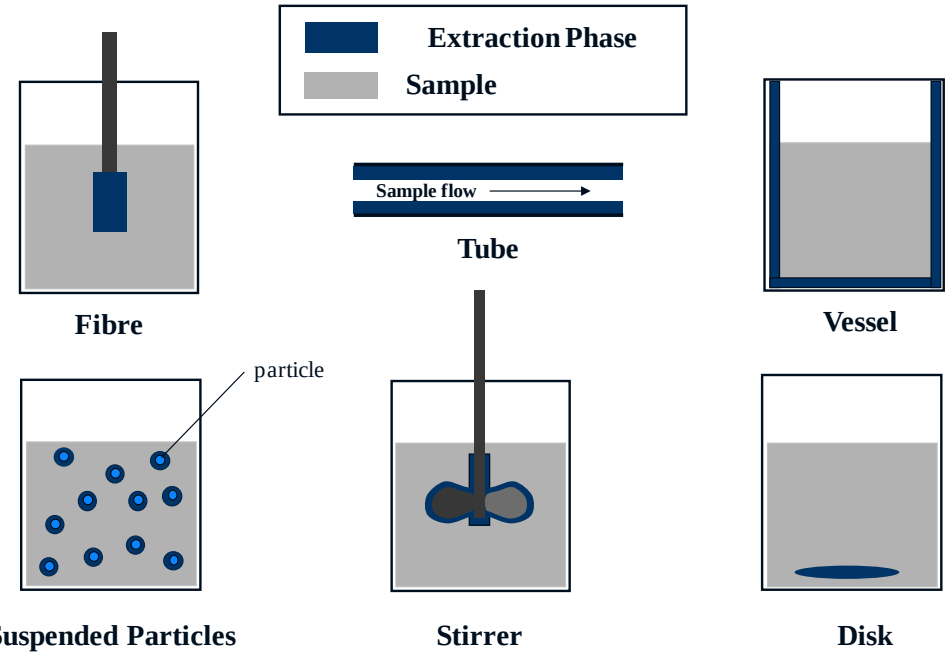


Figure 1.1 Configurations of solid phase microextraction. Figure reproduced from reference with the permission of the publisher. [35]

Since SPME is an equilibrium-based extraction method and utilizes a small volume of the extractive phase, it takes advantage of the selective extraction of analytes from the sample matrix. This extraction selectivity in SPME provides a high degree of clean-up of unwanted interferences from samples, which minimizes the possibility of matrix effect (a critical concern in LC–MS applications). Therefore, the choice of extractive phase and its selectivity toward the analyte of interest are important factors in SPME method development.

In addition, SPME is an open-bed system where an extractive phase is coated on the outer surface of a solid support. One of the most beneficial features of the open-bed system is the opportunity for the application of a large sample volume without the limitation of breakthrough volume. This feature provides selective equilibrium-based extraction of the analytes of interest, even those that are not successfully retained in an equivalent packed-bed system. Another beneficial aspect of an open-bed sorbent is the ability to perform direct extraction from complex matrixes (including dense fluids and colloidal suspensions) without any need for sample pretreatment or concerns regarding clogging or contamination (which is common in conventional packed-bed extractive systems).[29]

The traditional methods for studying free and total concentrations of analytes and ligand-receptor binding (i.e., equilibrium dialysis, ultrafiltration, and ultracentrifugation) involve the physical separation of unbound and bound ligands, followed by the analysis step.[36] Comparatively, SPME, as a microextraction sample preparation technique, offers the determination of both free and total concentrations of the analytes from a single biofluid sample using proper calibration strategies. This technique results in significant time and cost savings compared to traditional methods where two separate analyses are performed.[1, 37]

In addition, proper configuration of SPME can be applied for direct *in vivo* sampling from a living system (e.g., sampling from plants or circulating blood of an animal) without the need to isolate a defined sample volume. This format of SPME is particularly attractive for metabolomics studies as it decreases the overall number of experimental steps and also eliminates the need for the metabolism quenching step.[38]

1.4 Introduction to the fundamentals of solid phase microextraction

Since the work described in this thesis deals with the analysis of non-volatile species from biological fluids, direct immersion extraction and solvent desorption modes were utilized throughout the study; therefore, the main focus of the theoretical principles described in this section is based on these application modes.

1.4.1 Solid versus liquid coatings

There are substantial differences between the performance of liquid (absorbent) and solid (adsorbent) SPME coatings. The extraction of an analyte into the liquid SPME coating [e.g., polydimethylsiloxane (PDMS) and polyacrylate] is based on partitioning, in which the analyte is absorbed by the coating. In this case, if the coating is thin and a reasonable amount of time is spent for the extraction, the molecules are able to penetrate the whole volume of the coating. Figure 1.2 (a) demonstrates the mechanism of sorption for liquid SPME coatings.

In contrast, solid SPME coatings [e.g., divinylbenzene (DVB)/PDMS, Carboxen/PDMS, and Carbowax (CW)/DVB] have a "glassy" or "crystalline" structure that results in considerably lower diffusion coefficients for the analytes within the coating (Figure 1.2 (b and c)).[17, 35] In solid SPME coatings, the extraction mechanism is based on physical interactions/trapping of the analytes on the pores or high surface areas of the coating; therefore, analytes can only be adsorbed on the pores and cannot diffuse within the entire volume of the coating. The micropores (<20 Å) and mesopores (20–500 Å) are ideal for trapping small and mid-size analytes and usually retain the analytes until either energy is applied or they are displaced by a solvent. However, macropores (> 500 Å), which exist

primarily on the surface of the materials, can adsorb larger analytes through different interactions such as hydrogen bonding, pi-pi bonding or van der Waals. Due to the limited sites of adsorption on the solid coatings, they have a competitive extraction mechanism; therefore, if a long extraction time is applied, compounds with poor affinity toward the coating are frequently displaced with those with a higher affinity or those present in the sample at high concentrations.[39]

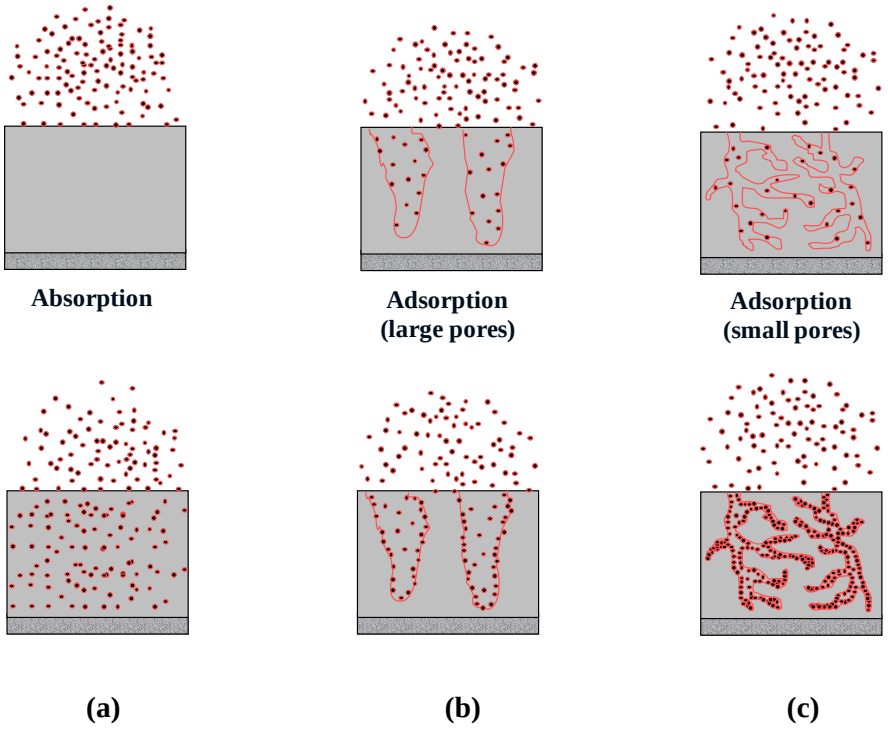


Figure 1.2 Sorption mechanisms for (a) liquid SPME coating, (b) solid SPME coating with large pores, and (c) solid SPME coatings with small pores. Figure reprinted from reference with the permission of the publisher.[35]

One way to overcome the limitation of solid SPME coatings is the application of pre-equilibrium extraction, so that the total amount of analytes accumulated by the porous coating is substantially less than the saturation value. In such cases, proper calibration

methods should be applied in order to determine the initial analyte concentration in the sample.

In general, liquid SPME coatings are more frequently used because of the linear dynamic ranges associated with their linear absorption isotherms. Solid coatings have chemisorption properties that result in irreversible adsorption of analytes to the surface of the coating, and possible loss or modification of the analytes during extraction and/or desorption. On the other hand, solid SPME coating exhibits a high degree of selectivity and sensitivity for some groups of compounds (such as carbon-based SPME for the extraction of volatile compounds).[32, 35]

1.4.2 Theoretical principles of solid phase microextraction

Equilibrium extraction for liquid SPME coatings: A microextraction system is considered to reach equilibrium between the sample and the coating when an increase in extraction time does not result in any further increases in the amount of analyte extracted by the coating (within experimental error). In the case of direct immersion extraction using the liquid based SPME coating, the amount of analyte extracted by the coating at equilibrium can be expressed as Equation 1.1:

$$n_e = \frac{K_{fs} V_f V_s C_s^0}{K_{fs} V_f + V_s} \qquad \text{Equation 1.1}$$

where C_s^0 represents the initial concentration of a given analyte in the sample, V_s represents the sample volume, V_f represents the SPME coating volume, and K_{fs} represents distribution constant of the analyte between the SPME coating and sample matrix. The

distribution constant (K_{fs}) is defined as the ratio of the analyte concentration in the coating to the analyte concentration in the sample at equilibrium (Equation 1.2).

$$K_{fs} = C_f^{\infty} / C_s^{\infty} \qquad \text{Equation 1.2}$$

The magnitude of K_{fs} depends on the nature of the analyte and the SPME extractive phase, and some specific properties of the sample matrix such as temperature, pH, and ionic strength. Therefore, it is critical to control and preserve these factors during extraction.[17]

Equilibrium extraction for solid SPME coatings: In case of solid adsorptive coatings the amount of analyte extracted at equilibrium can be calculated using Equation 1.3:

$$n_e = \frac{K_{Afs} V_f V_s C_s^0 (C_{f\max} - C_f^{\infty})}{V_s + K_{Afs} V_f (C_{f\max} - C_f^{\infty})} \qquad \text{Equation 1.3}$$

where $C_{f\max}$ represents the maximum concentration of active sites on the solid coating, C_f^{∞} represents the equilibrium concentration of a given analyte on the fiber, and K_{Afs} represents the analyte's adsorption constant at equilibrium. The adsorption constant is defined as the ratio of the surface concentration of the adsorbed analyte on porous solid extractive phase to the concentration of the analyte in the sample at equilibrium ($K_{Afs} = S_{Af}^{\infty} / C_s^{\infty}$). Equation 1.3 demonstrates that when the equilibrium concentration of the analyte is much lower than the concentrations of the active sites on the coating ($C_f^{\infty} << C_{f\max}$), the amount of the analyte extracted is linearly proportional to the initial sample concentration.

However, if C_f^{∞} is sufficiently large, it results in the saturation of the active sites, and consequently nonlinear adsorption isotherms.[35]

Equilibrium extraction from heterogeneous samples: When extraction is performed from a multi-phase system or a heterogeneous sample which consists of an aqueous phase with suspended solid particles (having various interactions with the analyte), Equation 1.1 can be further modified to Equation 1.4.

$$n_e = \frac{K_{fs} V_f V_s C_s^0}{K_{fs} V_f + \sum_{i=1}^{i=m} K_{is} V_i + V_s} \qquad \text{Equation 1.4}$$

where K_{is} represents the distribution constant of the analyte between the *i*th phase and the sample matrix with volume of V_i, and the rest of the terms are the same as defined for the previous equations. For instance, for the extraction of samples containing volatile species, the *ith* phase can be headspace, and contribution of this phase should be taken into account for volatile compounds. In complex matrixes, the *ith* phase can represent a binding matrix such as proteins and macromolecules present in biological samples (e.g., plasma and blood). In case of the multi-phase matrix the microextraction process can be considered complete when the analyte has reached equilibrium with all the existing phases.

It is clear from Equation 1.4 that when dealing with complex matrixes the analytes in the heterogeneous sample partition/interact with multi-phase systems and are less available for extraction. This effect depends on the analyte's affinity to the competing phases and the volume of the competing phases. Therefore, the amount of analyte extracted by SPME is influenced by the sample matrix and is proportional to the unbound (free) concentration of

the analyte under study. This result is considered to be the reason for the aforementioned advantage of SPME for the determination of both free (unbound) and total (bound + unbound) concentrations of analytes from a single biofluid sample. In this case, utilization of individual matrix-free and matrix-matched calibrations is required for determining the free and total concentrations of the analytes, respectively.[35]

Pre-equilibrium extraction: If analytical sensitivity is not an issue, the extraction time less than the equilibrium time can be used to increase the sample throughput. The amount of analyte extracted in pre-equilibrium extraction is based on the time of accumulation of the analyte in the coating and can be determined using Equation 1.5:

$$n = (1-e^{-at})\ n_e = (1-e^{-at})\ \frac{K_{fs}V_fV_sC_s^0}{K_{fs}V_f + V_s} \qquad \text{Equation 1.5}$$

where *t* represents the time for pre-equilibrium extraction and *a* represents a time constant. The magnitude of time constant depends on various factors such as the distribution constant, the mass transfer coefficients, sample volume, and volume and surface area of the SPME coating. As with the case of the equilibrium extraction, the equation of pre-equilibrium extraction indicates that the amount extracted by SPME at the time of pre-equilibrium is proportional to the initial sample concentration. Therefore the initial concentration of the analyte in sample can be calculated, provided that agitation conditions and extraction time are controlled precisely and kept constant.[40, 41]

Negligible extraction: When the sample volume is too large or the amount of analyte extracted by SPME coating is negligible (i.e. $V_s >> K_{fs} V_f$), Equation 1.1 can be modified to:

$$n_e = K_{fs} V_f C_s^0$$ Equation 1.6

Equation 1.6 is applied in the case of direct on-site or *in vivo* sampling (such as direct sampling from a lake, or sampling from circulating biological fluid in a living system, respectively), where the sample volume is too large and the amount of analyte extracted becomes independent of the sample volume.[17]

Exhaustive extraction: When the product of the distribution constant and coating volume is much larger than the sample volume (i.e. $K_{fs} V_f >> V_s$), Equation 1.1 can be modified to:

$$n_e = V_s C_s^0$$ Equation 1.7

Equation 1.7 indicates that the entire concentration of the analyte in the sample matrix is extracted into the SPME coating. In this case, the initial concentration of the target analyte can be easily calculated through determining the amount extracted by SPME coating and the sample volume.[35]

1.4.3 Kinetics of solid phase microextraction

Theoretically, the time required to reach equilibrium is infinite, but from a practical point of view the equilibrium time is defined as the required time to extract 95% of the analyte. The speed of extraction in SPME is determined by the kinetics of the extraction process. The kinetics theory identifies the extraction rate as the "bottleneck" of SPME, and indicates strategies for improving the rate of extraction.[35] For static extraction systems, the analytes must first diffuse through an ever-broadening depleting layer in the sample matrix and then through the fiber coating. In this case, the mass transfer of analytes from the

progressively thicker depleted layer to the fiber coating determines the overall extraction speed; as a result, very long extraction times are expected. However, agitation of the sample increases the mass transfer of the analytes and results in an improved rate of extraction and reduction in extraction time. In practical agitation conditions, independent of the degree of agitation in the system, there is always a stationary thin layer of sample around the SPME coating. As the distance from the SPME coating surface rises, the movement of the sample fluid slowly increases until it corresponds with the bulk flow in the sample.

To model the mass transport under practical agitation conditions, the progression in fluid motion and molecules' convection in the space surrounding the coating surface can be described as a static layer zone with distinct thickness, called a boundary layer (δ). The thickness of the boundary layer is determined by both the rate of convection (agitation) in the sample and the diffusion coefficient of the analyte. When the extraction rate is determined by diffusion in the boundary layer, equilibration time can be estimated by Equation 1.8.[35]

$$t_e = t_{95\%} = \frac{3K_{fs}\, b\, \delta}{D_s} \qquad \text{Equation 1.8}$$

In this equation, δ represents the thickness of the boundary layer, D_s represents the diffusion coefficient of the analyte in the sample matrix, b represents the coating thickness, and K_{fs} represents the distribution constant. According to Equation 1.8, the time required to reach equilibrium depends on the agitation conditions (thickness of boundary layer), affinity of the analyte toward the coating (distribution constant), physicochemical properties of the analyte (diffusion coefficient), and the thickness of the SPME coating. Therefore, thin coatings and efficient agitation conditions are recommended for the best sample throughput. It should be

noted that an analyte with a high K_{fs} value will have a long equilibrium time, even with a thin boundary layer (rapid agitation).[35]

1.4.4 Calibration in SPME

Calibration is a process that relates the measured analytical signal to the concentration of the analyte in the sample matrix. Since SPME is a non-exhaustive sample preparation technique, in order to obtain accurate and reliable quantitative results, it is critical to employ appropriate calibration methods. The choice and suitability of a SPME calibration technique depends on various factors such as the type of applications and analytical instruments. Several calibration approaches have been proposed for SPME including: traditional calibration methods (external, internal, and standard addition calibrations), equilibrium extraction, exhaustive extraction, pre-equilibrium extraction, and diffusion-based calibration. Diffusion-based calibration methods consist of Fick's first law of diffusion, interface and cross-flow models, and kinetic calibration methods (standard in-fiber and standard-free calibration). The details regarding each calibration system are well documented in the literature.[40, 41] For the studies discussed in this thesis, external, internal or standard addition calibration techniques have been employed, depending on the type of applications.

1.5 Automation of sample preparation for LC applications

Sample preparation is known as the 'bottleneck' in most analytical protocols. A survey on chromatographic analysis shows that sample preparation contributed to approximately two thirds of the total time of analysis.[42] Many biological and environmental applications deal with numerous samples for analysis, and the manual analysis of the samples in a linear

sequence would be impractical. Improving sample throughput is therefore vital in order to achieve fast sample preparation methods. High sample throughput can be achieved by analyzing several samples simultaneously or via increasing the sampling rate.[43] Over the past several decades, the automation of sample preparation has been an important objective for laboratories to address the limitations of demanding sample preparation strategies.[44] Automation of the analytical methods provides a number of advantages including reduced analysis time (both for routine analysis and method development), faster sample throughput, and greater reproducibility through reduction of human error.[45] Depending on the required task, the choice of the automation may differ in complexity.

During the past twenty years, the applications of the automated high-throughput SPE methods have increased dramatically.[34, 46-53] The introduction of an automated 96-well SPE system in 1996 was a milestone for bioanalysis and provided significant improvements in parallel sample preparation techniques.[54, 55] However, 384 and 1536 well-plates are also available to further improve the sample throughput,[43, 56, 57] but the 96-well format is commonly used for the parallel sample preparation. Since the introduction of the 96-well format SPE system, various automatic workstations have been commercialized with different extractive phase chemistries and diverse complexities and applications.[44, 55, 58-63]

Online SPE format is another approach for the automation of the SPE system. In this format, the sample is directly injected into an SPE cartridge, followed by direct elution of the analytes into an analytical LC column and subsequent MS analysis. The on-line SPE techniques are beneficial in terms of strong selectivity and sensitivity; but, they have lower sample throughput than the 96-well formats and can suffer from carryover effects.[64] The success of the 96-well format SPE has inspired the application of this format for other

sample preparation techniques such as LLE, which had typically been considered too difficult to automate.[46, 52, 65-70]

1.5.1 Automation of solid phase microextraction

The solvent-free nature and the syringe device format of SPME facilitate its combination with gas chromatography (GC) and permit the direct introduction of an SPME device into a GC injector. Since the 1990s, commercial autosamplers capable of completing the automatic performance of all SPME steps and the introduction into the GC system have been available.[45]

In 1995, the first coupling of SPME to an LC system was achieved through the manual introduction of an SPME device into the path of the LC mobile phase (using an injection tee for solvent desorption).[71] However, this method suffers from a lack of automation, the chance of sample leak at the LC interface, and the possibility of the loss of analytes. Automation of SPME in conjunction with LC analysis was primarily introduced through the development of the automated in-tube SPME approach by Eisert and Pawliszyn in 1997.[49] The principle of automated in-tube SPME involves the placement of a coated capillary within the path of the LC system (typically between the injection needle and the loop of the autosampler). Then, in several cycles the sample is automatically drawn into and ejected from this in-tube SPME coating. The process of circulating the sample facilitates agitation in the system in order to enhance mass transfer and partitioning of the analytes into the extractive phase. After the extraction, the analytes can be desorbed by directing the flow of the LC mobile phase through the capillary and toward the column for separation and

analysis.[49] However, in-tube SPME provides a high degree of automation and sensitivity, the process suffers from a lack of high-throughput.[72]

Typically the extraction and desorption steps are the most time-consuming steps of an SPME-LC analytical process. Therefore, it is more efficient to perform parallel extraction and desorption steps for multiple samples. To address this requirement, during previous years, high-throughput SPME-LC analysis has been successfully achieved via the development of robotic autosamplers and the utilization of multi-well plate formats.[1, 37, 43, 73, 74]

In 2005, O'Reilly *et al.* first proposed the approach of performing high-throughput parallel SPME analysis on a 96-well format.[45] In 2007, a simple semi-automated proof-of-concept study was reported using an array of eight commercial PDMS-DVB SPME coatings. This study evaluated the applicability of automated SPME for bioanalysis by comparison against the accepted automatic 96-well format LLE.[75] The results indicated that SPME achieved a good performance with higher accuracy and precision as compared to LLE. However, due to the high expense of a single commercial SPME fiber, building a 96-fiber array is considered cost-prohibitive. This limitation was overcome by Hutchinson *et al.* through the introduction of a commercial 96-pin-tool replicator device, used as the support to immobilize 96 stainless steel pins coated with PDMS hollow fiber membrane.[43] The performance of the 96-pins was tested against other possible configurations (e.g. membrane, multi-fiber "brush", and commercial SPME metal fiber), and the 96-pins device was found to be the most suitable configuration for the automated 96-well format SPME-LC system because of higher reproducibility and robustness. The in-house multi-fiber SPME device not only addressed the limitations of the expensive commercial fiber assemblies, but it also took

advantage of controlling the length and thickness of the extraction phase over the metal substrate. O'Reilly *et al*. reported on the evaluation of different agitation methods (such as sonicating, magnetic stirring, and orbital shaking), and they showed that the application of an orbital shaker provided more uniform agitation.[43] Based on the results of O'Reilly's study, Vuckovic *et al.* reported on the application of a prototype SPME autosampler named Concept 96 (designed by Professional Analytical System (PAS) Technology) for determining drugs from whole blood using octadecyl silica-based 96-fiber SPME coating (Figure 1.3 (a)).[74]

Meanwhile, Xie *et al*. reported on an automated in-tip fiber SPME approach in 96-well format coupled with LC–MS/MS for high-throughput clinical application.[76] In Xie's research, SPME fibers were fitted in the tips of pipets and a commercially available Tomtec Quadra 96-workstation was used to aspirate and dispense the sample solution and desorption solvent to the pipet tips. However, the in-tip fiber SPME system has limitations when applied to complex and viscous samples (a result of the narrow opening of the pipet tips, and difficulties in expelling all sample residues).

In recent years, different geometries (rod fiber, disk, thin-film) and various coating chemistries have been designed and developed for the Concept 96-fiber SPME system, and used for the high-throughput analysis of drugs from complex biological samples (urine, plasma, and whole blood) with high efficiency and precision.[73, 74, 77-79]

1.6 Thin-film microextraction

SPME was originally introduced in rod fiber geometry and it has been the most common format since its introduction. However, the main limitation of the SPME fibers is

their relatively low sensitivity due to the small amount of sorbent: the amount of analyte extracted by SPME is typically small, especially when extracting analytes with a high degree of matrix binding (e.g., drug-protein binding in plasma or whole blood matrixes). For a given application, this may result in low absolute recoveries and necessitate the use of highly sensitive analytical instrumentation to ensure an adequate limit of quantitation is achieved. As a result, during the past several years, improving the extraction capacity and consequently the overall method sensitivity has been one of the main focuses of SPME method development.

According to the fundamental principles of SPME (Equation 1.1), the amount of analyte extracted by SPME is proportional to the volume of the extraction phase (V_f). Therefore one approach to improve the extraction recovery is to increase the volume of the extraction phase. This improvement can be accomplished by either increasing the thickness of the stationary phase coating as is accomplished in stir-bar sorptive extraction[19, 80] or increasing the surface area.[81] The use of thicker coatings increases the extraction equilibrium time leading to lower sample throughputs (Equation 1.8). On the other hand, increasing the surface area can be achieved by increasing the diameter of the SPME fiber or using thin-film geometry. A large increase in the fiber diameter has the limitation of occupying a large space (volume) in the sample well. This space causes displacement of the sample solution and limits the maximum sample volume that could be placed in the wells. In contrast, the application of thin-film geometry addresses this limitation by occupying less space and facilitating the use of larger sample volumes.

In addition to the increased extractive phase volume, and consequently the improved sensitivity, thin-film geometry offers two other advantages over the traditional rod fiber geometry. First, the thinner SPME coating provides significant improvements in the convection and mass transfer of the analyte, which results in a faster equilibrium extraction time (Equation 1.8). Secondly, the application of thin-film geometry is beneficial for the pre-equilibrium extraction as the initial rate of SPME extraction is proportional to the surface area of the extraction phase (Equation 1.9).[81]

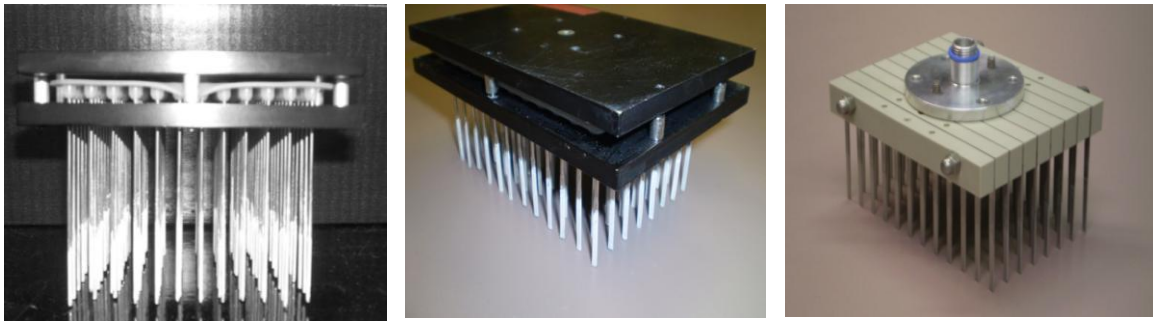
$$dn/dt = \frac{D_s A}{\delta} C_s^0 \qquad \text{Equation 1.9}$$

In Equation 1.9, *dn/dt* represents the rate of extraction, *A* represents the surface area of the extraction phase, δ represents the thickness of the boundary layer, D_s represents the diffusion coefficient of the analyte in the sample matrix, C_s^0 represents the initial concentration of analyte in the sample, and *t* represents the extraction time. Based on Equation 1.9, by using very short pre-equilibrium extraction times, when the analyte uptake by the coating is linear at the time of extraction, thin-film SPME coating is able to extract a larger amount of analyte in comparison with rod fiber geometry.

The Pawliszyn group has reported on the demonstration of the theoretical concept of thin-film SPME coupled with LC–MS/MS application by the immobilization of a layer of C18 coating on a flattened end of a stainless steel rod (Figure 1.3 (b)). This report indicates that an increase in the surface area of the SPME coating resulted in a significant improvement of the extraction rate and up to a 2-fold improvement in the analytical sensitivity compared to the rod fiber configuration.[73]

1.7 Concept 96-blade (thin-film) SPME device and 96-autosampler

In order to overcome the limitations of the in-house multi-fiber devices (e.g. instability of the immobilized fibers during the agitation) and the difficulties in manually flattening the fibers to make thin films, the study of this thesis is focused on the application of a commercial 96-blade (thin-film geometry) device designed by PAS Technology. The 96-blade SPME device is composed of eight rows of blade sets (stainless steel) where each blade set consists of 12 thin-film pins. The eight rows of the blade sets are held together using nine inter-blade holders, creating a 96-blade SPME device (Figure 1.3 (c)) that works as a part of Concept 96-autosampler. The robotic Concept 96-autosampler is designed to directly place the 96-blade device into the 96-well plates prefilled with samples/solvents, enabling the performance of preconditioning, extraction, washing, and desorption steps. The detailed description of the Concept 96-autosampler is provided in Chapter 2, Section 2.2.4.



(a) **(b)** **(c)**

Figure 1.3 (a) In-house 96-fiber SPME, (b) In-house 96-thin-film SPME, and (c) Concept 96-blade SPME device. Figures (a) and (c) are reprinted from reference [35] and Figure (b) is reprinted from reference [73] with the permission of the publishers.

1.8 Introduction to available SPME coatings for LC applications

To date, the application of SPME with GC analysis is predominant over SPME-LC applications. One of the main reasons for this preference is the lack of automation of SPME in LC applications, which has been addressed in recent years. Another main reason for the prevalence in the use of SPME in GC applications is the limited number of available extractive phases suitable for use with LC applications.[82] Currently, there are eight commercially available SPME coatings including PDMS, PDMS/DVB, polyacrylate, Carbowax-polyethylene glycol (PEG), Carbowax/templated resin (CW/TPR), CW/DVB, Carboxen (CAR)/ PDMS, and DVB/CAR/PDMS. These coatings are basically designed for GC applications and thus roughly cover the scale of polarity applied in LC analysis. In addition, they occasionally exhibit some limitations such as physical instability, swelling in organic solvents, lack of inter-lot reproducibility, and high cost (>$150 per fiber). Even though these fibers are reusable, because of their high cost their initial application in the multi-fiber design is prohibitive. Recently, the first SPME fiber probe with designated design for LC applications has become commercially available. This SPME fiber, which is made of C18-silica based stationary phase, is disposable yet still expensive (>$18 per fiber).

During the past decades many efforts have been made toward developing and evaluating improved SPME-LC coatings, and many in-house-made SPME coatings have been discovered and tested in various applications. Wu *et al.* have shown a wide applicability for a biocompatible polypyrrole polymer coating for the extraction of several classes of analytes due to its inherent multi-functionality.[83-87] Liao *et al*. reported on the development of poly (acrylic acid) coated fused silica to extract proteins.[88] Polycrystalline graphites have

been used as alternative sorbents for the SPME-LC applications for the extraction of non-ionic surfactants and various organic pollutants in water.[89-91] Li *et al*. reported on the development of single-walled carbon nanotube SPME coatings by electrophoretic deposition and applied them for the determination of phenols in aqueous samples.[92] In addition, sol-gel-based SPME coatings have also been studied in an effort to improve the chemical and mechanical stability of the SPME coatings in LC application.[93-95] The incorporation of different functional groups has lead to the development of various monolithic silica coatings [96, 97] and polymer monolithic coatings [98, 99] with the focus on improvements in the extraction of polar compounds.

Molecularly imprinted polymer (MIP) SPME coatings have been introduced based on the mechanism of molecular recognition and as a selective tool for analytical recognition and quantitation of compounds.[100] Furthermore, the development of biocompatible coatings based on restricted access materials (RAM) facilitates the extraction and enrichment of low molecular weight compounds and exclusion of proteins based on size exclusion process.[101, 102]

Recently variations in the extractive phases for SPME-LC applications have increased by the development of diverse coating chemistries based on mixtures of SPE sorbents with a biocompatible binder.[1, 37, 73, 79, 103-106]

1.9 Coating requirements for the 96-blade SPME system for the analysis of biological samples

The properties of a given SPME coating depend on the extractive phase materials and the applied method for the deposition of the coating on the substrate. To date, several SPME

coating preparation procedures have been explored, including physical deposition, sol-gel technology, and electrochemical procedures. Typically, physical deposition of the coating on the SPME fiber and lack of chemical binding between the coating and the substrate are the main reasons for poor mechanical and chemical stabilities of the coatings.[107] Chemical stability in contact with aqueous and organic solvents and mechanical stability are two main requirements for the SPME extractive phases used in LC applications. The deficiency of the SPME coating to meet these requirements leads to classification of the coating as 'disposable'. Therefore, the coatings need to be renewed prior to the next analysis: a process that is time-consuming and cost-prohibitive especially in the case of a multi-fiber SPME system.

As a result, a proper coating deposition method should be applied to ensure the preparation of good quality coatings with a high degree of stability and robustness. On the other hand, since the 96-blade device is a commercial product, the ultimate goal is the industrialization of the coating preparation procedure. Therefore, the procedure of SPME coating preparation should be reproducible, cost-effective, and easy to automate. The reproducibility of the coating preparation procedure leads to high fiber-to-fiber reproducibility, and consequently the precision of the analytical method. Biocompatibility of the coating (described in detail in Chapter 3) is another important factor in the application of the automated 96-blade SPME system for the analysis of biological samples.

In addition to the above-mentioned properties, extraction recovery and a relatively short equilibrium time are two other desired characteristics for the SPME coating, which can be obtained by the proper choice of extractive material and geometry of the extractive phase.

The overview of the desired coating properties for the 96-blade SPME system for the analysis of biological samples is available in Figure 1.4.

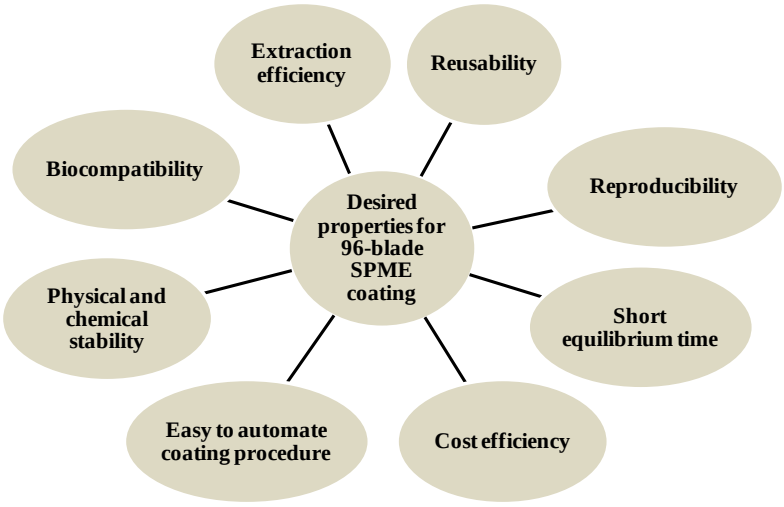


Figure 1.4 Desired coating properties for 96-blade SPME system for bioanalysis

1.10 Research objective

The research objectives of this thesis include (i) improvement in throughput, sensitivity, and efficiency of the SPME method through the application of a 96-blade (thin-film) SPME system, (ii) development and evaluation of different coating preparation strategies, and (iii) coating chemistries for the automated 96-blade SPME system (with the desired properties discussed in Section 1.9, Figure 1.4), and (iv) application of the developed 96-blade SPME-LC–MS/MS system for the analysis of low molecular weight compounds from biofluids.

To address the limitations of the current commercial SPME coatings (e.g., instability of the coating for long-term handling, limited extraction recovery for a wide range of polarity, and lack of compatibility with complex matrixes), the main focus of this thesis is

the evaluation of different coating procedures for the development of biocompatible and long-lasting coatings, which can be used for the analysis of a wide class of compounds from complex biological matrixes. Thus, Chapter 2 consists of a report on the application of sol-gel technology for the preparation of highly-stable thin-film octadecyl (C18)-silica gel coating for the 96-blade SPME system. In Chapter 3, three different methods: dipping, brush painting, and spraying are evaluated for the preparation of biocompatible particle-based 96-thin-film SPME coatings. In Chapter 4 the evaluation of three different biocompatible polymers is discussed, with the main focus to improve the biocompatibility of the SPME coatings for direct immersion extraction from whole blood matrix. The optimized coating strategy with the best biocompatible characteristic for whole blood was then used for the application in a novel approach named 'Extracted Blood Spot' (EBS) sampling. The evaluation of this approach was accomplished using two different analytical methods of LC–MS/MS and direct analysis in real time (DART)-MS/MS.

In order to address the requirement for simultaneous analysis of compounds from different classes and polarities, the development and the evaluation of biocompatible polystyrene-divinylbenzene-polyacrylonitrile (PS-DVB-PAN) and phenylboronic acid-polyacrylonitrile (PBA-PAN) coatings for the extraction of a wide polarity range of analytes from biological fluids were the focuses of the studies in Chapter 5. In Chapter 6 the method development and the application of the PS-DVB-PAN 96-blade SPME-LC–MS/MS system for the analysis of grape, berry, and wine samples are discussed, along with a comparison of SPME with the solvent extraction method. Finally, Chapter 7 summarizes the main research findings of the work presented in this thesis and makes recommendations for future consideration.

Chapter 2

Thin-film octadecyl-silica gel coating for automated 96-blade SPME coupled with LC–MS/MS

2.1 Preamble and introduction

2.1.1 Preamble

This chapter has been published as a paper: Fatemeh S. Mirnaghi, Maria Rowena N. Monton, Janusz Pawliszyn, Thin-Film Octadecyl-Silica Glass Coating for Automated 96-Blade SPME Coupled with LC–MS/MS for Analysis of Benzodiazepines, *J. Chromatogr. A*, 2012, 1246, 2-8. The materials of the current chapter are reprinted from this publication with the permission of Elsevier (Copyright Elsevier 2012). The contribution of co-author Maria Rowena Monton to the work described within this chapter was technical advice at the early stage of preparation of sol-gel coating. All of the work reported within this chapter has been performed solely by the author.

I, Maria Rowena Monton, authorize Fatemeh Mirnaghi to use the material for her thesis.

2.1.2 Introduction

The main reason for physical and chemical instability in most commercially available SPME coatings is the physical deposition of the coating on the SPME substrate which results in instability of the coating after several uses.[108-110] One solution to overcome this common

problem is the creation of chemical binding between the sorbent and the SPME substrate. Over the past twenty years, the development and application of sol-gel SPME coatings has increased to address this issue.[109, 111-116] The sol-gel process consists of three main steps: hydrolysis, condensation, and polycondensation reactions. The sol is a colloidal suspension which is developed through condensation reactions of the hydrolyzed products of a silicon alkoxide. The polycondensation of the sol promotes further cross-linking, resulting in a three-dimensional network, and leads to the creation of a gel. Eventually, evaporation of solvents in the gel during the drying step results in the final silica gel.[109, 117-119] The sol-gel process has been broadly utilized for the production of different types of products including bulk glasses and films,[109, 117-119] in which the latter is usually made by dipping or spinning methods.[120] As many studies have reported on the development and application of sol-gel based SPME coatings, the chemical bond between the silica gel sorbent and the metal SPME substrate significantly improves the thermal and mechanical stability of the coating.[109, 111-116]

Organically modified silica (ORMOSIL) is an organic-inorganic hybrid material which is also synthesized through the sol-gel process. For the preparation of ORMOSIL, a second precursor containing an organic substituent is added to the sol-gel reaction, which leads to the fabrication of a hybrid complex. The blending of an organosilicon with an inorganic precursor in ORMOSIL gel facilitates the development of unique functionalities to the final material.[109, 117-119, 121, 122] The properties of coatings made from ORMOSIL gel can be optimized through the selection of different sol-gel precursors, providing an appealing flexibility for analytical applications.[118, 119]

Depending on the type of sol-gel chemistry, the stability of the sol-gel coating in desorption solvents can be challenging in LC applications. Up to now, many sol-gel SPME

coatings have been developed and applied for thermal desorption in gas chromatography applications, but only a few sol-gel SPME coupled with LC applications have been reported for the solvent desorption of compounds.[93-95, 110]

The work completed for this chapter is focused on the development, optimization, and evaluation of high-quality octadecyl (C18)-silica gel coating with high chemical and physical stability and long-term reusability, which can be used in conjunction with an LC system. Referring to the required coating properties for automated 96-blade thin-film SPME systems (already discussed in Chapter 1) and owing to the above-mentioned characteristics of sol-gel based coatings, the main goal of this work was the development of thin-film C18-silica gel coatings for the 96-blade SPME system.

The sol-gel technique provides a chemically bonded extractive phase on the surface of stainless steel blades and presents a successful means of chemical immobilization for the SPME coating. This provides good mechanical and chemical stability and prevents instability and stripping of the coating in long-term use.

In addition, apart from the universal advantages of thin-film geometry for SPME coating (i.e., improvement in extraction sensitivity and rate, and shorter extraction time[35, 81]) the use of thin-film geometry in the sol-gel based SPME coating provides additional benefits. These benefits include preparation of uniform coatings, straightforward control of coating thickness, and minimal cracks and shrinkage during the silica gel coating preparation, which are the main challenges in the sol-gel process.[93]

In this study, octadecyl (C18) was selected as the stationary phase due to its extraction recovery for a wide range of compounds. Several variables such as the sol-gel aging time, the speed of the dipping step, drying atmosphere, and drying temperature were evaluated and

optimized for the preparation of high-quality C18-silica gel thin-film coatings. The performance of the C18-silica gel 96-blade SPME system, coupled with LC–MS/MS, was evaluated for high-throughput analysis of benzodiazepines (diazepam, nordiazepam, oxazepam, and lorazepam) from phosphate-buffered saline solution (PBS) and human plasma. Various factors such as reusability, reproducibility, and validity of the system were evaluated. This study can be used as a preliminary investigation for the preparation of other sol-gel derived thin-film SPME coatings with diverse properties and functionalities. The chemical structure of the benzodiazepines under study is demonstrated in Figure 2.1.

2.2 Experimental

2.2.1 Chemicals and materials

Tetraethyl orthosilicate (TEOS), sodium chloride, potassium chloride, potassium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma-Aldrich (MO, U.S.). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Caledon Labs (ON, Canada). Ethanol (HPLC grade) was purchased from Fisher Scientific (NJ, U.S.). N-Octadecyl triethoxysilane (C18-TEOS) was purchased from UCT Specialties LLC (PA, U.S.). Diazepam, lorazepam, oxazepam, nordiazepam, and diazepam-*d5* were purchased from Cerilliant (TX, U.S.) as 1 mg/mL standards in methanol with the exception of lorazepam, which was purchased as a 1 mg/mL standard in acetonitrile. Human plasma was purchased from Lampire Biological Laboratories (PA, U.S.). Aluminum oxide sand paper (220 grit) was purchased from Benchmark (ON, Canada). Polypropylene Nunc 96-well plates were purchased from VWR International (ON, Canada).

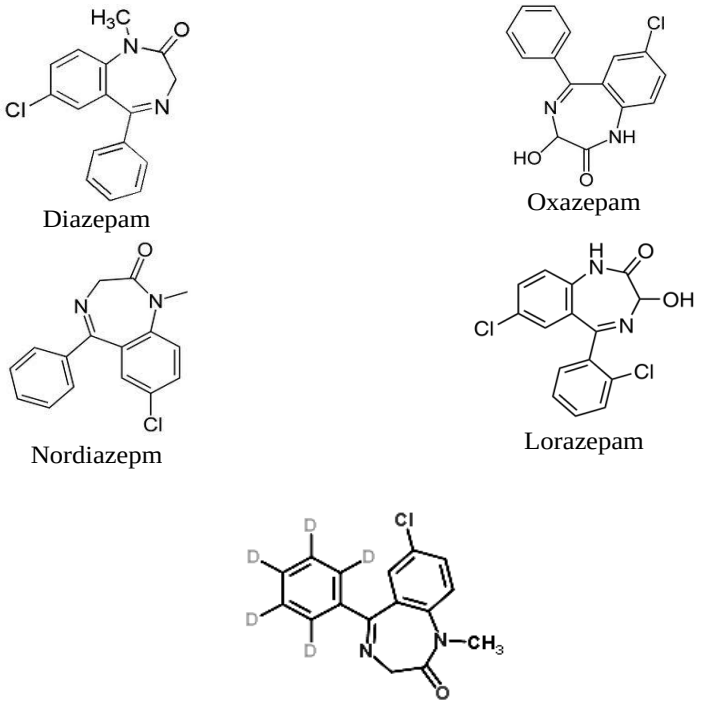


Figure 2.1 Chemical structure of diazepam, nordiazepam, oxazepam, lorazepam, and diazepam-*d5*

2.2.2 Preparation of standard solutions and samples

Working standards were prepared monthly from stock solutions using acetonitrile/water (50:50 v/v) as the diluent and were stored at 4 °C. To ensure good instrumental performance, quality control (QC) standards were injected at the beginning, end, and periodically throughout the sample run. For the instrumental calibration, the calibration range standards were injected in duplicate at the beginning and the end of each sample run.

Phosphate-buffered saline (PBS) solution was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate in 1 L of nanopure water with the pH adjusted to 7.4. The spiked PBS and plasma samples were prepared daily. The concentration of organic solvents in samples was always kept at less than 1%. After spiking the analytes in plasma, samples were incubated overnight to ensure drug-plasma protein binding equilibria was reached prior to extraction.

2.2.3 Liquid chromatography and mass spectrometry conditions

A Waters Symmetry Shield RP18 (Waters Associates, Inc., MA, U.S.) with 2.1 mm × 50 mm dimensions and a 5 μm particle size was used as the chromatographic column. An LC-10 AD Shimadzu high-pressure liquid chromatography system (Shimadzu, Kyoto, Japan) was used for the separation of compounds. Chromatographic conditions used for the separation of benzodiazepines were previously reported by Lord *et al*.[123] An API 3000 triple quadrupole mass spectrometer (Applied Biosystems, CA, U.S.) equipped with a TurboIonSpray source was used for quantitative analyses of the compounds.

A CTC PAL autoinjector from Leap Technologies (CTC Analytics, NC, U.S.) was used for the injection of 20-μl sample volumes into the LC–MS/MS system. The MS/MS analysis was performed in positive mode under multiple reaction monitoring (MRM) conditions and using instrument settings described in Table 2.1. The MS conditions were optimized at the following values: nebulizer gas = 12, curtain gas = 8, collision gas = 12 (arbitrary units), ionspray temperature = 450 °C, and ionspray voltage = 5300 V. Analyst 1.4.2 software was used for data acquisition and processing. When analyzing biological samples, a bypass pump and a switching valve (Waters Corporation, MA, U.S.) were used to

divert the flow of column effluent into the waste in the first minute of the chromatographic run time.

Table 2.1 Summary of MS/MS parameters for the analysis of benzodiazepines

Analytes	Q1 mass (amu)	Q3 mass (amu)	DP (V)	FP (V)	EP(V)	CE (V)	CXP (V)
Diazepam	285.1	154.1	100	270	13	37	10
Nordiazepam	271.1	140.1	90	165	15	40	11
Oxazepam	287.1	241.1	80	180	13	31	23
Lorazepam	321.0	275.1	90	210	9	30	20
Diazepam-*d5*	290.2	198.2	80	200	11	45	40

*DP=Declustering potential, FP=Focusing potential, EP=Entrance potential, CE=Collision energy, and CXP=Collision cell exit potential

2.2.4 Automated Concept 96-blade SPME system

The Concept 96-blade SPME device and autosampler were provided by Professional Analytical System (PAS) Technology (Magdala, Germany). The thin-film blades are made of stainless steel (1.4310 grade) with the following dimensions: length: 50 mm, width: 2.5 mm, depth: 0.7, and each blade set consists of 12 thin-film pins. The 96-blade SPME device is composed of eight rows of blade sets, holding together with the use of inter-blade holders, creating a 96-blade SPME system (Figure 1.3 (a)). The Concept 96-autosampler, which was used for this study, contains three integrated arms, three separate orbital agitators, and one static stage. The entire system is fully controlled by the Concept software.

The 96-well-plates can be fitted on top of the agitators and the static stage. One robotic arm is designed to automatically hold, move, and place the 96-blade SPME device into the 96-well-plates, enabling the performance of the steps of preconditioning, extraction,

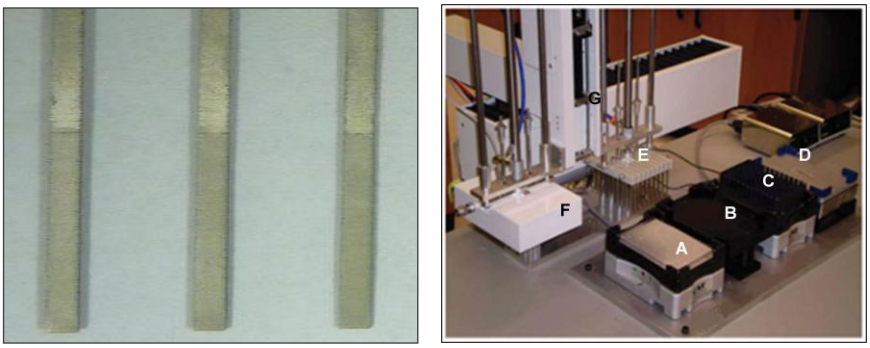
washing, and desorption. The orbital agitators are assigned to shake the 96-well-plates at a specific speed controlled with Concept software. The second arm is equipped with a nitrogen blow-down device in order to perform solvent evaporation and analyte pre-concentration in cases when enhanced sensitivity is important and reconstitutions and/or pre-concentration are required. Furthermore, the third arm is equipped with a syringe for dispensing a specific volume of the solvent into the individual wells of the 96-well-plate. In addition, the initial prototype of the Concept 96-autosampler has the functionality to inject the samples into the HPLC port for on-line chromatographic separation and analysis. However, the application of this feature resulted in a significant reduction of the sample throughput since simultaneous sample preparations and injections were not feasible. Therefore, the unit was operated as an off-line sample preparation station for high-throughput SPME analysis, and a separate commercially available HPLC autosampler was used to perform LC injections from the multi-well plate.

Figure 2.2 (a) demonstrates a close-up view of three optimized C18-silica gel coated blades, and Figure 2.2 (b) shows the Concept 96 autosampler from PAS Technology.

2.2.5 Preparation of C18-silica gel solution

The C18-silica gel was made using tetraethyl orthosilicate (TEOS) and n-octadecyl triethoxysilane (C18-TEOS) as precursors under hydrolytic conditions and acidic catalysis (hydrochloric acid (HCl)). The sol was prepared by mixing the appropriate amounts of reaction components (44.5: 19: 28: 8.5 % (v/v) for TEOS: C18-TEOS: ethanol: acidified water, 0.01% HCl) to obtain a C18-TEOS/TEOS molar ratio of 0.2.[113] The mixture was then aged for 3 h at 25 °C and 1000 rpm stirring speed. After 3 h of stirring, the coating

preparation was performed immediately in order to prevent any viscosity changes in the sol and to preserve the reproducibility of the final silica gel. The bare silica gel was prepared in absence of C18-TEOS in the sol mixture (63.5: 28: 8.5 % (v/v) for TEOS: ethanol: acidified water, 0.01% HCl), and all subsequent steps of the coating preparation followed the same procedure as the C18-silica gel coating.



(a) **(b)**

Figure 2.2 (a) Close-up view of three optimized C18-silica gel coated blades (b) The Concept 96-autosampler and its labeled components: A, C, and, D are orbital agitators for extraction, desorption, and conditioning, respectively. B is the wash station. E is the 96-blade device. F is the nitrogen evaporation device. G is the syringe arm.

2.2.6 Preparation of C18-silica gel 96-blade SPME coating

In order to obtain effective immobilization and chemical binding of the silica gel coating to the SPME stainless steel substrate, the surface of the thin-film stainless steel blades needed to be treated prior to the coating immobilization. First, the steel blades were polished with sand paper and cleaned by sonicating in acetone for 10 min. Then, the stainless steel blades were oxidized through 1 h of sonication in concentrated hydrochloric acid. In the next step the steel blades were immediately washed with plenty of water, rinsed with nanopure water, and dried in an oven for 1 h at 150 °C. After cooling to room temperature,

the dipping method was used to coat 2 cm of the length of the blades using the prepared sol. For the coating preparation, the blades were immersed in the dipping solution and were withdrawn vertically at a 90° angle.

In order to attain the proper film thickness without any shrinkage or cracking, the speed of the coating withdrawal was optimized to 1 mm/s. The prepared coatings were then immediately dried for 1 h at room temperature in a desiccator under running nitrogen. Then, the coatings were dried in an oven using a temperature gradient starting at 40 °C (kept for 1 h) and increased at a rate of 0.5 °C/min to 150 °C (for 5 h). After cooling the coatings to room temperature and before initial use, the C18-silica gel thin-film coatings were preconditioned overnight in methanol/water (1:1 v/v) prior to the first use.

2.2.7 Automated 96-blade SPME procedure

Prior to each extraction, a 30 min preconditioning of the C18-silica gel coating in methanol/water 1:1 v/v (with agitation) was required. After preconditioning, a precisely controlled volume (1.5 mL) of the sample was used for 40 min equilibrium extraction at an agitation speed of 1000 rpm (2.5 mm amplitude). In order to remove the attached salts, proteins, and biological particulates onto the coating surface, a 15 s static washing step in water was performed after extraction from human plasma. Then, desorption of analytes was performed in 1.5 mL of acetonitrile/water 1:1 v/v solvent (spiked with 10 ppb diazepam-*d5* as an internal standard) for 40 min at an agitation speed of 1200 rpm (1 mm amplitude). The carryover was evaluated by performing a second desorption using fresh portions of acetonitrile/water 1:1 v/v solvent. Finally, the 96-well plate containing the final extract was transferred to the LC–MS/MS system for separation and quantitation. Each step

(preconditioning, extraction, washing, and desorption) was performed automatically using the Concept autosampler and software with pre-set time and agitation speeds.

2.2.8 Data analysis and calculations

The isotopic label calibration was drawn by plotting the peak area ratio of the analyte to internal standard versus analyte concentration. The unknown sample concentrations were calculated from the equation y = mx + b, as determined by the standard line. In order to ensure the accuracy of low concentration standards, 1/y linear weighted regression was used for the calibration. Percent absolute recovery or extraction recovery was calculated by a ratio of the amount (ng) of analyte extracted by the SPME coating versus the total amount (ng) of analyte originally present in the spiked sample, multiplied by 100. Percent carryover was determined by dividing the ng extracted from the second desorption with the total ng extracted from the first and second desorptions, multiplied by 100.

2.3 Results and discussion

2.3.1 Sol-gel reaction

The sol-gel is a process in which a solution or sol goes through a sol-gel transition**.** As a specific example of the silica sol-gel process, an alkoxysilane (TEOS and C18-TEOS in this study), undergoes hydrolysis, condensation, and polycondensation reactions in the presence of ethanol and water, and under acidic or basic conditions (acidic conditions in this study). Finally, sol-gel polymerization yields a network of Si-O-Si chemical linkage and the gel is processed by drying. Hydrolysis and condensation reactions of most metal alkoxides have an extremely fast rate and can be carried out without a catalyst, but the reactions of

alkoxysilanes proceed more slowly, requiring the addition of either an acidic or basic catalyst.[117] The schematic illustration of a silica sol-gel process is shown in Figure 2.3.

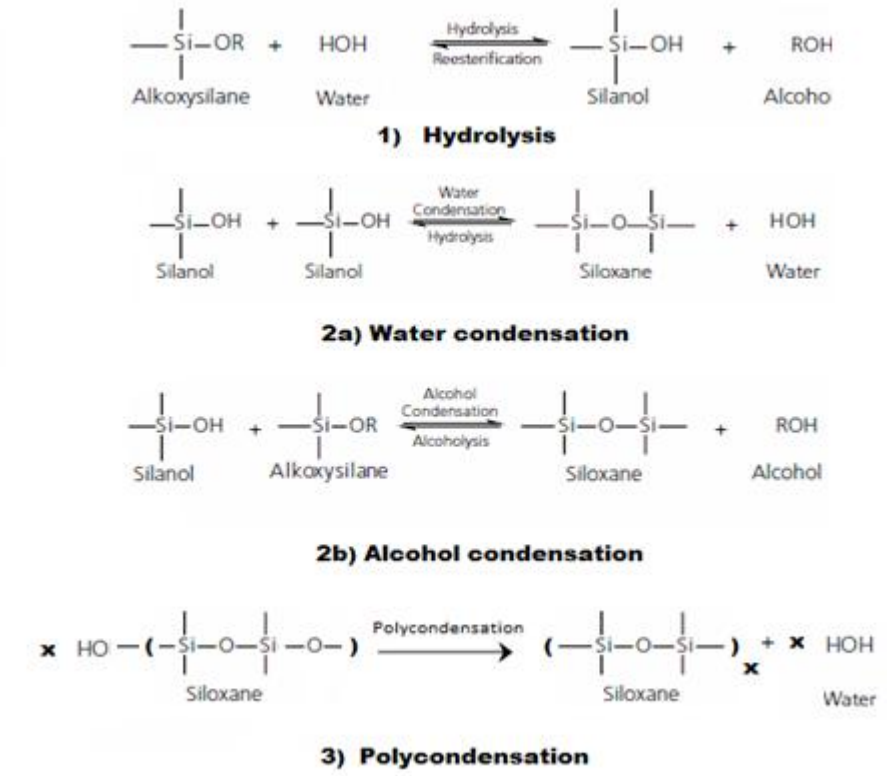


Figure 2.3 Schematic illustration of the sol-gel process. Figure reproduced from reference with the permission of the publisher.[125]

2.3.2 Binding the silica gel coating to the metal substrate

Binding the C18-silica gel coating to the stainless steel substrates involves the creation of a -M-O-Si- chemical bond between the oxidized metal substrate and the silicon oxide in the coating film.[124] The schematic illustration of the chemical binding of silica gel coating to the metal substrate is shown in Figure 2.4

Figure 2.4 Schematic illustration of the chemical binding of the silica gel coating to the metal substrate. Figure reproduced from reference with the permission of the publisher.[126]

2.3.3 Cracking and shrinkage

As reported in many studies, one of the most challenging issues in the preparation of sol-gels is the creation of undesirable shrinkage and cracking, which are driven through capillary forces and originate from solvent evaporation during the drying of the gel.[93, 118] As Figure 2.5 shows, the preparation of a thin-film silica gel coating without the optimization of the parameters is not exceptional and it also results in shrinkage and cracks on the coating. Since the main goal of this study is the development of a high quality C18-silica gel thin-film SPME coating with improved stability and reusability, it is vital to overcome the challenging limitations of the cracks and shrinkage in the thin-film silica gel coating. The results of this study show that shrinkage and cracking of sol-gel thin-film are controllable through the optimization of the thickness of thin-film coating and drying conditions.

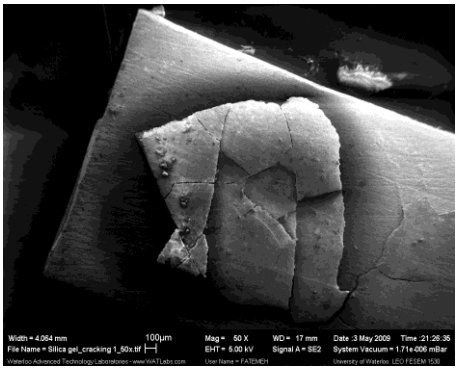


Figure 2.5 Typical cracks and shrinkage in thin-film silica gel coating without the optimization of the thickness of thin-film coating and drying conditions

2.3.4 The critical thickness of the silica gel thin-film

The critical thickness is ~1 µm for inorganic sol-gels and as high as 10 µm for ORMOSIL gels. The problem of shrinkage and cracking is more significant for sol-gel films thicker than the critical thickness. Usually, the organic-inorganic hybrid films (ORMOSIL) lead to less shrinkage and lower rigidity of the film as compared to inorganic sol-gels. This result is achieved because of the presence of organic materials in the organic-inorganic hybrid gel that provide stress relaxation through plastic deformation.[109, 110, 124] Because the ORMOSIL gel has been used as an SPME coating in this work, it is critical that the thickness of the coating be maintained as low as 10 µm to obtain structural integrity and high-quality thin-film silica gel without shrinkage or cracking. Even though the application of a very thin SPME coating may result in a low extraction volume (V_f) and, consequently, leads to lower extraction recovery (Equation 1.1), it is preferable to obtain high-quality thin coatings with a lower extraction recovery rather than low-quality thick coatings with a larger extraction recovery.

The thickness of sol-gel thin-film is controlled via two main factors: the viscosity of the sol-gel solution and the withdrawal speed of the dipping step in the coating preparation procedure.[93, 124] The coating thickness increases with the withdrawal speed and viscosity at approximately $\eta v^{2/3}$, where η represents the viscosity of the solution and v represents the withdrawal speed.[124] The results show that the viscosity of the C18-silica gel itself is a function of the sol-gel composition (C18-TEOS/TEOS ratio) and the aging time of the sol, which are individually described in the following sections.

2.3.5 Optimization of C18-TEOS/TEOS ratio

The ratio of C18-TEOS/TEOS is one of the contributing factors to the viscosity of the sol, and consequently the thickness of the silica gel coating. Three different ratios of C18-TEOS/TEOS (i.e., 0.2, 0.3, and 0.5) were evaluated for the preparation of C18-silica gel coating. As expected, the results showed that the sols made with C18-TEOS/TEOS ratios of 0.3 and 0.5 were more viscous and resulted in thicker gels that led to considerable cracking and shrinkage of the coating. In contrast, sols made with a C18-TEOS/TEOS ratio of 0.2 resulted in optimal viscosities to produce a coating with thickness lower than the critical thickness and a final silica gel free from cracks and shrinkage. Therefore, the C18-TEOS/TEOS ratio of 0.2 was considered the optimum ratio for sol-gel coating preparation in this study.

2.3.6 Optimization of sol-gel aging time

Sol-gel aging time is another contributing factor to the viscosity of the sol and thickness of the silica gel coating and plays an important role in the characteristics and the quality of the final product. Insufficient aging times cause inadequate polycondensation of

the sol which adversely influences the coating properties. On the other hand, long aging times result in a greater extent of polycondensation and cross-linking of the sol, which finally ends up with the transition to a rigid gel. As the viscosity of the sol exceeds a critical limit, it leads to the coatings which are thicker than the critical thickness and cause cracks and shrinkages. So, it is very important that the aging time be properly optimized.

The results show that for the preparation of the C18-silica gel a minimum of 3 h aging time is required for sufficient polycondensation of the sol, and aging times longer than 6 h result in a cross-linked gel which is too viscous to be used for coating preparation. Therefore, three aging times of 3, 4, and 4.5 h were evaluated. The aging time of 3 h produced the most high-quality coating with no evidence of shrinkage. In contrast, aging times of 4 and 4.5 h resulted in coatings which exceeded the critical thickness with apparent cracks and shrinkage on the surface. Therefore, 3 h was used as the optimum aging time for the rest of the study.

2.3.7 Optimization of withdrawal conditions

The speed of withdrawal of the substrate from the solution during the dip-coating procedure is another significant factor that affects the thickness of the coating. In this work, a range of withdrawal speeds (0.5–10 mm/s) was studied, the results of which indicate that the speed of the withdrawal is very critical to the quality of the coating. A withdrawal speed of >1 mm/s results in a much thicker coating with surface cracks and shrinkage, but withdrawal speeds of ≤1 mm/s resulted in higher quality coatings free of cracks or shrinkage. The withdrawal speed of 1 mm/s was optimum both in terms of coating quality and extraction recovery (thicker than those made with speeds of <1 mm/s) and was used for the remainder of the study.

In addition, the angle of the withdrawal can affect the thickness of the coating. In order to achieve an even layer of thickness on both sides of the thin-film substrate, the substrate is dipped into the solution and is drawn up vertically at 90°.[124] Any variance from 90° may cause variation in thickness of the coating from one side of the substrate to the other.

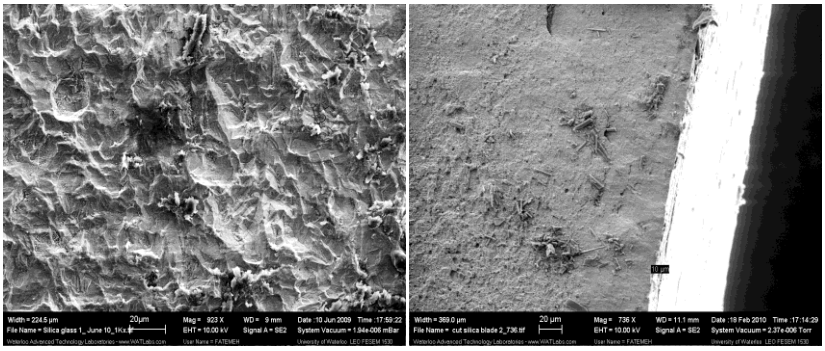
2.3.8 Optimization of drying conditions

The origin of the shrinkage and cracking comes from the drying process. Therefore, one of the solutions to prevent this limitation and to obtain a high-quality silica gel thin-film is to optimize the drying conditions. According to the literature, the formation of cracks is more likely to occur when the silica gel is completely dried at room temperature or when the temperature is lower than at which the bonds between the thin-film gel and the substrate are formed.[124] However, the results of this thesis show that the immediate and complete drying of the coatings at high temperatures can also cause cracks and shrinkage.

In this work various drying conditions were evaluated and the best quality silica gel films were obtained when the coating was immediately dried for 1 h under running nitrogen flow at room temperature and then transferred to an oven for complete drying. It is assumed that drying of the sol-gel coating in a neutral atmosphere at room temperature prevents boiling of the ethanol and water present in the gel (which usually occurs at high temperatures): this method minimizes the formation of the fractures and cracking in the coatings. Next, a temperature gradient was used for complete drying of the silica gel thin-film SPME coating (started at 40 °C, maintained for 1 h, then increased to 150 °C at a rate of 0.5 °C/min, and kept for 5 h).

2.3.9 Evaluation of surface topography and thickness of the coating

The surface topography and the thickness of the optimized C18-silica thin-film sol-gel coating was evaluated using scanning electron microscopy (SEM). Prior to taking the SEM images, a 10 nm layer of gold was deposited on the surface of the coating. The SEM images were obtained using LEO 1530 field emission SEM (Carl Zeiss NTS GmbH, Germany). As shown in Figure 2.6 (a) the SEM images indicate that the flow of nitrogen over the surface of the coating during the initial drying step creates a rough and wrinkled coating surface, resulting in an improved surface area of the coating. The average thickness of the optimized C18-silica gel thin-film coating is estimated at about 10 μm, as illustrated in Figure 2.6 (b).



(a) **(b)**

Figure 2.6 SEM images of the C18-silica gel thin-film. (a) Surface morphology of the optimized C18-silica gel thin-film (dried at room temperature under N_2 followed by complete drying under a temperature gradient in an oven) using 923 × magnification. (b) Estimation of the coating thickness of the optimized C18-silica gel thin-film using 736 × magnification.

2.3.10 Optimization of the C18-silica gel 96-blade SPME system

The automated optimization of all SPME steps, (i.e., preconditioning, extraction, washing, and desorption) was performed using the Concept autosampler. The optimization of

the SPME conditions was performed using the optimized C18-silica gel thin-film coating (C18-TEOS/TEOS = 0.2, aging time = 3 h, withdrawal speed = 1 mm/s, and drying 1 h under nitrogen at 25 °C followed by a gradient temperature: 40-150 °C).

Preconditioning: Prior to each extraction, the C18-silica gel coatings were preconditioned in methanol/water 50:50 (v/v) to properly activate (swell) the silica gel coatings and to achieve the efficient partitioning of the analytes into the extractive phase.

Extraction: In order to evaluate and monitor the extraction recovery of the coating and to obtain the maximum sensitivity of the system, all experiments were performed at equilibrium extraction. The evaluation of extraction time profiles of the benzodiazepines under study was performed in PBS and human plasma matrixes, and no significant difference was observed between the two profiles for all four analytes. Using the C18-silica gel 96-blade SPME system, a 40 min extraction time was required to reach equilibrium for all four compounds and applied for the rest of the study. However, this time of analysis can be significantly decreased using pre-equilibrium extraction: owing to the computer-controlled timing and high precision of the automated 96-blade SPME system, pre-equilibrium extraction can be precisely performed without any loss of accuracy. The extraction time profile for all four benzodiazepines from PBS is illustrated in Figure 2.7.

A 1.5 mL volume of the sample was used for the extraction and this volume was carefully controlled throughout the study. The agitation speed was optimized based on the highest applicable speed resulting in optimum extraction recovery without a chance for spilling or cross contamination of the samples. Using the agitator with the amplitude of 2.5 mm for extraction, the optimal agitation speed was 1000 rpm.

Wash: After extraction and before transferring the blades to the desorption solvent, a fast wash step in water is required to remove loosely attached particles and salts from the coating surface. For this study a 15 s wash step (immersion of the blades in 1.5 mL of nanopure water pre-filled in the wells of a 96-well plate) was optimized to obtain efficient rinsing of the coating with a minimal loss of analytes. The possibility of the analytes' loss during the wash step was evaluated by analyzing the wash solution and the amount was determined to be <1% for all four benzodiazepines.

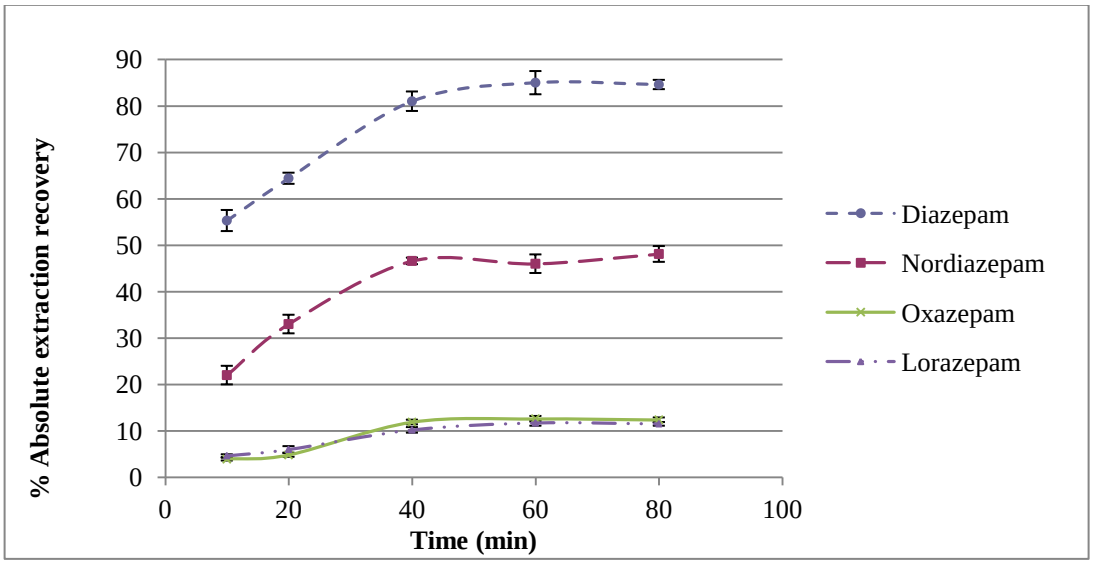


Figure 2.7 Extraction time profile for the extraction of benzodiazepines from PBS (pH = 7.4) using the C18-silica gel 96-blade SPME system. The extraction of 50 ng/mL benzodiazepines, extraction speed = 1000 rpm, desorption time = 40 min, and desorption speed = 1200 rpm.

Desorption: Desorption conditions including desorption solvent, time, speed, and volume was precisely optimized. The desorption solvent was selected to be compatible with the composition of the mobile phase in order to achieve proper chromatographic peak shape. Three different desorption solvents including acetonitrile/water 50:50 (v/v), methanol/water

50:50 (v/v), and acetonitrile/water 80:20 (v/v) were used for solvent optimization. The results showed that acetonitrile/water 50:50 (v/v) resulted in the best recovery and lowest carryover. The evaluation of desorption time and speed demonstrated that a minimum of 40 min desorption time and 1200 rpm (1 mm amplitude) agitation speed provided the best recovery and minimal carryover (< 1% for all four benzodiazepines). A desorption time of longer than 40 min did not provide any improvement in the amount of recovery. A minimum of 1.5 mL of desorption solvent was required to cover the coating for efficient recovery. Using an agitator with 1 mm amplitude, the agitation speeds larger than 1200 rpm caused spilling and cross contamination of the samples. A speed of 1200 rpm was chosen to achieve the highest level of efficiency.

In order to remove the trace of analytes remaining from the previous extraction and to prepare the coating for the next use, a second 30 min desorption step, called a "carryover" was used prior to the next extraction. The efficacy of the 30 min carryover step for cleaning the trace analytes (< 1%) was evaluated by analysis of the third desorption. No trace amounts of analytes were detected for the third desorption, indicating that a second 30 min desorption was sufficient to clean the coating. In order to compensate for any possible variation in the injection volume, and the ionization and detection of the analytes, 10 ng/mL of diazepam-*d5* was added as an internal standard to all the samples and the desorption solvents.

2.3.11 Evaluation of the extraction recovery of C18-silica gel 96-blade SPME coating

The absolute recoveries for the extraction of diazepam, nordiazepam, oxazepam, and lorazepam from PBS and human plasma matrixes are shown in Table 2.2. The results indicate that the C18-silica gel coating appears to have higher selectivity for non-polar compounds

(i.e., diazepam with log of octanol-water partition coefficient (log P) = 2.8 and nordiazepam with log P = 2.7) rather than more polar ones. However, by selecting the proper precursors in the sol-gel reaction, the optimized coating preparation procedure can also be used to prepare other types of thin-film silica gel coatings for the extraction of more hydrophilic compounds. Since there is a high binding affinity between benzodiazepines and plasma proteins,[127] when extracting from plasma only a small concentration of analyte remains unbound (free) to be extracted with SPME coating.[35] Therefore, a significant difference in the amount of absolute recoveries from PBS and human plasma can be observed.

Table 2.2 Percent of absolute recovery for the extraction of selected benzodiazepines from PBS and human plasma using the C18-silica gel 96-blade SPME-LC–MS/MS system.

Analytes	PBS	Human plasma
Diazepam	83 ± 3.5	2.5 ± 0.20
Nordiazepam	48 ± 1.5	1.5 ± 0.15
Oxazepam	11 ± 1.2	0.55 ± 0.05
Lorazepam	11 ± 1.1	1.7± 0.18

The extraction volume (V_f), distribution constant (K_{fs}), and fiber constant ($K_{fs.}V_f$) of the thin-film C18-silica gel 96-blade SPME coating for extracting benzodiazepines from PBS is illustrated in Table 2.3. The volume of the thin-film blade coatings was determined based on the width ($w = 2.5\ mm$) and depth ($d = 0.7\ mm$) of the blade substrates and the length ($l = 20\ mm$) and thickness ($b = 10\ \mu m$) of the coating, and were calculated based on Equation 2.1:

$$V_f = 2[lb\ (w+2b)] + 2[lb\ (d+2b)] + [b\ (d+2b)\ (w+2b)] \quad \text{Equation 2 .1}$$

Through reducing the volume of desorption solvent and/or the evaporation of the final extract, further concentration of analytes in the final extract can be obtained.

Table 2.3 Evaluation of thin-film C18-silica gel coating parameters when extracting 100 ng/mL benzodiazepines spiked in 1.5 mL PBS.

	Diazepam	*Nordiazepam*	*Oxazepam*	*Lorazepam*
%Absolute recovery	83	48	11	11
Coating volume (V_f), mL	1.30×10^{-3}	1.30×10^{-3}	1.30×10^{-3}	1.30×10^{-3}
Distribution constant (K_{fs})	56.33×10^{2}	10.65×10^{2}	1.42×10^{2}	1.42×10^{2}
Fiber constant ($K_{fs}.V_f$), mL	7.32	1.38	0.18	0.18

2.3.12 Evaluation of non-specific extraction of silica gel coating

The non-specific extraction of sol-gel coating was studied through evaluating the extraction recovery for a bare silica gel coating prepared in the absence of the extractive precursor (C18-TEOS). The results demonstrate that the amount of non-specific extraction was <0.05% for all four benzodiazepines.

2.3.13 Evaluation of the stability and reusability of the C18-silica gel 96-blade SPME coating

The chemical and physical stability and reusability of the C18-silica gel 96-blade SPME coating were evaluated. For evaluating the chemical stability, three sets of coatings were soaked in pure ethanol, methanol, and acetonitrile overnight, and their physical appearance and extraction recovery were compared before and after exposure to the solvents. The results of the evaluation showed no difference in the coating properties before and after long-term organic solvent exposure, indicating the high chemical stability of the C18-silica

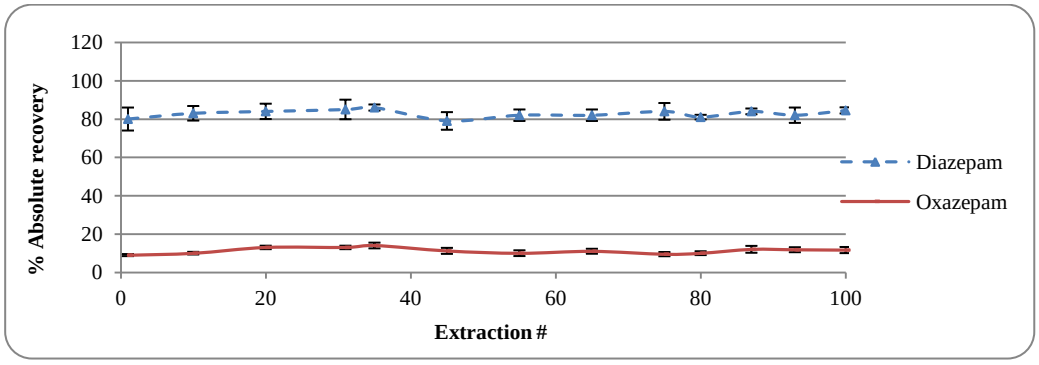
gel 96-blade SPME coating. For testing the physical stability, reproducibility, and reusability of the system, a set of C18-silica gel coatings were used for 100 sequential extractions from PBS. The coatings displayed reproducible extraction recovery for at least 100 uses in PBS (3% RSD for diazepam and 11% RSD for oxazepam for n = 13 experiments and n = 12 coatings) with no changes in physical appearance, indicating high physical and chemical stability, reproducibility, and reusability of the C18-silica gel coatings. Figure 2.8 (a) illustrates the evaluation of the extraction recovery and reproducibility of the coating for 100 times extraction from PBS.

It is assumed that the high chemical and mechanical stability of C18-silica gel thin-film coating is due to the chemical -M-O-Si- bond between the metal substrate and the sol-gel coating. The reusability of the coating was also evaluated for human plasma matrix, and the results (Figure 2.8 (b)) showed good physical stability and reproducible recovery for at least 20 extractions (8% RSD for diazepam and 11% RSD for oxazepam for n = 5 experiments and n = 12 coatings). After twenty extractions the coating was still presenting reproducible extraction recovery; however, due to the complexity of the biological matrix in human plasma some physical changes were observed on the surface of the coating.

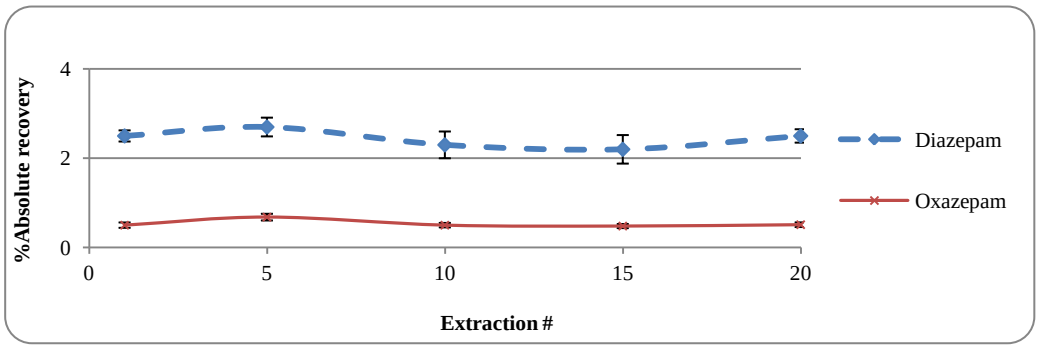
It is assumed that the observed physical changes are possibly due to the adsorption of plasma proteins to the untreated silanol sites on the C18-silica gel coating, especially because extraction and desorption are performed at neutral pH. In future studies the long-term biocompatibility of the C18-silica gel coating in a biological matrix such as human plasma might be improved by end-capping the silanol groups.

The results show that the washing step after extraction is a critical factor in preserving the stability of the coating in the human plasma matrix. As a preliminary assessment, the

stability of the C18-silica gel coating in a whole blood matrix was also evaluated for three trials, and no red blood cell (RBC) attachment was observed. Since most particle-based SPME coatings suffer from a lack of biocompatibility and reusability in whole blood due to RBC attachment, whole blood stability is one of the principal advantages of the silica gel coating.



(a)



(b)

Figure 2.8 Reusability of the C18-silica 96-blade SPME system for equilibrium extraction of (a) 50 ng/mL diazepam and oxazepam from PBS (n = 12) and (b) 100 ng/mL diazepam and oxazepam from plasma.

2.3.14 Inter- and intra-blade reproducibility of the C18-silica gel 96-blade SPME coatings

The inter-blade relative standard deviation (RSD) of the coating was investigated to evaluate the reproducibility of the dipping process and to study the variations between the extraction recoveries of different coated blades. In addition, in order to inspect the reproducibility of the extraction recovery of the individual C18-silica gel 96-blade SPME coatings and the entire SPME-LC–MS/MS method, the intra-blade standard deviation was tested. The results of the evaluation of inter-blade and intra-blade relative standard deviation of the system for the extraction of benzodiazepines from PBS and human plasma are reported in Table 2.4. The reproducibility of the system can be further improved through the automation of the dip-coating process.

Table 2.4 Inter- and intra-blade reproducibility of C18-silica gel 96-blade SPME coating for three extractions and n = 12 (50 ng/mL of benzodiazepines for PBS extraction and 300 ng/mL of benzodiazepines for human plasma extraction).

Extraction matrix	Diazepam	Nordiazepam	Oxazepam	Lorazepam
PBS inter-blade RSD (%)	3.8	5.6	7.4	8.5
PBS intra-blade RSD (%)	3.5	5.3	9.1	7.2
Plasma inter-blade RSD (%)	8.8	9.8	9.7	10.6
Plasma intra-blade RSD (%)	8.3	9.1	13.0	12.2

2.3.15 Limits of detection and quantitation

For the entire study of this thesis, the limit of detection (LOD) was calculated based on 3×S/N (signal to noise), and the limit of quantitation (LOQ) was calculated based on

10×S/N. The signal to noise ratio was determined manually on chromatogram printout and was confirmed based on four replicated analyses at the LOD and LOQ levels. The LOD of C18-silica 96-blade SPME-LC–MS/MS for the extraction of all four benzodiazepines from human plasma was estimated to be in the range of 0.4–0.7 ng/mL, and the LOQ was in the range of 1.5–2.5 ng/mL. The lowest and highest LOD and LOQ were obtained for the analysis of diazepam and oxazepam, respectively. The method displayed a good linear response with a linear regression coefficient in the rage of 0.987-0.996 for all four compounds.

2.4 Conclusions and future directions

The incorporation of thin-film geometry and the optimized conditions for the preparation of sol-gel coatings has facilitated effective control of the coating thickness and minimized the occurrence of cracks and fractures during the drying process. As a result, a sol-gel extractive phase is obtained with high physical and chemical stability and improved reusability; these are the most important properties of the sol-gel as it relates to SPME-LC applications. High chemical stability of the C18-silica gel 96-blade SPME coating overcomes the challenge of stabilizing the sol-gel coating in desorption solvents, and facilitates new applications for the sol-gel based SPME-LC system for the extraction of non-volatile and semi-volatile compounds. The C18-silica gel 96-blade SPME system allows for simultaneous analysis of up to 96 samples, automatically.

The high-throughput format is time efficient, requiring approximately 1.5 min analysis time per sample. Taking advantage of the good precision of the automated system, this analysis time can be reduced even further via pre-equilibrium extraction. The open-bed C18-

silica gel 96-blade SPME system can be applied for the analysis of complex matrixes containing particulate matters (i.e., biological tissue, food homogenates, and suspensions) without any need for sample pre-treatment. The proposed system has the potential to be applied to high-throughput analysis in different areas of science including biological, pharmaceutical, environmental, food, and clinical studies.

The optimization of C18-silica gel SPME coating provides valuable insights into the critical aspects of fabricating high-quality sol-gel-based coatings in thin-film format. The incorporation of optimized parameters for the preparation of high quality silica gel coating facilitates the preparation of other sol-gel-derived coatings with different properties (using different types of precursors). Future applications of this study include the preparation of sol-gel coatings containing entrapped biomolecules to be used for diverse applications such as sample preparation (as outlined in the present work), or for solid-phase assays such as drug-protein binding studies (using protein-doped thin-films).

2.5 Addendum

The text of this chapter was rewritten in comparison to the published research article.

Chapter 3

Optimization of the coating procedure for high-throughput 96-blade solid phase microextraction system coupled with LC–MS/MS

3.1 Preamble and introduction

3.1.1 Preamble

This chapter has been published as a paper: Fatemeh S. Mirnaghi, Yong Chen, Leonard M. Sidisky, Janusz Pawliszyn, Optimization of the Coating Procedure for High-Throughput 96-Blade Solid Phase Microextraction System Coupled with LC–MS/MS for Analysis of Complex Samples, *Anal. Chem*. 2011, 83, 6018-6025. The materials of the current chapter are reprinted from this publication with the permission of the American Chemical Society (Copyright 2011 American Chemical Society). The contributions of Yong Chen and Leonard M. Sidisky were the preparation of three sets of coatings which were used in one part of this study for evaluation of the coating preparation procedure.

I, Yong Chen, authorize Fatemeh Mirnaghi to use the material for her thesis.

I, Leonard M. Sidisky, authorize Fatemeh Mirnaghi to use the material for her thesis.

3.1.2 Introduction

During the last two decades, the high selectivity and sensitivity of LC–MS/MS compared to other techniques has led to its widespread application in the quantitative

determination of drugs and metabolites in different biological samples.[128-131] However, the direct introduction of samples to the LC–MS/MS without sample pre-treatment is very challenging and can result in critical limitations (Section 1.1, Chapter 1). The necessity of the application of sample preparation techniques prior to LC–MS/MS analysis has been addressed through the introduction of different methods. However, in spite of the application of sample preparation techniques, a reliable and accurate determination of analytes in very complex matrixes, such as plasma and whole blood, is still challenging. Many reports have indicated that the matrix effect phenomenon (ion suppression or enhancement) is more likely to be observed with the application of less selective sample preparation techniques.[132-135] As a result, the choice of an appropriate sample preparation technique is critical for the effective sample clean-up and isolation of analytes from complex matrixes.

SPME is an equilibrium-based sample preparation technique. The clean-up in SPME-LC is performed by selective extraction of analytes that have a high affinity toward the extractive phase, followed by liquid desorption of extracted analytes in a desorption solvent. Theoretically, SPME can provide cleaner sample extracts than PPT and LLE and equal or better sample clean-up than that obtained by SPE. This superiority is a result of the use of a small volume of sorbent while the absolute amounts of analytes of interest and potential interferences extracted by SPME are much smaller and depend on the magnitude of their distribution constant. In fiber/thin-film geometry of SPME, the sample is exposed to an open-bed coating, addressing the limitations of clogging and/or contamination of the extractive phase that is common in conventional packed-bed systems.

However, in open-bed extractive phases the adhesion of macromolecules such as particulate and proteins to the coating surface can become problematic. The adsorbed

macromolecules can act as a diffusion barrier, which influences the kinetics of extraction and affects the amount of analytes extracted by the coating.[79] In addition, it may affect the recovery of the coating for the subsequent extractions and could limit the reusability of the coating. One of the solutions to dealing with the issues involved in open-bed extractive phases is the application of biocompatible coatings. The application of biocompatible coatings can help to minimize the attachment of the macromolecules on the surface of open-bed SPME coatings and to preserve the efficiency of the coating.

In SPME, 'biocompatibility' can be understood to mean two different terms: (i) prevention of adverse and/or toxic reactions in the living system (which is relevant for *in vivo* applications), and (ii) reduction of the adhesion of biological molecules such as proteins or blood cells to the surface of the coating (applicable in both *in vivo* and *in vitro* applications).[79] Application of biocompatible coatings can preserve the efficiency of the coating via minimizing the attachment of macromolecules on the surface of the SPME coating. To date, several studies have been reported on the development and evaluation of biocompatible SPME coatings for *in vivo* and *in vitro* applications using biocompatible polymers. The development of biocompatible SPME coatings can be obtained via the application of biocompatible extractive phases (e.g., PDMS,[136] polypyrrole,[137, 138] and the coatings based on restricted access materials[101, 102]) or modification of the conventional SPME coatings with biocompatible polymers such as polyacrylonitrile[104] and poly(ethylene glycol)[139]. The former type benefits from the development of an unlimited number of biocompatible coatings via the modification of available SPME extractive phases with biocompatible polymers.

The choice of a proper biocompatible polymer for the development of a stable and long-lasting SPME coating can be challenging. Polyacrylonitrile (PAN) is a biocompatible polymer that is used extensively in biomedical studies such as dialysis and ultrafiltration and has shown good biocompatible properties in different applications.[140, 141] Most importantly, in addition to its biocompatible characteristics, PAN can act as a chemically and mechanically stable glue. These properties make PAN a perfect choice for use as a binder for the immobilization of particles and a biocompatible phase for minimizing the adhesion of macromolecules on the surface.

A previous study demonstrated the application of PAN as a biocompatible binder for the preparation of SPME coatings via a dipping method, and has proved the biocompatibility of the coatings by X-ray photoelectron spectroscopy.[104] Since the main goal of this thesis is the preparation of the coating with biocompatibility, high stability, and log-term reusability for the automated 96-blade SPME system, PAN was chosen as the binder for immobilizing the stationary phase on the substrate. The C18 silica-based stationary phase was chosen for the extraction phase because of its capability and applicability for efficient extraction of a large variety of analytes. The main focus of the work described in this chapter is the evaluation of different coating preparation procedures in order to achieve an optimum coating strategy for the 96-blade SPME system. Three different methods, namely dipping, brush painting, and spraying were evaluated, and the spraying method was established as optimum in terms of stability and reusability.

The performance of the coating prepared with the optimized preparation method (spraying) was evaluated for high-throughput analysis of benzodiazepines (diazepam, lorazepam, oxazepam, nordiazepam) from phosphate-buffered saline solution (PBS) and

human plasma. Reusability, reproducibility, pH stability, and reliability of the C18-PAN 96-blade SPME system were evaluated. The structures of the analytes under study are shown in Figure 2.1.

3.2 Experimental

3.2.1 Chemicals and materials

Polyacrylonitrile, trichloroacetic acid, sodium hydroxide, and a flask-type sprayer were purchased from Sigma-Aldrich (MO, U.S.). N, N-Dimethylformamide (DMF) was purchased from Caledon Labs (ON, Canada). Discovery silica-based-C18 particles (5 µm) were obtained from Supelco (PA, U.S.). The remainder of the materials was purchased from the same sources reported in Chapter 2 (Section 2.2.1).

3.2.2 Preparation of standard solutions and samples

The information for this section was described in Chapter 2 (Section 2.2.2).

3.2.3 Coating procedures for the preparation of C18-PAN 96-blade SPME coating

Surface treatment of the stainless steel blade was performed through sonication of individual sets of blades with concentrated hydrochloric acid for about 60 min. The blades were then washed thoroughly and rinsed with nanopure water. Then, they were dried at 150 °C for 30 min, and finally cooled to room temperature. The blades were coated using three different methods: spraying, dipping, and brush painting. In all three cases the C18 particles were immobilized on the surface of the stainless steel blades using biocompatible PAN glue. Since a previous study showed that 10% w/w PAN in DMF resulted in optimum properties of the required glue,[104] the same combination was used for the preparation of PAN glue.

Then, the PAN-DMF mixture was heated in the oven at 90 °C for 60 min until a yellowish clear solution was obtained (PAN does not dissolve in DMF at room temperature). The mixture was then cooled to room temperature and C18 particles were added (20% of the total volume). All three coatings were prepared using the same slurry of the C18-PAN mixture.

For the spraying method, a flask type sprayer (250 mL Erlenmeyer flask with a sprayer head) was used for spraying the slurry on the thin-film stainless steel surface. After transferring the mixture into the flask-type sprayer, the source of nitrogen gas was connected to the sprayer head to provide the required pressure for spraying. Then, the coating preparation was performed by spraying very thin layers of C18-PAN slurry on the first 2 cm of the length of the blades. After each layer, the coatings were immediately cured in the oven at 180 °C for 2 min, and then cooled to room temperature.

For the dipping method, the first 2 cm of the length of the blades were dipped into the C18-PAN coating slurry and were then slowly withdrawn. Next, the coatings were dried immediately at 180 °C for 2 min.

A painting brush was used to prepare the coatings with the brush painting method: thin layers of the coating slurry was spread onto the first 2 cm of the length of the blades using a fine brush with a width approximately equal to the width of the blades; the coating on the blades was dried under the same conditions as the other two coatings.

For all three types, the sequential steps of the coating and thermal drying were repeated for 10 layers in order to fully cover the blade surface and to obtain proper thickness of the C18-PAN coatings.

3.2.4 Automated Concept 96-blade SPME system

The Concept 96-blade SPME device and autosampler were both obtained from PAS Technology (Magdala, Germany). A detailed description of the Concept 96-blade SPME device and autosampler was provided in Chapter 2 (Section 2.2.4). Figure 3.1 demonstrates the C18-PAN coated 96-blade SPME device.

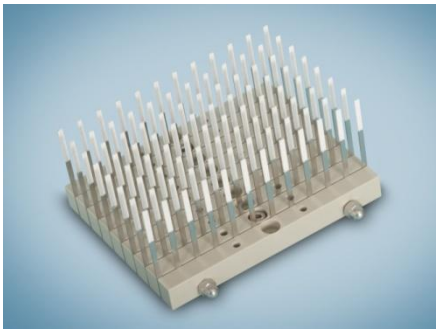


Figure 3.1 C18-PAN coated 96-blade SPME device.

3.2.5 Automated SPME procedure for high-throughput analysis

The Concept software was adjusted to automatically perform sequential steps of preconditioning, extraction, washing, and desorption with the preset times and agitation speeds. Prior to extraction, preconditioning of the C18-PAN coating was performed via 30 min agitation in methanol/water 50:50 (v/v).

The extraction was performed from 1 mL of the spiked samples placed in each well of the 96-well plate with a 1000 rpm agitation speed (2.5 mm amplitude). The time of extraction was set for 1 h in order to achieve equilibrium extraction for all four analytes. After extraction from the plasma, the coating was washed for 15 s (static) to remove salts, macromolecules, and proteins from the coating surface. The extracted analytes were then

desorbed in 1 mL of acetonitrile/water 50:50 (v/v) spiked with 10 ppb diazepam-*d5* (as an internal standard) for 40 min and with a 1200 rpm agitation speed (1 mm amplitude). Finally, the 96-well plate containing the final extract was directly transferred into the LC–MS/MS autoinjector for analysis. The evaluation of the extraction recovery, reusability, reproducibility, and pH stability were performed at the equilibrium extraction following the same SPME procedure described in this section.

3.2.6 LC–MS/MS conditions

The LC–MS/MS conditions for this study are identical to those described in Chapter 2 (Section 2.2.3). A typical SPME-LC–MS/MS chromatographic data set for equilibrium extraction of benzodiazepines from human plasma is illustrated in Figure 3.2.

3.2.7 Calibration and calculations

Internal standard calibration was used to compensate for any possible variation during the desorption step and the LC–MS/MS injection and run. It should be noted that the internal standard was purposely added during the desorption step rather than the extraction step in order to verify and monitor any variation in the coating extraction recovery during the coating reproducibility and reusability test. Therefore, a 10 ng/mL diazepam-*d5* was used as an internal standard for all the standards and desorption solvents for the entire study. The unknown sample concentrations were calculated from the equation of isotopic label calibration, and a 1/y linear weighted regression method was used for the calibration to ensure the accuracy of low concentration standards. The methods for calculation of the percent absolute recovery and carryover were reported in Chapter 2 (Section 2.2.8)

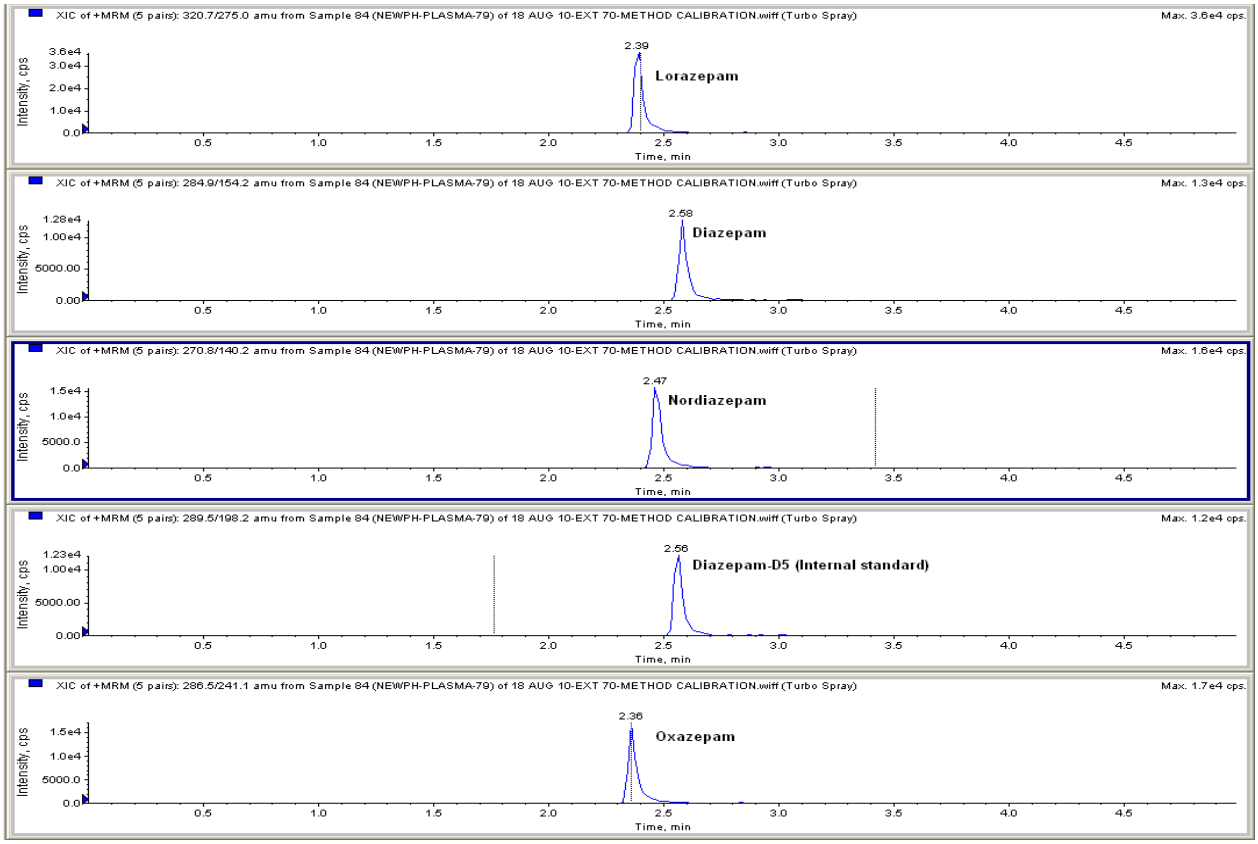


Figure 3.2 Examples of XIC chromatograms (321.0 → 275.1, 285.1 → 154.1, 271.1 → 140.1, 290.2 → 198.2, and 287.1 → 241.1 respectively) for SPME-LC–MS/MS analysis of 100 ng/mL of benzodiazepines from human plasma.

3.3 Results and discussion

3.3.1 Optimization of the coating preparation procedure

In order to achieve the most stable and reproducible procedure for making C18-PAN 96-blade coatings, three different methods of coating preparation were studied. In this part of the experiment, three different sets of blades were prepared by Supelco using dipping, brush painting, and spraying methods. The blades were then tested by the author at the University of Waterloo for sequential extractions from spiked PBS samples and were evaluated based

on their physical stability, extraction recovery, and reproducible extraction recovery. The results of the evaluation of all three sets of coatings for long-term extraction of diazepam from PBS are demonstrated in Table 3.1. These results indicate that the coatings prepared by dipping and brush painting resulted in poor stability in long-term usage; in 70 trials most of the coatings prepared by dipping and brush painting peeled off from the substrate surface, resulting in loss of recovery. In contrast, the spraying method resulted in the production of significantly more stable and robust coatings without any loss of recovery or any changes in physical condition. The reason for improved stability of the sprayed C18-PAN coating is because of the effective deposition of the multi thin-layers of small particles of the coating slurry on the blades. The etching step provides a rough surface on the substrate and improves the available surface area on the metal substrate. The spraying method spreads fine particles of the coating slurry on the pores of the roughened surface of the blades with high pressure. Repeating the spraying procedure for several layers, followed by thermal drying at high temperatures after each layer, provides a strong attachment of the coating to the stainless steel substrate. This strong connection prevents detachment of the coating from the surface in long-term use. Therefore, the spraying method was selected as the optimal method for the preparation of the C18-PAN coating and was used for the entire study.

The optimized thickness of the sprayed C18-PAN coating was obtained over 10 layers of spraying (60 μm) which resulted in optimum robustness and stability of the coating. The study showed that much thinner coatings resulted in lower physical stability, and consequently less reusability.

In another part of this study, an evaluation was performed for studying the efficiency of pre-treatment (acid etching) of the stainless steel blades on physical stability and

robustness of the coating. Two sets of pre-treated and untreated blades were coated with the spraying method and used for the sequential extractions of diazepam from PBS for more than 140 usages. The untreated blade set showed stripping and detachment of the coating from the surface after several extractions; however, the pre-treated blades demonstrated very stable properties and no changes in the physical condition of the coatings (for up to 140 extractions). The stability of the pre-treated sets is a result of the pre-treatment process of the stainless steel substrate with highly concentrated acid, which provides oxidation of the metal blades and promotes chemical adhesion of the C18-PAN coating on the surface of thin-film stainless steel substrate.

Table 3.1 Comparison of different methods of coating preparation for C18-PAN 96-blade SPME coatings when extracting 50 ng/mL diazepam from PBS.

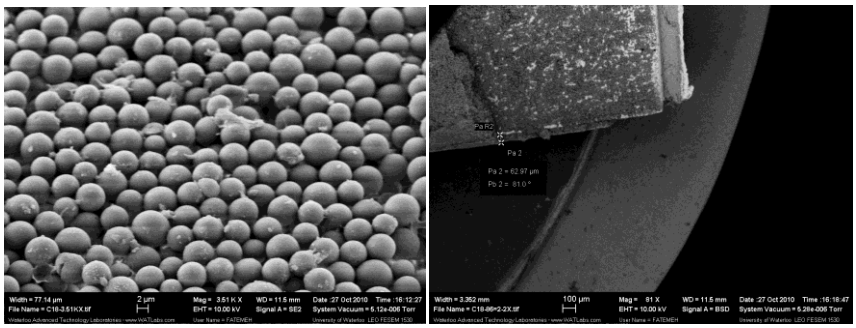
Coatings	*Average absolute recovery for n=12 after 15 extractions*	*Average absolute recovery for n=12 after 35 extractions*	*Average absolute recovery for n=12 after 70 extractions*	*Physical stability after 70 extractions (n=12)*
Spraying	97% (RSD=5%)	98% (RSD=5%)	94% (RSD=4%)	Good
Brush Painting	80% (RSD=35%)	77% (RSD=46%)	32% (RSD=147%)	Weak
Dipping	55% (RSD=80%)	40% (RSD=110%)	16% (RSD=230%)	Poor

3.3.2 Characterization and scanning electron microscopy of the sprayed C18-PAN coating

The octadecyl-silica (C18) 5 μm particles are made of octadecyl as the bonded phase and silica as the platform. These spherical particles have a pore size and surface area of around 180 Å and 200 m^2/g, respectively. The process of spraying and thermal drying (evaporation of DMF solvent at high temperatures) results in the creation of a highly porous

C18-PAN surface. The porosity of the structure is a critical factor influencing the efficiency of the adsorption process because the porous structure significantly increases the effective surface area of the coating; therefore higher extraction recovery can be expected.[83]

The topography of the coating surface was studied with scanning electron microscopy (SEM) using the same instrument and procedure reported in Chapter 2 (Section 2.3.9). As shown in SEM micrographs in Figure 3.3, the surface of the coating looks uniform and dense with a porous structure. The SEM was also used to study the average thickness of the coating which was estimated at 60 μm.



(a) **(b)**

Figure 3.3 SEM images of the C18-PAN SPME prepared using the spraying method. (a) Surface morphology using 3500 × magnification, and (b) estimation of the coating thickness using 80 × magnification.

3.3.3 Optimization of the automated 96-blade SPME system

Preconditioning: The results of the evaluations indicate that the preconditioning step has a significant influence on the extraction recovery of the C18-PAN coating. Preconditioning aids in activating the C18-PAN coatings for the extraction. In order to achieve the efficient partitioning of the analytes into the extractive phase, prior to each extraction, the coatings

were preconditioned in methanol/water 50:50 (v/v) and prevented from drying until the time of extraction.

*Extraction***:** Practically, equilibrium time is achieved when no additional increase in the amount of extracted analyte is obtained by increasing the time of extraction.[35] Therefore, the amount of analyte extracted at equilibrium time achieves the maximum method of sensitivity and demonstrates the extraction recovery of the coating. The evaluation of the extraction time profiles of the analytes under study in PBS and human plasma matrixes showed no significant differences between the two profiles. The equilibrium time for all four benzodiazepines was estimated at about 60 min. Therefore, 60 min equilibrium extraction was used for the entire study (this time of analysis, however, can be significantly shortened using pre-equilibrium extraction). The extraction time profile for the extraction of benzodiazepines from PBS is illustrated in Figure 3.5.

A 1 mL volume of the sample was used as the extraction volume, and it was precisely controlled through the study. The optimization of the agitation speed was performed based on the highest applicable speed, which resulted in the greatest recovery without the chance of spilling and cross contamination of the sample. The optimal speed was 1000 rpm using an agitator with 2.5 mm amplitude.

*Wash***:** When extracting from human plasma, a fast wash step should be employed between the extraction and desorption steps in order to minimize the transfer of any adsorbed salts and proteins onto the coating surface to the desorption solvent. The solvent and time for the wash step should be optimized to achieve efficient rinsing of the coating and minimal loss of the analytes. In the current study, a 15 s wash in purified water (no agitation) was effective for removing any attached proteins and particles from the surface with minimum

loss. In order to study the possibility of analyte loss, the wash solution was directly injected into the LC–MS/MS system. The amount of loss during the 15 s washing step for all four benzodiazepines was determined to be about 1-1.5%. In order to prevent the injection of the particulates and proteins from the wash solution into the LC–MS/MS system, it is strongly recommended that the pre-column and post-column filters are incorporated, and a bypass switching valve is used to divert the first 1.0 min of chromatographic run time to the waste.

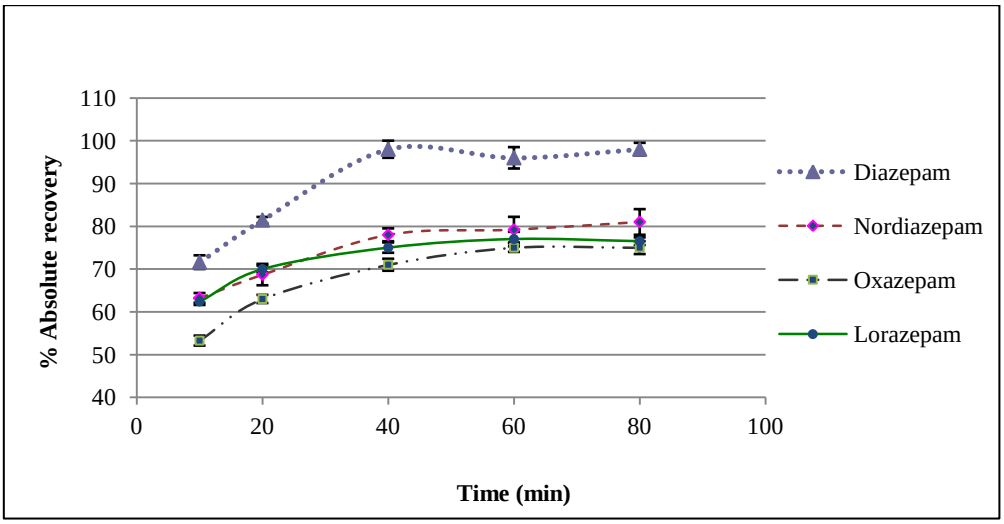


Figure 3.4 Extraction time profile for the extraction of benzodiazepines from PBS using the C18-PAN 96-blade SPME system. Extraction time profiles were constructed using 50 ng/mL of benzodiazepines in PBS, pH = 7.4 (1000 rpm extraction agitation speed, 40 min desorption time, 1200 rpm desorption agitation speed, and n = 12).

*Desorption and carryover***:** The optimization of the desorption conditions (solvent, time, volume and speed) was performed to obtain efficient desorption of the analytes from the coating and to minimize any remaining trace of the analytes on the coating. Three desorption solvents (methanol/water 50:50, acetonitrile/water 50:50, and acetonitrile/water 80:20 v/v) were evaluated to optimize the proper desorption solvent. For all four analytes,

the desorption efficiency of both compositions of acetonitrile/water (50:50 and 80:20 v/v) resulted in significantly improved absolute recovery and lower carryover (74-97% absolute recovery and 1-1.5% carryover) when compared to that of methanol/water 50:50 v/v (45-60% absolute recovery and 2-5% carryover). In spite of the similarity of desorption efficiency for both acetonitrile/water compositions, the composition of acetonitrile/water 50:50 (v/v) was selected as the optimal desorption solvent for the following three reasons: (i) it minimizes the consumption of organic solvent (a greener method), (ii) it decreases the possibility of solvent evaporation during the desorption step, and (iii) it obtains the most compatibility with the composition of the LC–MS/MS mobile phase (preventing tailing and bad peak shape).

The comparison of the absolute recovery of the three desorption solvents for equilibrium extraction is demonstrated in Figure 3.6. Furthermore, the optimal desorption volume was found to be 1 mL to ensure the entire length of the coating is covered with the solvent and to prevent spilling/cross contamination. Desorption profile studies showed that a minimum of 40 min desorption time and a 1200 rpm agitation speed (1 mm amplitude) is required to obtain the most efficient desorption of the analytes and the lowest carryover.

To study the amount of analytes carried over from the previous analysis, a second desorption step was performed for 30 min in acetonitrile/water 50:50 (v/v) and analyzed using the LC–MS/MS system. The analysis of the second desorption solution showed < 2% carryover for all four analytes; a desorption efficiency of >95% is acceptable for quantitative analysis. In the case of reusable application of the coatings, such as in the current study, any remaining trace of the analytes in the coating must be removed prior to the next extraction using a second desorption step. Ideally, the preconditioning procedure can be used to remove

the carryover from the previous extraction; but, in the current study, the preconditioning solvent is methanol/water 50:50 (v/v), and it does not completely remove the remaining trace of the analytes from the previous extraction. Therefore, after the main desorption step, a 30 min second desorption (carryover) step is always required to entirely eliminate the trace amounts of analytes. In order to optimize the carry over step, a third desorption step was also performed and analyzed: no remaining traces of analytes were detected. Therefore, a 30 min second desorption was found to be effective to clean any trace of the analytes.

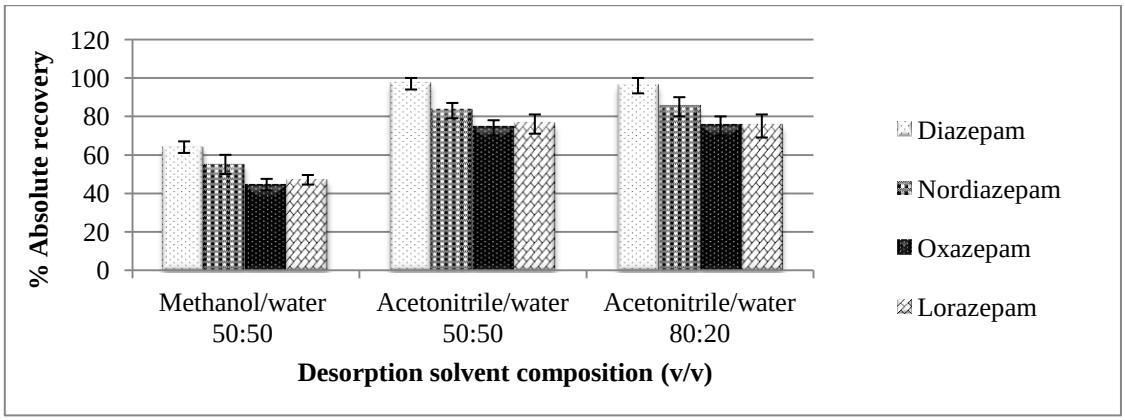


Figure 3.5 C18-PAN 96-blade desorption solvent optimization using 1 mL desorption solvents, when extracting 50 ng/mL benzodiazepines from PBS (60 min extraction time, 40 min desorption time, 1200 rpm desorption agitation speed, and n = 12).

3.3.4 Efficiency of C18-PAN 96-blade SPME coating

SPME is basically a non-exhaustive sample preparation technique that functions based on the partitioning of analytes between the sample and the extractive phase. The small amount of extraction recovery and low sensitivity of the traditional geometries of SPME are considered to be limiting factors of SPME (as a result of the small volume of the extractive phase). However, by taking advantage of the enhanced extraction volume in thin-film

geometry, when extraction has reached the equilibrium, the sprayed C18-PAN 96-blade coating could provide almost exhaustive extraction in the case of diazepam and >74% absolute recovery for the extraction of nordiazepam, oxazepam, and lorazepam from PBS.

Table 3.2 demonstrates the absolute recoveries of the analytes under study for the extraction from PBS and plasma using the C18-PAN 96-blade SPME system. The differences in the amount of absolute recovery of the analytes for the extraction from PBS and plasma are explained in terms of the high affinity binding of benzodiazepines to human plasma proteins.[127, 142]

Table 3.2 Percent of absolute recovery for the extraction of selected benzodiazepines from PBS and human plasma using a sprayed C18-PAN 96-blade SPME-LC–MS/MS system (the average for n = 6 and five experiments).

Analytes	PBS	Human plasma
Diazepam	96.7 ± 5.3	4.5± 0.40
Nordiazepam	82.3 ± 4.0	4.6 ± 0.30
Oxazepam	74.3 ± 4.6	4.0 ± 0.30
Lorazepam	74.8 ± 4.3	10.0 ± 0.50

3.3.5 Comparison of recovery of C18-PAN blades with that of C18-PAN rod fibers

As part of this thesis, the recovery of the sprayed C18-PAN thin-film 96-blade system was compared with that of the C18-PAN rod fiber, a study previously published by the Pawliszyn research group.[143] In the previous work, the 1.5 cm length of the stainless steel rod fibers (254 µm wire diameter) were coated with C18-PAN slurry using the dipping method, and a thickness of 60 µm was achieved. Table 3.3 compares the surface area, extraction volume (V_f), distribution constant (K_{fs}), fiber constant ($K_{fs}.V_f$), and extraction recoveries of

the sprayed C18-PAN thin-film blades with those of dipped C18-PAN rod fibers for the equilibrium extraction of nordiazepam and diazepam from PBS. The coating volume of the thin-film and the distribution constants were calculated based on Equations 2.1 and 1.1, respectively. For the rod fiber coatings, the volume of the coating can be determined from the coating length (*l*) and thickness (*b)*, and the radius of the support wire (*r*) using Equation 3.1:

$$V_f = \pi\, l\, [(r + b)^2 - r^2] \qquad \text{Equation 3.1}$$

The thin-film geometry of the 96-blade resulted in a 9.4-fold increase in the volume of the coating when compared to rod fibers (Table 3.3). For the extraction of nordiazepam, the distribution constant for sprayed blades and dipped fibers were comparable (5.43×10^2 vs. 5.60×10^2). The absolute recovery for the dipped fibers was determined as 23% versus 82% for the sprayed blades. Since for both systems the volume of the sample, initial concentration of the analytes in the sample, and coating thickness were the same, the 3.6-fold increase in the extraction recovery of nordiazepam corresponds to the effect of increasing the volume in the blade system (Equation 1.1).

For the extraction of diazepam, in addition to the 9.4-fold increase in the volume of the coating, the application of the sprayed blade system resulted in a 3.4-fold enhancement in the distribution constant. Therefore, the maximum improvement in the extraction recovery was obtained (40% recovery for the dipped fibers versus exhaustive recovery for the sprayed blades). It should be noted that, due to of the large extraction phase to sample volume ratio, $K_{fs}V_f$ in the denominator of the Equation 1.1 could not be neglected; therefore, the amount of the analyte extracted (*n*) is not linearly proportional to the fiber constant ($K_{fs}V_f$).[81]

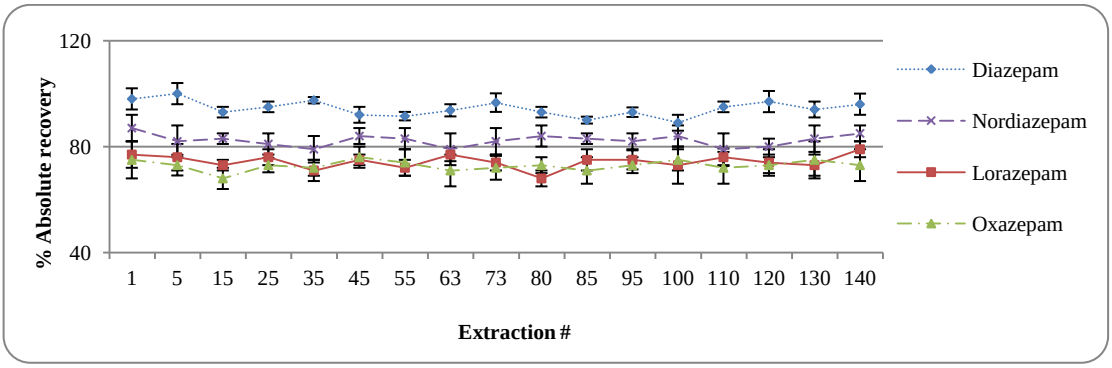
Table 3.3 Comparison of the sprayed C18-PAN 96-blade with dipped C18-PAN rod fibers[143] (Equilibrium extraction from PBS)

Analyte	*Nordiazepam*		*Diazepam*	
Parameters	Sprayed C18-PAN 96-blade (2 cm)	Dipped C18-PAN rod fibers (1.5 cm)	Sprayed C18-PAN 96-blade (2 cm)	Dipped C18-PAN rod fibers (1.5 cm)
Surface area (mm^2)	140	18.8	140	18.8
Coating volume (V_f), mL	8.38×10^{-3}	8.87×10^{-4}	8.38×10^{-3}	8.87×10^{-4}
% Absolute recovery	82	23	97	40
Distribution constant (K_{fs})	5.43×10^{2}	5.60×10^{2}	3.88×10^{3}	1.14×10^{3}
Fiber constant ($K_{fs}.V_f$), mL	4.50	0.49	32.50	1.01

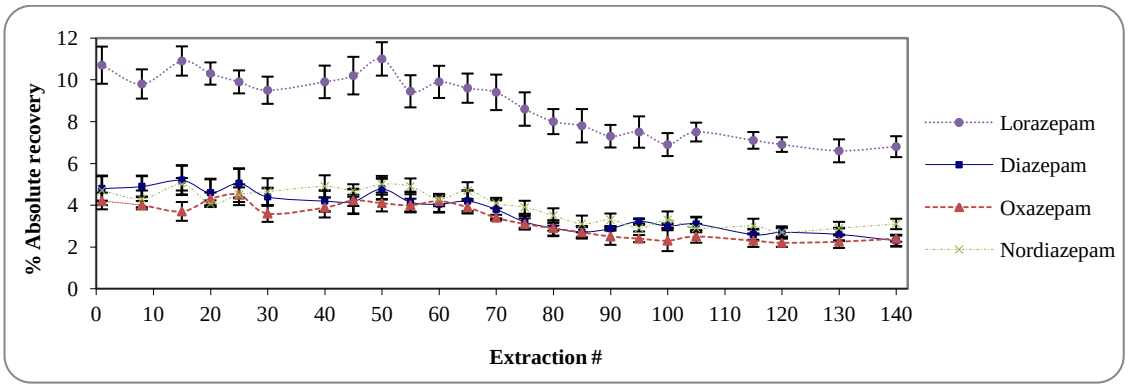
3.3.6 Reusability and robustness of the C18-PAN 96-blade SPME coating

The robustness and reusability of the sprayed C18-PAN 96-blade SPME coating was evaluated using two sets of coated blades (n = 12) for 140 independent extractions from spiked PBS and plasma (Figure 3.7). The coatings showed good physical stability and reproducible recovery for at least 140 extractions from PBS (RSD = 3–5% for all four benzodiazepines for n = 17 experiments and n = 12 coatings) and 70 extractions from human plasma (RSD = 7–10% for all four benzodiazepines for n = 13 experiments and n = 12 coatings). Even though the complex biological matrix of plasma caused a drop in the extraction recovery after the 70^{th} extraction, the amount of extraction recovery was still reproducible from the 70^{th} to the 140^{th} extractions (RSD = 10-14% for all four benzodiazepines for n = 12 experiments and n = 12 coatings). Therefore, if a proper

calibration technique is employed, the coatings could also be reused at least 140 times in plasma. The above-mentioned reusability test for plasma extraction was re-confirmed by evaluating another set of C18-PAN coatings where similar results were observed.



(a)



(b)

Figure 3.6 Reproducibility and reusability of the C18-PAN 96-blade SPME system for equilibrium extraction of (a) 50 ng/mL of benzodiazepines from PBS (n = 12) and (b) 100 ng/mL of benzodiazepines from human plasma (n = 12).

The chemical stability of the sprayed C18-PAN coating was also studied by soaking three sets of coatings (n = 5) overnight in pure acetonitrile, methanol, and DMF. The efficiency of these coatings was compared to another untreated set of coatings as reference. The evaluation of the physical appearance and extraction recovery of all three sets of

coatings in contact with solvents did not show any differences when compared to those in the reference set, indicating the high chemical stability of the sprayed C18-PAN coating.

3.3.7 Intra- and Inter-blade reproducibility of the C18-PAN 96-blade SPME system

The reproducibility of the coating preparation procedure and variations in the extraction recovery of the spray-coated blades were studied by evaluating inter-blade RSD for the recovery of n = 96 manually-made C18-PAN coatings. Through an evaluation of intra-blade variations of the coatings, the repeatability of the extraction recovery of the individual coatings and reproducibility of the optimized SPME-LC–MS/MS method were studied. Table 3.4 illustrates the results of inter- and intra-blade RSD of the coatings when extracting benzodiazepines from PBS and plasma. The spraying procedure in this study was performed manually: it is expected that the inter-fiber reproducibility of the system can be significantly improved through the automation of the coating procedure.

Table 3.4 Inter- and intra-blade reproducibility of the C18-PAN 96-blade SPME system when extracting benzodiazepines from PBS and plasma (n = 96 and three extractions).

Extraction matrix	PBS	Plasma
Inter-blade RSD (%)	5-7	9-11
Intra-blade RSD (%)	5-8	8-9

3.3.8 Evaluation of pH stability

The pH stability of the C18-PAN 96-blade SPME coating was evaluated in the pH range of 1-12. The study of the pH stability was performed by evaluating the physical appearance and extraction recovery of n = 3 C18-PAN coatings which were used for 10

sequential extractions in PBS, adjusted to a specific pH. The results of the evaluation indicate that C18-PAN coating preserves physical and chemical stability and reproducible extraction efficiencies in the pH range of 2-10. However, for extractions outside this range of pH, the harsh chemical effect of highly acidic and basic conditions results in a significant reduction of the coating thickness after several extractions, and consequently a considerable loss of extraction recovery.

3.3.9 Evaluation of LOD, LOQ, and linearity

The methods for determining the LOD and LOQ were reported in Chapter 2 (Section 2.2.8). The LOD was in the range of 0.1-0.3 ng/mL for all four benzodiazepines when extracting from human plasma. The limit of quantitation was obtained in the range 0.5-1 ng/mL for the extraction of all four analytes from human plasma. The highest and lowest LOD and LOQ were obtained for lorazepam and diazepam, respectively. The method demonstrated good linearity in the concentration range of 0.5-500 ng/mL for all four analytes. The linear regression coefficients of the constructed calibration curves for the extraction of all four benzodiazepines from human plasma ranged between 0.985-0.993.

3.3.10 Sensitivity improvement via drug-protein binding disruption

As discussed in Chapter 1, the amount of analyte extracted by SPME is influenced by the sample binding matrix. As a result, when extracting from complex biological samples (e.g., plasma or whole blood) the amount of recovery corresponds to the free concentration of the analyte in plasma. This amount of recovery can be increased through precipitation of the binding matrix (e.g., plasma proteins), and consequently disruption of ligand-receptor binding prior to SPME analysis. This method provides an enhancement in the free portion of

analytes in the sample matrix, and consequently an improvement in the sensitivity of the assay.

Many studies have reported on different approaches for the precipitation of plasma proteins.[9, 144, 145] The most common method includes the addition of an organic solvent to the plasma sample. This method suffers from dilution of the sample by adding a substantial volume of organic solvent, which may lead to changes in the characteristics of the original sample.[145]

In order to study the applicable methods for drug-plasma binding disruption with minimal dilution, several approaches (e.g., heating, freezing and thawing, and acid precipitation) were explored in this thesis. Among the tested methods, acid treatment resulted in the successful precipitation of the proteins and provided the best recovery. Typically, the available methods of acid precipitation in the literature include a considerable volume of the acid (and base for neutralization), which dilute the sample.[144] In this study the method of acid precipitation was optimized with minimum dilution (<5%).

This evaluation showed that 30 µL of saturated trichloroacetic acid solution in water (4 g/mL) is adequate for complete plasma protein precipitation (PPT) in 1 mL of plasma. The procedure for acid precipitation is optimized as follows: After the addition of the acid, the sample is immediately vortexed (1200 rpm speed, 30 s). The mixture is then kept in ice for 5 min. After allowing the mixture to warm to room temperature, it is centrifuged at 4000 rpm for 25 min. The supernatant is then separated and neutralized to pH 7.4 with 10 µL of 10 M NaOH.

The final product was used for SPME extraction, followed by LC–MS/MS analysis. The evaluation of the percent recovery was performed using pre-spiked and post-spiked plasma samples (spiked before and after protein precipitation, respectively). The results for the SPME-LC–MS/MS analysis of pre- and post-spiked protein precipitated plasma samples are demonstrated in Table 3.5.

The results indicate that there is some loss of analyte for the pre-spiked samples during the acid treatment, which is a common phenomenon in acid protein precipitation methods.[146] This loss can be attributed to incomplete disturbing of the analyte-protein bindings before precipitation. It was reported that this issue can be resolved through another acidification step (with hydrochloric acid) for complete drug-protein disruption prior to protein precipitation.[146]

But, in the case of this study, the addition of another acidification step provides a greater degree of dilution, which is not optimal for the proposed system. As a result, as long as it is experimentally proven that the amount of recovery is reproducible (Table 3.5), the current optimized method with minimum dilution can be used for disturbing the analyte-protein binding and improving the free concentration of analytes in the sample.

With a maximum dilution of 4 % (including acid and base addition), a 55% recovery was achieved for the SPME extraction of diazepam for a pre-spiked plasma compared to 83% for a post-spiked plasma sample. The comparison of the recovery for the pre-spiked protein-precipitated sample with that of untreated plasma (4.5%, Table 3.2) exhibits a 12-fold increase in the sensitivity of the SPME for the extraction of diazepam from plasma.

Table 3.5 SPME extraction recovery and reproducibility for the extraction of diazepam from protein precipitated plasma.

	% Absolute recovery	Intra-day reproducibility (% RSD)	Inter-day reproducibility (% RSD)
Post-spiked acid PPT*	83	7	9
Pre-spiked acid PPT	55	9	12

*Plasma protein precipitation

3.4 Conclusions and future directions

The automated C18-PAN 96-blade SPME system allows for the high-throughput simultaneous sample preparation of 96 samples in a total of 160 min which corresponds to <1.7 min per sample. This length of analysis is based on the time required for equilibrium extraction, and it can be further decreased via pre-equilibrium extraction without loss of precision (using an automated system). In addition to the improved stability and reusability of the analysis of biological samples, the C18-PAN coating resulted in significant improvements in the extraction recovery of oxazepam and lorazepam when compared to the C18-silica gel coating. On the other hand, the time of equilibrium extraction was longer for the C18-PAN coating, which can be attributed to the differences in the properties of the two extractive phases including the thickness of the coatings (60 versus 10 μm).

The thin-film geometry of the blades resulted in a significant enhancement in the absolute extraction recovery of the system and provided low levels of detection and quantitation. The proposed C18-PAN 96-blade SPME system has the potential to be employed for high-throughput analysis in a wide variety of research areas such as food, clinical, biological, pharmaceutical and environmental sciences, and metabolomics studies.

A reported example for the clinical application of the automated C18-PAN 96-blade SPME system is its utilization for high-throughput therapeutical monitoring of tranexamic acid concentration in human plasma in patients undergoing cardiopulmonary bypass surgery.[147] Furthermore, determination of selected pharmaceutical residues in wastewater and *in vivo* determination of selected pharmaceuticals in fish muscle are the two pharmaceutical and environmental applications for the developed C18-PAN SPME coating that have been recently reported by the Pawliszyn research group.[148, 149]

The next steps of study for the reported work in this chapter are the modification and evaluation of the system for direct extraction from whole blood matrix, and the development of other types of extractive phases capable of extracting different classes of compounds in a wide range of polarity (which are discussed in Chapters 4 and 5, respectively).

3.5 Addendum

The text of this chapter was rewritten in comparison to the published research article.

Chapter 4

Reusable SPME coating for direct immersion whole blood analysis and extracted blood spot sampling coupled with LC–MS/MS and DART–MS/MS

4.1 Preamble and introduction

4.1.1 Preamble

This chapter has been published as a paper: Fatemeh S. Mirnaghi, Janusz Pawliszyn, Reusable SPME Coating for Direct Immersion Whole Blood Analysis and Extracted Blood Spot Sampling Coupled with Liquid Chromatography−Tandem Mass Spectrometry and Direct Analysis in Real Time−Tandem Mass Spectrometry, *Anal. Chem*. 2012, 84, 8301–8309. The materials of the current chapter are reprinted from this publication with the permission of the American Chemical Society (Copyright American Chemical Society 2012).

4.1.2 Introduction

Packed-bed extractive phases usually suffer from clogging and contamination of the system in contact with complex biological matrixes. The open-bed extractive phase in SPME addresses this issue and facilitates the direct handing of complex matrixes. However, there is a possibility of the adhesion of macromolecules onto the outer surface of the SPME coating, which can be resolved via the application of biocompatible coatings.

These biocompatible coatings preserve the efficiency of the SPME coating for long-term exposure to complex biofluids by reducing the attachment of macromolecules and particulate matters onto the surface.

As described in Chapter 3, many contemporary studies have investigated the development and evaluation of biocompatible SPME coatings.[79] When dealing with whole blood matrix, the adsorption of protein and adhesion of blood cells on the surface of the SPME coating are two different issues that must be resolved using biocompatible coatings. Not all biocompatible coatings that minimize protein adsorption are also capable of the effective prevention of blood cell attachment; most available lab-made or commercial biocompatible SPME coatings lack reusability in whole blood (despite their compatibility with plasma matrix). This lack of reusability is due to irreversible attachments of blood cells to the coating surface, which affects the kinetics of the extraction and results in a loss of coating recovery after several uses in whole blood. While many studies have reported on the development of biocompatible coatings for long-term use in the human plasma matrix,[74, 104, 139] no study thus far has addressed the development of the biocompatible coatings for 'long-term reusability' in whole blood matrix.

In Chapter 3 the development and optimization of the thin-film C18-PAN coating for the extraction from human plasma was discussed, demonstrating the coating's high degree of reusability for direct extractions from human plasma. The evaluation of this coating for several extractions from whole blood, however, showed irreversible red blood cell attachment on the coating surface and loss of extraction recovery. The current chapter, therefore, discusses some modifications to the C18-PAN coating in order to improve the biocompatibility with whole blood in long-term use.

The surface chemistry and topography of the coating are important parameters that influence the intensity of the protein adsorption and interaction of blood cells on the coating surface.[150] Fabrication of a hydrophilic polymer layer on the surface of the extractive phase is one common solution for minimizing the adsorption of blood cells. Theoretically, the polar polymer layer acts as a barrier on the surface via the formation of a hydration layer with the available water molecules in the matrix. This layer protects the coating surface against the interactions with the blood cells and proteins.[151]

This chapter is focused on the modification of the thin-film C18-PAN coating using two different polymers: polyacrylonitrile (PAN),[104] and 2-methacryloyloxy ethyl phosphorylcholine (MPC)[152-155]. Polyacrylonitrile polymer is one of the most common and widely-used biocompatible polymers. The application of PAN for the development of biocompatible surfaces has already been reported in many studies, indicating the significant effect of PAN on reducing the interaction of matrix components with the exposing surface.[140, 156-158] Moreover, MPC has the same structure as the phosphatidylcholine polar group that forms the cell membrane; it can therefore form a membrane structure with anti-thrombus properties which controls the adhesiveness of blood platelets to the surface. The structures of PAN and MCP are shown in Figure 4.1.

For the purpose of this study, a new washing strategy after extraction was employed in order to improve the reusability of the modified coatings in whole blood. The modified coatings were evaluated in terms of the extent of blood cell attachment, reproducible extraction recovery, and reusability for direct immersion whole blood analysis in long-term usage.

This chapter also includes a discussion of the employment of the optimal modified C18-PAN for the evaluation and optimization of extracted blood spot sampling coupled with LC–MS/MS and direct analysis in real time (DART)–MS/MS. For the entire study, diazepam and diazepam-*d5* were used as the model analyte and isotopic-labeled analogue, respectively.

4.1.2.1. Introduction to extracted blood spot (EBS) sampling

Since the introduction of dried blood spot sampling (DBS) in the 1960s, DBS has been commonly used for the population screening of newborns, and many applications have been reported for the qualitative and quantitative screening of metabolic disorders.[159, 160] Over the years, this technique has been successfully applied for the screening of individuals for clinical purposes and detecting numerous biological markers in epidemiological studies.[161-163] It has been also extended to therapeutic drug monitoring, pharmacokinetics and toxicokinetics studies.[162, 164]

DBS offers a number of advantages over conventional whole blood, plasma or serum sample collections, including: (i) being a less invasive sampling method, (ii) requiring smaller blood volumes (reducing the use of animals and cost of analysis), (iii) offering easier storage and shipment to laboratories with no requirement for freezers and dry ice (cost efficient), and (iv) minimizing the risk of biohazard infection.[159, 164] However, the application of DBS (filter paper deposition) has some disadvantages, including: (i) possible hematocrit and chromatographic effect (uneven distribution of the analyte and variation in assay result when punching the blood spot), (ii) error and/or contamination due to punching device, (iii) instability of some compounds on filter paper, and (iv) analyte signal suppression/enhancement due to the blood and filter paper matrixes. [165-168]

In order to overcome the above mentioned limitations, this work for the first time introduces the 'Extracted Blood Spot' (EBS) approach. EBS includes the utilization of a biocompatible SPME coating (instead of filter paper/card in DBS) for spot sampling of blood or other biofluids. With the EBS approach, a small volume of the blood sample is deposited on a customized SPME coating. In a short sampling time (based on the kinetics of the extraction), analytes are selectively extracted into the coating. Then, in a short time interval, before the blood spot dries on the coating, a fast wash step rinses the blood matrix from the coating surface. In next step, the analytes can be immediately desorbed or the coating can be stored and transported to a laboratory for analysis.

EBS integrates all four steps of sampling, extraction, analyte pre-concentration, and sample clean-up in one single step and offers the following advantages over the conventional DBS technique: (i) extraction and preservation of the analytes against possible variation (e.g., oxidation), (ii) prevention of the adverse ion suppression/enhancement caused by filter paper and blood matrixes (the blood spot is rinsed and removed in the wash step), (iii) no limitation with filter paper punching, hematocrit and chromatographic effect, (iv) a simple sample preparation step with fewer experimental steps, and (v) automation and high-throughput.

4.1.2.2. Introduction to direct analysis in real time (DART)

Direct analysis in real time (DART) is a fast method of analysis which provides the immediate analysis and screening of samples without the need for sample pre-treatment.[169-171] The DART ionization source facilitates the desorption of analytes directly from the sample substrate placed in the open atmosphere between the exit of the source and the inlet

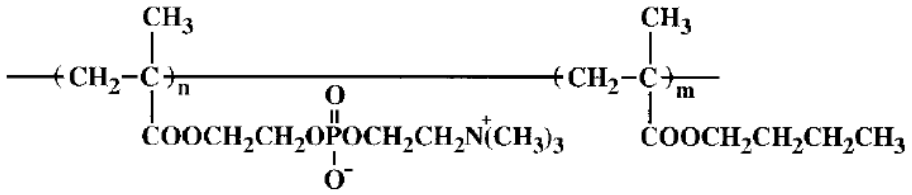
of a mass spectrometer. The DART source works via the direction of a heated gas flow (containing metastable–electronic or vibronic excited-state–atoms of helium and nitrogen) to the sample. This step results in the heating of the sample, and desorbing the molecules of interest into the gas state where ionization occurs.[171]

The major limitation of DART is the possibility of the matrix effect, which may happen in the absence of sample pretreatment, though the introduction of complex samples containing numerous interferences to the system.[172, 173] This limitation impacts sensitivity, accuracy, and precision and makes qualitative and quantitative analysis difficult.[172] Therefore, in order to obtain accuracy and reliability in a short analysis time, an appropriate sample preparation method that features efficient and fast sample clean-up is required. For the first time, in this study, the SPME coating is utilized for the extracted blood spot sampling coupled with DART–MS/MS. The application of the modified biocompatible SPME coating for EBS sampling provides efficient and fast sample clean-up, isolation, and pre-concentration of compounds from complex matrixes prior to DART.

4.2 Experimental

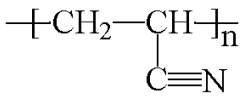
4.2.1 Chemicals and materials

Stainless steel (1.4310 grade) thin-film substrates were obtained from PAS Technology. The 74×74 stainless steel meshes were obtained from IonSense Inc. MPC (Lipidure®-CM) was purchased from NOF Corporation (Japan). Discovery silica-based-C18 particles (5 µm) were obtained from Supelco (PA. U.S.). The remainder of the materials were purchased from the same sources reported in Chapters 2 and 3 (Sections 2.2.1 and 3.2.1).



n=3 and m=7

(a)



(b)

Figure 4.1 Structure of (a) 2-methacryloyloxy ethyl phosphorylcholine (MPC), and (b) polyacrylonitrile

4.2.2 Preparation of standard solutions and buffers

The information for this section was described in Chapter 2 (Section 2.2.2), with the exception of using human whole blood instead of human plasma.

4.2.3 Preparation of modified biocompatible C18-PAN SPME thin-film coatings

C18-PAN SPME coatings were prepared with the spraying method following the same formula and procedure reported in Chapter 3 (Section 3.2.3). C18-PAN coatings were then modified using two different polymers as described below. For all the modifications, the dipping method was used for covering the original C18-PAN, since it provides more uniform and reproducible coverage compared to spraying and brush painting methods.

MPC-modified C18-PAN: An MPC solution was prepared by dissolving MPC particles in ethanol (5% w/v). The C18-PAN coatings were dipped in the MPC solution for 60 s,

followed by drying at 50 °C for 4 h. The coating and drying steps were repeated three times to obtain full coverage on the surface.

PAN-over C18-PAN: PAN glue was prepared with the same procedure described in Chapter 3 (Section 3.2.3). Different approaches were tried and evaluated for the preparation of the modified PAN-over C18-PAN coating, including: (i) dipping the C18-PAN coatings (n = 6) in PAN glue for 20 s, and drying at 180 °C for 2 min, (ii) dipping two sets of C18-PAN coatings (n = 6 each) in PAN glue for 20 and 60 s, followed by drying at 70 °C for 4 h, and (iii) preparation of two sets of C18-PAN coatings (n = 6 for each set) in different thicknesses (10 s dipping for thin and 60 s dipping for thick cover), followed by drying under an ultraviolet (UV) lamp light [emitting UVA (320-400 nm) and UVB (280-320 nm) regions] for 30 min on each side.

4.2.4 LC–MS/MS conditions

The LC–MS/MS conditions were identical to those of explained in Section 2.2.3 (Chapter 2). The summary of MS/MS parameters for diazepam and diazepam-*d5* is provided in Table 2.1.

4.2.5 Automated 96-blade (thin-film) SPME system

This study utilizes the Concept 96- blade (thin-film) SPME device and 96-autosampler. The detailed description of the original system is provided in Chapter 2 (Section 2.2.4). However, in this study the static wash station of the original Concept 96-autosampler was modified to an agitating wash station, and an updated version of agitator was used for the desorption. Figure 4.2 illustrates a picture of the modified Concept 96-autosampler including four orbital agitators.

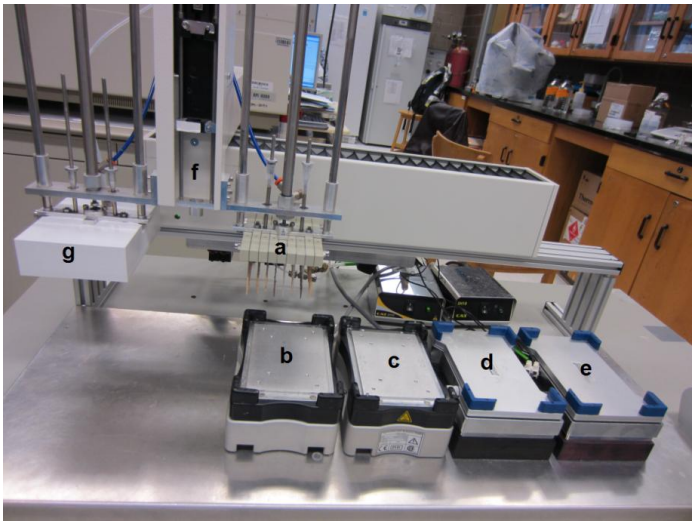


Figure 4.2 The modified Concept 96-autosampler including the following labels: a: 96-thin-film SPME device, b, c, d, and e: orbital agitators for extraction, desorption, wash, and conditioning, respectively. f: syringe arm, and g: nitrogen evaporation device

4.2.6 Automated SPME-LC–MS/MS procedure for direct immersion blood analysis

The spiked whole blood samples were incubated to reach equilibrium between the drug and the blood binding matrix. Each step (preconditioning, extraction, wash and desorption) was carried out automatically with a preset time and speed. Prior to extraction, the modified C18-PAN coatings were preconditioned while being agitated in methanol/water 50:50, v/v (30 min). Equilibrium extraction (60 min) was performed from 1 mL of spiked whole blood or PBS (pH = 7.4) at a speed of 1,000 rpm and with 2.5 mm amplitude. After being extracted from the blood, the coatings were rinsed for 20 s in nanopure water along with agitation. Desorption was performed in 1 mL of acetonitrile/water 50:50 (v/v) for 40 min (1500 rpm, 1 mm amplitude). Prior to the next extraction, the carryover of analyte on the coating was cleaned using a 30 min second desorption in 1 mL of acetonitrile/water 1:1, v/v (1500 rpm, 1

mm amplitude). The above-mentioned procedure was performed for the evaluation of all the types of modified C18-PAN coatings.

4.2.7 The procedure for extracted blood spot sampling coupled with LC–MS/MS

The optimized UV-dried thin PAN-over C18-PAN coating was utilized for the extracted blood spot experiment. Five µL of the spiked whole blood was spread on the PAN-over C18-PAN coatings using a micropipette. Then, the blood spots were left for 2 min to enable the extraction of the analytes by the coating. The blades were then immediately assembled into the 96-blade holders and mounted on the 96-autosampler to be washed for 20 s in nanopure water while agitating. This washing step helped to clean the attached blood cells from the surface before they dried on the coating. Next, the coatings were desorbed in 1 mL of acetonitrile/water 1:1 (v/v) for 40 min (1500 rpm speed, 1 mm amplitude). The analysis of the samples was carried out using the same LC–MS/MS conditions described for the direct immersion whole blood analysis (Section 4.1.4). If any transportation was necessary, the coatings could also be frozen and stored for a later desorption and analysis. A second desorption in 1 mL of acetonitrile/water 1:1 v/v (30 min, 1500 rpm, 1 mm amplitude) was performed to clean the carryover from the previous desorption prior to reusing the coatings.

4.2.8 Coating preparation for the EBS-DART system

The preparation of the coating for the EBS-DART system was completed in two main steps. Initially, the 74×74 stainless steel bare meshes were coated with a layer of C18-PAN using the brush painting method. The C18-PAN slurry was prepared following the same procedure described in Chapter 3 (Section 3.2.3). The stainless steel bare meshes were

prepared to have dimensions of approximately 1.2 × 15 cm, which fit into the DART transmission module. Then, using a painting brush with the approximate width of the mesh, a thin layer of the coating slurry was spread on the surface of the mesh. Next, the meshes were dried in the oven (180 °C) for 2 min. In next step, a layer of PAN was placed on top of the coatings using the brush painting method, followed by drying each side under UV light for 30 min. Figure 4.3 (a and b) compares the bare stainless steel mesh versus PAN-over C18-PAN coated mesh.

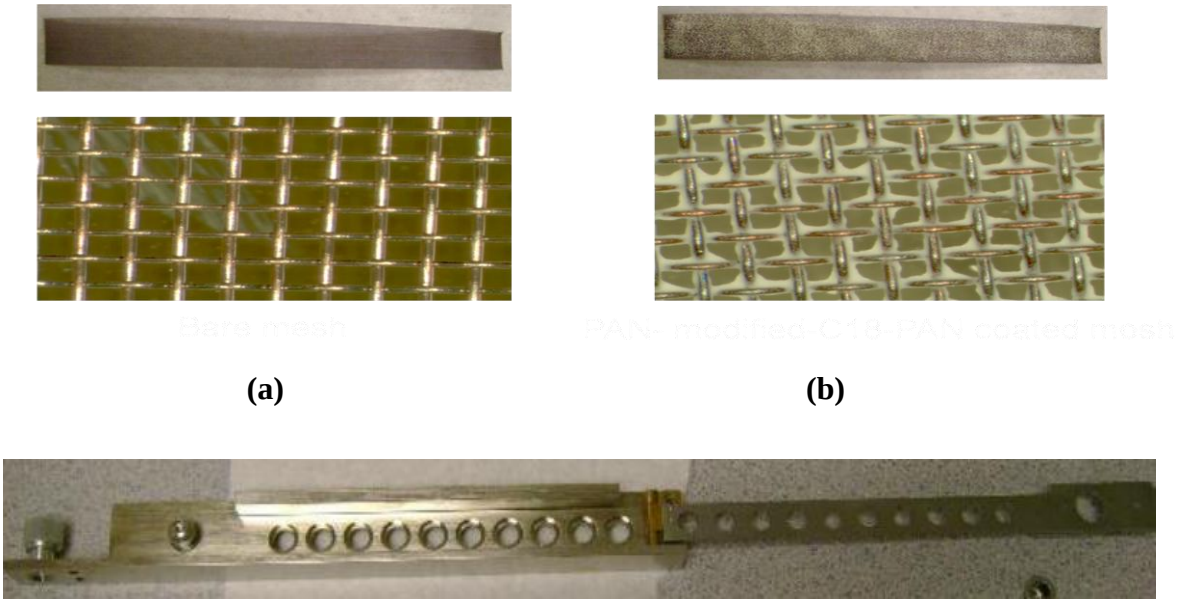
4.2.9 EBS-DART–MS/MS conditions

A DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Inc., Saugus, MA) was coupled with an Applied Biosystems API 4000 triple quadrupole mass spectrometer via a Vapur interface (IonSense, Inc.). The Vapur interface is necessary for (i) the efficient collecting of the desorption gas, and (ii) the transfer of the gas to the atmospheric-pressure interface inlet of a mass spectrometer. The utilization of the Vapur interface improves the sensitivity and reproducibility of the analysis.

The DART-SVP was fitted with a single dimensional motorized linear rail and was controlled through the DART-SVP web-based software for the automatic introduction of the samples to the source. DART software was set to control the gas selection, ionization polarity mode, contact closure to the mass spectrometer, rail speed for sample introduction, and the gas heater temperature for the analytes' desorption.

The optimization of the parameters and analysis of the samples were performed by spotting the samples on the PAN-over C18-PAN coated mesh (in case of EBS-DART–

MS/MS analysis) and bare mesh (in case of DART–MS/MS analysis); the meshes were held in a sampling module called the 'transmission module' (IonSense, Inc.).



(a) **(b)**

(c)

(d)

Figure 4.3 Pictures of (a) stainless steel bare mesh, (b) PAN-over C18-PAN coated mesh, (c) transmission module, and (d) DART–MS/MS.

The transmission module includes a series of 10 holes with a 7 mm diameter (placed every 14 mm) which sandwiches and holds the meshes so that samples can be pipetted (Figure 4.3 (c)).[174] The linear rail is capable of moving the module through the gap between the DART source and the ceramic transfer tube of the Vapur interface where the ionization takes place (Figure 4.3 (d)). In this study, 5 μL aliquots of the sample were deposited on the meshes (bare and coated) using a micropipette.

In the case of the coated meshes, the blood spots were left on the meshes for 5 min to allow for the extraction of the analytes. Then, they were immediately rinsed for 5 s with nanopure water. Then, coated meshes containing extracted analytes were fully dried (using a blow drier) before being introduced to the DART source for analysis. In the case of the bare meshes, the blood spots were left to completely dry (following the routine procedures), prior to the time of analysis.

The DART parameters for the analysis of diazepam are optimized in the following conditions: source gases = helium and nitrogen, source gases pressure = 80 psi, desorption temperature = 250 ºC, discharge needle voltage = 3000 V, grid voltage = 350 V, sampling time = 30 s, gap time = 5 s, the distance between the DART gun and ceramic transfer tube of the Vapur interface = 11 mm, transmission module sampling speed = 0.2 mm/s. The MS/MS analysis in API 4000 mass spectrometer was performed in positive mode under multiple reaction monitoring (MRM) conditions at 285.1 → 154.1 and 290.2 → 198.2 for diazepam and diazepam-*d5*, respectively. Collision gas was optimized at 12 (arbitrary units). Declustering potential, entrance potential, collision energy, and collision cell exit potential were set at 75, 15, 40, and 15 V for diazepam, and 100, 15, 50, and 17 V for diazepam-*d5*, respectively.

4.2.10 Data analysis and calculations

The methods for calculating the percent absolute recovery and carryover are reported in Chapter 2 (Section 2.2.8). A 1/y linear weighted regression method was used for drawing the calibration curves. The absolute matrix effect was calculated based on the ratio of the peak area of the blank blood extract spiked with the known concentration of the analyte to the peak area obtained from the standard solution of the analyte with the same concentration, multiplied by 100. Matrix effects larger than 120% and smaller than 80% represent significant ionization enhancement and suppression for a given analyte, respectively.[7, 175] The methods for determining the LOD and LOQ were already reported in Chapter 2 (Section 2.2.8).

4.3 Results and discussion

4.3.1 Coating topography and blood biocompatibility

Figure 4.4 shows the scanning electron microscopy (SEM) micrographs of the original and all the modified C18-PAN coatings (details for SEM instrument and procedure were reported in Chapter 2, Section 2.3.9).

In the original C18-PAN coating, the amount of stationary phase particles is much higher than the PAN content, with PAN mainly acting as a porous glue to bind the stationary phase particles together. Thus, there is no coverage layer of polar PAN preventing the blood cells from attaching to the coating surface (Figure 4.4 (a)). The addition of another biocompatible layer on the top surface of the coating is therefore required in order to improve the blood biocompatibility. This additional biocompatible layer acts as a protective layer on the coating's outer surface and minimizes the adsorption of blood cells on the

surface. The SEM micrograph of the UV-dried PAN-over modification (Figure 4.4 (b)) indicates that it properly provides the required protective layer on the C18-PAN coating surface. In contrast, this chapter's studies indicate the weaker biocompatible characteristics of MPC-modified C18-PAN coatings. This weaker biocompatibility can be explained by the lack of a uniform full-coverage protective layer on the surface to protect the coatings against interactions with blood cells (Figure 4.4 (d)).

The main characteristic of the polyacrylonitrile [poly(vinyl cyanide)] as a biocompatible polymer is the presence of strongly polar nitrile groups. The cyano functional groups (C ≡ N) in polyacrylonitrile interact with water molecules contained in blood via hydrogen bonding, and form a hydration layer that inhibits the interaction of the blood cells and proteins with the coating surface.[176]

4.3.2 Application of direct blood analysis for the evaluation of modified biocompatible C18-PAN SPME coatings

Evaluation of the modified C18-PAN coatings made with all the types of modifications showed that UV-dried thin PAN-over C18-PAN provided the best results in terms of biocompatibility and reusability (at least 30 reproducible extractions from whole blood). A detailed description of the evaluation and performance of all the modifications are described in the following section:

MPC-modified C18-PAN: The evaluation of thin-film MPC-modified C18-PAN coatings showed considerable enhancement of biocompatibility in direct whole blood analysis.

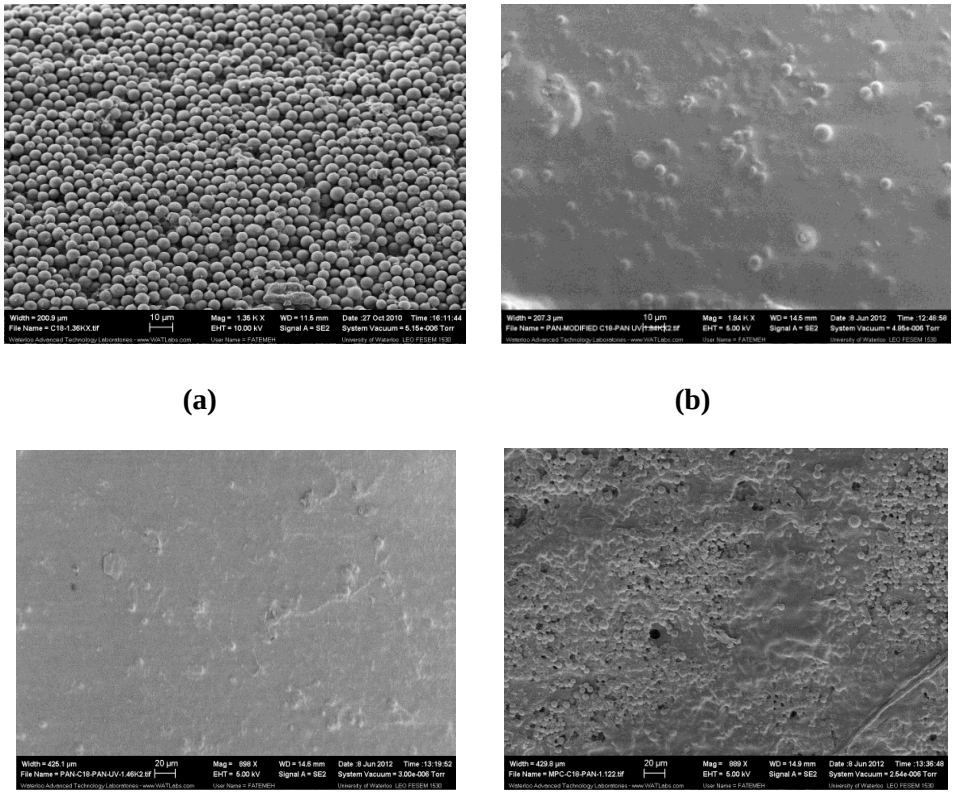


Figure 4.4 Scanning electron microscopy (SEM) micrographs of (a) original C18-PAN, (b) UV-dried thin PAN-over C18-PAN, (c) UV-dried thick PAN-over C18-PAN, and (d) MPC-modified C18-PAN thin-film SPME coating.

This coating demonstrated improved reusability for at least 20 direct blood analyses with negligible blood cell attachment and no change in the extraction recovery of the coating (RSD= 11%). After the 20th extraction, some localized attachment of the blood cells started appearing, mainly in the tip areas of a number of thin-film coatings (4 out of 12). The absolute recoveries for 20 extractions are shown in Figure 4.5.

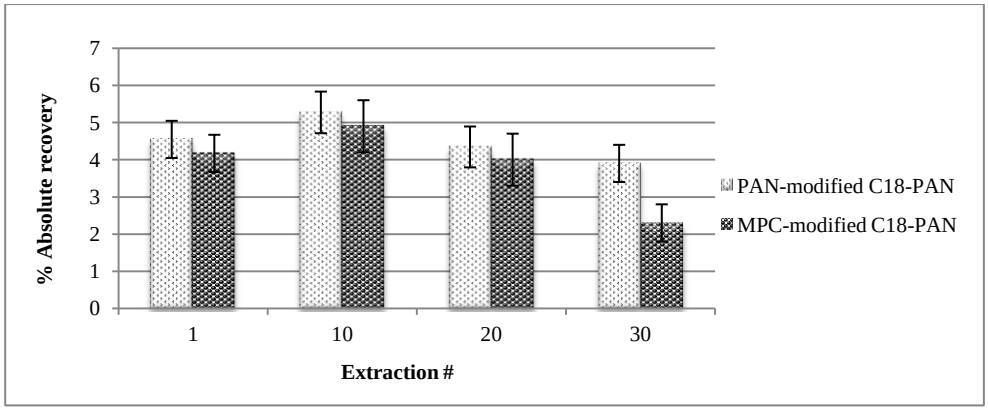


Figure 4.5 Direct immersion SPME-LC–MS/MS analysis using UV-dried thin PAN-over C18-PAN and MPC-modified C18-PAN for the extraction of 100 ng/mL of diazepam from whole blood, n = 6 (quantitation based on external calibration).

PAN-over C18-PAN: Three different drying strategies were tested for the preparation of the modified PAN-over C18-PAN coating. Among all methods, drying with UV light had the best results and the UV-dried thin PAN-over C18-PAN showed the best performance in terms of blood compatibility. The performance of all the PAN-over coatings under different drying conditions is compared as follows:

a) Drying at 180 °C: although this modification decreased the amount of interactions of the blood cells with the coating surface, it resulted in localized red blood cell attachments in some small holes on the surface. The creation of small holes on the surface of the coatings is likely due to the vigorous evaporation of the solvents while drying at high temperatures.

b) Drying at 70°C: two sets of coatings (20 s and 60 s dipping in PAN) were dried using this method. However, both sets presented improved prevention of the blood cells' attachment on the surface of the coatings up to 16 extractions, this drying strategy resulted in some unsatisfactory changes in the coating structure.

c) Drying under UV light: This drying method was used to address the limitation of unwanted coating changes from high-temperature drying. UV light was used for curing two sets of thin and thick PAN-over coatings (20 s and 60 s dipping in PAN) for 30 min on each side. For both sets of coatings (n = 6 for each), the improvement of biocompatibility with whole blood was significant. However, the thinner PAN layer provided much better coating recovery and quality. As shown in Figure 4.5, the thin PAN-over C18-PAN coating resulted in reproducible efficiencies (RSD = 12%) for at least 30 extractions from whole blood without any blood cell attachment.

In addition, the thinner modified coating provided higher extraction recovery. The extraction recovery of the thin UV dried PAN-over C18-PAN coating (95 ± 5% for the extraction of diazepam from PBS) is comparable to the original C18-PAN; however, the thicker modification provided a decrease in extraction recovery when they are compared for equilibrium extraction in PBS (80 ± 7%). The coating recoveries of the original and all the modified C18-PAN coatings are reported in Figure 4.6. It seems that the thin PAN layer acts as a porous bag which holds the C18-PAN coating (Figure 4.4 (a and b)) without changing the extraction recovery of the original coating. However, it is assumed that the thicker PAN layer changes the efficiency of the original coating by blocking the available surface pores on the coating (Figure 4.4 (c)). This thick layer can act as a barrier for the analytes and results in lower recoveries even when a longer extraction time is provided.

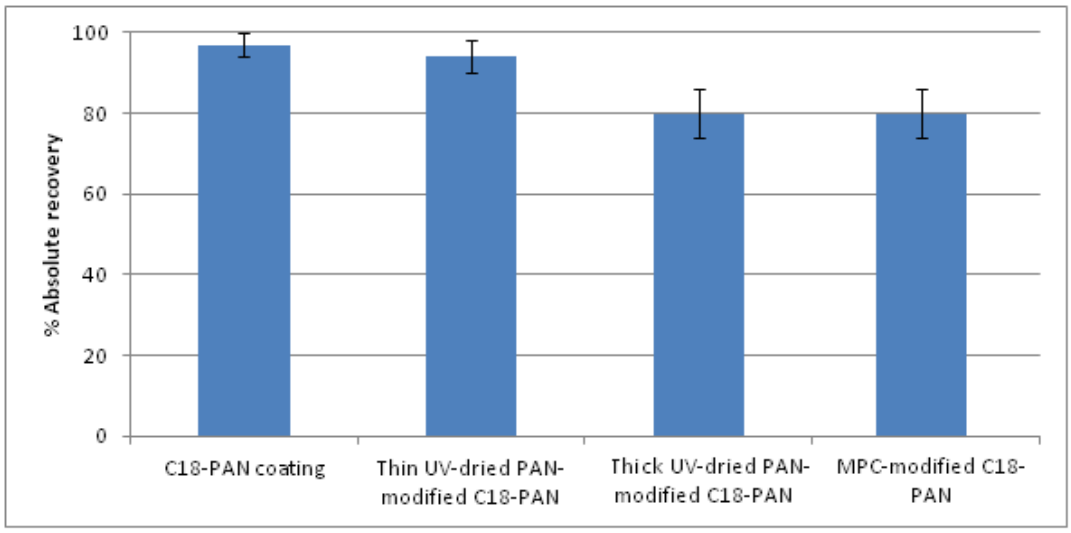


Figure 4.6 The comparison of the extraction efficiencies of the original and modified C18-PAN coatings for equilibrium extraction of 100 ng/mL diazepam from phosphate buffered saline (pH = 7.4), n = 6.

4.3.3 Optimization of SPME parameters for the UV-dried thin PAN-over C18-PAN SPME coating for direct immersion whole blood analysis

The optimized SPME parameters of the PAN-over C18-PAN coating were comparable to that of the original C18-PAN coating for the extraction of diazepam (with the exception of the wash step). A detailed description of the SPME parameter for C18-PAN coating can be found in Chapter 3 (Section 3.3.3). In summary, 30 min preconditioning (methanol/water 1:1 v/v), 60 min equilibrium extraction (comparable for PBS and whole blood), and 40 min desorption (acetonitrile/water 1:1 v/v) were found to be as the optimal SPME parameters for the extraction of diazepam from whole blood. A 30 min second desorption was needed to clean the trace residual in the coating from the previous desorption (< 2% carryover).

The optimization of the wash step showed that the application of the agitation during wash is vital for the removal of adsorbed blood cells on the surface. A 20 s wash step in nanopure water along with agitation was enough to clean the proteins and blood cells from

the surface of the coating. A shorter wash step resulted in some red blood cell attachments. In addition, a longer wash step was not optimal, since it provides a higher chance of analyte loss. The possibility of the loss of analyte during the 20 s wash was evaluated through the direct injection of the wash solution into the LC–MS/MS system. The amount of absolute loss of diazepam during the 20 s wash step (with agitation) was 0.3% (RSD = 13%, n = 6).

4.3.4 Reproducibility and validation for direct immersion whole blood analysis coupled with LC–MS/MS

Evaluation of the absolute matrix effect was performed by comparing the blank blood extract spiked with the 50 ng/mL of diazepam with the same concentration of the standard solution. The reproducibility of the coating preparation step and repeatability of the extraction recovery in long-term use were studied through inter- and intra-blade RSD, using two different calibration methods (isotope dilution and external calibration methods). Table 4.1 reports on the absolute recovery and matrix effect, inter- and intra-day reproducibility, LOD, LOQ, and linearity for direct immersion whole blood analysis of diazepam (equilibrium extraction).

Table 4.1 Recovery, reproducibility, and validation for direct immersion SPME-LC–MS/MS

	%Absolute recovery	Inter-day (RSD %) isotope dilution/ external calibration	Intra-day (RSD %) isotope dilution/ external calibration	LOD (ng/mL)	LOQ (ng/mL)	%Absolute matrix effect	Linearity (R^2)
Direct blood SPME-LC-MS/MS	4.8*	4/12*	1/10*	0.2	0.5	105	0.996

* n = 4 for 100 ng/mL diazepam in whole blood

Extracted Blood Spot Sampling (EBS)

4.3.5 Optimization of EBS parameters for LC–MS/MS analysis

By applying the current blade geometry for the preparation of the coating, a blood spot volume as low as 3 up to 20 µL can be used for EBS sampling. However, through the application of other geometries with higher surface areas, the available surface area of the coating can be improved and a larger sample volume can be used. A 5 µL sampling volume was used to evaluate the limit of quantitation of the method for small volumes of the sample. After the deposition of the blood spot on the surface of the coating, the blood and the coating needed to be in contact for a period of time to allow for the extraction of compounds by the coating. It is important that this period – referred to as the "extraction time" – be optimized.

Since the coating is designed to be reusable, the extraction time should be short enough to prevent the complete drying of the proteins and blood cells on the coating and long enough to get a reproducible amount of extraction recovery. The results of the studies on the extraction time show that a 2 min extraction addresses both above-mentioned issues. The wash, desorption, and carry over steps for the extracted blood spot sampling were similar to those of the direct immersion procedure (20 s wash along with 40 min desorption in acetonitrile/water 1:1 v/v and 30 min second desorption to clean < 2 % carryover).

4.3.6 Quantitation, reproducibility, and validation for EBS-LC–MS/MS

Taking advantage of the concept of microextraction, EBS can benefit from having a short pre-equilibrium extraction time and, consequently, achieves a small but reproducible amount of extraction recovery. It is highly recommended that a proper quantitative calibration method is applied for the EBS-LC–MS/MS analysis in order to obtain reliable

and precise quantitative results. Matrix-matched external calibration and isotope dilution are the proposed quantitation methods to be used for the EBS-LC–MS/MS analysis. The reproducibility of the assay was compared using both quantitative methods. As expected, the isotope dilution calibration resulted in large improvements in reproducibility. Therefore, the isotope dilution method was used as the quantitation technique for this study in order to compensate for any possible variation in the sampling time in the pre-equilibrium extraction method, and to achieve higher precision.

The recovery of diazepam for tested spiking levels of 0.5 and 50 µg/mL were found to be 97 % (±2) and 102 % (±3) when matrix-matched isotope dilution was used for quantitation of n = 4 samples. Table 4.2 reports on absolute recovery and matrix effect, reproducibility, LOD, LOQ, and linearity of EBS-LC–MS/MS analysis for a 5 µL volume of blood and 2 min extraction time. The limits of detection and quantitation can be obtained through the application of a larger volume of the sample.

Table 4.2 Recovery, reproducibility, and validation for the extracted blood spot-LC–MS/MS (reproducibility evaluated using isotope dilution and extrenal calibration methods).

	%Absolute recovery	Inter-day (RSD %) isotope dilution/ external calibration	Intra-day (RSD %) isotope dilution/ external calibration	%Absolute matrix effect	LOD (ng/mL)	LOQ (ng/mL)	Linearity (R^2)
Extracted blood spot-LC–MS/MS (5 µL, 2min)	2.1[§]	5/14[§]	2/12[§]	98	70	200	0.993

[§] n = 4 for 5 µL of 1 µg/mL diazepam in blood

Direct Analysis in Real Time (DART)

4.3.7 Mechanism of desorption and ionization in DART

As briefly explained in the "Introduction" section of this chapter, the desorption of analytes from the coating and their ionization are made by directing a flow of heated gases containing metastable species into the sample.[169]

As inert gases flow through the DART chamber, an electrical discharge creates plasma that contains ions, electrons, and excited neutral atoms and molecules. Electrostatic lenses remove the charged particles (ions and electrons) from the gas stream, carrying metastable species. The gas temperature is increased through a heater coil as it travels toward the exit orifice of the source. The heated gas **(**containing neutral but highly energetic atoms and molecules**)** exits the source heading toward the sample, which is placed along the path between the DART insulator cap and MS inlet, for thermal desorption and ionization.

Even though the DART ionization mechanism is not fully characterized, it is assumed that the ionization in the positive mode is initially performed by the reaction of gas metastables with some ambient atmospheric species such as water to form reactive protonated clusters. Then, the formation of $[M+H]^+$ ions is achieved by subsequent proton transfer from these species to the analyte's molecule (M).[169, 171, 172]

4.3.8 Optimization of the coating preparation for EBS-DART–MS/MS

Typically, stainless steel meshes are used as the substrate for the deposition of samples in the DART-SVP system. These meshes are used because the substrate needs enough paths to facilitate transmission of the heated desorption gas through the sample to allow for the efficient desorption and ionization the target analytes. When combining EBS with DART, it

is preferable to prepare the coating on similar stainless steel meshes for the same reason. The coated mesh should be very thin in order to prevent complete blocking of the mesh holes with the coating. In addition, the method of coating preparation should be reproducible.

In this study, thin layers of the UV-dried PAN-over C18-PAN extractive phase was coated on the stainless steel meshes using three different methods of coating preparation (dipping, brush painting, and spraying). Brush painting was found to be the most applicable and reproducible method for the preparation of EBS-DART systems. In order to guarantee the easy transmission of desorption gas through the coating, 10 tiny holes were made on the spotting area of the coated meshes using a very small needle. The patterns of the holes for all the coatings were kept similar for the entire study.

4.3.9 Optimization of EBS sampling for DART–MS/MS

As previously discussed, it is crucial to optimize the extraction time for EBS in order to prevent the complete drying of the blood cells on the coating and to obtain reproducible recovery. The results show that an extraction time longer than 5 min results in difficulties in washing the blood from the surface of the coated mesh. Since the maximum extraction time was preferable in order to obtain higher sensitivity (without the risk of blood cell attachment), 5 min was used as an optimal extraction time for the entire EBS-DART–MS/MS study. After a 5 min extraction, the application of a pressured stream of water (using a wash bottle) for 5 s was found sufficient to wash the blood from the surface of the PAN-over C18-PAN coated meshes. Then, in order to prevent any variation in analysis, the coated meshes should be fully dried before being introduced to the DART source. Higher RSD values were obtained for the samples which were not fully dried.

For the drying step, four different methods were evaluated: (i) 30 min drying at room temperature, (ii) drying with KimWipe paper tissue through gentle wiping to absorb water, and drying with (iii) hot or (iv) cold air using a blow dryer (3 min). The evaluation of the drying approaches demonstrated that all four methods of drying were comparable in terms of extraction recovery and reproducibility for the analysis of diazepam; however, the last three methods were much faster.

The short 30 s sampling (desorption and ionization) time in DART does not result in complete desorption of the analytes from the coating. Therefore, in cases when the coated meshes are reused for the analysis, the coatings can be vortexed in acetonitrile/water 1:1 (v/v) for 40 min and allowed to dry thoroughly before usage, or longer exposure time in DART can be used to achieve complete desorption of the analytes from the coating.

4.3.10 Optimization of the desorption/ionization gas temperature in DART–MS/MS

Desorption/ionization of gas temperature is one of the key parameters that influence the result of DART–MS analysis. In order to obtain the most efficient thermal desorption of the diazepam, a wide range of temperature (150–400 °C) was tested, and the result were evaluated in terms of signal intensity and reproducibility. The optimal temperature was found at 250 °C; it provided the highest peak intensity and lowest RSD for the desorption and ionization of diazepam. The lack of efficiency for higher temperatures is a result of the instability of the C18 particles and polyacrylonitrile at the temperatures >250 °C.

4.3.11 DART–MS/MS sample presentation speed

Sample presentation speed or the speed that the sample is pushed through the desorption/ionization region has a significant effect on the process of the desorption and

ionization of analytes in DART. In order to evaluate the optimum sampling speed, a range of speeds (0.2 to 1 mm/s) was tested. The results showed that a sample presentation speed of 0.2 mm/s (minimum available speed) provided the highest signal intensity and reproducibility for diazepam. A slower sampling speed allows the sample spots to remain in the heated ionization region longer, resulting in more efficient desorption and ionization.

4.3.12 Quantitation and validation of EBS-DART–MS/MS

Matrix-matched external calibration is usually used for the quantification of analytes in DART–MS analysis; however, depending on the analyte of interest and the matrix under study, many studies have reported on the limitations of ambient MS analysis in DART due to the relatively high RSD for replicated DART–MS measurements.[172, 177]

In contrast, isotopic-labeled internal standards can significantly improve reproducibility by compensating for the signal fluctuation and unavoidable matrix effects that usually occur with ambient ionization techniques.[172, 177] Therefore, matrix-matched isotope dilution was employed for quantitation of EBS-LC–MS/MS analysis in this study, and calibration curves were plotted daily. The isotope dilution calibration for diazepam in whole blood for EBS-DART–MS/MS system is illustrated in Figure 4.7.

In this part of the study, the recovery, limits of detection and quantitation, and linearity of the EBS-DART–MS/MS (coated mesh) sampling was compared with regular DART–MS/MS (bare mesh) for the extraction of diazepam (Table 4.3).

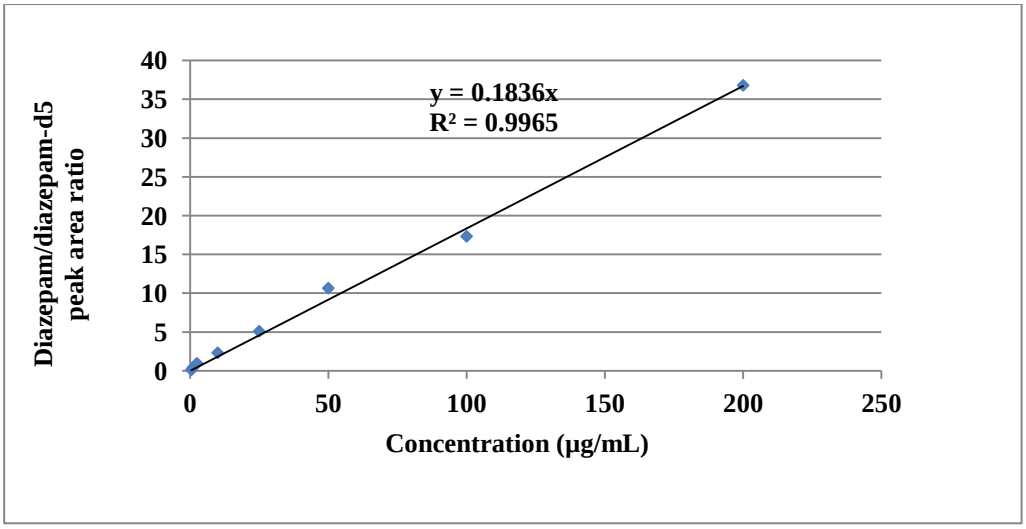


Figure 4.7 Isotope dilution calibration for diazepam in whole blood (diazepam-*d5* as internal standard) using EBS-DART–MS/MS (UV-dried PAN-over C18-PAN coated mesh).

Table 4.3 Recovery and validation for EBS-DART–MS/MS and DART–MS/MS.

	UV dried PAN-over C18-PAN	Bare mesh
Recovery * (%) (2 µg/mL spiking level)	96	108
Recovery * (%) (50 µg/mL spiking level)	102	93
LOD (µg/mL)	0.3	0.3
LOQ (µg/mL)	1	1
Linearity (R^2)	0.9965	0.9981
Linearity (µg/mL)	1-200	1-50

* Recoveries were calculated based on isotope dilution matrix-matched calibration when extracting 5 µL of 5 µg/mL diazepam in whole blood (n = 4).

Recoveries were calculated based on the matrix-matched isotope dilution calibration. EBS-DART sample preparation (coated mesh) provided an improvement in the linear range

when compared to the bare mesh. It should be noted that the reported LOD and LOQ values are based on 5 µL of blood sample and considering >95% diazepam-plasma protein binding.

4.3.13 Reproducibility of EBS-DART–MS/MS

The evaluation of inter- and intra-day reproducibility in three different matrixes (acetonitrile/water 50:50 (v/v), PBS, and blood) was performed for bare and C18-PAN coated mesh using external calibration and isotope dilution (Table 4.4). The results of the analysis for both bare and coated meshes and using all three matrixes reveal that the normalization of the analyte's response using isotope dilution calibration provides a significant improvement in quality and reproducibility of the data.

Table 4.4 Reproducibility for DART–MS/MS and EBS-DART–MS/MS.

	Isotope dilution/external calibration reproducibility (RSD, %).			
Matrix	*Bare mesh*		*PAN-over C18-PAN coated mesh*	
	Inter-day (n=4)	Intra-day (n=4)	Inter-day (n=4)	Intra-day (n=4)
Acetonitrile/water (1:1, v/v)	4/24	3/21	3/20	3/22
PBS buffer	5/30	4/33	4/25	3/30
Whole blood	4/40	5/35	3/21	2/20

* 5 µL of 5 µg/mL diazepam (and diazepam-*d5* in case of isotope dilution) in whole blood.

The relatively high RSD for the external calibration (which is corrected by isotope dilution calibration) could be explained by the following reasons: (i) variations in position of the mesh in the transmission survey, (ii) random distribution of the sample spot on mesh, (iii) variation of the thickness of the coated meshes, and (iv) the possibility of leaky Vapur interface on MS. However, even when external calibration is used, the reproducibility of the assay is improved using the coated mesh in comparison to the bare mesh.

4.3.14 Evaluation of matrix interferences in EBS-DART–MS/MS

Matrix effects (ion suppression/enhancement) provided by interfering matrix components can lead to significant errors in the quantification of the analytes, and can influence detection capability, precision, and accuracy of the method.

The main goal for the integration of EBS with DART is to minimize the matrix effect by cleaning up the sample and normalizing the matrix. For the purpose of evaluating matrix effects, the total ion chromatogram for the analysis of 5 µL blood spot samples (10 ppm diazepam) on PAN-over C18-PAN coated mesh was compared with that of bare mesh. As shown in Figure 4.8 (a), in the absence of EBS sampling, the existence of matrix inferences resulted in a significant suppression of the diazepam's signal (m/z = 285.1). In contrast, the selective extraction of analyte and the wash of the blood spot in EBS sampling resulted in the elimination of ion suppressing components, and a much cleaner mass spectra was obtained (Figure 4.8 (b)).

As another part of study, matrix-matched external calibration curves of DART–MS/MS (bare mesh) and EBS-DART–MS/MS (coated mesh) in whole blood was compared in their overlapped linear range (1–50 µg/mL). As shown in Figure 4.9, a significant shift in the matrix-matched external calibration curve of the bare mesh was observed, when it is compared with that of coated mesh. This observation can be explained by the adverse matrix effect on the ionization of analytes on the bare mesh or the possible pre-concentration of analytes on the coated mesh, or both. Further investigations on this matter are underway in our laboratory.

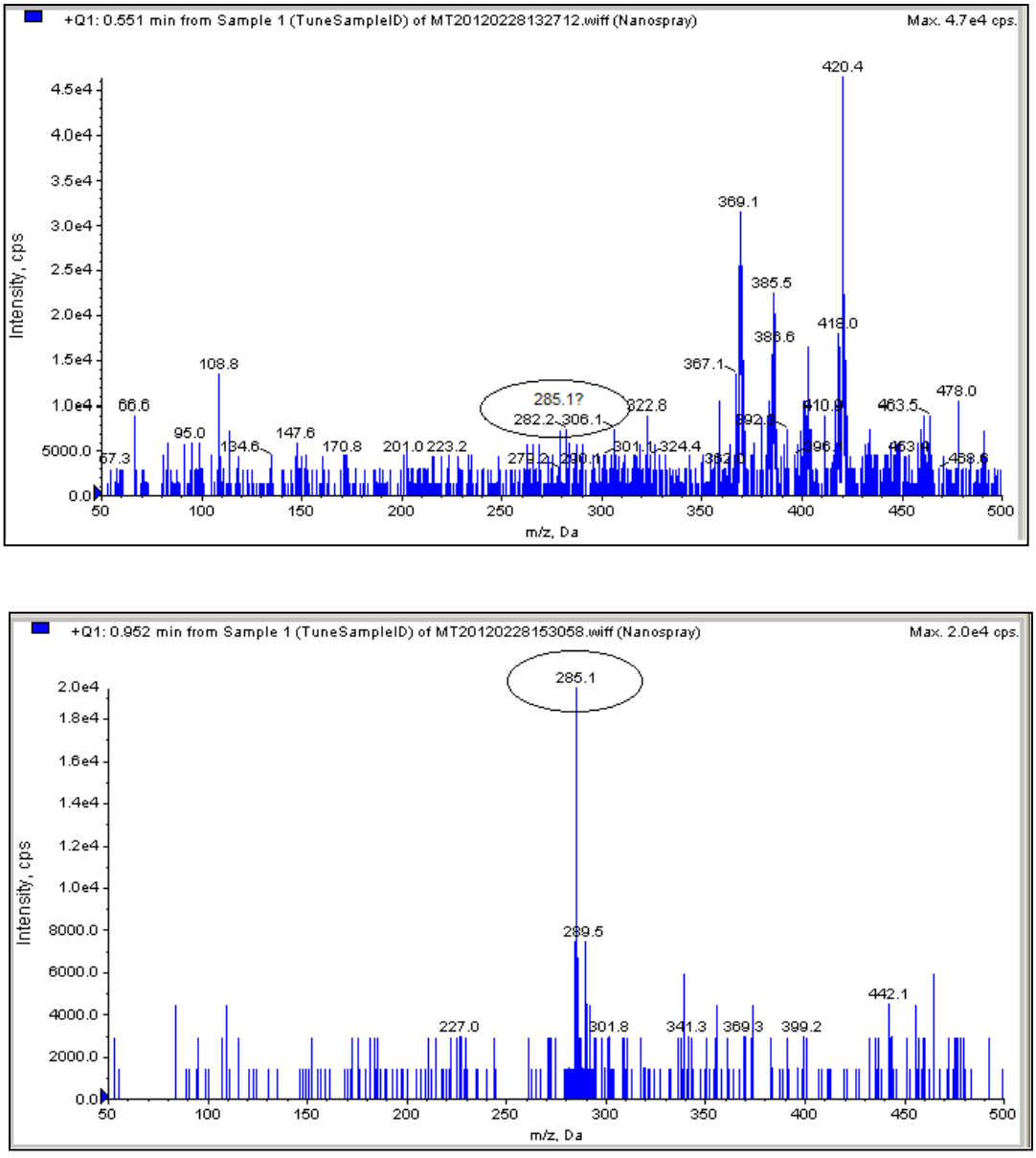


Figure 4.8 Evaluation of matrix interferences for blood analysis. Full scan Q_1 mass spectra for (a) DART–MS using bare mesh, and (b) EBS-DART–MS using PAN-over C18-PAN coated mesh (2 min extraction and 5 s washed after 5 min extraction).

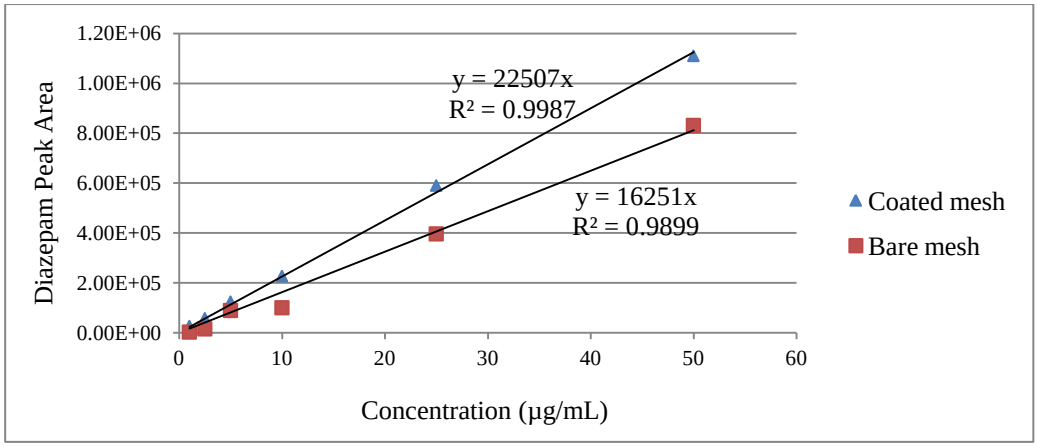


Figure 4.9 Comparison of diazepam matrix-matched external calibration curve for DART–MS/MS (using bare mesh) with that of EBS-DART–MS/MS (using PAN-over C18-PAN coated mesh) for 5 µL of blood spot.

4.4 Conclusions and future directions

This optimized biocompatible coating has the potential to be applied for the analysis of other complex biological matrixes such as tissue and food samples. As a future approach, the biocompatible PAN-over C18-PAN SPME offers the chance for reliable *at vivo* (instant sampling of biofluids in field at the time they leave the living system) and *in vivo* applications (using other geometries of coating), through minimizing the risk of cell coagulation on the surface and adverse and toxic reactions in the living system.

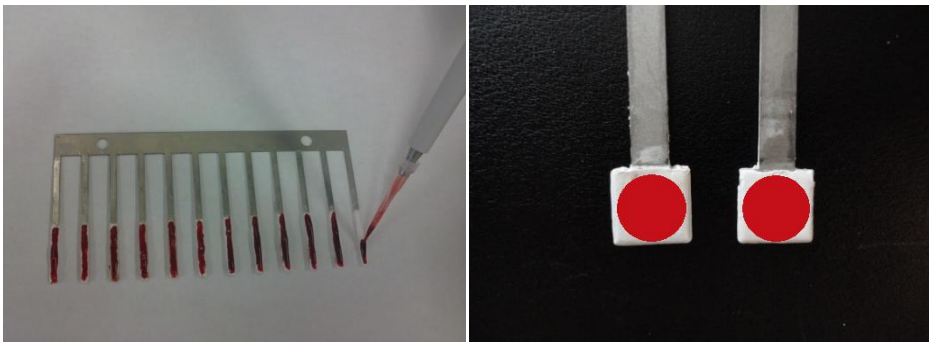
EBS benefits from the advantages of DBS and presents solutions for the limitations of DBS. In this work, EBS-LC–MS/MS integrates sampling and sample preparation into a single step. In addition, the application of an automated high-throughput system aided the preparation of 96 EBS samples in approximately 42 min, corresponding to < 0.5 min per sample.

EBS-DART–MS/MS is a novel approach which improves the reliability of DART by cleaning up the interfering matrix without requiring time-consuming sample treatment. DART–MS/MS is a very fast analysis method with no need for any chromatographic separation, and it can achieve high reliability through being combined with EBS sampling, without sacrificing time. The application of the high-throughput automated transmission survey in this study resulted in a total of 14 min for the EBS-DART–MS/MS analysis of 10 samples (5 min extraction, 3 min blow drying, and 6 min mass spectrometry), which corresponds to 1.4 min per sample. This analysis time can be reduced by the application of EBS-DART in 96-format.

The reported range for the therapeutic and toxic concentrations of diazepam in the literature differ based on different individual patient conditions, various dose intakes in different treatment, and the plasma/whole blood distribution ratio of the analyte.[178-181] The average ranges for the therapeutic and toxic concentrations of diazepam in whole blood are 0.05-1.45 and 1.4-8.6 µg/mL, respectively.[182] For a 5 µL blood spot in EBS-LC–MS/MS and EBS-DART–MS/MS studies, a LOQ of 0.2 and 1 µg/mL were obtained, respectively. These limits of quantitation can match the entire toxic concentration range of diazepam and medium-to-high levels for the therapeutic concentrations range. However, the enhancement of sensitivity can be achieved via an increase of the sample volume (from 5 µL up to 50-100 µL for instance) and/or by increasing the available free concentration of the analyte in the matrix (such as the disruption of drug-protein binding which was discussed in Chapter 3, Section 3.3.5).

The application of the thin-film coating with a higher surface area can facilitate the enhancement of the volume of the blood spot in EBS sampling. Therefore, for future studies,

square thin-film geometry (length: 10 mm, width: 10 mm, thickness: 0.8 mm) with a 2-fold increase in surface area is proposed by the author as an alternative to the current geometry (Figure 4.10). The proposed geometry will facilitate an even distribution of blood spot with larger volumes and individual applications of EBS coatings (instead of blade rows). In addition, it will provide a faster extraction rate, higher recovery, and improved LOD in a limited extraction time.



(a) **(b)**

Figure 4.10 (a) Current geometry, and (b) newly proposed geometry for the extracted blood spot sampling using UV-dried thin PAN-over C18-PAN thin-film coating.

The application of the EBS-DART–MS/MS is very valuable for analyzing short-lived metabolites and unstable compounds, and it is beneficial for monitoring and determining unstable metabolites for on-site clinical and biological analysis. The application of the presented EBS-DART–MS/MS system was recently reported in the Pawliszyn research group for the analysis of cocaine and methadone in urine samples.[183] As a future approach, further investigations are required for the application of the EBS methodology for the analysis of other classes of compounds with different polarities and characteristics.

4.5 Addendum

The text of this chapter was rewritten in comparison to the published research article. The author thanks IonSense Inc. and VBM Consulting Ltd. for their collaboration and the provision of the DART SVP system.

Chapter 5

Development of coatings for the automated 96-blade SPME system, capable of extracting a wide polarity range of analytes from biofluids

5.1 Preamble and introduction

5.1.1 Preamble

This chapter has been published as a paper: Fatemeh S. Mirnaghi, Janusz Pawliszyn, Development of Coatings for Automated 96-Blade Solid Phase Microextraction-Liquid Chromatography–Tandem Mass Spectrometry System, Capable of Extracting a Wide Polarity Range of Analytes from Biological Fluids, *J. Chromatogr. A, 2012,* 1261, 91-98. The materials of the current chapter are reprinted from this publication with the permission of Elsevier (Copyright Elsevier 2012).

5.1.2 Introduction

Since the introduction of SPME in 1990,[31] this technology has been expanding to different areas such as clinical, biological, pharmaceutical, food, environmental and metabolomics studies.[1, 32, 34, 38, 41, 147, 184] The successful and reliable utilization of SPME technique for different applications relies on addressing the requirements for each individual study, including the construction of new SPME devices, the development of high-throughput automated SPME systems, and the discovery of new stationary phases. Some studies require the analysis of compounds from different classes and with various physiochemical properties (e.g., metabolomics studies). This requirement can be addressed through (i) the development of different chemistries of SPME extractive phases for individual polar or non-polar groups

of compounds, or (ii) the development of proper SPME coatings to enable parallel extractions of compounds in a wide range of polarity. The latter category takes advantage of the simultaneous extraction of analytes with different properties and hydrophobicity in a single experiment. This advantage is valuable when it is not feasible to design two separate experiments for the extraction of polar and non-polar analytes (i.e., the amount of sample is too small and/or the metabolites are not stable). The simultaneous extraction of analytes results in significant time saving as well as improvements in the accuracy of the data by avoiding the possible variations in different experimental conditions.

Most commercially available SPME coatings exhibit limited efficiency toward the extraction of a wide range of polarity. In addition, they suffer from the lack of chemical and physical stability, long-term reusability,[110] and biocompatibility (the fouling of the extraction phase upon exposure to biological samples).[79] As reported in Chapters 2 and 3, so far C18-silica gel and C18-PAN are the only developed extractive phases for the 96-blade SPME system. As expected from their C18- functional group, they are mainly capable of the extraction of non-polar and mid-polarity compounds, and are limited in their ability to extract highly polar analytes.

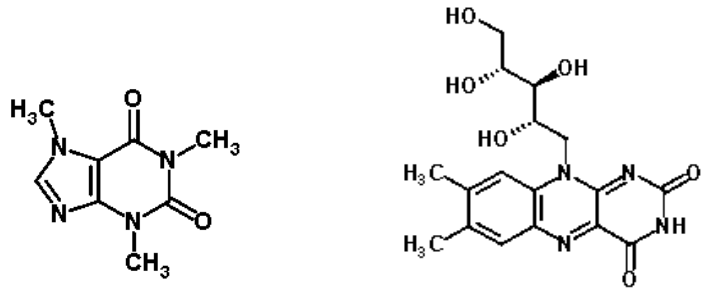
Therefore, the main focus of this chapter is the development and evaluation of alternative coatings for the 96-blade SPME system capable of the simultaneous extraction of both hydrophilic and hydrophobic compounds in a single experiment. Modified polystyrene-divinylbenzene copolymer with a weak anion exchanger (PS-DVB) and phenylboronic acid (PBA) are two candidate stationary phases which can address the discussed requirements. The combination of the reversed phase mechanism of PS-DVB and ion exchange interactions

of copolymerized weak anion exchanger groups provides a unique extraction recovery for this mixed-mode sorbent for a wide range of compounds.[185, 186]

In addition, PBA contains different functional groups which are involved in multiple interaction mechanisms with compounds, and provides the efficient extraction of different classes of analytes.[187-189] A previous study of the Pawliszyn group reported on the successful application of these two extractive phases for the efficient extraction of 36 metabolites with a wide range of polarity.[105] However, in the previous work the stability, reusability, and biocompatibility of the coatings were not the main focus. The coatings were prepared in single rod fiber geometry, and commercial glue was used for the immobilization of particles on the substrate. The current study consists of the development of PS-DVB and PBA stationary phases for the 96-blade SPME system with four main improvements upon the previous work: (i) high stability and reusability, (ii) improved biocompatibility, (iii) thin-film geometry and correspondingly increased sensitivity, and (iv) the application of an automated high-throughput 96-format SPME system, with the main focus on its ability to extract analytes in a wide range of polarity.

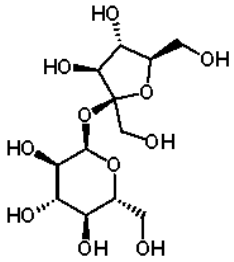
In order to achieve the required biocompatibility and stability properties, PAN was used as the biocompatible binder for the immobilization of the particles on the coatings. The efficiency of the PS-DVB-PAN and PBA-PAN 96-blade SPME systems were evaluated for the LC–MS/MS analysis of diazepam, oxazepam, caffeine, riboflavin, and sucrose with a wide range of polarity (log P = 2.8 to -3.7)[190] from PBS and human plasma. Extraction recovery, reusability, reproducibility, absolute matrix effect, and the validity of the system were evaluated and/or optimized for both proposed coatings. The structures of diazepam and

oxazepam are shown in Figure 2.1, and the structure of caffeine, riboflavin, and sucrose are shown in Figure 5.1.



Caffeine

Riboflavin



Sucrose

Figure 5.1 Chemical structure of caffeine, riboflavin and sucrose.

5.2 Experimental

5.2.1 Chemicals and materials

Caffeine, riboflavin, sucrose, and acetic acid were purchased from Sigma-Aldrich (MO, U.S.). *Easy* modified polystyrene-divinylbenzene (Macherey-Nagel) particles were purchased from VWR International (Mississauga, Canada). Phenylboronic acid particles

were purchased from Varian Inc. (CA, U.S.). The remainders of the materials were purchased from the same sources reported in Chapters 2 and 3 (Sections 2.2.1 and 3.2.1).

A concentration of 1 mg/mL of analytes was purchased or prepared as the stock solution. The working standards were prepared daily (due to the instability of sucrose) from stock solutions using acetonitrile/water (1:1 v/v) as the diluents, and all stocks and working standards were stored at 4 °C in a refrigerator. Other information regarding the preparation of standard solutions and samples was provided in Section 2.2.2 (Chapter 2).

5.2.2 Automated Concept 96-Blade SPME system

This study used the Concept 96-blade SPME system along with the original configuration of Concept 96-autosampler described in Chapter 2 (Section 2.2.4).

5.2.3 Preparation of PS-DVB-PAN and PBA-PAN coatings

In Chapter 3 it was reported that the spraying method resulted in the highest levels of stability and reusability among other tested methods (spraying, dipping, and brush painting). As a result, the spraying method (reported in Section 3.2.3, Chapter 3) was chosen as the optimal method of coating preparation. In this chapter, the same formula and procedure was followed for the preparation of the coatings except that the spraying and drying steps were repeated 15 times in order to uniformly cover the surface of the substrates (due to the larger particle size). Finally, in order to improve the biocompatibility of the coatings and to minimize the attachment of the plasma proteins on the surface, a very thin layer of PAN glue was sprayed on the surface of the final coatings.

5.2.4 Liquid chromatography and mass spectrometry conditions

The liquid chromatography and mass spectrometry instrumentations used in the experiments of this chapter were the same as those reported in Chapter 2 (Section 2.2.3). A reverse phase pentafluorophenyl column (Supelco Discovery HSF5 column, 2.1 × 150 mm, and 3 μm particles) with gradient elution of mobile phases A (water/acetic acid 99.9/0.1, v/v) and B (acetonitrile/acetic acid 99.9/0.1, v/v) were used for the chromatographic separation of the analytes under study. The gradient elution program was optimized as follows: it started at 10% B and kept for 2 min (0.01–2.00 min), then, sharply raised to 90% B and kept for 3 min (2.01–5.00 min). After 5 min, the gradient was sharply changed to 10% B where it remained for another 5 min (5.01-10.00 min). The following were optimized as mass spectrometry parameters: ionspray temperature = 450 °C, ionspray voltage = 5300 V, nebulizer gas = 8, curtain gas = 6, and collision gas = 12 (arbitrary units). A summary of other MS/MS parameters is provided in Table 5.1. A typical SPME-LC–MS/MS chromatographic peak for all the analytes under study is provided in Figure 5.2.

Table 5.1 Summary of MS/MS parameters

Analytes	Q1(mass amu)	Q3(mass amu)	DP (V)*	FP (V)*	EP (V)*	CE (V)*	CXP (V)*
Diazepam	284.9	154.2	100	270	13	37	10
Oxazepam	286.5	241.1	80	180	13	31	23
Riboflavin	377.3	243.2	120	290	8	33	20
Caffeine	194.9	138.2	65	270	12	27	11
Sucrose	365.1[§]	203.0	160	210	11	28	15

* DP = Declustering potential, FP = Focusing potential, EP = Entrance potential, CE = Collision energy, and CXP = Collision cell exit potential.

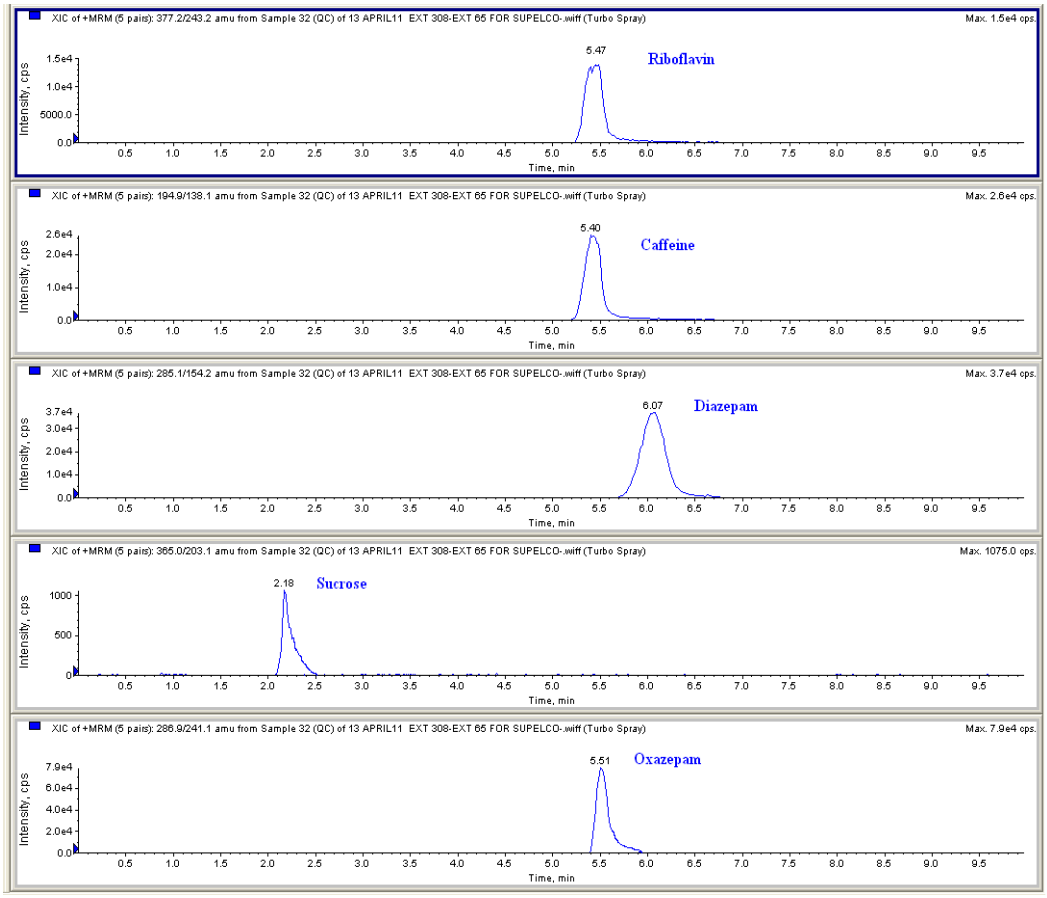


Figure 5.2 Typical SPME-LC–MS/MS chromatographic data of the model analytes

5.2.1 Optimized procedure for the automated 96-blade SPME analysis

Prior to extraction, the PBA-PAN coatings were conditioned for 30 min in methanol/water 1:1 v/v (along with agitation). The preconditioning step was not found necessary for the PS-DVB-PAN coating. Equilibrium extraction was performed for 60 and 120 min from 1 mL of spiked samples for the PS-DVB-PAN and PBA-PAN coatings, respectively (1000 rpm and 2.5 mm amplitude). A 10 s wash in nanopure water was carried out for both coatings after extraction from human plasma. The optimized desorption conditions for PS-DVB-PAN and PBA-PAN coatings were found to be 40 and 60 min

(respectively), using 1 mL of acetonitrile/water 1:1 (v/v) and 1200 rpm speed (1 mm amplitude). For both coatings, a 40 min second extraction was used to clean the residual trace of analytes from the coatings.

5.3 Results and discussion

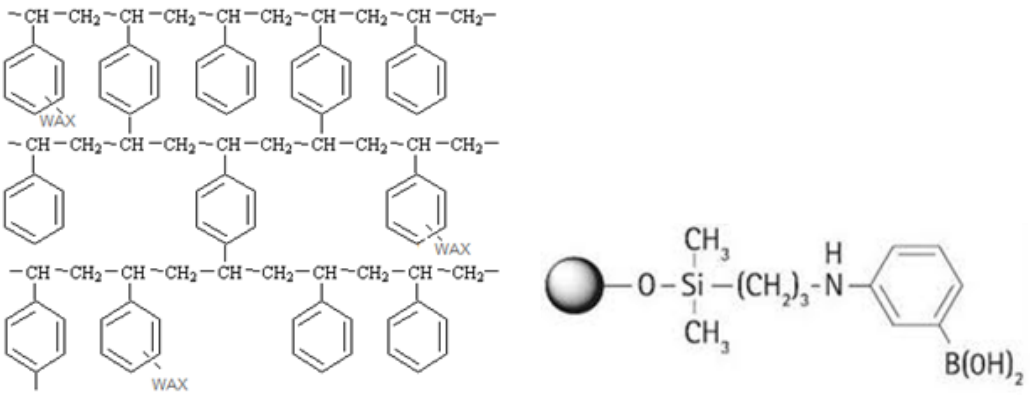
5.3.1 Characterization and scanning electron microscopy of the coatings

Figure 5.3 (a) demonstrates the chemical structure of modified polystyrene-divinylbenzene (PS-DVB) copolymer with an unknown weak anion exchanger (WAX). According to the manufacturer, it has a particle size of 80 µm, a specific surface area of 650 –700 m^2/g, and a pore size of 50 Å. The modified PS-DVB copolymer with WAX exhibits more polar properties than conventional PS-DVB polymers because of its bifunctional characteristic as a mixed mode sorbent.[185, 186]

As shown in Figure 5.3 (b), the PBA coating consists of phenylboronic acid covalently linked to a silica gel surface. This silica-based extractive phase consists of irregularly shaped acid-washed silica with a particle size of 40 µm, a mean porosity of 60 Å and a specific surface area of 500 m^2/g.

Due to the multiple functional groups in the PBA coating, different types of interactions and extraction mechanisms, depending on the nature of the analyte and the pH of the sample matrix, are involved. Boronic group functionality facilitates the covalent bonds of the phenylboronic acid with cis-diol containing compounds. However, for those analytes lacking cis-diol groups the extraction is performed via secondary interaction mechanisms, including hydrogen bonding interactions (with hydroxyl groups in neutral and anionic forms), ionic interactions (in case of the formation of the boronate form in alkaline

conditions), charge transfer (anionic boronate functionality), and van der Waals and pi-pi interactions (with phenyl and propyl groups). The exhibition of the anticipated secondary interactions is demonstrated in Figure 5.4. The capability of PBA to covalently bond with cis-diol containing compounds provides the opportunity for the efficient extraction of compounds that are not easily extracted by other sorbents via a universal extraction mechanism (e.g., nucleic acids, low molecular weight proteins, carbohydrates, and catechols).[187-189]



(a) **(b)**

Figure 5.3 Structure of (a) polar modified polystyrene-divinylbenzene copolymer with an unknown weak anion exchanger, and (b) phenylboronic acid sorbent consisting of phenylboronic acid covalently linked to a silica gel surface.

Scanning electron microscopy **(**SEM) was used for the evaluation of the topography of the coatings' surface using the same instrument and procedure reported in Chapter 2 (Section 2.3.9). The results of scanning electron microscopy of the coatings are shown in Figure 5.5. The thickness of the PS-DVB-PAN and PBA-PAN coatings were estimated to be approximately 230 and 185 µm, respectively.

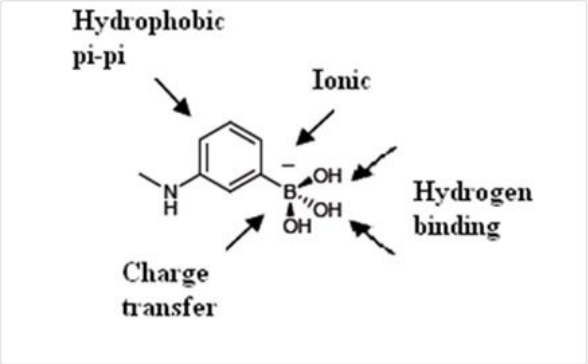
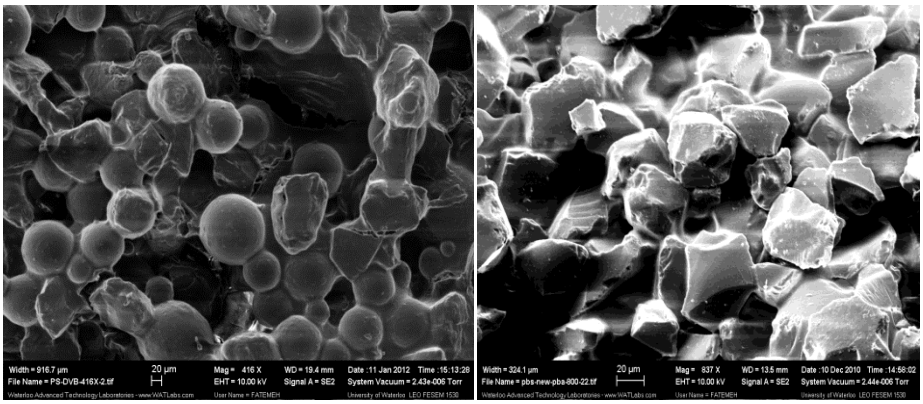


Figure 5.4 Illustration of the predicted secondary interactions of the phenylboronic acid extractive phase.



(a) **(b)**

Figure 5.5 SEM images of (a) PS-DVB-PAN SPME coating (80 µm particles) using 416 × magnification, and (b) PBA-PAN SPME coating (40 µm particles) using 837 × magnification.

5.3.2 Development of the automated 96-blade SPME method

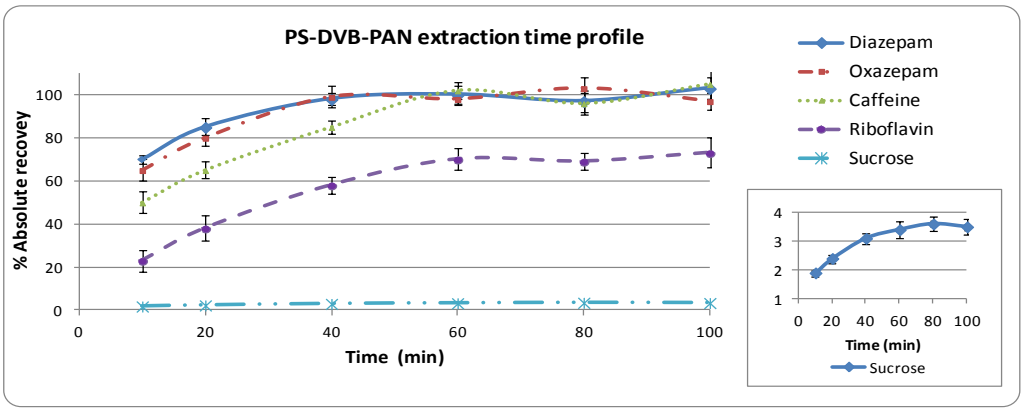
Preconditioning: The effect of preconditioning on the extraction recovery of the coatings was evaluated through the extraction recovery and reproducibility of the system with and without a preconditioning step (30 min agitation in methanol/water 1:1 v/v). The evaluation showed no considerable difference in the extraction efficiencies of PS-DVB-PAN coatings in both conditions. In contrast, the preconditioning of silica-based PBA-PAN thin-film coating resulted in very significant improvements in the extraction recovery. A 30

minute preconditioning step, with agitation, was found optimal for the preparation of PBA-PAN coating prior to the extraction.

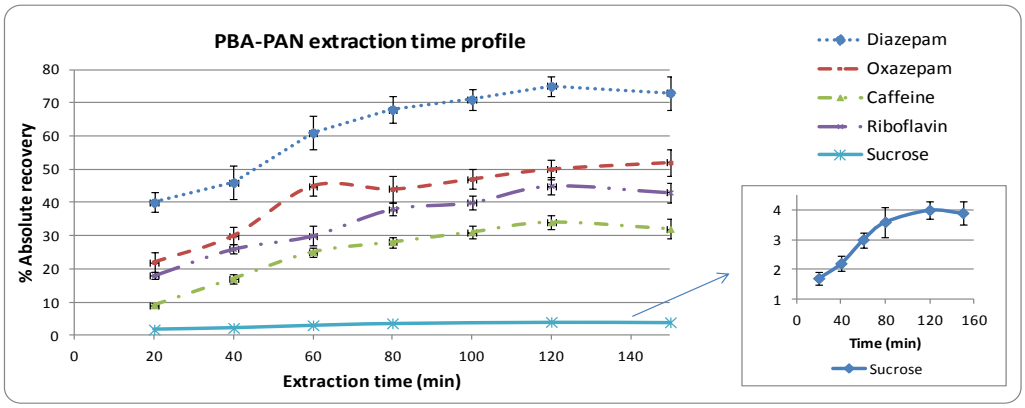
Extraction: The extraction time profiles demonstrated showed that 60 and 120 min extractions are required for equilibrium extraction of all five analytes when extracting with PS-DVB-PAN and PBA-PAN coatings, respectively (Figure 5.6). The evaluation of the extraction time profiles for extraction from human plasma compared to that of PBS showed no significant difference. The extraction was performed from 1 mL of spiked PBS or human plasma, and this volume was controlled precisely through the entire study. The extraction agitation speed was set to 1,000 rpm (2.5 mm amplitude), as the maximum available speed without spilling the sample.

Wash: A 10 s static wash step in nanopure water after extraction from human plasma was optimized as the minimum washing time with no protein attachment and the least amount of loss of analytes for the PS-DVB-PAN and PBA-PAN coatings. The evaluation of loss of analytes during the 10 s washing step showed that the amount of loss was in the range of 0.2 - 1.5% for all five compounds for both coatings.

Desorption: The desorption solvent was optimized via the evaluation of three different solvents (acetonitrile/water 50:50, methanol/water 50:50, and acetonitrile/water 80:20 v/v). For both PS-DVB-PAN and PBA-PAN coatings, acetonitrile/water 50:50 v/v was the optimal desorption solvent (it had the best recovery and lowest carryover) for all five analytes (Figure 5.7). Minimum desorption times of 40 and 60 min were found to be optimal for the efficient desorption of all five analytes, with the lowest carryover from PS-DVB-PAN and PBA-PAN coatings, respectively.



(a)

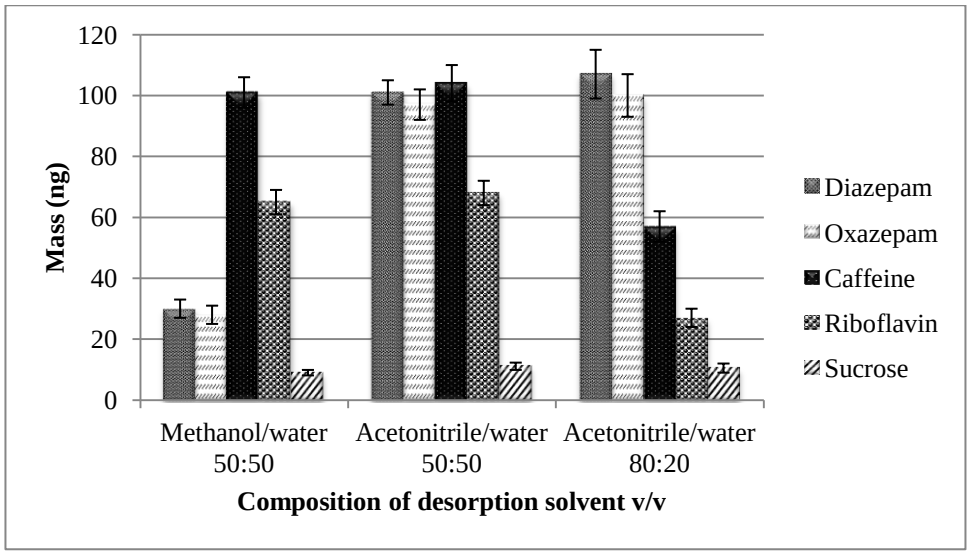


(b)

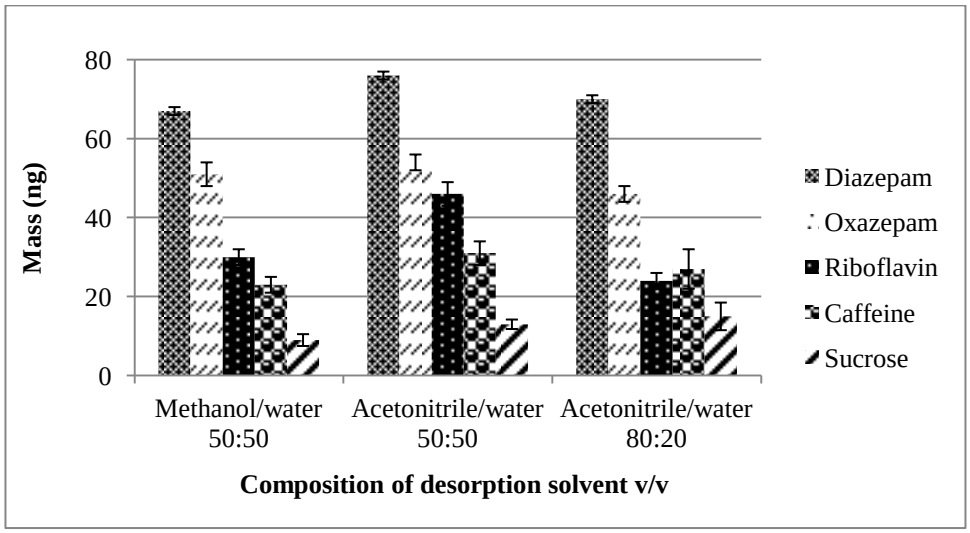
Figure 5.6 Extraction time profile of 100 ng/mL diazepam, oxazepam, caffeine, and riboflavin, and 300 ng/mL sucrose spiked in PBS, pH = 7.4 for (a) PS-DVB-PAN (n = 6) and (b) PBA-PAN coatings (n = 6).

In order to obtain a full coverage of the coatings and efficient desorption without the risk of spilling/cross contamination, 1 mL of the desorption solvent and 1200 rpm agitation speed (1 mm amplitude) was optimized. For the PBA-PAN coating, the effect of pH on the desorption of the compounds containing cis-diol functional groups (disruption of the covalent bindings with the boronic group) were evaluated through the comparison of desorption in acidic and neutral conditions. The comparison of the efficiency of the acidic desorption solvent (99% acetonitrile/water (50:50, v/v) + 1% acetic acid) with that of

acetonitrile/water 50:50 (v/v) showed no significant differences for the desorption of the compounds under study.



(a)



(b)

Figure 5.7 Desorption solvent optimization using optimal SPME conditions for the extraction of 100 ng/mL diazepam, oxazepam, caffeine, and riboflavin, and 300 ng/mL sucrose spiked in PBS (pH = 7.4) for (a) PS-DVB- PAN (n = 6), and (b) PBA-PAN coatings (n = 6).

Carryover: For both coatings, a second 40 min desorption step in fresh acetonitrile/water 50:50 (v/v) was found optimal to remove any remaining traces of the analytes from the previous desorption. The results of the analysis of the second desorption (demonstrating the amount of carryover after the first desorption) for the PS-DVB-PAN and PBA-PAN coatings are shown in Table 5.2. Desorption efficiencies of ≥ 95% are suitable for the quantitative analysis. The evaluation of the third desorption step (40 min) resulted in no detectable signal, indicating that a second 40 min desorption was enough for removing the trace of analytes from the coatings.

Table 5.2 Percent carryover for PS-DVB- PAN and PBA-PAN SPME coatings for the equilibrium the extraction of 100 ng/mL diazepam, oxazepam, caffeine, and riboflavin, and 300 ng/mL sucrose spiked in PBS, pH = 7.4 (n = 6).

Analyte	PS-DVB-PAN % Carry over	PBA-PAN % Carry over
Diazepam	3.1 ± 0.2	2.1 ± 0.2
Oxazepam	2.8 ± 0.15	1.5 ± 0.2
Caffeine	1.5 ± 0.05	0.5 ± 0.1
Riboflavin	2.0± 0.08	0.7 ± 0.1
Sucrose	ND	ND

ND = Not Detected

5.3.3 Efficiency of PS-DVB-PAN and PBA-PAN 96-blade SPME coatings

Table 5.3 demonstrates the extraction recovery of the PS-DVB-PAN and PBA-PAN coatings for the extraction of the compounds under study from PBS (pH = 7.4) and human plasma. Taking advantage of bifunctional properties and thin-film geometry, the PS-DVB-PAN 96-blade SPME coating provided exhaustive extraction recovery for non-polar and

polar compounds (diazepam: log P = 2.82, oxazepam: log P = 2.24, and caffeine: log P = –0.07), and >70% recovery for more polar compounds such as riboflavin with log P = –1.9. In addition, the coating demonstrated successful extraction (3.5%) for sucrose (log P = –3.7), which could not easily be extracted using pre-existing 96-blade SPME coatings. As shown in Table 5.3, PBA-PAN coating was also able to extract both types of polar and non-polar compounds with good efficiencies (4–74% absolute recovery). It is noteworthy that SPME is an equilibrium-based and non-exhaustive sample preparation technique. Consequently, the demonstration of exhaustive or high extraction recovery for this wide range of polarity of compounds is a remarkable benefit, obtained from the improved efficiency of the system (thin-film geometry and stationary phases). This improved efficiency cannot be easily achieved with conventional existing SPME systems. In addition, the ~4% recovery for the extraction of very polar sucrose from an aqueous sample is valuable, since the previous study (rod fiber geometries) showed an extraction recovery of <0.8% using similar stationary phases and almost no recovery using the other types of extractive phases.[105]

In SPME, the degree of enrichment of the compounds in the coating can be shown through the evaluation of coating constant: the coating constant for all five compounds when using thin-film PS-DVB-PAN and PBA-PAN coatings for the extraction from PBS are reported in Table 5.3. Because of the restriction for the volume of the sample/solvent in well plates, a volume of 1 mL was used for both the extraction sample and the desorption solvent. However, further pre-concentration of the analytes can be obtained through the evaporation of the final extracts.

For the PBS-PAN coating, the effect of pH on the extraction recovery of the compounds containing cis-diol group (sucrose and riboflavin) were studied at alkaline pH

(i.e., pH = 8.5). For this part of the experiment, except for the regular preconditioning step in methanol/water 50:50 (v/v), the coating was preconditioned for 30 min in phosphate buffer (pH = 10) to obtain the reactive boronate form (i.e., $B(OH)_3$). Then, the extraction was performed for 120 min in phosphate buffer (pH = 8.5), followed by a 60 min desorption in 99% acetonitrile/water 50:50 (v/v) + 1% acetic acid (to ensure disruption of the covalently bounded cis-diol groups to the coating). The evaluation showed no significant difference in the amount of recoveries for the extraction from alkaline pH in comparison with physiological pH, as it has been shown for sucrose in the previous study.[105]

The differences in the absolute recoveries of some analytes between the extraction from PBS and human plasma are due to the high affinity binding of these drugs to the human plasma proteins.[127]

Table 5.3 Absolute recovery and coating constant for PS-DVB-PAN and PBA-PAN 96-blade SPME coatings for equilibrium extraction (100 ng/mL diazepam, oxazepam, caffeine and riboflavin, and 300 ng/mL sucrose spiked in PBS (pH = 7.4), and 300 ng/mL of all five compounds spiked in human plasma, n=6 and four experiments).

			PS-DVB-PAN			PBA-PAN		
Analyte	**Log P***	**Pka ***	**%PBS recovery**	**%Plasma recovery**	**Coating constant (mm^3)**	**% PBS recovery**	**%Plasma recovery**	**Coating constant (mm^3)**
Diazepam	2.82	3.4	98.1 ± 4.2	5.3 ± 0.6	5.16E+4	74.1 ± 5.5	1.5 ± 0.2	2.87E+3
Oxazepam	2.24	12.4	97.4 ± 3.3	6.7 ± 0.5	3.75E+4	50.1 ± 2.3	1.8 ± 0.2	1.01E+3
Caffeine	-0.07	10.4	98.9 ± 5.4	30.2 ± 2.1	8.99E+4	33.7± 2.7	15.1 ± 1.5	5.09E+2
Riboflavin	-1.46	10.2	71.4 ± 2.9	42.6 ± 3.3	2.50E+3	44.6 ± 3.9	19.5 ± 1.8	8.06E+2
Sucrose	-3.70	12.6	3.5± 0.3	3.6± 0.4	3.63E+1	4.0 ± 0.4	3.8 ± 0.5	4.17E+1

* Syracuse Research Corporation PhysProp Database

5.3.4 The 96-blade SPME system and the extraction from human plasma matrix

Understanding the drug-protein interactions is essential in basic life sciences since protein binding plays an important role in intermolecular interactions in living organisms. Only free concentration of the drug can transfer through membranes, induce therapeutic response, or be metabolized and eliminated from the body. Therefore, in order to understand the drug distribution and pharmacological activity of the pharmaceuticals, determining the free and bound concentration of drugs in a living system is crucial.[191, 192]

Human plasma consists of different transport proteins. Albumin is the most abundant protein that plays a transportation role in the circulatory system. It includes three homologous domains (I-III), each divided into two sub-domains (A and B). The principle regions of the ligand binding for this protein are located in the hydrophobic cavities in sub-domain IIA and IIIA, which have similar chemistry.[193] In addition to these two sub-domains (considered as high affinity binding sites), albumin has a number of lower affinity binding sites. Furthermore, there are nonspecific interactions between the ligands and the polypeptide chain of the protein.[194, 195] Depending on the type, characteristic, and extent of the ligand, it can be bound to the protein molecule at various binding sites. Consequently, multiple ligand-protein equilibria are established.

When dealing with SPME systems with negligible depletion (usually considered as <5% extraction recovery),[196] the amount of analyte extracted by SPME coating is proportional to the initial free concentration of the ligand (analyte) in the plasma matrix (Figure 5.8). [35]

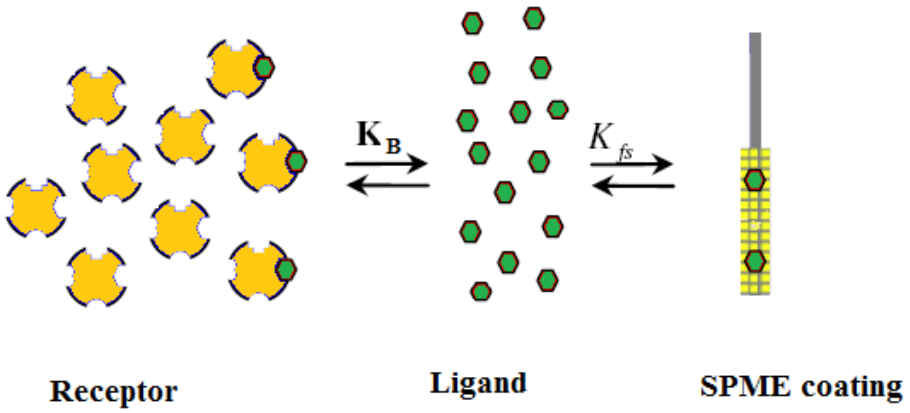


Figure 5.8 Schematic illustration of ligand-protein binding and ligand-SPME coating equilibria. Figure reproduced from the reference with the permission of the publisher.[192]

However, in the SPME systems with significant depletion where free molecules of the analyte of interest (ligand) are considerably removed from the system, the free (unbound) concentration of the ligand in the extraction matrix drops. As a result, the system may attempt to re-establish the disturbed equilibrium by dissociating a portion of the ligand molecules from the ligand-protein complex to compensate for the change in equilibrium.

In this case, a fraction of the dissociated analyte may be extracted with the SPME coating (depending on the presented SPME and the ligand-binding conditions), consequently increasing the amount extracted by SPME coating. The feasibility and degree of the occurrence of the changes in ligand-protein equilibria depend on a variety of factors, including: (i) the number and extent of available low- and high-affinity binding sites for the specific analyte, (ii) ligand-protein binding constants for each site, (iii) the ligand to protein concentration ratio, (iv) kinetics of ligand-protein binding, (v) distribution constant of ligand for specific SPME coating, and (vi) kinetics of SPME equilibrium extraction. Among these

parameters, the number and degree of affinity for different binding sites and their binding constants toward the ligand are very important.

The detailed principle and equations for the calculation of free, total, and percent ligand-protein binding for the SPME systems with negligible and significant depletion have been extensively reported in literature.[1, 143, 146, 191, 192, 197, 198] In this thesis, one of the analytes under study was selected as an example for studying the experimental results of protein binding in case of a significant depletion in the 96-blade SPME system. For instance, in the case of the PS-DVB-PAN coating, when extracting diazepam from human plasma the percent plasma protein binding (PPB) is calculated as 95 % using Equation 5.1,[104] when the slope for plasma and PBS calibration curves were determined as 5.1 and 97.9, respectively. The calibration curves were constructed based on the optimized SPME conditions for the extraction from spiked PBS (5-100 ng/mL) and plasma (50-1000 ng/mL).

$$\%PPB = 1 - \frac{slope\ calibration\ plasma}{slope\ calibration\ PBS} \times 100 \qquad \text{Equation 5.1}$$

The % PPB for the extraction of diazepam is reported in the range of 96-98 % in the litreature.[127] This minor difference can be explained as follows: According to the reported studies, human serum albumin contains several binding sites with different affinities for most benzodiazepines, such as diazepam, resulting in multiple ligand-protein equilibria.[192, 199] In this case, the ligand-binding constant in the high affinity binding site ($k_1 = 1.74\times10^6$ for diazepam) are orders of magnitude larger than those of the low affinity binding sites ($k_2 = 3.31\times10^4$ and $k_3 = 8.10\times10^3$ for diazepam). When dealing with SPME systems with significant depletion, the change of the free concentration in the first place influences the ligand binding in its low affinity binding sites because of their smaller binding constants.

Therefore, by removing the ligand molecules from the system in such cases, the ratio of the bounded ligand to the free concentration does not change considerably (Figure 5.9) and, consequently, the amount of diazepam extracted by SPME coating does not change significantly. Figure 5.9 shows the schematic of a typical Scatchard plot (the ratio of the concentration of bound ligand to unbound ligand (B/L_F) versus the bound ligand concentration (B)) for the drugs with multiple binding sites.

This condition can be explained for any other SPME extractive phase that provides significant depletion of the analyte with multiple ligand-receptor equilibria.

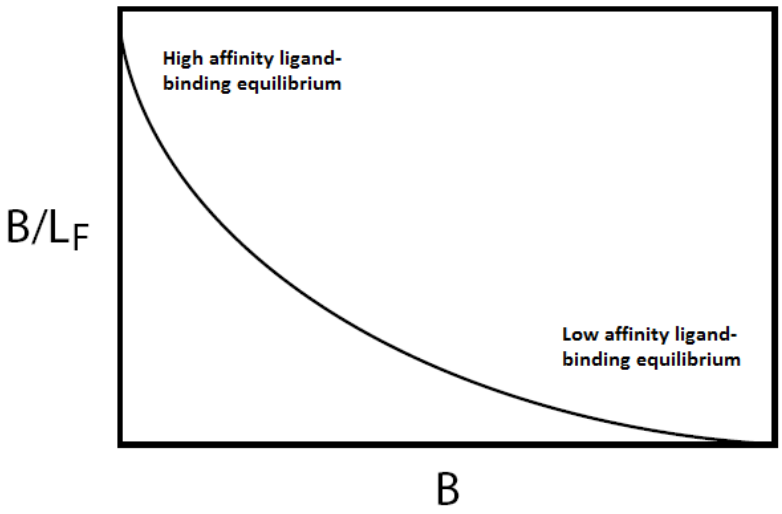


Figure 5.9 The schematic of a typical Scatchard plot for ligands with multiple protein binding sites.

5.3.5 Robustness and reusability of PS-DVB-PAN and PBA-PAN coatings

The evaluation of the robustness and reusability of the developed SPME systems was performed by monitoring the extraction recovery and physical stability of the coatings over

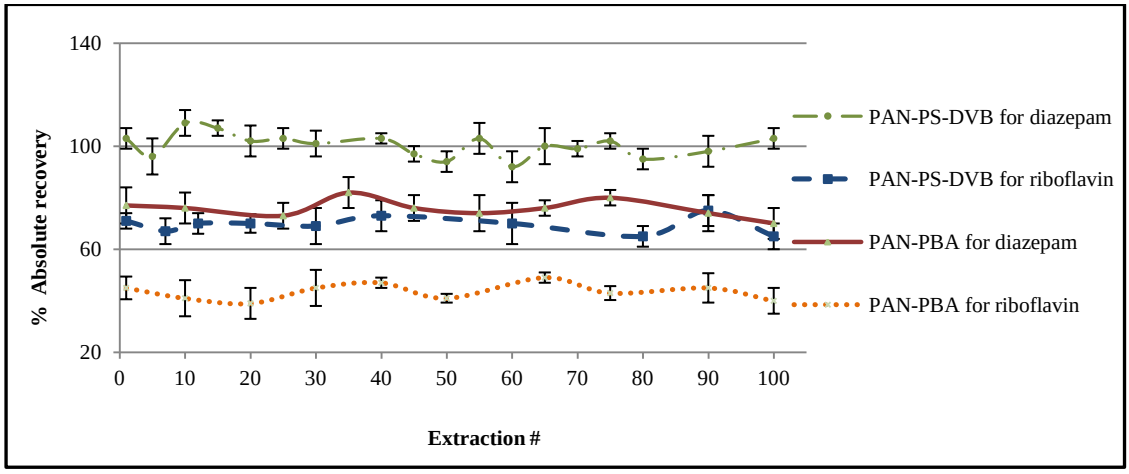
one hundred uses for extraction from PBS and human plasma. The results showed that both PS-DVB-PAN and PBA-PAN coatings presented high physical and chemical stability and reproducible extraction efficiencies in PBS and plasma matrixes in long-term use. The assessment of the reproducibility of the extraction recovery was carried out for the sequential extraction of diazepam and riboflavin from PBS and human plasma for 100 times (Figure 5.10). The PS-DVB-PAN coating provided 4 and 5% RSD (n = 6 coatings and n = 18 experiments), and PBA-PAN coating presented 5 and 7% RSD (n = 6 coatings and n = 10 experiments) for the extractions of diazepam and riboflavin from PBS, respectively. The evaluation of the reusability and reproducibility of the coatings for the extraction from human plasma also showed that the extraction efficiencies of the coatings remained stable for more than 100 extractions. For n = 6 coatings, PS-DVB-PAN coating resulted in 11 and 6% RSD, and PBA-PAN coating provided 10 and 9% RSD for sequential extractions of diazepam and riboflavin from human plasma, respectively (n = 10 experiments).

The chemical stability of the coatings was also assessed by soaking the coatings in different organic solvents (acetonitrile, methanol, ethanol, and DMF) overnight. No changes in the physical properties and extraction recoveries of the coatings were observed after long-term exposure to the tested organic solvents.

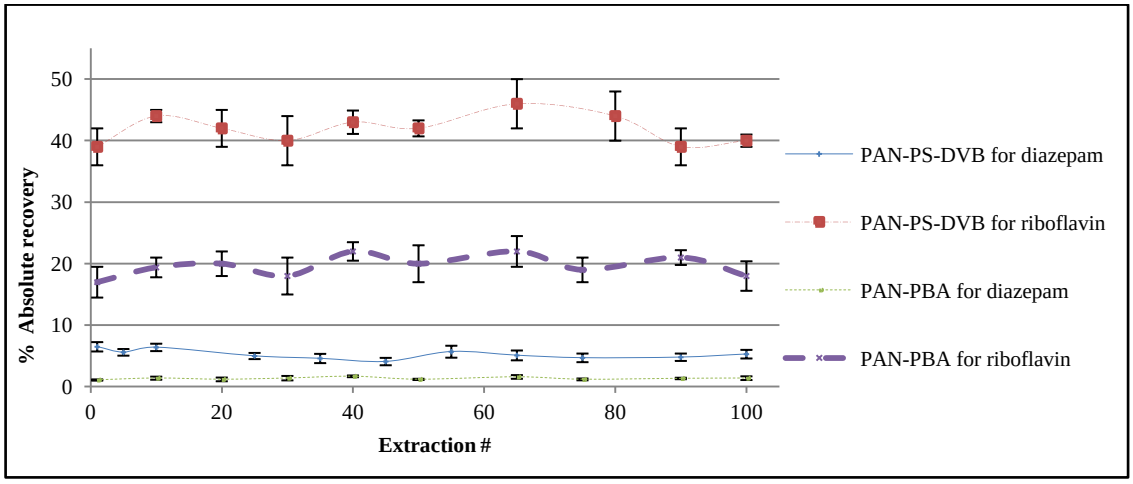
5.3.6 Reproducibility of the PS-DVB-PAN and PBA-PAN 96-blade SPME-LC–MS/MS systems

The reproducibility of the coating preparation method was studied via the evaluation of inter-blade RSD. Furthermore, the intra-blade RSD of the coatings was used to test the repeatability of the performance of individual coatings in the SPME-LC–MS/MS method.

The results of the assessment of inter- and intra-blade RSDs of the PS-DVB-PAN and PBA-PAN 96-blade SPME coatings are summarized in Table 5.4. Further improvements in inter-blade reproducibility of the coatings can be achieved via the automation of the coating procedure.



(a)



(b)

Figure 5.10 Reusability of PS-DVB-PAN and PBA-PAN thin-film SPME coatings (n = 6) for 100 times extraction using optimal SPME-LC–MS/MS conditions for (a) 100 ng/mL diazepam, oxazepam, caffeine and riboflavin, and 300 ng/mL sucrose spiked in PBS (pH =7.4) and (b) 300 ng/mL of all five compounds spiked in human plasma.

5.3.7 Evaluation of matrix effects

The details of the procedure for the calculation of the absolute matrix effect was provided in Chapter 4 (Section 4.2.10). In this study, the blank plasma extract was spiked with 50 ng/mL of analyte and the peak areas were compared to those of pure standards at the same concentration. Figure 5.11 demonstrates the evaluation of the absolute matrix effect for both coatings and all five compounds.

Table 5.4 Inter-blade and intra-blade reproducibility of PS-DVB-PAN and PBA-PAN 96-blade SPME systems for the equilibrium extraction of 100 ng/mL diazepam, oxazepam, caffeine and riboflavin, and 300 ng/mL sucrose spiked in PBS (pH = 7.4) for n = 6 coatings

Coating	***PS-DVB-PAN***				***PBA-PAN***			
Reproducibility	***% Inter-blade RSD***		***% Intra-blade RSD***		***% Inter-blade RSD***		***% Intra-blade RSD***	
Matrix	***PBS***	***Plasma***	***PBS***	***Plasma***	***PBS***	***Plasma***	***PBS***	***Plasma***
Diazepam	4.4	8.2	4.3	10.6	6.4	10.1	6.9	11.7
Oxazepam	4.6	9.3	4.5	8.7	5.6	9.5	5.2	10.6
Caffeine	5.3	8.4	5.4	6.7	7.2	8.7	8.1	9.9
Riboflavin	5.4	6.2	4.8	6.9	8.6	9.7	7.7	9.2
Sucrose	8.3	10.3	8.5	12.0	9.8	13.2	10.2	14.1

No significant matrix effects were determined using PS-DVB-PAN and PBA-PAN coatings, indicating the effectiveness of the selective extraction mechanism and the biocompatibility of the PS-DVB-PAN and PBA-PAN coatings. The biocompatibility of the coating prevented fouling of the coating by the adsorption of salts, proteins, and other macromolecules during extraction from biological samples. Consequently, the chance of ion

suppression/enhancement of the analytes with the matrix interferences is decreased. The efficiency of the wash step after extraction is another important factor in the successful cleaning of the interferences from the coatings and preventing their transfer to the final extracts.

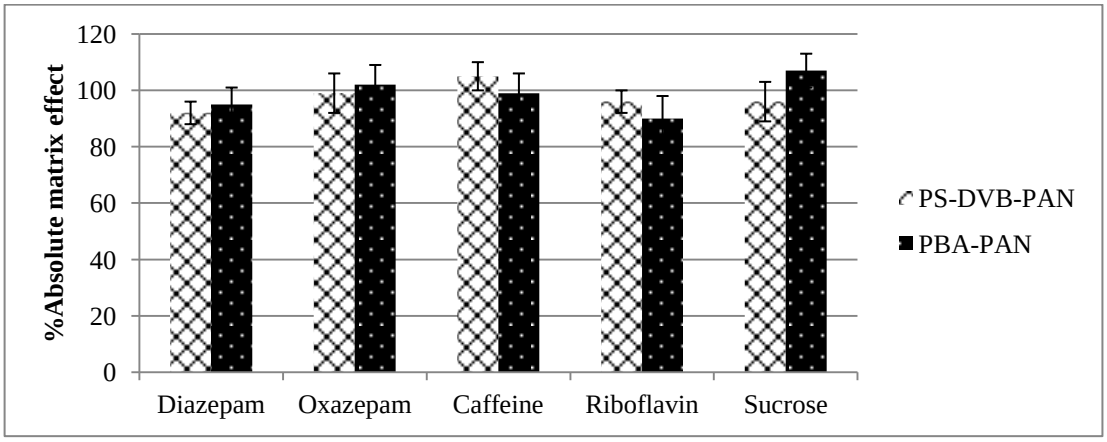


Figure 5.11 Absolute matrix effect for PS-DVB-PAN and PBA-PAN coatings (n = 4).

5.3.8 Limits of detection and quantitation

The evaluation of the LOD and LOQ for the PS-DVB-PAN and PBA-PAN 96-blade SPME-LC–MS/MS systems for the extraction from human plasma is shown in Table 5.5. The methods for determining the LOD and LOQ were reported in Chapter 2 (Section 2.2.8). Oxazepam and sucrose provided the lowest and highest LOD and LOQ values for both coatings, respectively. The low signal intensity for the sucrose was due to very low mass spectrometry sensitivity and the small extraction recovery for this compound.

5.4 Conclusions and future directions

The 96-blade SPME system provides high-throughput simultaneous analysis of up to 96 samples in a total of 100 and 210 min for the PS-DVB-PAN and PBA-PAN coatings, which corresponds to approximately 1 and 2.2 min per sample, respectively. However, these lengths of analysis can be significantly decreased using pre-equilibrium extraction. Furthermore, the 100 x reusability of the developed coatings reduces their cost down to a few cents per coating.

Table 5.5 Limits of detection and quantitation for PS-DVB-PAN and PBA-PAN 96-blade SPME-LC–MS/MS analysis of all five compounds from human plasma.

Coating	*PS-DVB-PAN*		*PBA-PAN*	
Analyte	*LOD (ng/mL)*	*LOQ (ng/mL)*	*LOD (ng/mL)*	*LOQ (ng/mL)*
Diazepam	0.3	1.0	0.4	1.5
Oxazepam	0.1	0.5	0.2	1.0
Caffeine	0.3	1.0	1.0	3.0
Riboflavin	0.5	1.5	1.0	3.0
Sucrose	25	100	25	100

The PS-DVB-PAN and PBA-PAN 96-blade SPME systems demonstrated the successful extraction of a variety of compounds from a single complex sample with a high degree of efficiency and reproducibility. The sensitivity and the extraction recovery for the samples containing binding matrix (such as plasma) can be significantly increased through disrupting the drug-protein binding, without the need for any additional complicated sample pre-treatment step (Chapter 3, Section 3.3.10).

These systems have the potential to be used in various areas of science including biological, clinical, pharmaceutical, food, and metabolomics studies for the high-throughput

analysis of various exo- and endogenous analytes with different polarities. Currently the PS-DVB-PAN 96-blade SPME system is being used for the high-throughput sample preparation of biological samples for the global analysis of metabolites with a wide range of polarity (manuscripts under preparation). The application of the PS-DVB-PAN 96-blade SPME system for determining phenolic compounds from grape, berry, and wine samples is discussed in Chapter 6. The application of the PBA-PAN 96-blade SPME system for the extraction of proteins from biological samples is being investigated by the Pawliszyn research group.

5.5 Addendum

The text of this chapter was rewritten in comparison to the published research article.

Chapter 6

Automated determination of phenolic compounds in grape, berry, and wine samples using 96-blade solid phase microextraction system

6.1 Preamble and introduction

6.1.1 Preamble

This chapter has been accepted for publication in the Journal of Chromatography A as a paper entitled: F. S. Mirnaghi, F. Mousavi , S. M. Rocha, J. Pawliszyn, Automated Determination of Phenolic Compounds in Grape, Berry, and Wine Samples Using 96-Blade Solid Phase Microextraction System Coupled with Liquid Chromatography-Tandem Mass Spectrometry *(2012).*

The contributions of co-author Fatemeh Mousavi involved assistance in the analysis of the grape, berry, and wine samples. In addition, the contribution of Dr. Sílvia M. Rocha involved the provision of the wine and the dried berry and grape samples from Portugal.

I, Fatemeh Mousavi, authorize Fatemeh Mirnaghi to use the material for her thesis.

I, Sílvia M. Rocha, authorize Fatemeh Mirnaghi to use the material for her thesis.

6.1.2 Introduction

Since food samples contain complex non-homogeneous mixture of a range of chemical substances, the isolation and determination of analytes of interest in food matrixes is

challenging. Even with the introduction of advanced techniques of separation and identification during the past several decades, it is rarely possible to analyze food samples without sample preparation. The sample preparation procedures often take up two thirds of the total analysis time, and significantly contribute to the total cost of analysis.[42] An inappropriate method of sample preparation may provide significant analytical errors, which could result in unreliable quantitative data.[200] Traditional sample preparation methods for food analysis are time-consuming and lack efficiency.[201-204] Liquid-liquid extraction (LLE) exhibits a lack of sample throughput and automation, large volumes of organic solvent consumption, the possibility of loss of analyte during extraction, and a high degree of matrix effect (LC–MS applications). Solid phase extraction (SPE) may cause clogging in the packed bed stationary phase when dealing with complex matrixes containing macromolecules and particulate matters, and, consequently, requires time-consuming pre-treatment steps prior to extraction.[29]

Growing emphases on improved food safety and the quality of the nutrient content of food crops demand more rapid and automated procedures to address the increase in the number of samples to be tested. This demand necessitates the utilization of proper sample preparation techniques which offer fast, solventless, inexpensive, and easy-to-automate procedures. In addition, the challenge for the analysis of complex food samples containing particulate matters increases the need for systematic sample preparation strategies with minimum sample preparation steps, reproducible recovery, high accuracy, and minimal loss of analytes.

SPME is a fast sample preparation technique with minimal consumption of organic solvents. The biocompatible open-bed stationary phases in SPME provide direct handling of

complex matrixes without the need for sample pre-treatment. In addition, it resolves the limitation of clogging the extractive phase with particulate matters (common in pack-bed systems) and the attachment of macromolecules on the surface of the coating. As a result, the chance of co-extraction of undesired interferences, and consequently the possibility of the matrix effect is reduced. This is a very important feature in LC–MS food analysis, since the co-elution of complex matrix interferences with the analyte of interest may result in suppression/enhancement of the analyte's signal, and consequently cause significant errors in quantitative data.[205, 206] Furthermore, the automated 96-blade SPME system facilitates the simultaneous preparation of up to 96 samples, and results in significant time savings and improved precision.

Chapter 6 of this thesis reports, for the first time, on the application of an automated SPME-LC–MS/MS system for food analysis. Grape, berry, and wine samples were selected as food matrixes, and phenolic compounds were chosen as analytes of interests due to their importance in food analysis.

Phenolic compounds are secondary plant metabolites that are categorized in two large groups of flavonoid compounds (flavanones, flavonols, flavanols, and anthocyanins) and non-flavonoid compounds (acids, hydroxycinnamates, and stilbenes), based on their carbon skeleton.[201, 207, 208] These compounds are synthesized by grapes and a large number of other plants (e.g., vegetables, fruits) during normal growth and as a defense reaction to situations of stress (e.g., microbial infections and UV irradiation).[201, 202, 209-211] Phenolic compounds play a crucial role in the quality of wine including aging behavior, taste, bitterness, color, and haziness.[203, 212]

Numerous studies have reported on the beneficial effects of phenolic compounds which are associated with human health (e.g., anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial, and anti-aging properties).[212-216] The flavonoids and stilbenes groups are the main contributors to the anti-oxidant activities of the phenolic compounds due to their ability to block free radical reactions by the quick donation of a hydrogen atom to lipid radicals.[212, 217, 218] These groups help restrain platelet aggregation and low-density lipoprotein (LDL) oxidation,[202, 219-222] provide anti-aging properties,[223, 224] and reduce oxidation-related human diseases (e.g., cancer,[225, 226] inflammation,[227, 228] and brain dysfunction[229, 230]). As a result of the anti-oxidant activity of the phenolics, many studies have confirmed that the regular consumption of red wine in moderate amounts reduces the risk for coronary heart disease (CHD).[218, 231, 232]

In this thesis, the 96-blade SPME-LC–MS/MS system was used for determining naringenin and taxifolin (flavanone), trans-resveratrol (stilbene), quercetin-3-D-glactoside and rutin hydrate (flavonols), catechin hydrate and epicatechin (flavanol) and caffeic acid (phenolic acid) from Portuguese grape, berry, and wine samples. The chemical structure of the model analytes are demonstrated in Figure 6.1. The classified categories and log P of the analytes under study are shown in Table 6.1.

In order to obtain reliable analytical data, standard addition calibration was used as the quantitation method for determining unknown phenolic compounds from grape, berry, and wine samples. Furthermore, the results of SPME analysis and its efficiency in reducing the matrix effects were compared with solvent extraction.

6.2 Experimental

6.2.1 Chemicals and materials

L-(+)-Tartaric acid, potassium-L-tartarate monobasic, rutin hydrate, quercetin-3-D-glactoside, naringenin, taxifolin, trans-resveratrol, (+) catechin hydrate, (–)-epicatechin, and caffeic acid were purchased from Sigma-Aldrich (MO, U.S.). The remainder of the materials were purchased from the same sources reported in Chapters 2, 3 and 5 (Sections 2.2.1, 3.2.1, and 5.2.1).

6.2.2 Preparation of working standards and buffers

Stock solutions were prepared daily at a concentration of 1 mg/mL of analytes in methanol and kept at –30 ^{0}C. Working standards were also prepared daily from these stock solutions using acetonitrile/water (50:50 v/v) as diluents. Tartaric buffers were made of proper molar ratios of potassium tartarate monobasic and tartaric acid in 1L of water and the pH of the buffers were adjusted accordingly (pH= 2.5 and 3.7). Synthetic wine was prepared as 85% tartaric buffer and 15% ethanol (v/v).[233] The concentration of organic solvents in buffers and spiked real samples was always kept at less than 1%. After spiking the analytes in grape, berry, and wine matrixes, samples were incubated for 1 h (2400 rpm agitation) to allow for establishing the equilibrium of phenolic compounds with the binding matrix prior to extraction.

6.2.3 Grape, berry, and wine samples: origin and preparation

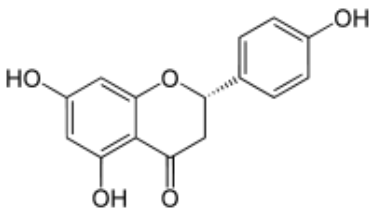
Sercial white and Tinta Negra red grapes were chosen from the V. vinifera L variety, which belong to a group of the world's largest fruit crops and are mainly used to produce

table wine.[207] Sercial and Tinta Negra grapes are used to produce the world-famous Madeira wine. Tinta Negra, the main variety of grape cultivated in the Madeira Island (around 90%), was studied in terms of its phenolic compounds in both whole grape and skin. The *vérasion* Sercial white grape and *maturity* Tinta Negra red grape were harvested in the early ripening stage (0 days) and late ripening stage (42 days), respectively.

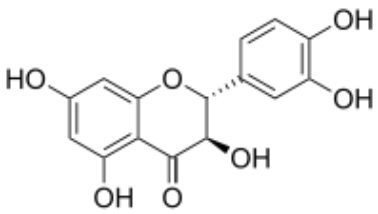
In addition, elderberry from the Sambucus Nigra L variety was considered for the analysis of phenolic compounds in this study. A 5-year-old sweet Madeira wine (Tinta Negra mole) was also selected for evaluation of phenolics in wine.

After being picked up, the grapes and berries were immediately transported to the laboratory and refrigerated at 4 ºC. For the preparation of grape skin, the Tinta Negra red grapes were pealed and the skin was manually separated from the pulp. All berry, grape, and skin samples were then freeze-dried using a VirTis bench top K freeze dryer (SP Industries, NY, U.S.), packed into vacuum bags, and kept under desiccators until the time of analysis. At the time of analysis, a blender machine was use to grind the freeze-dried samples. Then, aliquots of samples were weighed precisely, dissolved in optimized volumes of nanopure water, and mixed properly to be ready for the analysis.

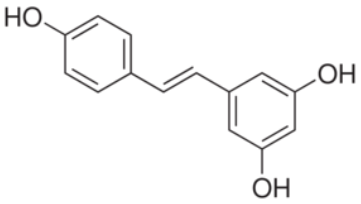
It should be noted that the volumes of dilution for each sample were optimized based on a pre-test analysis to estimate the approximate concentration of the analytes in the samples; accordingly, different dilution volumes were precisely adjusted for the final analyses of different analytes to prevent exceeding the linear concentration range of the extractive coating and to avoid falling below or beyond the limit of quantitation of LC–MS/MS.



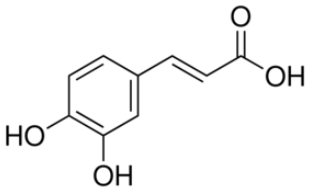
Naringenin



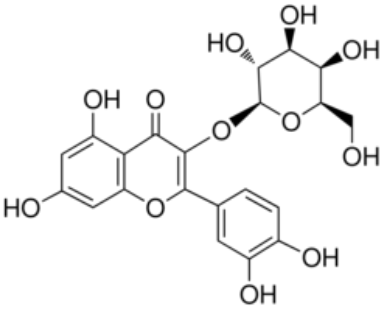
Taxifolin



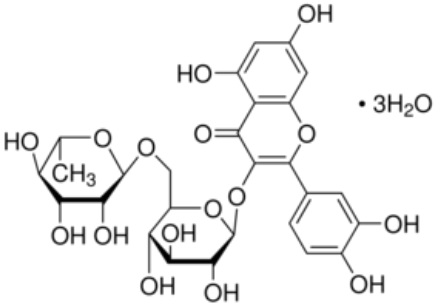
trans-Resveratrol



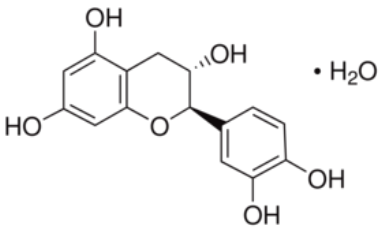
Caffeic acid



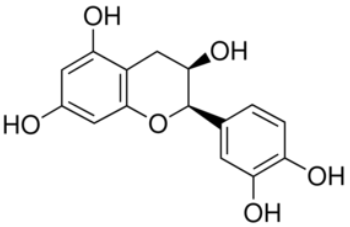
Quercetin-3-D-glactoside



Rutin hydrate



Catechin hydrate



(-)-Epicatechin

Figure 6.1 Chemical structure of the phenolic compounds under study.

Twenty-five, 24, 16, and 12 mLs of nanopure water were used to dissolve each gram of red grape, white grape, elderberry, and red grape skin, respectively. The exceptions were the analysis of catechin and epicatechin in elderberry, white and red grapes, quercetin-3-D-glactoside and rutin in white grape, and quercetin-3-D-glactoside and rutin in elderberry in which further dilution of the samples were required in order to fit in the linear range of the coating. The pH of Sercial white grapes (*vérasion*) was estimated at around 2.5 while the pH of elderberry, Tinta Negra whole grapes and skin, and wine were in the range of 3.5–4.

6.2.4 Preparation of the coating for the 96-blade SPME system

The process for the preparation of C18-PAN and, PS-DVB-PAN and PBA-PAN coatings have been discussed in Chapter 3 and 5, respectively. Mixed-mode (C18+ strong cation exchanger, benzenesulfonic acid) and diol-based coated blades were obtained from Supelco (PA, U.S.).

6.2.5 Liquid chromatography and mass spectrometry conditions

Liquid chromatography and mass spectrometry instrumentations were the same as those reported in Chapter 2 (Section 2.2.3).

The MS/MS analysis was performed in negative mode under multiple reaction monitoring (MRM) conditions. The ionization source parameters were optimized as follows: nebulizer gas = –14, curtain gas = –6, collision gas = 12, spray ionization voltage = –4500 V and temperature = 400 °C. A summary of other MS/MS parameters is provided in Table 6.1.

A Gemini NX C18 column (3 × 250 mm and 3 μm particles, Phenomenex) was used for the chromatographic separation of compounds. A gradient elution using mobile phase A (99.9% water + 0.1% acetic acid, v/v) and B (99.9% methanol + 0.1 % acetic acid, v/v), and

a flow rate of 0.2 mL/min was used for the separation. The program of chromatographic gradient elution is shown in Table 6.2.

Table 6.1 Classified category, log P, and optimized mass spectrometry conditions for the phenolic compounds under study.

Category	Phenolic compounds	Log P*	Q1 mass (amu)	Q3 mass (amu)	DP (V)	FP (V)	EP (V)	CXP (V)	CE (V)
Flavonols	Rutin hydrate	-0.90	609.2	300.2	-100	-300	-10	-15	-53
	Quercetin-3-D-glactoside	-0.92	463.2	300.2	-90	-280	-10	-15	-40
Flavanones	Naringenin	2.63	271.2	151.2	-120	-300	-8	-10	-25
	Taxifolin	1.82	303.2	125.2	-100	-250	-15	-20	-30
Stilbenes	trans-Resveratrol	3.14	227.1	185.2	-100	-280	-15	-10	-25
Flavanols	Catechin hydrate	0.61	289.2	245.2	-100	-320	-15	-15	-20
	(-)-Epicatechin	0.61	289.2	245.2	-90	-300	-9	-15	-26
Phenolic acids	Caffeic acid	1.42	179.0	135.2	-105	-200	-8	-10	-25

DP=Declustering potential, FP=Focusing potential, EP=Entrance potential, CE=Collision energy, and CXP=Collision cell exit potential.

* Online chemical information resource: www.chemspider.com

Table 6.2 Chromatographic gradient elution program.

Time (min)	Mobile phase B (%)
0.01	25
3	25
7	80
10	80
17	25
20	25

6.2.6 Automated Concept 96-blade SPME system

The 96-blade system along with the modified configuration of the Concept *96* autosampler (Chapter 4, Section 4.2.5) was used for this study.

6.2.7 Automated SPME procedure for high-throughput analysis

For all experiments in this study, the extraction time was set at equilibrium for 2 h (1000 rpm agitation speed, 2.5 mm amplitude), and the volume of the sample was precisely controlled at 1 mL. A 20 s wash step after extraction from grape, berry, and wine samples was used to remove the macromolecules from the surface of the coating. The desorption conditions was optimized for 90 min in 1.5 mL of acetonitrile/water 1:1 (v/v) with a speed of 1500 rpm (1 mm amplitude agitator). When needed, the final extracts were diluted to fit in the linear response of the MS detector, and transferred to the autoinjector for LC–MS/MS analysis.

6.2.8 Procedure of solvent extraction for berry, grape and skin samples

Aliquots of the elderberry, grape, and skin samples were precisely weighed and transferred to proper containers. The extraction was performed by sonicating the individual samples with a methanol/water/formic acid 70:30:1 (v/v/v) solvent at 10 °C for 20 min using 10 times the volume of the sample weight. Extracted samples were then centrifuged for 20 min at 5 °C (14000 rpm), and supernatants were filtered via 0.45 µm syringe filters.[234]

6.2.9 Procedure of solvent extraction for wine

The solvent extraction for wine was performed with the addition of 5 mL of methanol/formic acid 99:1 (v/v/v) per mL of wine. The procedures for other steps of solvent

extraction (including sonication, centrifugation, and filtration) were similar to those of berry, grape, and skin samples.

6.3 Results and discussion

6.3.1 Optimization of chromatographic conditions

The choice of the chromatographic separation phase and the mobile phase was important in order to obtain good separation of phenolic compounds. Epicatechin and catechin are geometric isomers (cis and trans isomer, respectively), and proper chromatographic conditions were required for their separation. The optimized chromatographic conditions for the separation of phenolic compounds in this study were reported in Section 6.2.5. The typical chromatographic peaks of the phenolic compounds are shown in Figure 6.2.

In order to prevent the matrix effect and to avoid a decrease in sensitivity due to the contamination of the ion source by non-volatile components, a post-column switching valve, which directs the first minute column effluent to the waste, was used to maintain instrument sensitivity and signal reliability over long periods of analysis.[235]

6.3.2 Optimization of SPME coating

Five different extractive phases (including PS-DVB-PAN, PBA-PAN, C18-PAN, mixed–mode-PAN, and diol-PAN in 96-blade formats) were evaluated for the extraction of pheolic compounds under study. The evaluation of the recovery of the coatings was performed in tartaric buffer in two different pH (2.5 and 3.7) in order to mimic the pH and

ionic strengths of the grape, berry, and wine samples (2.5 for the white grape and 3.7 for the remainder).

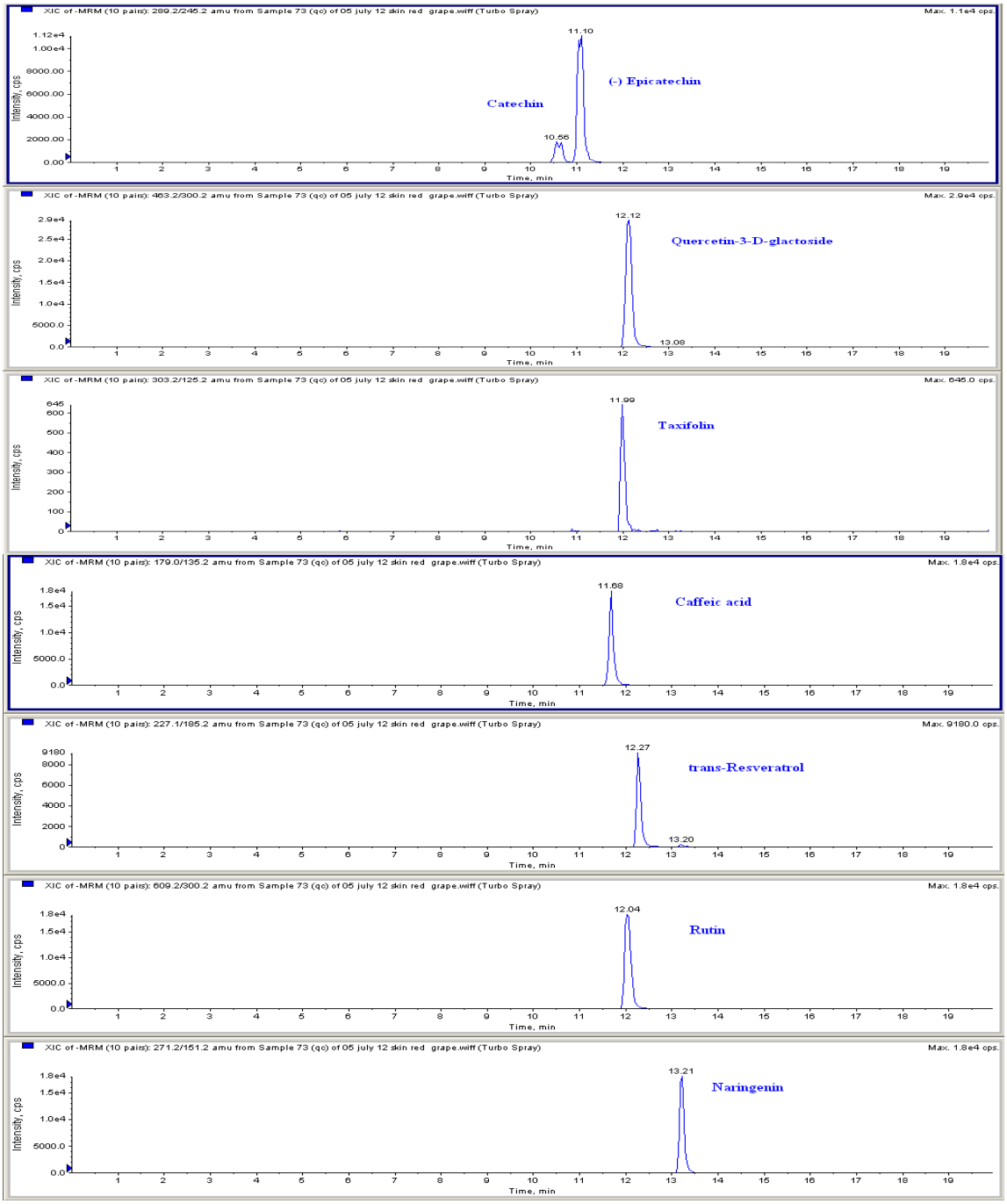


Figure 6.2 Typical chromatographic peaks of the phenolic compounds under study.

PS-DVB-PAN coatings had a significantly higher recovery for the extraction of phenolic compounds. No significant differences were observed in the extraction recovery of the compounds in both investigated pH. Therefore, PS-DVB-PAN was selected as the optimized coating for the remainder of the evaluations. Figure 6.3 compares the percent of absolute extraction recovery of different coatings for the extraction of the phenolic compounds from tartaric buffer (pH = 3.7).

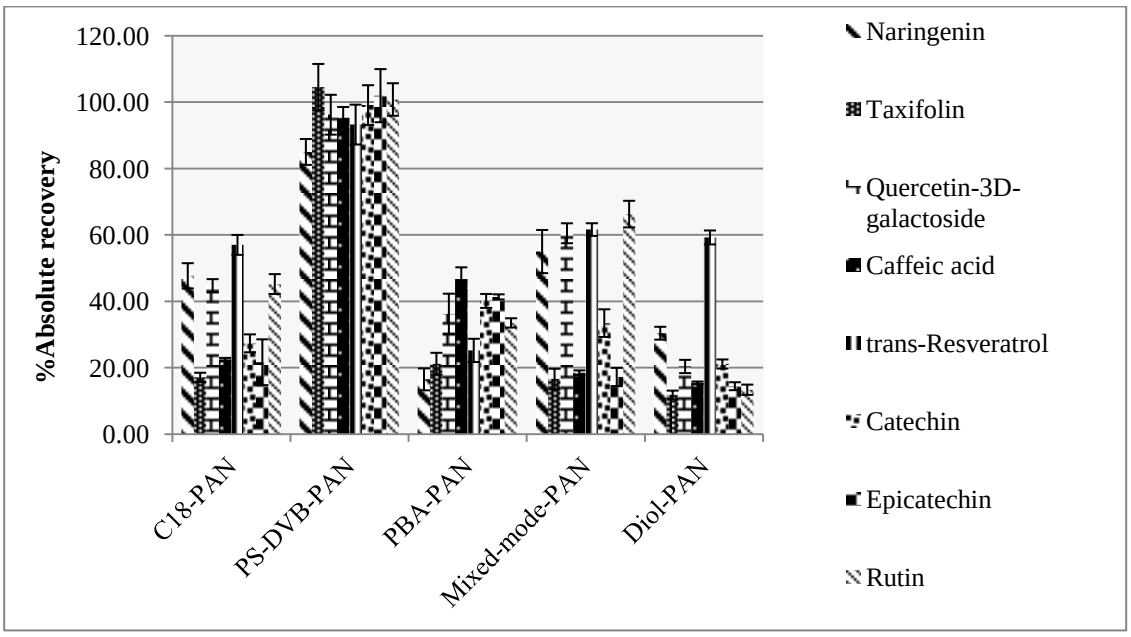


Figure 6.3 Evaluation of optimized coatings for the extraction of (200 ng/mL) phenolic compounds from tartaric buffer (pH = 3.7).

6.3.3 Optimization of automated 96-blade SPME system

Preconditioning: The extraction recoveries of two sets of PS-DVB-PAN coatings were evaluated with and without the preconditioning step (30 min agitating in methanol/water

50:50, v/v). No statistical differences were found in the amount of recovery of both sets of coatings for the extraction from either tartaric buffer or synthetic wine. The evaluation of the preconditioning step for the extraction of the phenolic compounds from tartaric buffer (pH = 3.7) is shown in Figure 6.4.

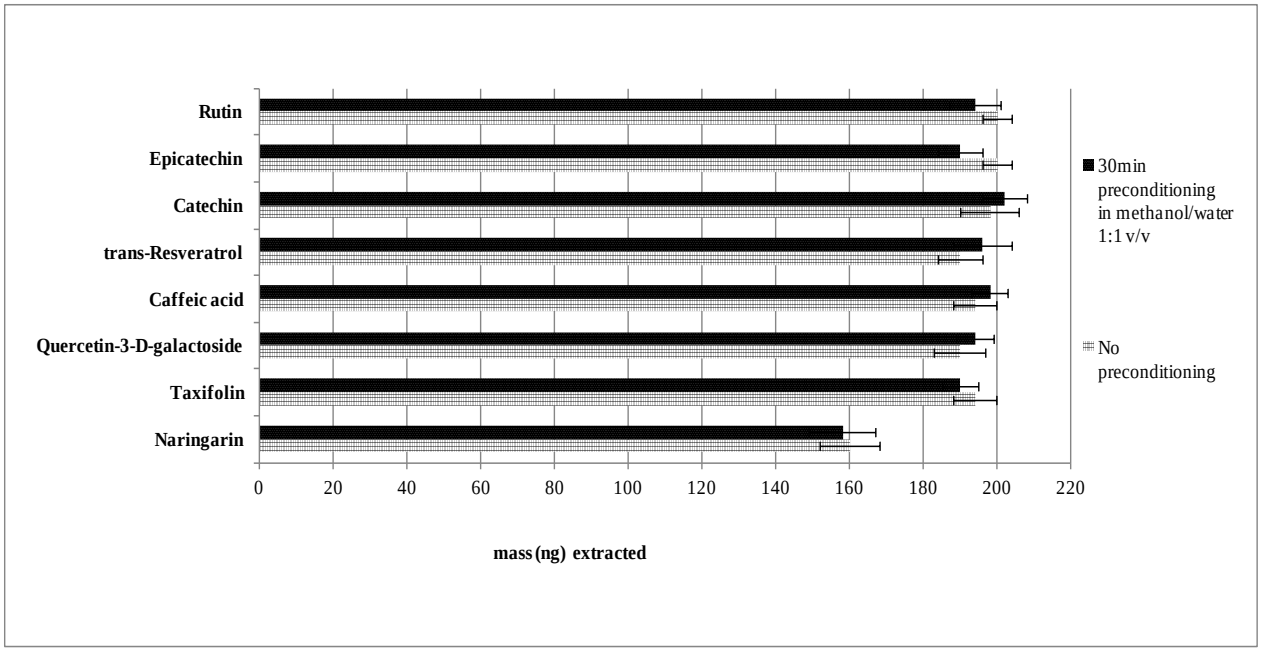


Figure 6.4 Evaluation of the effect of preconditioning on phenolic compounds extraction recovery using PS-DVB-PAN coating (200 ng/mL phenolics in tartaric buffer, pH = 3.7).

Extraction: An extraction time of 2 h was required to reach equilibrium extraction for all eight phenolic compounds in all the matrixes under study (i.e., tartaric buffer (pH = 2.5 and 3.7), synthetic wine, elderberry, grape, and wine matrixes). As a result, 2 hours was used as the optimized equilibrium extraction time for the entire study. The extraction time profile for the extraction of the phenolic compounds from tartaric buffer (pH = 3.7) using PS-DVB-PAN 96-blade SPME coating is shown in Figure 6.5. The highest applicable speed that did not risk spilling (1000 rpm, 2.5 mm amplitude) was used as the optimized agitation speed.

One mL of sample was used for the extraction volume, and accurately controlled during the entire study.

Wash: A wash step, after extraction, can prevent possible ion suppression/enhancement caused by interfering components during LC–MS/MS analysis and can preserve the efficiency and reusability of the coating for long-term use via minimizing the transfer of the attached macromolecules and particulates on the coating surface to the final extract. In this study, a 20 s wash (agitation) in nanopure water was considered optimal for proper cleaning of the coatings' surface from the food macromolecules. The evaluation of a possible wash loss during the 20 s wash step showed less than 1% loss of phenolic compounds.

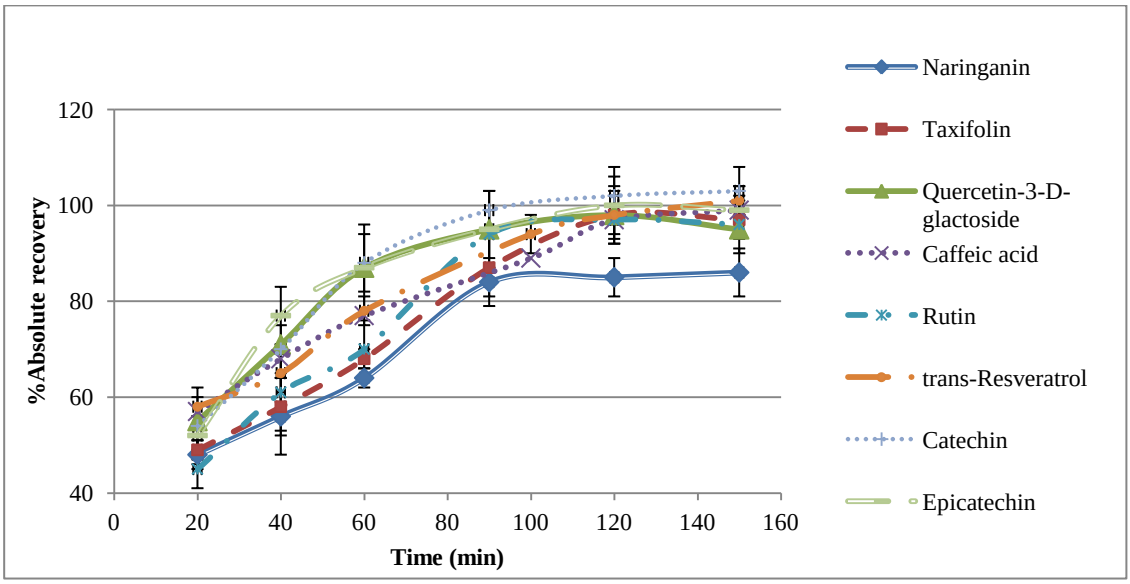


Figure 6.5 Extraction time profile for the extraction of (200 ng/mL) phenolic compounds from tartaric buffer (pH = 3.7).

Desorption: The evaluation of three different desorption solvents showed that the acetonitrile/water 50:50 (v/v) had the best recovery and the lowest carryover; therefore, it was selected as the optimal desorption solvent (Figure 6.6). A minimum of 90 min (1500

rpm agitation speed, 1 mm amplitude) was the optimal duration for the desorption of the analytes with the lowest carryover.

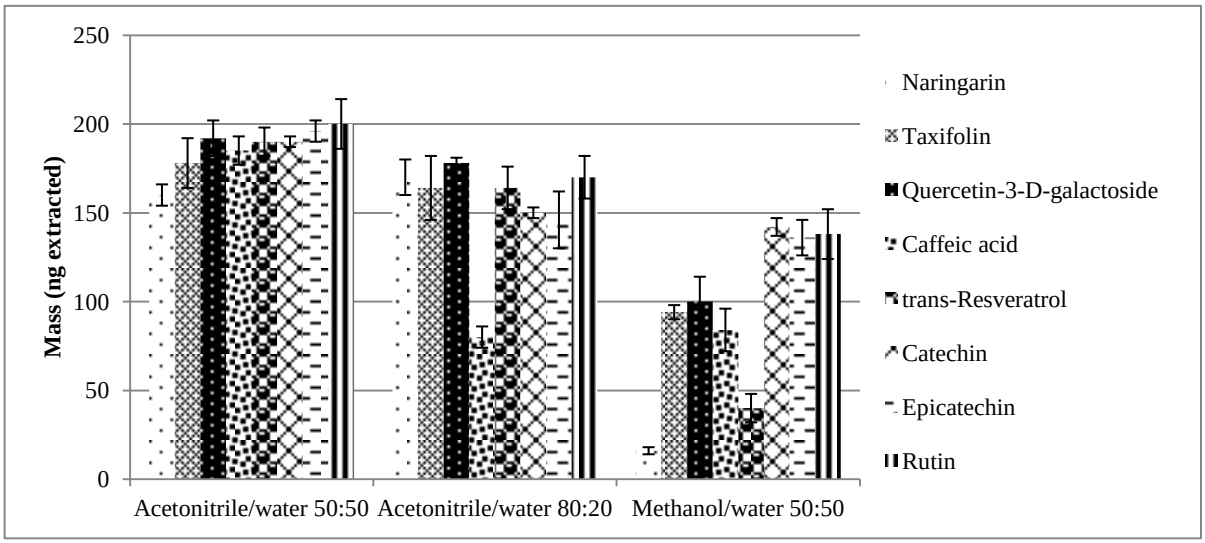


Figure 6.6 Evaluation of desorption solvent for PS-DVB-PAN coating for the equilibrium extraction of 200 ng/mL phenolic compounds from tartaric buffer (pH = 3.7).

The amount of carryover of the analytes on the coating from the previous desorption was evaluated through a second desorption step. A 40 min desorption with the optimized desorption conditions was established as optimal for the complete desorption of the residual analytes from the PS-DVB-PAN coating. The evaluation of a third desorption did not result in any detectable signal. The formula for the calculation of the carryover was discussed in Chapter 2 (Section 2.2.8). The amount of carryover of the phenolic compounds on the PS-DVB-PAN coating (after a first desorption of 90 min) is demonstrated in Table 6.3.

Table 6.3 Evaluation of the percent carryover of the phenolic compounds on PS-DVB-PAN coating (after 90 min first desorption).

Analytes	Naringenin	Taxifolin	Quercetin-3-D-galactosed	Caffeic acid	trans-Resveratrol	Rutin	Catechin	Epicatechin
Carryover (%)	4	2	3	2	3	3	2	2

6.3.4 Extraction recovery and reproducibility of PS-DVB-PAN for the extraction of phenolics from tartaric buffer and synthetic wine

The extraction recovery of the PS-DVB-PAN coating for the extraction of phenolics from tartaric buffer (pH = 3.7) is shown in Table 6.4. As previously discussed, no significant differences were observed in the extraction recoveries in pH of 3.7 and 2.5.

Previous studies have shown that the alcohol content in the sample matrix may affect the recovery of the SPME coating[236] due to the changes in distribution of the analytes between the coating and the sample matrix.[35] Since Madeira wine contains ethanol, as a matrix component it can induce some variations on the distribution constant of analytes between the coating and the wine matrix, and therefore, on the extraction recovery of SPME coatings.

As a result, the extraction recovery of PS-DVB-PAN coating for the extraction of phenolics was evaluated in synthetic wine (including 85% tartaric buffer and 15% ethanol, v/v) and was compared with that of pure tartaric buffer. As reported in Table 6.4, the alcohol content of synthetic wine caused a reduction in the absolute recovery of the phenolic compounds.

In addition, the reproducibility of the assay was studied through an evaluation of inter- and intra-day RSD. Table 6.4 shows that the intra- and inter-day RSDs for all eight compounds (n = 6 coatings and four experiments) were in the range of 4-8% and 7-13%, respectively.

The maximum saturation level of the adsorptive PS-DVB-PAN coating was studied through the evaluation of the calibration curve in a wide range of concentrations (0.05-40 mg/L) for the simultaneous extraction of all phenolic compounds from tartaric buffer (pH = 3.7). The results of the studies show that the limit of saturation for PS-DVB-PAN coating for the extraction of phenolics is at least 25 mg/L, except for quercetin-3-D-glactoside and rutin which had saturation limits of 15 and 10 mg/L, respectively (possibly due to their higher polarity). As a result, proper dilution volumes were adjusted for the preparation of freeze-dried samples prior to the extraction of the compounds with the SPME coating.

Table 6.4 Evaluation of % absolute recovery and reproducibility for the equilibrium extraction of 200 ng/mL phenolic compounds from tartaric buffer and synthetic wine (pH = 3.7).

	Tartaric buffer			Synthetic wine		
Analyte	**%Absolute recovery**	**% Intra-day RSD (n = 6)**	**% Inter- day RSD (4 trials)**	**%Absolute recovery**	**% Intra-day RSD (n = 6)**	**% Inter- day RSD (4 trials)**
Naringenin	80	5	8	69	6	9
Taxifolin	99	6	11	80	7	10
Quercetin-3-D-galactoside	96	8	13	74	5	13
Caffeic acid	95	4	7	82	5	8
trans-Resveratrol	93	4	8	77	4	10
Catechin	96	6	12	70	8	11
Epicatechin	97	7	9	78	8	10
Rutin	96	5	8	75	7	9

6.3.5 Method validation for PS-DVB-PAN 96-blade SPME-LC–MS/MS system

The linearity, sensitivity, LOD, and LOQ of the SPME-LC–MS/MS method were studied through the evaluation of the calibration curves for the extraction of phenolic compounds from tartaric buffer and synthetic wine (pH = 3.7). Ten–point concentration-response curves were used for constructing the extraction calibration of the compounds under study (3 replicates for each calibration point). The concentration of the calibration points were chosen in the ranges of the expected concentration of phenolics in the grape, berry, and wine samples. The least square linear regression method was used for the construction of the calibration curves.

Table 6.5 illustrates the linearity range, sensitivity (curve slope), linear regression coefficient (R^2), LOD, and LOQ for the SPME-LC–MS/MS analysis of the phenolic compounds under study. The methods for calculating the LOD and LOQ were reported in Chapter 2 (Section 2.2.8). The LOD and LOQ levels for both matrixes were obtained in a range of 0.2–3 and 0.5–10 ng/mL, respectively**,** indicating the proper sensitivity of the SPME-LC–MS/MS method for determining phenolic compounds from the matrixes under study.

The linear range was evaluated based on the applicable linear response when using LC–MS/MS for analysis. The upper limit of quantitation (ULOQ) is usually limited by the MS detector. For the analysis of those compounds whose concentrations exceed this limit, the final extracts were precisely diluted to fit to the linear concentration response of the detector. Then, the corresponding dilution factor was applied to normalize the quantitative data. Furthermore, linear regression coefficient (R^2) values were higher than 0.9963 for both matrixes.

Table 6.5 Sensitivity, linear regression coefficient, linearity, LOD, and LOQ for the PS-PAN-DVB SPME-LC–MS/MS.

	Tartaric buffer					Synthetic wine				
Analyte	Sensitivity	R^2	LOD ng/mL	LOQ ng/mL	Linearity ng/mL	Sensitivity	R^2	LOD ng/mL	LOQ ng/mL	Linearity ng/mL
Naringenin	0.84	0.9997	0.5	1.5	1.0-500	0.70	0.9989	0.5	2.0	2.0-500
Taxifolin	1.0	0.9972	2.0	7.0	7.0-1500	0.81	0.9979	3.0	10	10-1500
Quercetin-3-D-galactoside	0.97	0.9975	0.2	0.5	0.5-1000	0.75	0.9986	0.5	1.0	1.0-1000
Caffeic acid	0.97	0.9996	0.5	1.0	1.0-500	0.83	0.9991	0.5	1.5	1.5-500
trans-Resveratrol	0.95	0.9986	1.0	3.0	3.0-500	0.78	0.9978	1.5	5.0	5.0-500
Catechin	0.96	0.9969	1	5.0	5.0-1000	0.71	0.9963	2.0	7.0	7.0-1000
Epicatechin	0.98	0.9989	0.5	1.5	1.5-1000	0.78	0.9971	0.5	2.0	2.0-1000
Rutin	0.96	0.9964	0.2	1.0	1.0-1000	0.75	0.9984	0.5	1.5	1.5-1000

6.3.6 Evaluation of the matrix effect (ion suppression/enhancement)

When dealing with LC–MS/MS analysis, the phenomenon of the matrix effect is one of the most challenging issues for the quantitation of the phenolic compounds in complex food samples.[237] Necessary strategies must be employed to minimize the matrix effect in the first place, followed by the evaluation of the possibility of matrix effect during sample analysis. Many studies have reported on the applicable strategies for preventing the matrix effect in quantitative LC–MS analyses. These strategies include: the application of improved sample preparation techniques and optimized chromatographic separation, a reduction in the

sample injection volume, the employment of a post column switching valve, and the application of appropriate calibration techniques.[206, 235, 238, 239]

In this study, several approaches were applied to minimize this phenomenon, including: (i) applying selective biocompatible SPME coatings for improved isolation of the analytes from the complex matrixes, (ii) optimizing the chromatographic separation and utilizing post-column switching to reduce co-elution of interfering components, and (iii) using the standard addition calibration method as a proper quantitation technique for compensating any possible matrix effects.

The possibility of suppression/enhancement of the ionization of the phenolic compounds in this study were evaluated by calculating the absolute matrix effect, post-column infusion method,[7] and "sample extract dilution" technique.[234] Due to the unavailability of the blank grape, berry, and wine samples, tartaric buffer and synthetic wine were considered as analyte-free representative matrixes for the post-column infusion method and the absolute matrix effect evaluation.

Absolute matrix effect: A detailed description of the evaluation of the absolute matrix effect was discussed in Chapter 4 (Section 4.1.10). In this study, the evaluation was performed by comparing the response of the blank sample extract spiked with 150 ng/mL of phenolic compounds to the response of standard solution with the same concentration of. As shown in Figure 6.7, the results of this evaluation demonstrated that no significant matrix effect exists in the analysis of the phenolic compounds from tartaric buffer and synthetic wine (pH = 3.7).

Post-column infusion method: The details of the experimental procedure for this method have already been reported in several studies.[7] In this thesis, the evaluation was

performed through the simultaneous T-infusion of the mixture of (a) a 20 µl/min flow rate of 1 mg/L of analyte standard (in acetonitrile/water 1:1 v/v) and (b) LC effluent of the SPME extract of the blank sample, to the MS interface.

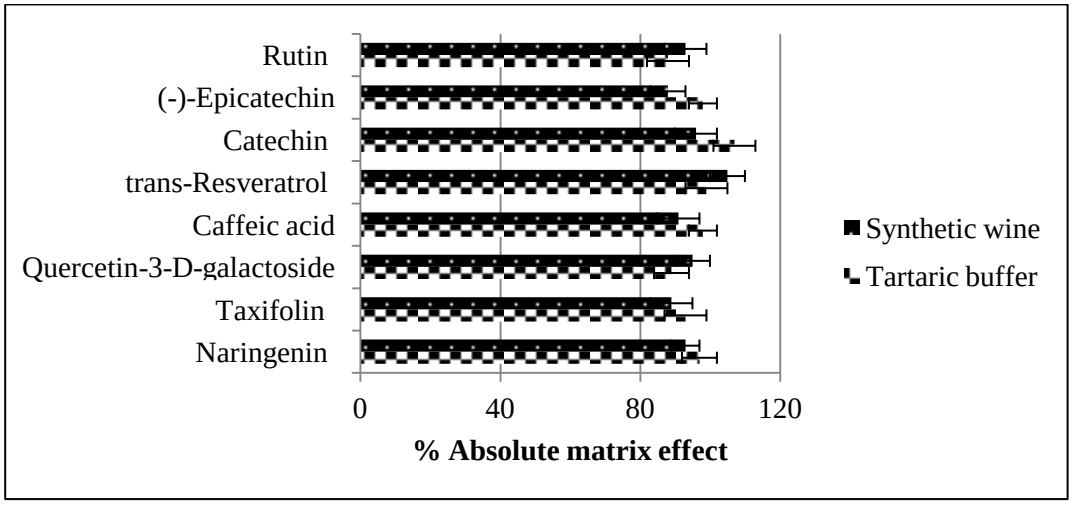


Figure 6.7 Evaluation of the absolute matrix effect for phenolic compounds in synthetic wine and tartaric buffer (pH = 3.7).

The comparison of T-infusion signals for both total and extracted ion chromatograms of the blank extracts (synthetic wine/tartaric buffer) with those of a pure mobile phase showed comparable results in the retention time of the analytes under study. No matrix effects were observed when extracting phenolic compounds using the optimized SPME-LC system.

"Sample extract dilution" method: The total number of ions formed during electrospray ionization (ESI) per unit of time is approximately constant. As a result, through sample dilution, the competition for ion evaporation from the droplet surface originated by the co-eluting compounds can be reduced significantly. For the evaluation of the matrix

effect using the sample extract dilution method, the final sample extracts are diluted with different dilution factors (1:0, 1:1, 1:2, 1:4, 1:9, 1:19, 1:49, and 1:99) using acetonitrile/water 1:1 v/v. Next, for each of the analytes under study, the plots of the normalized peak areas (peak area multiplied by dilution factor) were constructed against the corresponding dilution factor.[234, 240] When there is no matrix effect, the normalized response is the same for all the applied dilution factors (within the experimental error). The evaluation of the matrix effect using this method was studied for the final extract of the SPME method and it was compared to the solvent extraction method as a conventional sample preparation technique for food samples.

Table 6.6 shows a comparison of the minimum dilution level required for the reliable analysis of the final sample extracts obtained from SPME and solvent extraction methods with no matrix effects. For many of the phenolic compounds under study extreme dilution of the final extract (up to 1:49 dilution in some cases) was required for the solvent extraction technique, indicating a significantly higher ion suppression/enhancement of the analytes. This high matrix effect for the solvent extraction method can be explained by the complexity of the final extract and inefficient isolation of the analytes from the interfering components.

The results of the solvent extraction were compared with that of SPME with the consideration of the initial dilution volume for the preparation of extraction samples for each technique. In the case of SPME, in many cases reliable quantitative results were obtained with no need for the sample dilution; however, in some other cases the final extracts required a maximum dilution of 1:4 (< 15% cases). The application of a selective biocompatible extractive phase and utilization of the wash step in SPME minimizes the transfer of interfering macromolecules (such as polysaccharide, proteins, and particulates) into the final

extract and decreases the possibility of ion suppression/enhancement of the analytes. However, analysis of complex matrixes through further dilution of the final extracts should be considered, when necessary.

Table 6.6 Comparison of the matrix effect of SPME and solvent extraction methods using sample extract dilution method: the minimum dilution level required for the final sample extracts obtained from SPME and solvent extraction methods.

	Naringenin		Taxifolin		Quercetin-3-D-glactoside		Caffeic acid		trans-resveratrol		Catechin		Epicatechin		Rutin	
	SPME	*SE*	*SPME*	*SE*	*SPME*	*SE*	*SPME*	*SE*	*SPME*	*SE*	*SPME*	*SE*	*SPME*	*SE*	*SPME*	*SE*
Madeira wine	NA[£]	1:4[§]	NA*	1:1*	NA	1:4	NA	1:1	ND	ND	ND	ND	ND	ND	NA[£]	1:4
Red Grape	NA[§]	NA	NA[¥]	1:1	1:1	NA	NA	1:9	1:1	1:9	1:4	1:9	1:4	1:4	1:1	1:9
White grape	NA[¥]	1:1	NA[£]	ND	NA	1:4	NA	1:19	NA	1:9	NA	1:19	NA	1:19	1:1	1:4
Elderberry	NA[¥]	1:49	1:4	1:9	NA	1:49	NA	1:19	1:1	1:19	NA	1:9	1:1	1:49	NA	1:49
Skin red grape	1:2[¥]	1:19	NA	1:19	1:1	1:49	1:1	1:9	NA	1:9	1:4	1:4	1:2	1:9	1:4	1:19

*** Signal was detected for up to 1:1 dilution**
*** *Signal was detected for up to 1:2 dilution**
[£] Signal was detected for up to 1:4 dilution
[§] Signal was detected for up to 1:9 dilution
[¥] Signal was detected for up to 1:19 dilution

SE=Solvent extraction
ND=Not detected
NA=Not applicable (No dilution was required)

6.3.7 Quantitation of the phenolic compounds from grape, berry, and wine samples

Standard addition calibration is the best applicable quantitative method when an appropriate blank matrix (ideally the same as the sample) is unavailable, and is known as the most appropriate calibration method that compensates for any variation in analysis of complex matrixes.[241] The quantitation of phenolics in the grape, berry, and wine samples in this study was performed using standard addition calibration. For covering low and high

ranges of concentration of the analytes, depending on the expected concentration in the matrix, 10 concentration points were used to construct the standard addition calibration curves for each compound. Then, from the calibration curves, the unknown amounts of phenolics in grape, berry, and wine samples were extrapolated and quantified. The organic content volume of the added standard solutions was kept at <1% to avoid any variation of the sample. The concentration of analytes in pure sample (0 added) and the standard added calibration points were kept within the linear range of the adsorptive coating. After SPME analysis, the final extracts were diluted, as needed, before LC–MS/MS analysis in order to conform to the linear concentration response of the LC–MS/MS instrument and to prevent matrix effect. The calculated amounts of the phenolic compounds in grape, berry, and wine samples using SPME-LC–MS/MS analysis and its comparison with the solvent extraction-LC–MS/MS analysis are shown in Table 6.7.

In 60% of cases there was complete agreement between the results of the SPME and solvent extraction techniques (in the range of experimental error). In almost all other cases the calculated amounts for solvent extraction were larger than that of SPME. The following paragraphs discuss the interpretation of the obtained data.

The data showed that there is a chance for binding and adsorption/conjugation interactions between the analytes and the binding matrix of the food samples. The affinity and availability of the number of binding sites are different for various matrixes and analytes. For example, the binding mechanisms of the phenolics to the protein and sugar contents of fruits have been reported in previous studies.[242] Solvent extraction is a sample preparation method which involves the swelling of the matrix by the addition of an organic

solvent which aids to release the bound concentration of the analytes by disturbing the analyte-receptor bindings and adsorption/conjugations.

Comparatively, in SPME the amount extracted is proportional to the free concentration of analytes in the sample. In the complex matrixes under study, there is a possibility that different bindings/adsorption sites and mechanisms exist for the analytes. It is also possible that there is a chance for saturation of the high affinity binding sites with the initial concentration of the analytes in the pure sample. As a result of the saturation of high affinity binding sites (which can be irreversible), for the standard added concentration points of the standard addition calibration the binding/adsorption would occur in less specific sites with lower binding/adsorption affinities, or the added concentration may remain unbound. Therefore, the portion of free concentration extracted by SPME for the standard added points is much higher compared with that of pure sample (0 added). The larger % of free masses for the higher points of calibration cause variation in the slope of the standard addition calibration curve, and consequently, the final extrapolated concentration would be smaller than the total concentration (total minus high affinity bounded). In the other words, in cases of those analytes and matrixes where SPME shows disagreement with the solvent extraction, the standard addition method in SPME could be calibrated for only free concentration plus bound concentration to less specific binding/adsorption sites (the high affinity binding/adsorption sites are saturated).

To confirm the SPME results and the proposed hypothesis for the interpretation of the data, centrifugation of the samples was performed. In this part of the study, samples plus all the concentration points of their standard addition calibration were centrifuged at 14000 rpm for 25 min. As a result, the available binding matrix (macromolecules and particulates) in the

sample were precipitated along with the bound fractions of the compounds. Then, the supernatants, which only contained the free fraction of the analytes, were separated and after the required dilution were injected into the LC–MS/MS system for analysis. The results obtained from the centrifugation experiment closely matched those of the SPME analysis, indicating a validation of the data and proposed hypothesis.

Determining the free concentration is one of the advantages of the SPME method since it determines the bioavailable concentration of the phenolic compounds, which are extracted biologically. In contrast, traditional methods can only determine the total concentration of compounds; however, the bound concentration of the compounds would not be biologically available.

6.4 Conclusions and future directions

The proposed biocompatible SPME system can be used for quantitative analysis of other types of foods through direct immersion extraction without the need for any additional pre-treatment of the sample. Using the 96-blade SPME method, automated high-throughput analysis of the phenolic compounds in 96 food samples was achieved in 210 min (<2.2 min per sample). This method addresses the limitations of time-consuming sample preparation techniques for the analysis of large numbers of food samples. In addition, the developed method provided a good degree of recovery, reproducibility, and quantitation for the analysis of the compounds under study.

The matrix effect (ion suppression/enhancement) evaluation for SPME analysis was compared with that of solvent extraction, and the results indicated a significant reduction of matrix effects when using the SPME method. This reduction highlights the importance of the

sample preparation method for the isolation of analytes from interfering components in order to obtain reliable quantitative results.

Table 6.7 Calculated concentrations of the phenolic compounds using SPME (standard addition calibration method) and the comparison with solvent extraction, the value in brackets reports the % RSD for the SPME-LC–MS/MS analysis.

	Madeira wine		**Red Grape**		**White grape**		**Elderberry**		**Skin red grape**	
	SPME ***ng/mL (RSD)***	***SE*[§]** ***ng/mL (RSD)***	***SPME*** ***ng/g (RSD)***	***SE*** ***ng/g (RSD)***	***SPME*** ***ng/g (RSD)***	***SE*** ***ng/g (RSD)***	***SPME*** ***ng/g (RSD)***	***SE*** ***ng/g (RSD)***	***SPME*** ***ng/g (RSD)***	***SE*** ***ng/g (RSD)***
Naringenin	7* (9)	22 (7)	490 (9)	570 (7)	1177 (6)	1070 (8)	5800 (13)	6638 (8)	542* (13)	270 (9)
Taxifolin	10 (8)	12 (6)	2625* (4)	5700 (5)	1136 (9)	1230 (7)	4400 (8)	3700 (10)	690* (8)	2283 (7)
Quercetin-3-D-glactoside	54 (6)	59 (9)	6174* (11)	10023 (8)	104079 (7)	94000 (6)	83800 * (6)	438000 (9)	8910 (11)	8724 (10)
Caffeic acid	427 (6)	501 (10)	1894* (13)	6781 (9)	2827 (6)	2702 (4)	9116 (6)	9280 (5)	1120 (9)	987 (6)
trans-Resveratrol	<LOD	<LOD	4120* (4)	6043 (7)	4156* (7)	31200 (10)	713 (8)	648 (11)	9401 (12)	8708 (7)
Catechin	<LOD	<LOD	22553* (10)	449008 (12)	512050 (6)	506171 (7)	29183 (11)	29900 (7)	15704* (7)	55305 (11)
Epicatechin	<LOD	<LOD	63862* (8)	350105 (10)	660020 (11)	853905 (13)	12001* (5)	53600 (9)	4010* (9)	22303 (13)
Rutin	13* (10)	187 (8)	5173* (5)	14800 (8)	16872 (13)	20575 (9)	174000* (4)	351000 (9)	9374 (8)	9770 (9)

*As described in text, due to the complexity of binding interactions, in some cases the standard addition method can only be calibrated for free plus less specific binding/adsorption sites (high affinity binding/adsorption sites are saturated).

[§]SE=Solvent extraction

The study of determining the total concentration of analytes using standard addition calibration techniques provides valuable insights on the availability of different affinity binding sites and diverse interaction mechanisms in complex food matrixes, and consequently the bioavailable concentration of the analytes in foods. When standard addition calibrations are used for calculating the total concentration of analytes in a complex binding matrix, special care should be taken for microextraction methods where the amount extracted is proportional to the free concentration of the analytes.

6.5 Addendum

The text of this chapter was rewritten in comparison to the accepted manuscript. The author thanks Supelco for the provision of mixed-mode and diol coated blades.

Chapter 7

Summary and future directions

7.1 Summary

Since its introduction in 1990, theoretical and practical aspects of SPME have improved drastically, resulting in the production of new SPME devices, the commercialization of more robust and stable fiber assemblies, the discovery of new stationary phases, and the development of high-throughput automated SPME systems.[243]

However, to date, SPME has been used more predominantly in GC applications rather than LC. There are two main reasons for this trend: the lack of automation of SPME in LC applications, and the lack of proper stationary phases for those polarity ranges of analytes used in LC studies.

The first prototype 96-autosampler for SPME was designed by PAS Technology several years ago for evaluation by the Pawliszyn research group.[74] During the last few years, several in-house multi-fiber devices were modified and improved to develop the current Concept 96-blade device. However, the widespread application of an automated high-throughput SPME system in industrial and research labs is dependent upon ability of the system to fully address all the requirements of consumers in various fields. This dependency necessitates the design and development of various extractive phases (for different applications) with a focus on high quality, efficiency, and long-term reusability (to make it commercially economical). In addition, the system should be fully evaluated and validated to be ready for commercialization.

This thesis, to some extent, addresses the above-mentioned requirements for the commercial 96-blade SPME system through the following studies discussed: (i) the evaluation of different strategies for the development of highly stable coatings; (ii) the development of various chemistries of coatings to address the efficient extraction of analytes with different polarities; (iii) the evaluation of the proposed systems in terms of stability, sensitivity, reproducibility, and matrix effects; (iv) the evaluation of strategies to improve long-term reusability and biocompatibility for the extraction from complex biological samples; (v) the evaluation of different sampling strategies (i.e., direct immersion and spot sampling); and (vi) the validation of the system though its application for the analysis of complex food samples.

The evaluation of different coating preparation strategies showed that spraying method results in a highly stable coating with long-term reusability (more than 100 times) in biological samples. The spraying method spreads fine particles of the coating slurry on the pores of the pre-treated rough surface of the blades. Multi-layer spraying, followed by thermal curing at high temperature after each layer, provides a strong attachment of the coating to the stainless steel substrate.

The thin-film geometry of the blades resulted in significant improvements in the extraction recovery of the system. As a result, low levels of LOD and LOQ were obtained, enabling the successful and reliable analysis of trace concentrations of compounds from complex matrixes when coupled with LC–MS/MS systems.

The state-of-the-art 96-blade SPME system is comparable with the automated SPE and LLE techniques in terms of analysis time and precision. High-throughput simultaneous preparation of up to 96 samples takes ~1-2 min per sample for equilibrium extraction.

However, with the application of an automated highly sensitive 96-blade SPME system, this analysis time can be significantly reduced via pre-equilibrium extraction without sacrificing precision or limitation with sensitivity.

Utilization of polyacrylonitrile as a biocompatible binder with high mechanical and chemical stability provided long-term reusability of the coatings. This reusability can address the lack of inter-lot reproducibility that is common in disposable commercial SPME fibers. The cost of materials for the preparation of each thin-film coating in the lab was in the range of $1-6, depending on the type of coating. The coating's high degree of reusability (100-times) further reduces the cost for each coating down to a few cents. The cost of manufacturing and human labor should be added to this material cost, but even with these added costs, the final estimated cost of the 96-blade coatings would be still much cheaper than reusable/disposable commercial SPME fibers.

In addition, the open-bed biocompatible SPME system facilitates the efficient and reliable analysis of compounds from complex matrixes containing particulate matters (e.g., biological sample and food homogenates) without any need for sample pre-treatment.

The introduced extracted blood spot sampling method integrates sampling and sample preparation into a single step. It takes advantage of the beneficial features of the spot sampling method (e.g. uses a small blood volume and features easy storage and shipment) and presents solutions for the limitations of the conventional dried blood spot sampling technique. Using a SPME coating for the extraction of analytes in EBS sampling prevents the limitations of chromatographic, hematocrit, and matrix effects which are common in DBS. The automated high-throughput system facilitates the preparation of 96 EBS samples in approximately 42 min (which corresponds to < 0.5 min per sample). EBS presents a fast

sampling/sample preparation method for DART–MS/MS analysis and improves the reliability of DART by cleaning up the interfering matrix without sacrificing time (1.4 min per sample, for a simultaneous run of 10 samples).

Finally, the 96-blade SPME system was used for the extraction of phenolic compounds from grape, berry, and wine samples. The evaluation of the matrix effect (ion suppression/enhancement) for SPME versus solvent extraction resulted in a significant reduction of matrix effects by SPME.

Additionally, the use of standard addition calibration techniques to determine the concentration of the phenolic compounds in grape, berry, and wine samples provides valuable insights into the availability of different affinity binding sites and interaction mechanisms for the analytes in complex food matrixes. Furthermore, SPME method offers important information on bioavailable concentration of the analytes in food samples.

7.2 Future directions

Although the developed SPME coatings in this thesis were prepared manually, excellent inter-blade reproducibility was achieved for the systems under study. This reproducibility and precision can be further improved via the automation of the coating preparation procedures. For instance, the future approach for enhanced accuracy and reproducibility of the system includes the automation of dipping and withdrawing steps for the preparation of thin-film silica gel coatings, and the mechanization of the multi-layer spraying process for the preparation of particle-based coatings.

In future, the 96-well format SPME could be expanded to 384 and even larger formats in order to further increase the throughput of the system and shorten the required time for the analysis of each sample.

The information obtained on the fabrication of high-quality sol-gel based SPME coatings can be used for the preparation of other sol-gel-derived coatings such as entrapped biomolecular sol-gel coatings, which would be valuable for sample preparation approaches or ligand-receptor binding studies. Through the development of an appropriate methodology, the automated 96-blade SPME system can be used to obtain information on both free and total concentrations from a single bio-fluid sample. This possibility could provide accurate binding data such as binding curves and binding constants in a single automated experiment. In addition, it significantly increases the sample throughput and efficiency of the experiments of ligand-binding studies.

In cases where obtaining information about the total concentration is sufficient for the study, the sensitivity of the extraction from biological samples containing binding matrix can be significantly improved through this thesis's proposed method of disturbing ligand-receptor binding, without the need for any significant dilution or complicated sample pre-treatment steps.

The developed 96-blade SPME systems have the potential to be applied in various areas of research including biological, clinical, pharmaceutical, environmental, food, and metabolomics studies. The PS-DVB-PAN and PBA-PAN 96-blade SPME systems can be successfully applied for high-throughput analysis of different polarity ranges of analytes and exo- and endogenous compounds from a single biological sample. As the next step for this study, the PS-DVB-PAN 96-blade SPME system is being used for the sample preparation of

biological samples for the global analysis of metabolites, and the PBA-PAN 96-blade SPME system is under study for the extraction of proteins from biological samples. A future step of this thesis, which is currently under process in our laboratory, is the development and evaluation of other SPME coatings with more hydrophilic properties for selective extraction of highly polar compounds.

Based on the promising initial results of EBS sampling obtained in the study of this thesis, there is potential for various additional studies in the future. In order to increase the efficiency and sensitivity of the analysis, a larger volume of the blood can be used with the newly proposed square thin-film geometry described in this thesis (2-fold enhancement in surface area). In addition, in order to accelerate the kinetics of the extraction, the thickness of the coating for EBS sampling can be optimized to be prepared as thin as possible for future studies. This optimization will provide faster kinetics of the desorption and higher desorption efficiency for EBS-DART applications (resulting in less carryover and higher sensitivity) in a short period of time (10-30 s). In addition, the throughput of the EBS-DART system can be significantly improved via the application of 96-format DART systems.

The EBS-DART–MS/MS system can be used for monitoring/determining unstable compounds and short-lived metabolites for on-site and *at vivo* clinical and biological applications. The next step is the evaluation of developed EBS methodology for the analysis of other classes of compounds with different polarities. The extracted spot sampling approach can be used for the analysis of other types of biological samples such as plasma, urine, and saliva.

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Title: Thin-film octadecyl-silica glass coating for automated 96-blade solid-phase microextraction coupled with liquid chromatography–tandem mass spectrometry for analysis of benzodiazepines

Author: Fatemeh S. Mirnaghi,Maria Rowena N. Monton,Janusz Pawliszyn

Publication: Journal of Chromatography A

Publisher: Elsevier

Date: 13 July 2012

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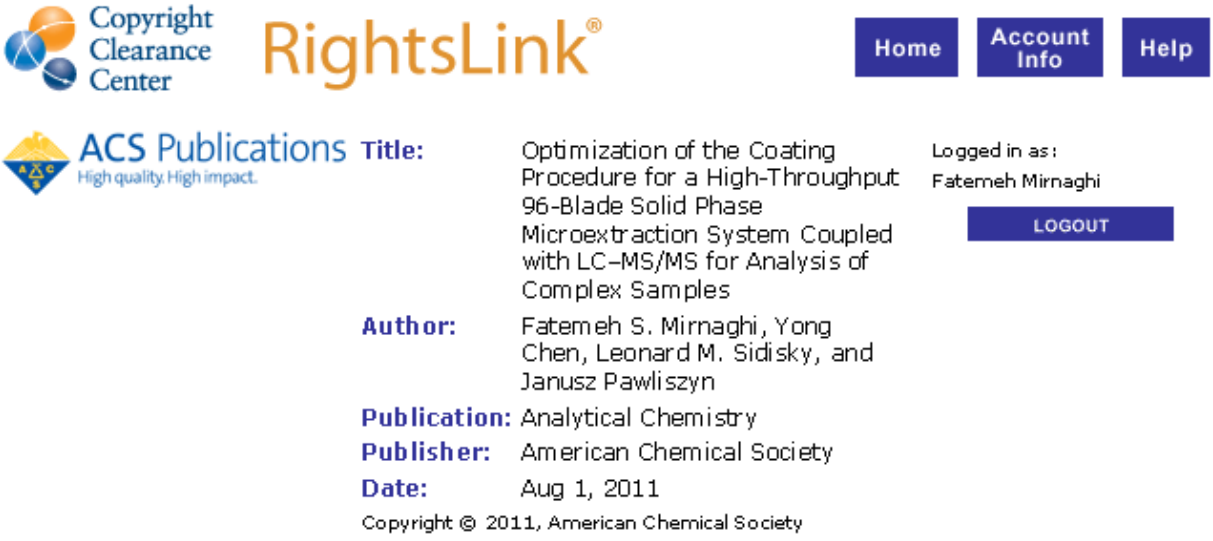

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Title: Reusable Solid-Phase Microextraction Coating for Direct Immersion Whole-Blood Analysis and Extracted Blood Spot Sampling Coupled with Liquid Chromatography–Tandem Mass Spectrometry and Direct Analysis in Real Time–Tandem Mass Spectrometry

Author: Fatemeh S. Mirnaghi and Janusz Pawliszyn

Publication: Analytical Chemistry

Publisher: American Chemical Society

Date: Sep 1, 2012

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Title: Development of coatings for automated 96-blade solid phase microextraction-liquid chromatography–tandem mass spectrometry system, capable of extracting a wide polarity range of analytes from biological fluids

Author: Fatemeh S. Mirnaghi,Janusz Pawliszyn

Publication: Journal of Chromatography A

Publisher: Elsevier

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