DEVELOPMENT OF MICROEXTRACTION TOOLS COMPATIBLE WITH THERMAL AND SOLVENT DESORPTIONS SUITABLE FOR TARGETED AND UNTARGETED ANALYSIS

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DEVELOPMENT OF MICROEXTRACTION TOOLS COMPATIBLE WITH THERMAL AND SOLVENT DESORPTIONS SUITABLE FOR TARGETED AND UNTARGETED ANALYSIS

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ABSTRACT

DEVELOPMENT OF MICROEXTRACTION TOOLS COMPATIBLE WITH THERMAL AND SOLVENT DESORPTIONS SUITABLE FOR TARGETED AND UNTARGETED ANALYSIS

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Solid phase microextraction (SPME) is one of the essential tools present in the analytical toolbox integrating the sampling and sample preparation into a single step. Because of this unique feature, SPME is distinct among other technologies as a tool suitable for *in-vivo*, on-site, and *in-vitro* applications. Up to date, a plethora of extractive phases suitable for targeted and untargeted studies have been developed for SPME. However, usually, they are only suitable for solvent or thermal desorption followed by liquid/gas chromatographic separation. This limits the applicability of the device for untargeted analysis where a system-representative chemical snapshot is needed. Therefore, the development of extractive tools and methods that can be used both with solvent and thermal desorption, providing wide-range analyte coverage is of great importance.

In this study, hydrophilic-lipophilic balanced particles providing wide-range analyte coverage were immobilized in the polyfluorinated amorphous polymer (PTFE-AF 2400) to have SPME devices that are biocompatible, with sufficient solvent and thermal stability. SPME devices with different geometries were prepared, optimized, and used in two different applications. The first type of device was a miniaturized SPME probe that is suitable for metabolomic, and pharmacokinetic profiling in cell cultures, and tissues where a spatial resolution of chemical information is crucial. It was shown that the developed SPME-LC-MS method using HLB/PTFE AF

microprobes, enables the extraction of wide range of molecules (Log P varies between -4.2 and 15.6) with acceptable method accuracy and repeatability. The second type of device was thin film SPME, capable of more sensitive analysis due to its larger surface area and on-site sampling. Using these thin film devices, a TFME-GC-MS method was developed for the determination of pesticides in apple juice. The developed TFME-GC-MS method is accurate and repeatable for the determination selected pesticides (except trifluralin at high-point concentration, 300.0 ng/mL). Preliminary studies also had shown that samplers are suitable for on-site sampling.

Keywords: solid phase microextraction, thin film microextraction, metabolomics, mass spectrometry, biocompatible materials

ÖZ

TERMAL VE SOLVENT DESORPSİYONA UYUMLU HEDEFLENMİŞ VE HEDEFLENMEMİŞ ANALİZLERDE KULLANILABİLECEK MİKROEKSTRAKSİYON FİBER VE İNCE FİLMLERİNİN GELİŞTİRİLMESİ

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Katı faz mikro ekstraksiyon (SPME) örnekleme ve örnek hazırlamayı tek adıma entegre eden önemli analitik uygulamalardan biridir. SPME'nin bu önemli özelliği, SPME'yi diğer uygulamalardan ayırmakta ve "yerinde" (*in-vivo*, on-site ve *in-vitro*) uygulamalarda kullanılmasını sağlamaktadır. Günümüzde SPME ile hedeflenmiş ve hedeflenmemiş örneklemeyi sağlayan birçok ekstraktif faz bulunmaktadır. Fakat, bu ekstraktif fazların çoğunluğu sıvı/gaz kromatografik ayırım sırasında yalnızca termal veya çözücü ile desorpsiyon sistemine uyumludur. Bu durum, geliştirilen ekstraktif probun hedeflenmemiş analiz sırasında sistemden çok hızlı kimyasal bilgi elde edilmesi gereken durumlarda kullanılmasını sınırlandırmaktadır. Bu sebeple, hem çözücü ile hem de termal yolla desorpsiyona uyumlu, geniş aralıkta analit ekstraksiyonunu sağlayacak ekstraktif cihaz ve metotların geliştirilmesi oldukça önem kazanmaktadır.

Bu çalışmada, geniş bir analit skalasında ekstraksiyon sağlayan hidrofilik-lipofilik dengelenmiş (HLB) parçacıklar florinlenmiş amorf polimeri (PTFE-AF 2400) içine sabitlenerek biyouyumlu ve aynı zamanda çözücü ile ve termal desorpsiyona uyumlu SPME probları geliştirilmiştir. SPME probları farklı geometrilerde hazırlanmış, optimize edilmiş ve iki farklı uygulamada kullanılmıştır. Ekstraktif fazın ilk uygulamasında, kimyasal görüntülemenin önemli olduğu metabolomik ve

farmakokinetik çalışmalarda hücre kültürü ve dokulardan doğrudan örnekleme yapılmasını sağlayacak minyatürleştirilmiş SPME probları geliştirilmiştir. Aynı zamanda HLB/PTFE AF bazlı minyatürleştirilmiş problar kullanılarak geliştirilen SPME-LC-MS metodu geniş fizikokimyasal özelliklere sahip (Log P değerleri -4.2 ve 15.6 arasında değişmekte) moleküllerin tayinini sağlamakta ve metot kesinliği ve tekrar edilebilirliği kabul edilebilir düzeydedir. Ekstraktif fazın ikinci uygulamasında ise daha hassas analizi ve yerinde (on-site) örneklemeyi sağlayan SPME ince filmi geliştirilerek elma suyundan çoklu pestisit yapılmıştır. Yapılan ön çalışmalar, ince filmlerin yerinde örneklemeye uygun olduğunu göstermektedir. Geliştirilen TFME-GC-MS metodu (yüksek nokta derişimde, 300.0 ng/mL, trifluralin dışında) kabul edilebilir kesinlikte ve tekraredilebilir sonuçlar sağlamıştır.

Anahtar Kelimeler: katı faz mikroekstraksiyon, ince film mikroekstraksiyon, metabolomik, kütle spektrometri, biyo-uyumlu malzemeler

This thesis is dedicated to my family.

with love and appreciation…

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
3R	Replacement, refinement, reduction
Am.Ac.	Ammonium acetate
ACN	Acetonitrile
AIBN	Azobisisobutyronitrile
BSA	Bovine serum albumin
CAR	Carboxen
CNT	Carbon nanotubes
COF	Covalent-organic framework
CT	Computerized tomography
CW	Carbowax
DI	Direct immersion
DLLME	Dispersive liquid-liquid microextraction
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DVB	Divinylbenzene
EPA	Environmental Protection Agency
ESI	Electrospray ionization
EU	European Union
FBS	Fatal bovine serum

FC-72	Perfluorohexane
F.A.	Formic acid
FTIR	Fourier transform infrared spectroscopy
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GHS	The Globally Harmonized System of Classification and Labelling of Chemicals
HLB	Hydrophilic-lipophilic balanced
HPLC	High-performance liquid chromatography
HS	Headspace
IVIVE	*In-vitro-in-vivo* extrapolation
LC	Liquid chromatography
LC-DAD	Liquid chromatography- diode array detector
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid liquid extraction
MIP	Molecularly imprinted polymer
MM	Mixed mode
MOF	Metal-organic framework
MRI	Magnetic resonance imaging
MRL	Maximum residual level
MS	Mass spectrometry
MAE	Microwave assisted extraction

MWCNT	Multi-walled carbon nanotubes
PA	Polyacrylate
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PET	Positron emission tomography
PFE	Pressurized fluid extraction
PP	Pollution prevention
PTFE-AF	poly[4,5-difluoro-2,2-bis(trifluoromethyl)-1,3-dioxole-co-tetrafluoroethylene] amorphous fluoropolymer
SPE	Solid phase extraction
SPME	Solid phase microextraction
SWCNT	Single-walled carbon nanotubes
TFME	Thin film microextraction
TGA	Thermogravimetric analysis
WHO	World Health Organization

LIST OF SYMBOLS

C_h^∞	equilibrium concentration in headspace
C_f^∞	equilibrium concentration in fiber
C_0	initial concentration of analyte
C_s^∞	equilibrium concentration in sample
D_s	diffusion coefficient
K_{fs}	distribution coefficient
V_f	volume of extractive phase
V_s	volume of sample
V_s	volume of sample
$t_{95\%}$	time required for the extraction of 95% of the analyte
t_e	equilibrium time
°C	degree Celsius
µL	microliter
δ	thickness of the boundary layer
A	surface area of extractive phase
$b - a$	thickness of the extractive phase
n	amount of extracted analyte

CHAPTER 1

INTRODUCTION

Solid phase microextraction (SPME) is a sample preparation technique that is capable of integrating the sampling and sample preparation into a single step. When volume of the sample is considerably large compared to the volume of the extractive phase, the extraction becomes non depletive, and this condition makes SPME suitable tool for *in-vivo* sampling. Because SPME can have different geometries (fiber, thin film (TFME), stir bar, etc.) and there are plenty of sorbents (polar, nonpolar, mix-mode) suitable for specific cases, wide range of applications have been reported up to date, including applications in clinical, bioanalytical, agricultural, environmental areas. However, especially in clinical applications, most of the studies conducted with SPME and its related techniques are proof of the concept studies and SPME is still not used for the routine clinical analysis because of the lack in translational research.

As stated before, many extractive phases have been developed or adapted up to date for SPME, but the most promising phase can be considered as hydrophilic-lipophilic balanced (HLB) polymer as it contains both polar and nonpolar moieties in its polymeric chain, enabling the extraction of the analytes with wide range of physicochemical properties with a single coating. In the market, HLB particles are available and there are various applications in different fields [1–6]. However, most of the applications are based on solvent desorption of analytes. When poly[4,5-difluoro-2,2-bis(trifluoromethyl)-1,3-dioxole-co-tetrafluoroethylene] amorphous fluoropolymer (PTFE AF 2400) is used as a binder for the preparation of the SPME probes, the device becomes suitable both for solvent and thermal desorption [7]. This

feature of the binder makes the resulting SPME device compatible both with liquid and gas chromatography and allows to determine both volatile and nonvolatile compounds present in a sample. This aspect is critical especially for untargeted screening. However, the capability of SPME devices prepared with this binder has not been scrutinized yet for different applications.

In this thesis, new SPME devices based on HLB/PTFE AF were prepared and optimized for use in two critical areas, namely, pre-clinical application for the cancer research and environmental application for multi-residual determination of pesticides.

In Chapter 2, HLB/PTFE SPME microprobes that will provide low invasiveness during the sampling and non-depletive extraction were prepared and optimized for their pre-clinical implementations. The applicability of prepared SPME microprobe devices for the sampling from cell cultures (2D and 3D) and *in-vivo* animal model (mouse) were on focus in this part.

In Chapter 3, the environmental implementation of HLB/PTFE AF based SPME in TFME geometry was discussed for the fast, reliable multi-class determination of pesticides. TFME samplers provide a larger surface area without thickening the extractive phase and thus increases the sensitivity of the analysis without sacrificing the extraction time. In this context, the extraction performance of the developed TFME samplers were evaluated and TFME parameters were optimized to enhance the extraction performance towards selected pesticides. Finally, the developed method was successfully validated for determination of pesticides in apple juice samples.

1.1 Analytical process

In an analytical study, information about any analyte can be obtained from any matrix that can be in solid, liquid, or gaseous phase by a proper analytical method.

Analytes can be found in major, minor, trace (analytes present in ppm level), and ultra-trace (analytes present lower than ppm level) amounts in their complex matrices [8]. Although they can be determined directly, a proper sample preparation technique is required for the extraction of analytes from their complex matrices, and an appropriate method should be designed to have sample representative, reasonable and reliable results.

There are several steps in analytical processes, and every step depends on each other like a chain. Also, each step is crucial to obtain the best results from the sample. Figure 1.1 shows typical steps involved in an analytical process; these are sampling, sample preparation, separation, quantification, statistical evaluation, and assessment of the results [9].

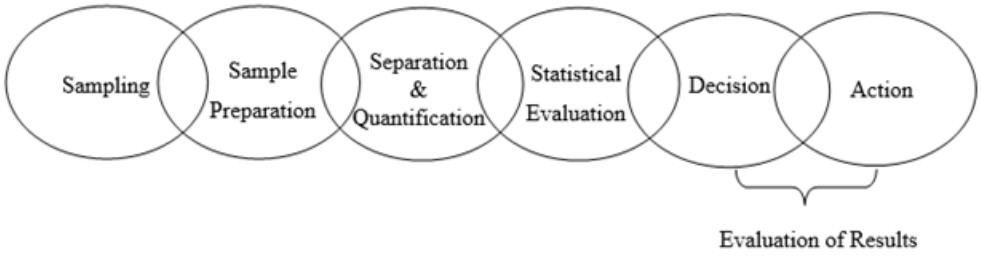


Figure 1.1. Steps in an analytical process

The first step of the analytical process, sampling, is the step in which a small representative amount of samples from its environment is collected. It is the most crucial step in an analytical approach since it causes the most significant error if not done properly. An adequate amount of samples should be collected and stored under suitable conditions to make the laboratory sample representative of the real ones. By a proper sample preparation technique, a trace amount of analytes can be isolated and even preconcentrated before their analyses. Sample preparation procedures

should be reproducible, easy to handle, easy to automate, and requiring a minimum number of steps [10].

Classical sample preparation techniques, such as liquid-liquid extraction (LLE) and Soxhlet extraction, require labor-intensive procedures and more organic solvent consumption. Also, these classical techniques cannot be automated and are unsuitable for *in-vivo* or on-site sampling due to the exhaustive extraction of analytes. However, microextraction techniques such as solid phase microextraction (SPME) combine sampling, isolation, and preconcentration steps into a single step. Besides, it does not require the use of hundreds of milliliters of organic solvent for the extraction of analytes, and it is suitable for the *in-vivo*/on-site applications where the sample volume is not known. After extraction of analytes, quantitative/qualitative analysis can be performed by various combinations of analytical instruments, including liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), thin layer chromatography- mass spectrometry (TLC-MS), gas chromatography-flame ionization detector (GC-FID), liquid chromatography-diode array detector (LC-DAD) or direct-to-MS. Following the instrumental analysis, statistical evaluation is performed, and an action is taken based on the results.

1.2 Sample preparation techniques

Most of the samples are complex and not suitable for direct analysis by an instrument, so there is a need for adequate sample preparation to eliminate the matrix interferences as possible. In general, 80% of the analysis time is spent on sampling and sample preparation steps in an analytical study [9]. Therefore, sample preparation in any analysis can be considered as the most time-consuming step. It includes separation of the analytes from their matrix, clean-up procedures to remove interfering species, preconcentration of analytes to make them detectable by an instrument, and derivatization of the analytes if necessary to change the properties

of the analyte for better isolation, or identification. Extraction techniques are summarized Figure 1.2 and explained below in detail [9].

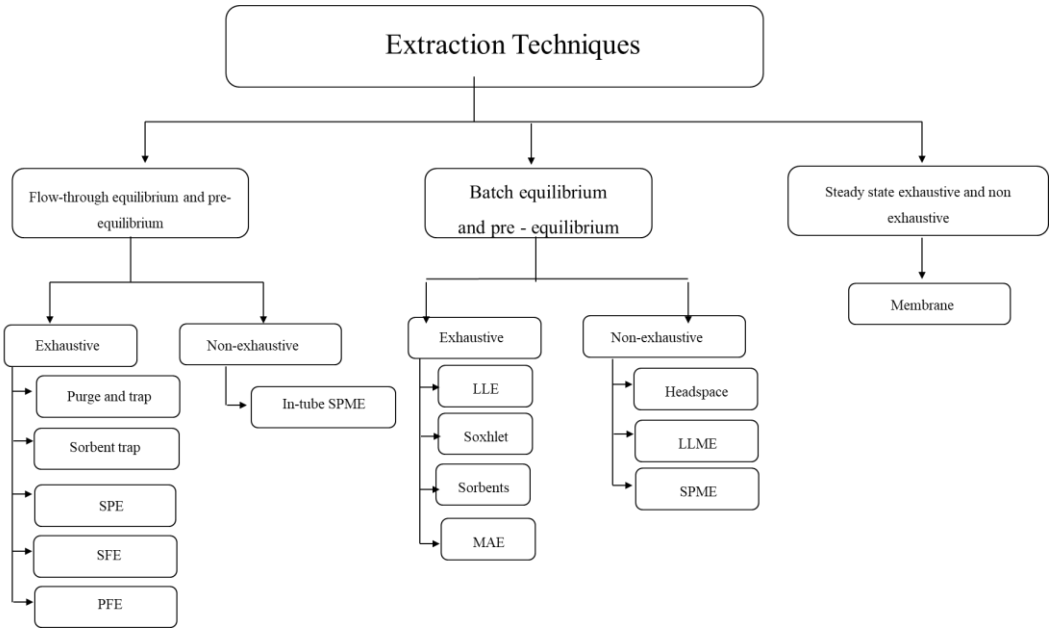


Figure 1.2. Extraction techniques (resketched from Pawliszyn, 2012) [9]

Extraction of the targeted analytes is the main interest of the sample preparation step. Various extraction techniques can be classified as exhaustive and non-exhaustive techniques. In an exhaustive extraction, all of the analytes are collected in the extractive phase [11]. In this technique, the analyte concentration can be found by dividing the extracted amount of analyte to the sample volume since all of the analytes are transferred to the extraction phase [9]. Therefore, it requires good control and knowledge about the sample volume.

Common exhaustive extraction techniques are liquid-liquid extraction (LLE), Soxhlet extraction, and solid phase extraction (SPE). LLE is an extraction technique based on the partition of analytes between two immiscible liquids. It aims to isolate the analytes from the aqueous phase into a suitable organic phase by mixing in separation funnel vigorously. The distribution of the analytes between the two phases

depends on their affinity, in other words their distribution coefficient (K_{fs}) between these two phases. The extracted amount of analyte can be maximized by multiple extractions with fresh portions of solvent. As a large volume of solvent needs to be used at this step, at the end of the extraction, the excess of the solvent is evaporated, and extract is reconstituted in a smaller volume before the instrumental analysis. In Soxhlet extraction, a solid sample containing solvent soluble analyte is placed in a thimble with a filter paper. A fresh organic solvent is continuously heated and condensed in a distillation flask. By continuous heating, solvent starts to boil and reaches to the thimble that contains the solid sample. The chamber is filled with the solvent until the chamber is emptied by siphon. In each cycle of solvent washing, a batch of nonvolatile compounds are dissolved in the organic solvent.

In Soxhlet extraction, extraction procedure takes approximately 8 or more hours, so that many cycles are achieved. The extracted compounds can be collected by evaporation of solvent after extraction is completed [12]. Until recent years, LLE and Soxhlet extractions were common techniques. However, they are labor-intensive due to the multiple extraction steps, requiring a large volume of toxic solvents such as benzene, carbon tetrachloride, or hexane, and in LLE may cause emulsion which may add extra steps to deal with. Being labor-intensive, time-consuming, and environmentally unfriendly does not align with the principles of green chemistry. Later, SPE was introduced to eliminate these drawbacks in classical exhaustive extraction techniques.

In SPE, the partition of analytes is achieved between a solid extractive phase and a liquid phase rather than the partition of two immiscible liquids as in LLE. A wide range of sorbents with different physicochemical properties are available for SPE studies to increase the selectivity towards specific group of analytes. In a typical set up of SPE, a solid sorbent is placed in a cartridge or on a disk to extract or preconcentrate the analytes or clean up the sample. The first step is conditioning of the sorbent with solvent to clean and wet the pores. The second step of SPE is loading the sample. As the sample passes through the cartridge, the analytes are retained by

the sorbent. Then, the cartridge is washed with water to remove the loosely retained unwanted species from the sorbent. The final step of SPE is elution; a solvent with higher affinity for the analytes than the sorbent is passed through the cartridge to collect the analytes. To achieve the best extraction of analytes from a sample, a sample can be manipulated under *in-vitro* conditions in terms of its volume, pH and salt concentration, temperature or sorbent type. SPE still has advantages compared to classical extraction techniques in solvent consumption. Although SPE based techniques can be applied to untargeted analysis [12-14], it is critical to control the breakthrough volume of the analyte. Breakthrough volume is defined as the highest sample volume that can be loaded to a SPE sorbent without losing the retention of analytes [16]. Because of this limitation of SPE, sample volume becomes an important parameter to not exceed the breakthrough volume. To able to report quantitative results using SPE, breakthrough volume of each analyte should be estimated, or if it is known that extraction is not exhaustive, quantification can be carried out by matrix-matched calibration. For example, Li et al. reported a simultaneous lipidomic and metabolomic investigation of small molecules using SPE. Different sample volumes were studied not to exceed the breakthrough volume. Some of the analytes cannot be detected because the volume of the loaded sample is limited in SPE [17]. On the other hand, in non-exhaustive extraction techniques such as solid phase microextraction (SPME), thin film microextraction (TFME), and dispersive liquid-liquid microextraction (DLLME), only a representative amount of analyte is extracted from the sample by using a very small volume of extractive phase that is in microliters (or micrograms) range and extraction of analytes depends on a partition equilibrium of these analytes between sample and extractive phase. Non-exhaustive techniques can be designed for pre-equilibrium or equilibrium conditions [9]. If equilibrium is established, and the sorption is non depletive, there is no need to know the exact volume of the sample [4, 8, 17–20] to perform a quantitative analysis. Moreover, non-depletive (less than 5% absolute recovery) extraction techniques allow to perform *in-vivo*, or on-site analyses without disturbing the system's equilibrium; contrary of the exhaustive extraction techniques which cannot

be used to monitor chemical changes directly in the living system since they disturb the equilibrium and extracts all of the analytes from the sample. For example, *in-vivo* blood analysis in a human body or on-site water analysis in a river can be performed using SPME based techniques. However, they require careful calibration and optimization, as well as needing more time and labor force. Still, once the method is optimized and calibrated, it is more convenient, and cost-effective than exhaustive extraction techniques, especially when coupled with chromatography and/or mass spectrometry. More details about SPME based techniques is given in the next section.

1.2.1 Solid phase microextraction (SPME)

SPME was developed in 1990 by Pawliszyn and his co-workers to extract and preconcentrate analytes from gaseous, liquid, or solid samples [9]. Today there is a plethora of methods based on SPME. In this approach, the extraction is carried out by adsorption or absorption of analytes from the sample matrix to the extractive phase based upon their partition coefficient. Unlike SPE, the volume of the extractive phase in SPME is very small, and recoveries are very low due to its non-exhaustive extraction feature. As mentioned in earlier sections, in an analytical study, the most time-consuming step is sampling and labor-intensive sample preparation procedures. However, SPME, coupled with modern instruments, can handle the complexity of the sample and provides high throughput analysis with less labor. The most common geometry for SPME is fiber format, but the extraction performance of the fiber is limited due to the small volume of the sorbent used. Sensitivity of the fiber can be enhanced by adjusting the thickness of the coating on the fiber [22]. Other parameters that affect the extracted amount of analyte(s) are extraction temperature, ionic strength, and pH of the sample. These parameters can be optimized to maximize the distribution coefficient between sample and extractive phase (K_{fs}) and so the sensitivity is increased. In Figure 1.3, a general SPME procedure is shown. As the first step, SPME probe is pre-conditioned to make the extractive phase ready for

extraction. Then sorption of the analytes is performed directly by immersion of the extractive phase to the aqueous sample or in headspace position of the sample for an optimized time. Later on, depending upon the purpose analytes can be desorbed into a suitable composition of organic solvents (solvent desorption), or analytes can be desorbed directly by thermal desorption to the injector port of GC.

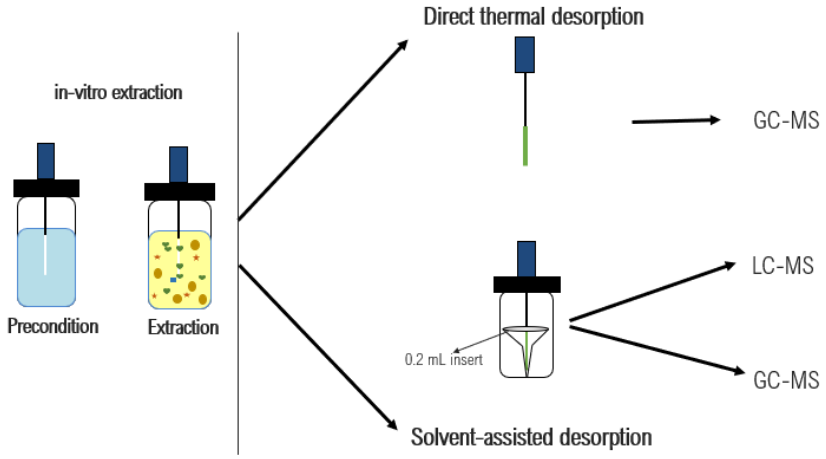


Figure 1.3. General SPME protocol

1.2.2 Thin film microextraction (TFME)

Thin film microextraction (TFME) was introduced in 2001 by Wilcockson to enhance the recovery of analytes during microextraction applications [23]. As mentioned in the SPME Section (1.2.1), one of the methods to enhance the sensitivity of SPME is to use larger volume of extractive phase. However, if this increase is achieved by increasing the thickness of the coating, the system reaches equilibrium extraction conditions at longer times [24]. The equilibrium extraction time conditions for SPME/TFME is described by Equation 1, in which the required time to reach equilibrium is shown as t_e. In this equation δ is the thickness of the boundary layer in the fluid, K_{fs} is the distribution coefficient of analyte toward the

extractive phase, D_s is the diffusion coefficient of the analyte in the fluid and $(b - a)$ is the thickness of the extractive phase.

As seen from Equation 1, by increasing the thickness of the coating, the equilibrium time is extended.

$$t_e = 3\delta K_{fs}(b - a)/D_s \qquad \text{Equation 1}$$

The other important parameter in SPME is the kinetic of the extraction. Equation 2 shows the description for the initial kinetic of the extraction. As can be seen from the equation the initial extraction rate ($\frac{dn}{dt}$) is directly proportional to the surface area of the extractive phase (A), diffusion coefficient (Ds) of the analyte in the sample matrix, and initial concentration of analyte ($C_{s)}$, and is inversely proportional to the thickness of the boundary layer (δ):

$$\frac{dn}{dt} = \left(\frac{DA}{\delta}\right) C_s \qquad \text{Equation 2}$$

Considering both equations, it can be concluded that to enhance the sensitivity of the SPME based extraction, a larger volume of the extractive phase is necessary. However, in order to not scarify the extraction time, the extractive phase should be spread in a larger surface area. This describes the basic principles of TFME.

1.2.3 Fundamentals of SPME and TFME

There are 3 basic extraction modes of SPME which are direct extraction, headspace extraction, and membrane protected SPME. Depending on the physicochemical properties of the analyte and the matrix, a suitable extraction mode should be chosen.

In direct extraction, extractive phase is dipped into the sample for a certain time (Figure 1.4).

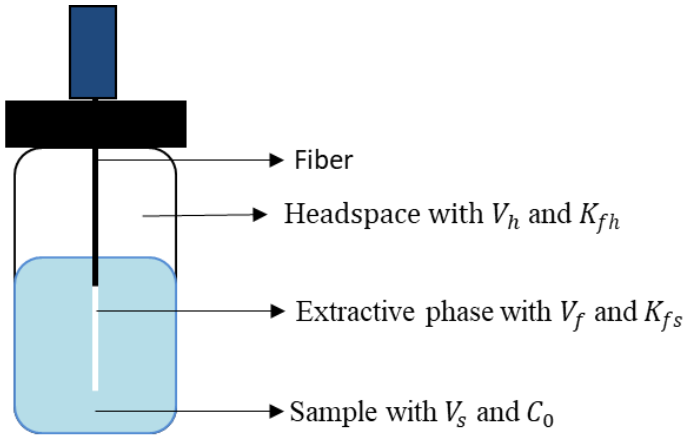


Figure 1.4. Direct extraction mode

When the system between the sample matrix and the extractive phase reaches equilibrium, no more analyte is extracted so, after equilibrium is established, the amount of the analyte on the fiber remains constant. For a given extractive phase, the equilibrium time can be controlled by adjusting stirring rate, coating thickness, and temperature. At equilibrium conditions, Equation 3 is obtained according to the law of conservation of mass when the system reaches equilibrium between the sample matrix and extractive phase:

$$C_0 V_s = C_s^{\infty} V_s + C_f^{\infty} V_f + C_h^{\infty} V_h \qquad \text{Equation 3}$$

Where, C_0 is the initial concentration of the analyte present in the sample with volume V_s, C_s^{∞}, C_h^{∞} and C_f^{∞} are equilibrium concentrations in sample, headspace and extractive phase, V_h and V_f are volume of the headspace and extractive phase,

respectively. In all extraction techniques, the analyte is extracted based on its partition coefficient or distribution constant (K_{fs}) which is the ratio of the concentration of analyte between extractive phase and sample matrix: $K_{fs} = \frac{C_f^{\infty}}{C_s^{\infty}}$. In the same manner, K_{hs} is the ratio of the concentration of analyte between headspace and sample matrix: $K_{hs} = \frac{C_h^{\infty}}{C_s^{\infty}}$

The extracted amount of the analyte by the extractive phase can be written as in Equation 4:

$$n = \frac{K_{fh}K_{hs}V_fK_{fh}C_0V_s}{K_{fh}K_{hs}V_f+K_{hs}V_h+V_s} \qquad \text{Equation 4}$$

K_{fh} is approximated by extractive phase/gas distribution coefficient (K_{fg}), while headspace/gas coefficient (K_{hg}) is approximated by gas/sample distribution coefficient. If the moisture is neglected Equation 5 is obtained:

$$n = \frac{K_{fs}V_fK_{fh}C_0V_s}{K_{fs}V_f+K_{hs}V_h+V_s} \qquad \text{Equation 5}$$

Further, if the sample is filled without headspace in a vial, there will be no effect of headspace and above equation can be written as below:

$$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + V_s}$$ Equation 6

In SPME, the volume of the extractive phase is in micrometers range. Equation 6 can be simplified further if the sample volume is much larger than the volume of the extractive phase ($V_s >> V_f$) and K_{fs} is significantly small compared to the affinity toward the sample matrix. By simplification of the Equation 6, Equation 7 is obtained. Extracted analyte without requiring knowing the volume of the sample can be calculated because $V_s >> V_f$, then V_s in the denominator is still much larger than the multiplication of $K_{fs} V_f$ in Equation 4, so $K_{fs} V_f$ can be neglected.

$$C_f^{\infty} = C_0 \frac{K_{fs} V_s}{V_s}$$ Equation 7

By simplification of V_s from both nominator and denominator, the final equation is obtained:

$$C_f^{\infty} = C_0 K_{fs}$$ Equation 8

The number of moles of analyte (n) present in the fiber can be calculated by multiplying the concentration by the volume of the fiber V_f:

$$n = C_f^{\infty} V_f = C_0 K_{fs} V_f$$ Equation 9

This feature of SPME makes the technique an all-purpose extraction technique and suitable for *in-vivo* and on-site analysis due to significant difference in the volume of extractive phase and sample. For example, extracted amount of analyte is related to the concentration of the analyte in the sample with an unknown volume of water (river, lake, sea, ocean, sewage), ambient air, or flowing blood can be calculated from the amount of extracted analyte without requiring knowing the volume of the sample. In Table 1.1 (given at the end of Section 1.3), various studies can be seen as examples for *in-vivo* analysis based on microextraction techniques.

1.2.4 SPME method development

To obtain the best sensitivity in the final method, various parameters effecting the recovery of the analyte should be evaluated critically, before using the method for routine analysis under *in-vitro* conditions. Basic parameters for SPME method development are selection of extractive phase (geometry and the type of the coating, thickness), extraction mode, extraction time, sample volume, agitation, desorption time, desorption solvent, sample modification (temperature, pH, ionic strength, derivatization), and calibration method. However, for *in-vivo* sampling, samples cannot be manipulated in terms of temperature, pH, ionic strength, and derivatization.

1.2.4.1 Extraction modes

To increase the selectivity and reliability of the extraction, the analytes of interest can be extracted from their matrices by direct immersion (DI) of the extractive phase to the sample, in headspace (HS) position, or by membrane-protected extractive phase as shown in Figure 1.5.

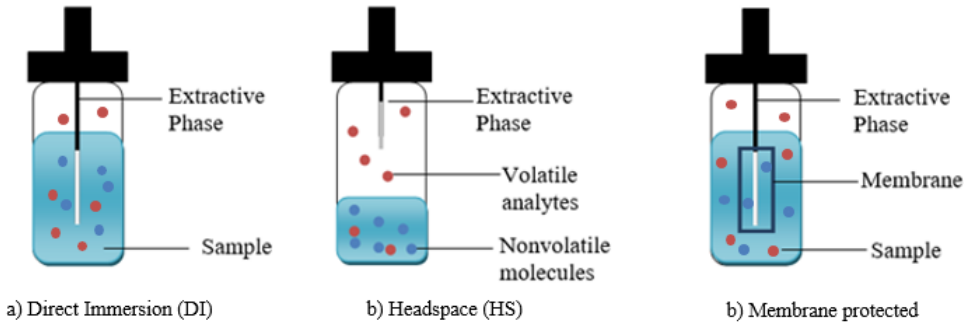


Figure 1.5. Extraction modes a) DI of the extractive phase b) HS extraction c) membrane protected extraction

With the direct immersion of the extractive phase, analytes are extracted directly from the sample. Diffusion of analytes is supported by the agitation of the sample to decrease the boundary layer mentioned in Section 1.2.4.3. DI mode is preferred for the extraction of non-volatile analytes, but the lifetime of the extractive phases is shorter in this mode of extraction, shown in Figure 1.5a. In the headspace mode of extraction which is represented in Figure 1.5b, the fiber is not immersed in the sample but, the sampling is performed indirectly from the gaseous phase (the headspace) formed by equilibration of analyte between liquid and the headspace. For example, for the extraction of volatile compounds from complex matrices such as biological fluids, or sewage water, headspace extraction is suitable to eliminate the extraction of nonvolatile compounds. It also protects the extractive phase from matrix related contamination. To increase the volatility of the analytes, extraction is supported by heating and agitation. The system reaches equilibrium faster for volatile analytes and shorter extraction times are required while longer times are required for semi-volatile analytes [11]. As its name implies, in membrane-protected extraction shown in Figure 1.5c, the extractive phase is protected by a porous membrane. In this extraction mode, extraction selectivity is increased especially for complex matrices containing nonvolatile high molecular weight molecules since their diffusion is prevented. In this mode of extraction, the diffusion of analytes through

the extractive phase slows down due to a barrier between the sample and the extractive phase, but it subsequently increases selectivity.

1.2.4.2 Extractive phases and coating methods used in SPME and TFME devices

Today there are various SPME devices prepared by different extractive materials, and coating methods. Figure 1.6 summarizes the chronological development in extractive phases and coating approaches used to immobilize the extractive phases. The first SPME fibers were made with silica fiber as supporting material which was chemically inert but fragile [25]. Today various supporting materials that provide durability and reproducible results are used as supporting materials for the extractive phase such as nitinol (NiTi) wires, stainless steel blades, stir bars, carbon mesh, etc.

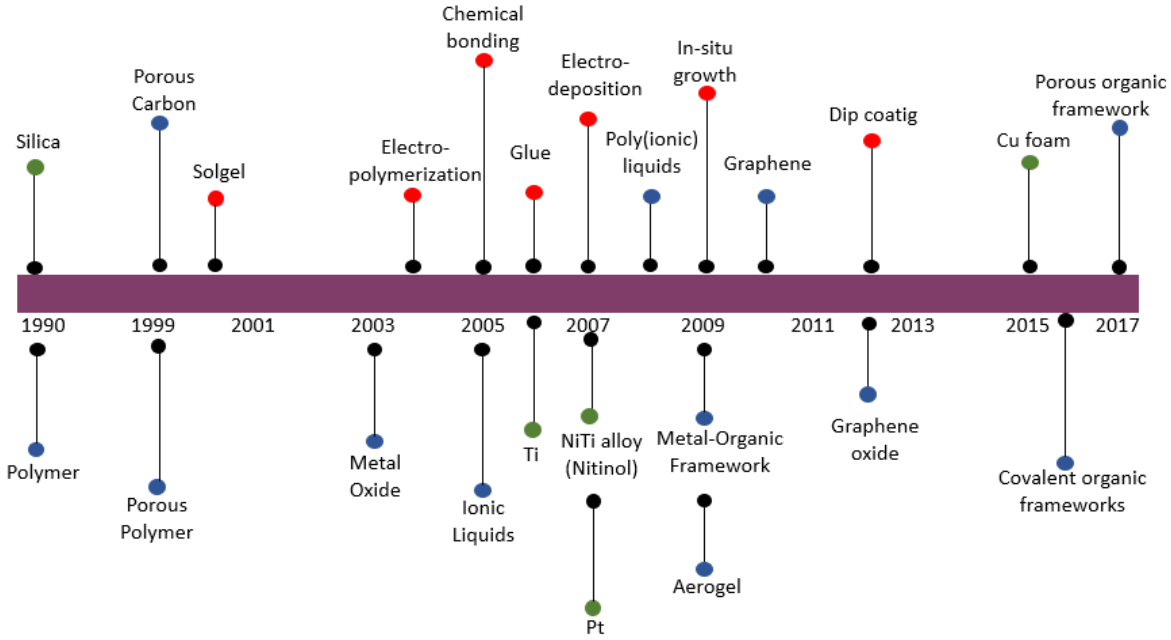


Figure 1.6. Chronological development of SPME coating materials (blue dots), coating support (green dots) and coating method (red dots) (resketched from (Peng,2022)) [26]

There are several commercial SPME devices with different thicknesses and combinations, which are polydimethylsiloxane (PDMS), polyacrylate (PA), polyethylene glycol (PEG), Carbowax, (CW), divinylbenzene (DVB), carboxen (CAR). Absorbent type extractive phases such as PDMS, PA act as a liquid extractive phase, and due to the absorption process of analytes, they have higher linear dynamic range for extraction compared to adsorbent (solid particle) type extractive phases such as PDMS-DVB, CW-DVB, CAR-PDMS, DVB/CAR, C18, HLB, etc. Figure 1.7 illustrates a general selection guide for commercial SPME fibers.

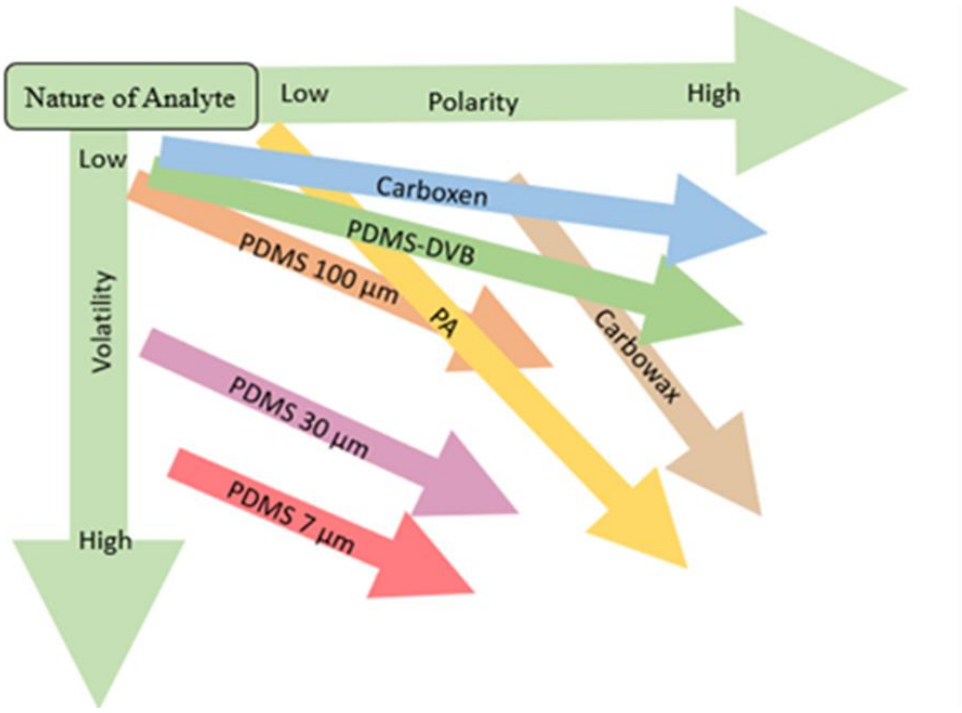


Figure 1.7. Extractive phase selection based on analyte polarity and volatility (resketched from Pawliszyn, 2012) [9]

In case of untargeted and metabolomic studies, extractive phases with the ability to extract a wide range of analyte coverage should be chosen to able to reveal what is present in the sample and take the snapshot of investigated system. For this purpose, HLB polymers gained attention. For example, copolymerization of DVB and N-

vinylpyrrolidone gives a hydrophilic-lipophilic balanced polymer that perfectly suits for the extraction of wide range of analytes.

The porosity of the extractive phase is also crucial because these coatings interact differently when their pore size is changed. Small molecules are extracted by internal micro-and mesopores while larger molecules are extracted by external macro-pores [27].

Rather than the porosity of the extractive phase, molecular weight of the analyte affects the extraction efficiency. Larger analytes are retained less on the surface of extractive phase, and thus require shorter extraction times while smaller analytes move through the extractive phase faster.

Due to the extraction property and high surface area of the porous materials, many novel extractive phases were developed as summarized below:

i) Molecularly Imprinted Polymers (MIPs): MIPs have specific cavities for the template molecule for the selective extraction of an analyte. A template molecule which is not retained strongly was used during the synthesis of polymer chain, is removed after polymerization process is completed. The resultant three-dimensional (3D) polymeric structure has selective extraction sites only for the target compound that making the extractive phase highly selective compared to other coatings [28].

ii) Carbon nanotubes (CNTs): Carbon-structured nanoparticles provide the extraction of organic molecules through non-covalent interactions. A graphene sheet is rolled into a tube to form single-walled carbon nanotubes (SWCNTs), or different layers of graphene sheets rolled to form multi-walled carbon nanotubes (MWCNTs). They provide thermal and chemical stability, durability, and a larger surface area compared to commercial coatings [29].

iii) Metal Organic Frameworks (MOFs): MOFs are produced by metal ions or clusters linked to organic ligands. Due to their uniform structure, they

provide enormous internal surface area that is perfectly fit for energy storage or suitable to be used as adsorption sites for extraction or preconcentration of compounds from their matrices. To prepare MOF coatings, a support which is acid-etched fiber is dipped into MOF precursor solution until desired thickness is obtained. In general, the resultant MOF-based extractive phase is used in the headspace position because, in the case of direct extraction, the extractive phases show low immobilization of the coating on the surface of the supporting material [30].

iv) Covalent Organic Frameworks (COFs): COFs are formed by the covalent bonding of elements such as carbon, boron, hydrogen, and nitrogen in certain conformations and classified according to the dimensions of the building blocks as two-dimensional (2D) COFs and 3D COFs [31].

v) Aerogels: Aerogels are prepared by the conversion of a liquid phase (sol) into a gel phase by the sol-gel method. They are porous materials with high surface area and the conversion of a sol into gel provides a 3D network making the aerogel an extractive phase. Today, different aerogels are available as SPME extractive phases such as silica, organic, metal-oxide, carbon, etc. [32].

1.2.4.3 Sample agitation

During the extraction process, extraction is controlled by the diffusion of the analytes from sample bulk to the extractive phase through the boundary layer (δ). Within the bulk of the sample, the boundary layer thickness of each analyte can vary because δ is determined by the diffusion coefficient of the analyte toward the extractive phase. The representative concentration gradient in a sample is shown in Figure 1.8. To decrease the thickness of the boundary layer formed around the extractive phase through which analytes travel via passive diffusion to reach the sorbent , sample is agitated. According to Equation 1, the thickness of the δ affects the equilibrium

extraction time, and the boundary layer decreases with increase of agitation. Therefore, by a proper agitation, equilibrium is established in a shorter time.

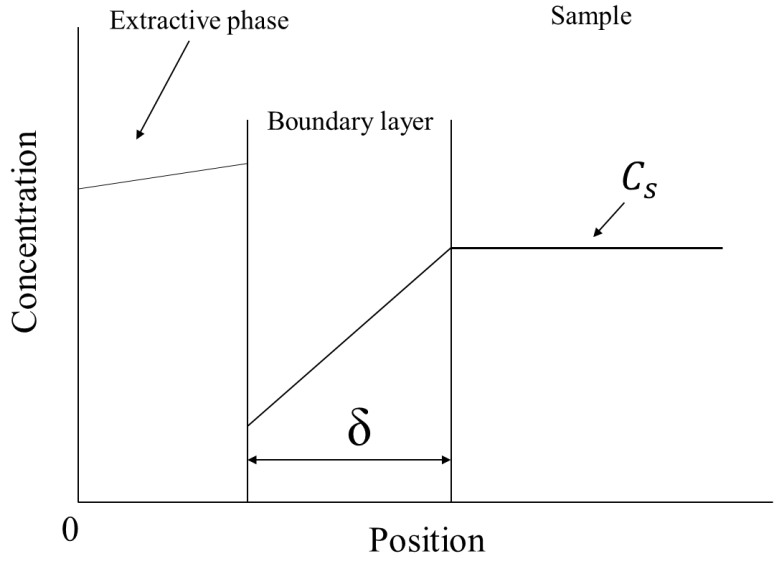


Figure 1.8. Boundary layer model (resketched from (Pawlizsyn,2003)) [33]

Because samples cannot be agitated during *in-vivo* or on-site sampling, the analytes should pass the symmetrical boundary layer by diffusion rather than convection. As agitation speed increases, the boundary layer around the solid support is compressed where molecules have shorter distance to travel through the extractive phase [32,33]. To have a reliable extraction process, optimum agitation conditions should be chosen. Agitation techniques are given as:

- Static agitation
- Magnetic stirring
- Vortex stirring
- Flow-through stirring

- Sonication
- Shaking
- Intrusive stirring
- Needle vibration

1.2.4.4 Extraction time

The extracted amount of analyte with an SPME device depends on the partition coefficient of the analyte for the sample and extractive phase. However, the extracted amount also depends on extraction time if it is at equilibrium or not. When extractive device is exposed to the sample long enough and the equilibrium is established, the extracted amount of analytes can be determined using mathematical Equation 9 derived in Section 1.2.3. A representative extraction process for SPME is shown in Figure 1.9.

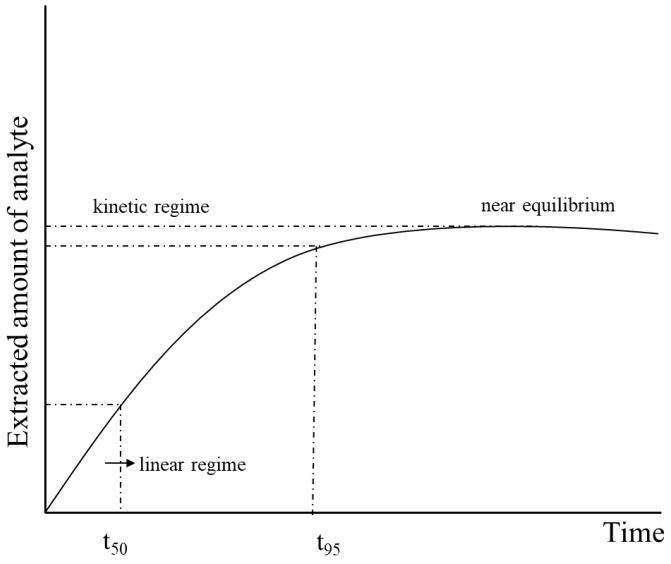


Figure 1.9. Extraction time profile in a typical extraction process (resketched from (Yu,2022)) [35]

As can be seen from the figure three distinct regions are present, linear regime, preequilibrium (kinetic regime) and equilibrium. In linear regime, the extracted amount of analyte is in linear relationship with the extraction time. In pre-equilibrium extraction conditions, any small sampling time differences results in low accuracy and precision, and extraction time should be controlled critically to have a reliable method.

In theory, the time required to reach equilibrium is infinite, but for any practical purposes, the equilibrium can be assumed to establish when 95% of the equilibrium extraction amount of the analyte is extracted from the sample. Using Equation 10, the required extraction time for equilibrium sampling can be calculated. In this formula, t_e is the equilibrium time and can be assumed to equal to $t_{95\%}$ which is the time required for the extraction of 95% of the analyte that would be extracted when equilibrium is reached, $(b-a)$ is the thickness of the coating and D_f is the diffusion coefficient of the analyte toward the extractive phase [9].

$$t_e = t_{95\%} = \frac{(b-a)^2}{2Df} \qquad \text{Equation 10}$$

A representative example of the relative error in pre-equilibrium conditions and equilibrium condition can be seen from Figure 1.10. For instance, in pre-equilibrium conditions, there is a comparable difference in the extracted amount of analyte in 30 min and 45 min sampling. Thus, any variation in extraction time will affect the extracted amount. However, in the equilibrium conditions, the extracted amount of the analyte will not change significantly if there is small variation in sampling time, therefore, less error will be present in extractions performed at equilibrium or close to equilibrium.

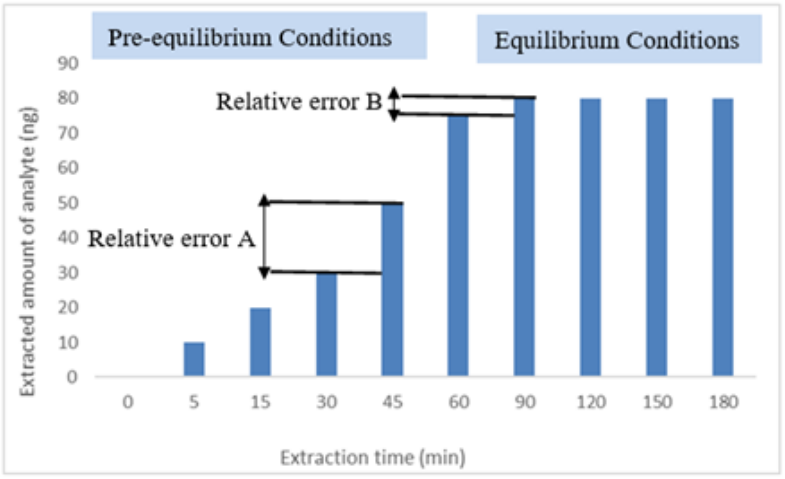


Figure 1.10. A representative extraction time profile showing relative error of the extracted amount of analytes at pre-equilibrium and equilibrium conditions

It is worth reminding that because each analyte has a different affinity toward the extractive phase, the equilibrium time will be different for each of them. Optimum time should be chosen to save the sampling time suitable for sufficient extraction of all analytes.

1.2.4.5 Extraction temperature

In a typical extraction, the amount of analyte extracted from the sample depends on the distribution coefficient K_{fs} as explained in Section 1.2.3. The temperature change affects the system both thermodynamically and kinetically. In thermodynamic viewpoint, analytes are retained on the extractive phase based on exothermic process (heat is released). As temperature increases, K_{fs} of an analyte decrease, so that the affinity of the analyte decreases toward the extractive phase. As a result of this phenomena, the system reaches equilibrium in shorter times; therefore, the required time to reach the equilibrium decreases. However, in kinetic viewpoint, the kinetic of extraction increases as temperature increases. Therefore,

the equilibrium is established at shorter time but less analyte is extracted at equilibrium [8, 35]. However, if the extraction is performed in short extraction times, especially in liner regime, compared to the extractions performed at lower temperature more analytes are extracted due to faster kinetic at higher temperatures. Therefore, the overall effect may change based on the extraction time selected for the study.

To have sensitive sampling during *in-vitro* sampling, optimum temperature should be chosen depending upon the physical states of the analytes. Especially the effect of sample temperature becomes critical for the systems with varying temperatures such as on-site or metabolomic analyses due to change of distribution coefficient of analytes toward the extractive phase and the change in temperature certainly affects the analyte recovery in such cases.

1.2.4.6 pH of sample

Another parameter to enhance the extraction of analytes from their aqueous matrix is the adjustment of sample pH. Depending upon pK_a/pK_b values of ionic analytes, the sample pH can be adjusted in a such a way that enhances the affinity of the analyte towards the extractive phase. For example, when an extractive phase is neutral, the ionic analyte should be neutralized to be extracted from its matrix in highest amount by the neutral extractive phase. In other words, if an ionic analyte has basic nature, the sample should be adjusted to have basic nature, whereas in the case of an acidic analyte sample should be acidified to make the analyte neutral within the sample. However, it should be kept in mind that if the sorbent has ion exchange capability the analyte with ionic character will be extracted in higher amount at pHs where the ionic interaction between sorbent and analyte is maximized.

As can be understood from above, when external matrix-matched SPME calibration is used, matrix pH normalization with a buffer is critical in cases when the analyte

extraction is effected strongly by sample pH and sample may have different pH than the calibration matrix.

1.2.4.7 Salt addition

The solubility of organic molecules in aqueous samples can be changed by altering the inorganic salt concentration as well as pH adjustment. In the case of the salting-out effect, the solubility of the analyte decreases in sample as salt concentration increases which at the same time increases the affinity of the analyte for the extractive phase. It should be noted that the sample should contain an optimum concentration of salt, otherwise high concentration of salt will cause to have a viscous sample that decreases the diffusion of the analytes through the boundary layer surrounding the extractive phase [9].

In overall, as shown above, to have a reliable, sensitive, and selective method, all parameters that improve the extraction efficiency should be evaluated critically before conducting on-lab analysis. However, it should be kept in mind that for *in-vivo* and on-site applications sample manipulation is not possible, therefore selection of the proper coating and geometry is imperative in such cases.

1.3 *In-vivo* applications of SPME and TFME

For the evaluation of a biological phenomenon, scientists conduct their first experiments in *in-vitro* conditions to eliminate the complexity of biological samples and ethical issues. *In-vitro* conditions indicate that the experiments are conducted in a controlled environment in a test tube or plate rather than a living organism. In most cases these controlled systems are quite simple and do not represent the complex biological systems so that the results obtained from *in-vitro* conditions frequently become insufficient and unrepresentative for living system.

To obtain information from the living system in detail, *in-vivo* sampling is a must. Any device that can be used for *in-vivo* sampling should be biocompatible, mechanically strong, provide reliable and reproducible results, extract the analytes in non-depletive amounts. In this context, SPME is one of the most promising techniques for *in-vivo* applications because of its non-exhaustive extraction nature and availability of biocompatible extractive phases. Meaning of being biocompatible in this context refers to use of devices that are non-toxic for the living system [25]. Depending upon the purpose of the study, the SPME device can extract representative amounts of a wide range of analytes for global metabolomics studies, or it can be tuned for selective extraction of a group of analytes.

Recent *in-vivo* applications are shown in Table 1.1.

Table 1.1. *In-vivo* applications of SPME and TFME

***In-vivo* Application**	**Extractive Phase**	**Geometry**	**Analyte(s)**	**Matrix**	**Sampling time**	**Instrumental Analysis**	**Year**	**Reference**
Exposome analysis	C18	Thin film blade	metabolites associated with CYP1A1 induction	Fish muscle	20 min	ULPC-HRMS	2016	[37]
Drug metabolomic analysis	Mix-mode (MM) C18 with benzenesulfonic acid	Fiber	Methylprednisolone and its metabolites	Pig lung and liver	For lung: 20 min For liver: 30 min	LC-MS	2014	[38]
Pharmacokinetic profiling	PPY bound to C_{18}-silica	Fiber	Diazepam and its metabolites	Rat nape	2 min	LC–MS/MS	2008	[39]
Drug metabolomic study	MM	Fiber	Carbamazepine and its metabolites	Circulating blood	2 min	LC-MS	2011	[40]
Pharmacokinetics profiling	C_{18} coated silica particle	Fiber	carbamazepine (CBZ) and its active metabolite carbamazepine-10,11-epoxide (CBZEP)	Circulating blood	2 min	LC-MS/MS	2011	[41]
Multidimensional analysis	C18, polyacrylate (PA), PDMS	Fiber	Human saliva compounds	Saliva	not given	ESI-MS, APCI-MS, LC-MS, GC-MS	2021	[42]

Table 1.1 (cont'd.). *In-vivo* applications of SPME and TFME

Targeted analysis	MOF	Fiber	polycyclic aromatic hydrocarbons (PAHs)	Fish muscle	30 min	GC-MS	2020	[43]
Pharmaceutical analysis	C18	Thin film blade	Pharmaceuticals (fluoxetine, venlafaxine, sertraline, paroxetine, and carbamazapine)	Fish muscle	30 min	LC-MS/MS	2012	[44]
Sampling of exhaled breath aerosol (EBA)	PDMS/DVB, CAR/PDMS, DVB/CAR/ PDMS, PDMS	Fiber	Analytes after chewing gum, banana garlic. Spraying the mouth, smoking	Exhaled breath	4 hours	DART-MS	2020	[45]
Untargeted exposome analysis	MM	Fiber	benzo[a]pyrene and its metabolites	Fish muscle	20 min	LC-MS/MS	2018	[46]
Sampling of oxylipins	MM	Fiber	Oxylipins	Rat brain	15 min	LC-MS	2020	[47]
Targeted metabolomics	(MIP)	Fiber	Hesperetin and its metabolites	Rat liver	10 to 360 min	UPLC-MS/MS	2019	[48]
Untargeted sampling	MM C18 and HLB	Fiber	Untargeted brain analytes	Human brain	4 min	LC-HRMS	2021	[49]
Sampling of VOCs	PDMS	Membrane	Volatile organic compounds (VOCs)	Human skin	5 min to 120 min	GC-MS	2008	[50]

1.4 Application of SPME for cancer diagnosis and future directions

1.4.1 Cancer

Cancer is defined as a genetic disease caused by abnormally and uncontrollably growing cells that can start in any organ within an organism according to the National Cancer Institute [51]. In normal conditions, cells are born, live, and die continuously but in some cases, this process cannot take place and these damaged/ old cells do not die, but they grow and divided to form tumor cells. These tumor cells can be cancerous. Cancer cells are quite different than normal cells as shown in Figure 1.11. They have an irregular shape, smaller cytoplasm, and multiple nuclei compared to normal cells.

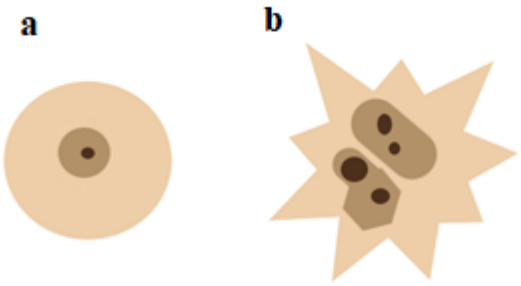


Figure 1.11. Structure of a) normal and b) cancer cells

According to the World Health Organization, cancer is the second most deathly disease in the World with 9.6 million deaths in 2018. Also, an online database showing cancer statistics, GLOBOCAN 2020 estimated the worldwide number of new cancer patients as 19.3 million and 10 million deaths in 2020 [52].

The diagnosis of cancer disease in earlier stages is very important for the treatment of the disease. At this point, the diagnosis and treatment of cancer cells becomes an important issue around the World to take an action against the disease. Although

some screening techniques such as computerized tomography (CT) scan, Positron emission tomography (PET) scan, or Magnetic resonance imaging (MRI) scan help to diagnose cancer, these techniques may not be enough for the determination of cells if they are cancerous or not. Although these screening techniques are not invasive, they may require taking certain imaging agents which discolor the target organ to ease the diagnosis. Near these practical screening techniques, the most common way to diagnose cancer is taking a biopsy. There are different biopsy techniques such as skin, needle, endoscopic, surgical, or bone marrow biopsy [53]. However, all biopsy techniques are invasive and require the removal of a certain tissue from the body by a trained person.

1.4.2 Use of SPME in cancer diagnosis

Many studies have reported the use of SPME in cancer diagnosis. For example, the use of SPME for detection of potential biomarkers for the diagnosis of the breast cancer was reported by Silav et al. In this study, the sampling was performed from the urine samples of breast cancer patients and healthy volunteers using 6 different extractive phases (PDMS, PA, DVB/CAR/PDMS, CW/DVB, CAR/PDMS, PDMS/DVB) targeting the volatile metabolites in the headspace of the sample. As the result of this study, 79 compounds including phenol, 3-heptanone, and (−)-4-carene were found statistically different in urine samples of cancer patients compared to healthy people [54].

Another interesting application of SPME was conducted by Jaroch et al. They developed a new approach for the metabolomic profiling of melanoma growth in mouse models. As seen in Figure 1.12, 2D cell culture, 3D cell culture, and *in-vivo* approaches were used for the untargeted profiling of compounds of B16F10 cancer cell line. In the light of the conducted experiments, biochemical changes during tumor growth was revealed. This study was the preliminary one that extrapolates the *in-vitro* conditions to *in-vivo* conditions for the untargeted profiling. Interestingly, in the present study, a greater number of metabolites was detected for *in-vivo* conditions

compared to 3D cell line [55], indicating that some of the information may not be transferred from *in-vivo* to *in-vitro* models.

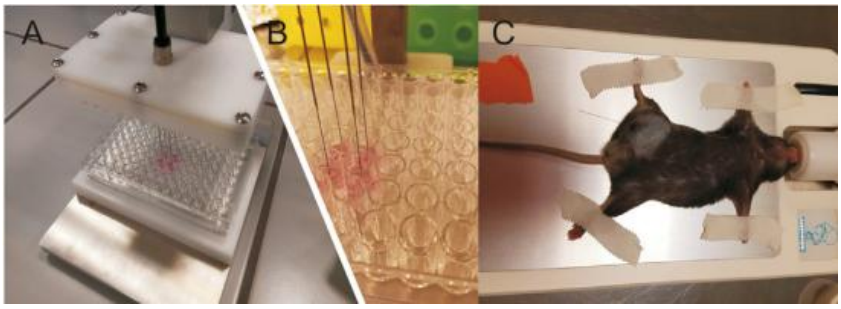


Figure 1.12. Sampling approaches a) 2D cell culture b) 3D cell culture c) *in-vivo* (reproduced with permission from [55] and Elsevier)

One of the applications of SPME fibers for *in-vivo* sampling was shown by Bogusiewicz et al. for the tumor phenotyping of gliomas which are defined as tumor cells in the brain and spinal cord. This was a preliminary study for *in-vivo* chemical biopsy of brain tissue in the operation room. The authors showed successfully that SPME can be used to obtain chemical information from the tissue (whether cells are cancerous or not) during the operation and in future can help with the decision to have a fast action during operations [42].

In summary, SPME probes are suitable for *in-vivo* sampling which is critical for metabolomic studies and they are promising tools for the evaluation of tissues in terms of being cancerous or not for the fast diagnosis of cancer. Rather than classical techniques such as chemical biopsy that requires the removal of the tissue, SPME probes provide noninvasive sampling so that the diagnosis and the treatment process become less painful.

1.4.3 *In-vitro-in-vivo* extrapolation (IVIVE) applications

In 1895, Russel and Burch proposed the three Rs (3Rs) rules for replacement, refinement, and reduction of animal use in research and development. [56]. The replacement aims to conduct the study without requiring animal tests, such as

adopting the study as *in-vitro* experiments, using animal/human cell cultures, cell, tissue, in-silico conditions (i.e. computer-based experiments) rather than using animal [56]. Refinement aims to minimize the use of stress agents or limitation of their physical/cognitive activities, or application of harm on the animals such as application of narcotics. The reduction aims to reduce the use of animals to a minimal level which is necessary to get satisfactory results from the conducted experiment. In fact, the reduction is not limited to the number of animals used during the study. It also involves the intensity of application of harm/stress agents on the animal and the time of using the animal should be reduced according to the 3Rs rule directed by the European Parliament and of the Council [57]. This approach became applicable around the world and in 2010 European Union (EU) accepted regulations under Directive 2010/63/EU for conducting a scientific experiment [57].

Recently to be in line with the 3R rules a new approach has been developed. In this approach the idea is to get the information from *in-vitro* conditions by relating *in-vivo* conditions to *in-vitro* conditions. This approach is known as *in-vitro-in-vivo* extrapolation (IVIVE). As the name indicates, IVIVE uses the data obtained under *in-vitro* conditions to predict what is happening *in-vivo*. IVIVE studies are quite important for animal welfare since classical animal studies are problematic because of ethical considerations due to being labor-intensive as well as being time-consuming. There are various IVIVE pharmacokinetic applications [54, 57–62]. IVIVE perfectly fits the 3Rs rule in terms of applicability for the cell line, cell or tissue sampling which meet the requirement of replacement principle while non-exhaustive and high-throughput analysis meet the reduction principle. Consequently, the combination of SPME with IVIVE studies meets the requirements of the 3Rs principle because prior to *in-vivo* experiments, *in-vitro* cell line studies can be performed. Moreover, the applicability of SPME to cell line studies provides a partial replacement. Also, since SPME is a non-exhaustive and non-depletive extraction technique, the same animal can be used for the sampling throughout the study. This advantage of SPME meets the requirement of reduction in the 3Rs principle.

Besides these advantages of SPME when it is used in IVIVE studies, it also can provide spatial resolution which becomes critical especially for the heterogeneous

samples such as cancer tissues. However, the sampling resolution in SPME is related to the size of the device.

Commercially available SPME fibers have higher volumes of coatings causing lower spatial resolution [64]. Miniaturized SPME can deal with the complexity and heterogeneity of the sample since the volume of the extractive phase can be lowered and extraction of analytes become non-exhaustive and non-depletive making the analysis suitable for bioanalytical, environmental applications. However, such miniaturized probes are not commercially available.

1.5 Cell culture models and applications of SPME

Cell culture is defined as the growing of cells obtained directly from the parental tissue, while cell line is defined as the growing cells obtained from cell culture itself. Growing cells *in-vitro* conditions started at the beginning of the 1900s and become important for metabolomic profiling, drug development, vaccine development, and cancer research. By growing eukaryotic or prokaryotic cells within the cell plate, pharmacokinetic and pharmacologic studies can be reliably conducted since cells can be manipulated [65]. Cell lines are commonly used to identify the compounds present in the cancerous cells, biomarkers, antigens, and abnormalities [66]. There are many applications of cell lines to investigate the effectiveness of the studied anticancer drug.

The most common way to grow the cells is in the 2D cell culture model, but this model has some limitations in practice. In 2D cell culture, cells can grow within a monolayer, so they have simpler structure compared to living morphology. Cells within the cell plate have an equal chance of reaching the nutrients or oxygen to survive. Because of these limitations, 2D cell culture does not provide representative information about living morphology. For this reason, to characterize cancerous cells, 3D cell culture is commonly used. In 3D cell culture, cells can grow in directions to form spheroids and organoids. Due to the complex morphology of 3D cell cultures, cells compete with each other to reach nutrients, oxygen and other

molecules present in the plate. Some cells within the spheroid/organoid can die due to an unequal chance to reach the resources that are important for their survival as in the case of living physiology. A schematic representation of 2D cells and 3D cells in a plate is shown in Figure 1.13.

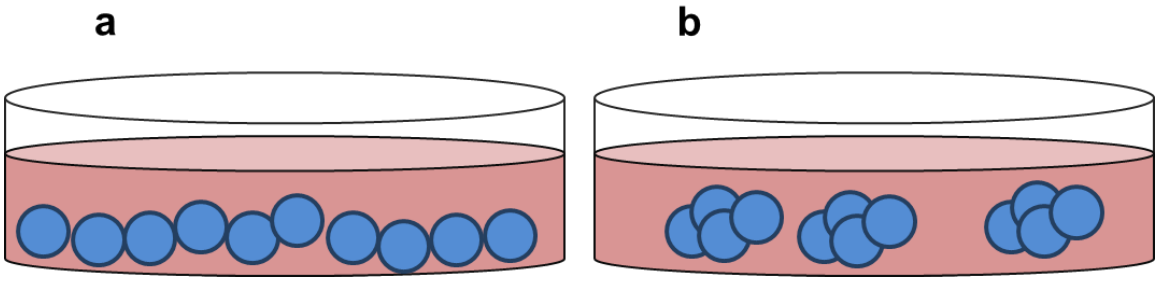


Figure 1.13. Schematic representation of a) 2D cell culture b) 3D cell culture

In a general point of view, the combination of SPME in an IVIVE application is a promising application for disease diagnosis, pharmacokinetics, and also cancer research. By combining SPME with an IVIVE study, the used animals in a study will be lowered since same animal can be used throughout the study due to non-depletive extraction and low invasive nature of SPME.

1.6 Environmental pollution and application of SPME and TFME

One of the most concerning problems around the world is environmental pollution, and there are uncountable agents causing the pollution of water, air, and soil [67]. As stated in Liang's study, urbanization brings economic growth but also environmental pollution because of industrialization [68]. For human and animal welfare and also to respect nature, it is critical to control environmental pollution. United States Environmental Protection Agency (EPA) defined a waste management hierarchy, shown in Figure 1.14, for pollution prevention (PP) that aims the use of nontoxic or less toxic compounds than toxic ones at least amount for multiple times. People are exposed to the pollutants mainly via air, water, food consumption or direct contact with contaminated products. As explained in earlier sections, SPME provides

substantial advantages due to applicability on-site and *in-vivo* analysis over classical techniques.

Also, thanks to the *in-vivo* application of SPME, various studies were conducted to show the human/animal exposure to pollutants to take an action for both environmental and human health. In fact, one of the critical groups of environmental pollutants is pesticides; more details is given about them in the next section.

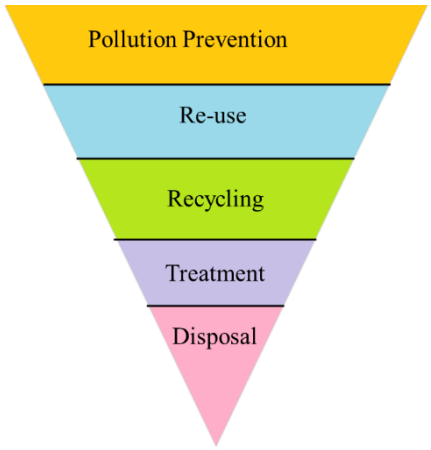


Figure 1.14. EPA Waste Management Hierarchy

1.6.1 Pesticides

The population around the world (currently ca.7.0 billion) reached to grow by 70 million per year reach to 9.2 billion by 2050 [69]. An increase in the human population brings more consumption of foods and the requirement to have healthy and high-quality crops become a critical issue especially due to limited sources of crops. Pests are any organisms that damage plants. At this point, to protect the plants from hazardous pests, the use of pesticides cannot be prevented. If pesticides are not applied, 35% of the crop is lost before harvesting, and later 35% of the harvest is lost during storage, transportation, and marketing, resulting in economic losses. Pesticides are specific natural or synthetic chemicals targeted to kill these pests and the types of pesticides were shown in Table 1.2 [70]. According to EPA, 70% of

applied pesticides are herbicides, target to kill the harmful pests but, they also affect the non-targeted organisms such as human and animals [70, 71].

Beyond Pesticides, which is a nonprofit organization published a document summarizing pesticide-induced disease database obtained from real exposure findings, animal and cell line/tissue studies. Some of the related diseases are shown below:

- Alzheimer's disease [72–74]
- Asthma [72, 75, 76]
- Birth and fetal defects [72, 77, 78]
- Cancer [72, 79–82]
- Parkinson's disease [72, 83–86]

Table 1.2. Pests and applied pesticides

Type of Pest	**Type of Pesticide**
Fungi	Fungicides
Insects	Insecticides
Plants	Herbicides
Rodents	Rodenticides
Spiders, mites	Acaricides/Miticides
Snails	Molluscicides
Bacteria	Bactericides
Algae	Algicides
Birds	Avicides

Bio-pesticides are obtained from natural sources, and they are host-specific meaning that they affect the specific pests while chemical pesticides affect a large group of organisms. This specificity of bio-pesticides makes them more effective and environmentally friendly toward the specific pest. However, the half-life of biopesticides are relatively short and decompose faster compared to agrochemicals.

Because of high selectivity and short shelf life, farmers do not prefer to use these biopesticides since they need to apply different kinds of biopesticides to control each of the pests [88]. Man-made chemical pesticides are obtained after certain chemical processes, and they effect broader spectrum of pests. These are the primary choices of the farmers as they do not require continuous application in the field due to their persistent nature in the soil.

Classification of chemical pesticides according to their chemical structure is shown in Table 1.3. Organochlorine pesticides contain five or more chlorine atoms within a hydrocarbon chain. Mostly, organochlorine pesticides are used as insecticides to affect the nervous system of pests. Organophosphorus pesticides are derived from phosphoric acid that inhibits the neural activity of pests. The phosphorus bond of phosphate ester makes them chemically inert and hard to decompose. They are quite common in the market with 40% sharing [89]. Carbamates are carbamic acid derivatives and they are easier to decompose in nature. Lastly, pyrethroids are derived from pyrethroic acids and can be obtained from flowers extract or produced synthetically. Most of this group of pesticides can easily be degraded with exposure to light [90].

Table 1.3. Classification of chemical pesticides (* indicates the pesticides used in the study)

Type of Pesticide	**Example**
Organochlorine	Dieldrin, lindane
Organophosphate	Parathion*, malathion*, chlorpyrifos-methyl*
Carbamate	Carbaryl*, carbofuran
Pyrethroid	Permethrin, furethrin
Dinitroaniline	Trifluralin, pendimethalin, benfluralin

In Table 1.4, classification for the pesticides proposed by The Globally Harmonized System of Classification and Labelling of Chemicals (GHS) is shown. The

classification is based on the calculation of single median lethal dose (LD_{50}) which is the lethal dose resulting in the death 50% of the test animals. This classification is still in use by World Health Organization (WHO) since 2009 [91].

Table 1.4. GHS classification of pesticides by hazard

Class		**Example**
Ia	Extremely hazardous	Aldicarb, parathion-methyl
Ib	Highly hazardous	Dichlorvos, primicarb
II	Moderately hazardous	Carbaryl, diazinon
III	Slightly hazardous	Chlorpyrifos-methyl, malathion
U	Unlikely to present acute hazard	Trifluralin, methoxychlor

Despite the necessity of pesticides for growing crops in agriculture, they are one of the critical pollutants. Uncontrollable excess use has serious effects on nontarget organisms, especially humans. In order to regulate their use, a residual concentration for each pesticide in crops is specified by the EU and called maximum residual level (MRL). Each country has certain regulations to reduce the use and adverse effects of pesticides. Because of the strict rules accepted by the EU, farmers may mix the agrochemicals to have stronger effects over the pests without exceeding the allowable concentration for a single pesticide. Because of the adverse effects of pesticides on nontargeted organisms and the environment, the multi-residual determination of them has become a crucial issue for public and environmental health. Some studies conducted to show human exposure for pesticides are given in Table 1.5.

1.7 Aim of the study

This thesis aimed to develop SPME-based devices suitable for thermal and solvent-assisted desorption, thus allowing the extraction of volatile, semi-volatile, and nonvolatile compounds with a wide range of physicochemical properties. To cover

wide range of analytes, HLB extractive phase was synthesized. To have SPME device suitable for both solvent and thermal desorption, the HLB particles were immobilized within thermally stable and inert PTFE-AF polymer. SPME devices were prepared in two geometries (fiber and thin film) to show the applicability of this extractive phase for studies with entirely different needs. In the first study, HLB/PTFE-AF SPME miniaturized fibers were produced with intention to use for small volume of samples (i.e., cell culture), *in-vivo* conditions, and spatial resolution from tissue (i.e., tumor profiling) for which the non-depletive and low invasive devices are needed. In the second study, opposite to the former study, high sensitivity was on focus for reliable determination of trace level of pesticides in agricultural products. Therefore, HLB/PTFE-AF thin films with larger extractive phase were produced to achieve high sensitivity in the final method. The studies in this thesis are preliminary applications of the developed samplers, showing the suitability of the extractive devices for untargeted global extraction and the suitability for complementary instruments such as LC and GC.

Table 1.5. Application of SPME for the determination of pesticides

Target group	Pesticide	Matrix	Sampling duration	Analyte	Effect of exposure	Instrumentation	Year	Reference
Pesticide applicators	Chlorpyrifos	Urine and blood	10 months	Urinary metabolites and enzyme activity molecules	Attention deficit hyperactivity disorder (ADHD)	GC-MS	2019	[92]
Farmers	Organochlorines, Organophosphates, pyrethroids	Urine and saliva	ng	Metabolomic investigation	Metabolic disturbances	GC-MS	2019	[93]
Children	Organophosphates	Urine	1 month after questionnaire	Urinary metabolites	Effect of dietary habits	GC-MS/MS	2019	[94]
Children	Organophosphates	Urine	6 day intervals over 4 months	Pesticide biomarkers	Asthma	Leukotriene E4 EIA Kit	2020	[95]
Farmers, construction workers, labors	Chloroacetamides, triazines, benzimidazoles, carbamates, pyrethroids, Organophosphates, avermictins, urea	Urine	First morning urine	Pesticides	not given	HPLC/MS/MS	2019	[96]
Men	Organophosphates, pyrethroids	Urine	not given	Pesticide biomarkers	Sperm concentration	GC-MS	2007	[97]

Table 1.5 (cont'd). Application of SPME for the determination of pesticides

Farmers	Organophosphates, triazines, pyrethroids	Plasma and urine	1,2,4 and 6 hours after spraying	Pesticides	not given	GC-MS	2019	[98]
Volunteers	Organochlorines, organophos-phates, dinitroanilin, nicotianilin, phenol, azole and pyrethroids	Hair	not given	Pesticides	not given	GC-MS	2012	[99]

CHAPTER 2

INNOVATION IN TRANSLATIONAL RESEARCH: BIOCOMPATIBLE MICROPROBE DEVICES FOR *IN-VITRO* AND *IN-VIVO* CANCER STUDY: MicroIVIVE

2.1 Experimental

2.1.1 Reagents and materials

All experiments were performed using ultrapure water (18.2 MΩ.cm at 25 °C, MilliPore). Analytical standards of creatinine, glutamine, glutamic acid, guanine, leucine, arginine, tryptophan, cholesterol, phenylalanine and nicotine were from Sigma Aldrich, riboflavin was from Merck and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was from Avanti Polar Lipids. For desorption of analytes, acetonitrile (ACN) and methanol (MeOH) were purchased from Sigma-Aldrich. MS-grade formic acid (F.A.), and MS-grade ammonium acetate (Am.Ac.) which were used during chromatographic separation were purchased from Sigma-Aldrich. Agarose purchased from Sigma-Aldrich was used to prepare a gel representing solid matrix and utilized to test the mechanical stability of the fibers. HLB particles were synthesized in the laboratory. For the synthesis of HLB particles, N-vinylpyrrolidone, divinylbenzene (DVB), and azobisisobutyronitrile (AIBN) were purchased from Acros Organics. PTFE-AF 2400 used as polymeric binder for the preparation of extractive phase and purchased from Sigma-Aldrich. Perfluorohexane (FC-72) was obtained from ABCR GmbH and used for the dissolution of PTFE-AF 2400. Nitinol wire was used as supporting material for the extractive phase and purchased from Aksöz ArGe.

1x phosphate buffer saline solution (PBS) (pH 7.4) was prepared using sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4) from Sigma-Aldrich and potassium dihydrogen phosphate (KH_2PO_4) from IsoLab. For the preparation of synthetic plasma, bovine serum albumin (BSA) was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was used during validation studies and obtained from Capricorn Scientific.

2.1.2 Instruments

For the quantification of analytes, LC-MS was used. Analyte separation was performed in Agilent 1260 Infinity II liquid chromatograph equipped with Agilent 1260 Infinity II series quaternary pump and Merck SeQuant ZIC-HILIC (column dimensions: 100 mm x 2.1 mm, 3.5 µm, 200 Å). Separated analytes were detected using Agilent single quadrupole mass analyzer equipped with electrospray ionization (ESI) source. ELMA Elmasonic S40(H) and ELMA LC 30 ultrasonic baths were used for degassing of the solvents prior to LC analysis. Extractions were performed using a mechanical shaker of CAT AEK-SH10. During method development for the separation of analytes two other columns were also tested, namely, Agilent, Poroshell 120 EC-C18, (4.6 x 150 mm, 4 µm) and Ascentis Express F5, (2.1 x 50 mm, 2.7 µm) column (Supelco). Bruker Alpha Fourier-transform infrared FT/IR spectrometer was used for characterization of synthesized HLB particles. QUANTA 400F Field emission scanning electron microscope (SEM) was used for morphological characterization of HLB particles.

2.1.3 Synthesis of HLB particles

Hydrophilic-lipophilic balanced (HLB) particles were synthesized in the laboratory using , the precipitation polymerization procedure described by Lendor et al. [100]. In a typical synthesis, 150.0 mL of acetonitrile and 50.0 mL of toluene were purged with nitrogen for 5 minutes in a three-necked round bottom flask with a magnetic

stirrer. The mixture was further mixed for 30 min at room temperature. Then 1.0 mL of N-vinylpyrrolidone, monomer, and 4.0 mL of divinylbenzene, crosslinker, were added to the solvent mixture, and 50.0 mg of azobisisobutyronitrile (AIBN) was added as an initiator and the mixture was purged with nitrogen. The resultant mixture was stirred at 100 rpm for 24 h at 80 °C in an oil bath for the reaction to occur. After the polymerization process was completed, particles were collected by vacuum filtration and washed with 100 mL portions of ethanol three times. The steps in the polymerization process are shown in Figure 2.1.

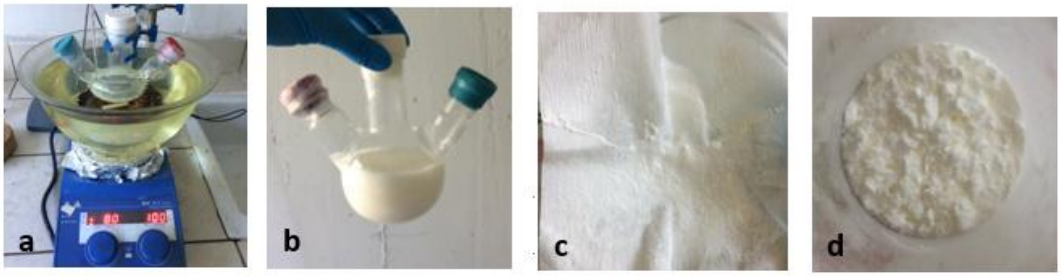


Figure 2.1. Synthesis of HLB a) the mixture before polymerization b) after precipitation polymerization completed c) after filtration of particles d) after drying the particles.

2.1.4 Preparation and optimization of SPME fibers

SPME devices were prepared in fiber format for this part of the study. HLB was used as extractive phase, where PTFE-AF 2400 was used as a binder for the immobilization of the particles on the surface of supporting material. For the dissolution of PTFE-AF 2400, perfluorohexane (FC-72) was used since PTFE-AF 2400 has a limited solubility in this solvent while HLB does not dissolve in it. For the preparation of extractive phase, the procedure of Gionfrioddo et al. was followed [7]. The preparation of slurry is shown in Figure 2.2.

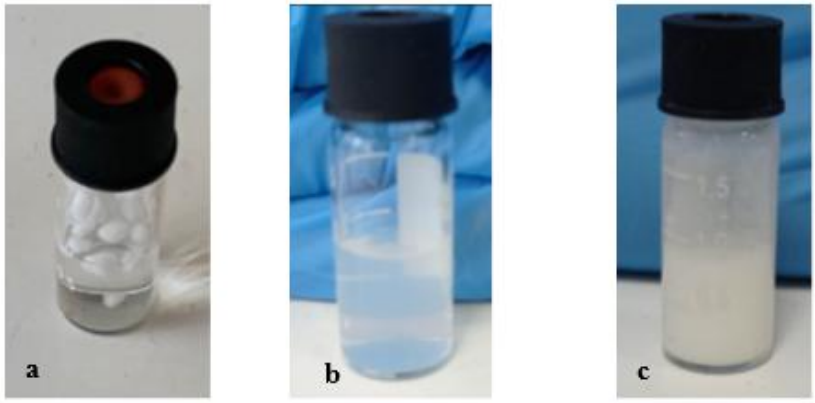


Figure 2.2. Preparation of HLB/PTFE slurry a) addition of PTFE-AF 2400 into FC-72 solvent b) after the dissolution of PTFE-AF 2400 in FC-72 c) after addition of HLB into the solution

Because fluorinated polymers are known for their chemical inertness and non-stick nature, the preparation of SPME fibers required an etching process to produce a groove in which the coating can be deposited. Schematic representation of the etching process is shown in Figure 2.3. A seal was also used to protect one end of the nitinol wire from etching. Etching conditions were optimized as follows: nitinol wire was immersed into 12 M HCl for 1 hour, then immersed into distilled water for 15 min and acetone for 15 min.

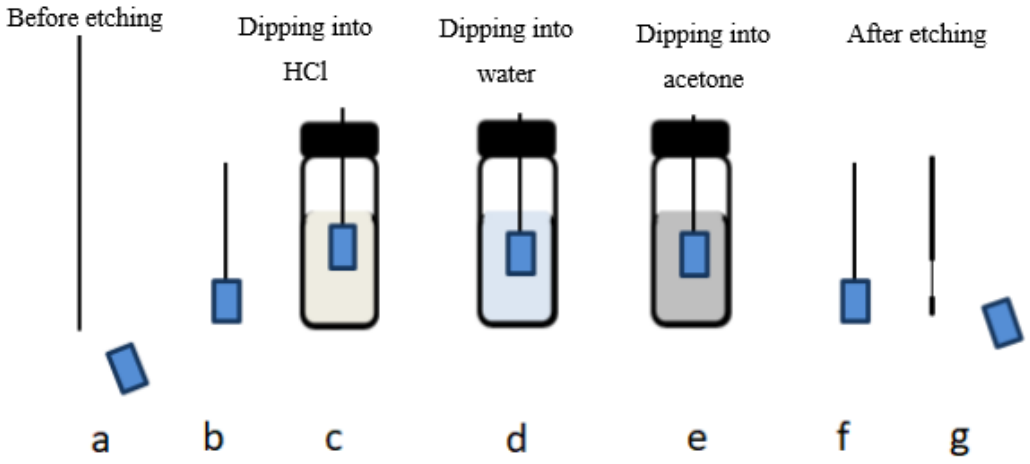


Figure 2.3. Etching process of an SPME fiber a) nitinol wire and a seal b) cutting and protecting one end of a nitinol wire by a seal c) immersion to 12 M HCl d)

immersion to water e) immersion to acetone f) removal of nitinol wire from acetone g) after the etching process

Different dip-coating rates were tested during fiber preparation. However, because the solvent used in the preparation of the HLB/PTFE slurry, FC-72, is very volatile and its boiling point is 56 °C, it evaporated quickly from the slurry during the coating procedure, changing the viscosity of the slurry. To decrease the evaporation of the solvent, the slurry was kept in an ice bath and HLB/PTFE SPME fibers were prepared at 8 °C. At the same time, 2 times coated, and 3 times coated fibers were prepared to optimize the number of coating layers. Then all fibers were tested to check their mechanical stability in aqueous and semi-solid samples. In these evaluations, one fiber was dipped into the water for 15 minutes, one was dipped into water and at the same time stirred at 200 rpm for 15 minutes and one of them was immersed into an agarose gel for 15 minutes to evaluate the stability of extractive probes for the sampling from solid samples.

2.1.5 Preparation of 2% agarose gel

The mechanical stability of the prepared HLB/PTFE fibers for the sampling from semi-solid samples were tested by immersion into 2.0% agarose gel. For this purpose, 2.0% agarose gel (w:v) was prepared as follows: 2.0 g of agarose was dissolved in water and diluted to 100.0 mL with water. Then the solution was boiled, and transparent solution was obtained. 50 mL portions of gel were poured into Petri dishes and the gels were cooled to room temperature and solidified.

2.1.6 Selection of analytes to represent the global extraction

In future studies, the proposed extractive devices will be used for the sampling of cancer cell lines and a mouse model for untargeted metabolomic investigations. Therefore, to evaluate the extractive performance of the new fibers, representative compounds for metabolites that can be found in a typical cell culture media and metabolites that have been reported as cancer biomarkers in different studies were selected as model analytes.

The properties of selected analytes are shown in Table 2.1. Creatinine is one of the molecules chosen as an analyte in the present study because it is added to the cell culture media to prevent bacterial growth [101]. Also, it is a standard molecule used to monitor the kidney function [102] and thus it will be used to monitor the kidney functions of mouse in further experiments. Cells in culture need 12 essential amino acids to synthesize protein. Amongst them is leucine -typical cell culture nutrient for the growth of the cells. Arginine is required for the growth of cells and also prevents the toxicity caused by excess amino acids and ammonia in the cell culture media [103]. Tryptophan is an essential amino acid in *in-vitro* cell culture and a cancer biomarker and there are several studies showing that there is an increase in the tryptophan concentration in cancerous cells [104–106]. Glutamine and glutamic acid are energy sources for the cells, and it provides the carbon and nitrogen required for the synthesis of nucleic acids and proteins [107–109]. Glutamic acid is added to the cell culture media for the formation of guanosine [110]. Due to many critical functions of amino acids, abovementioned ones were chosen in the present study. Cells in culture can require vitamins that can act as coenzymes. Riboflavin is often a component of cell culture media and also is a cancer biomarker because increased level of riboflavin increases the growth of cancerous cells [111]. For most of the cell lines, cholesterol is required for energy storage, transportation and is a signaling molecule [112, 113]. As phospholipids are what build up cellular membrane and dysregulations in phospholipids can also be associated with different cancers. It has been found that the concentration of phospholipids increase in tumor cells [114].

Other studies also reported an increase in phospholipid concentration in cancerous cells compared to non-cancerous cells [114,115]. For this reason, L-distearoyllecithin, also known as DSPC, was chosen as a representative of this class of compounds.

Table 2.1. Selected analytes and their properties

Analyte	**Molecular Weight (g/mol)**	**Log P**	**Polar Surface Area (Å²)**	**The function in cell culture and cell**
Creatinine	113.1	-1.76	58.7	Prevent the bacterial growth
Leucine	131.2	-1.52	63.3	Cell nutrient
Glutamine	146.1	-3.64	106.0	Energy source
Glutamic acid	174.1	-3.69	101.0	Energy source
Guanine	151.1	-0.91	96.2	Required for the synthesis of guanosine
Arginine	174.2	-4.20	128.0	Provides cell growth
Tryptophan	204.2	-1.06	79.1	Cancer biomarker
Riboflavin	376.4	-1.46	155.0	Cancer biomarker
Cholesterol	386.7	7.11	20.2	Supplement for the media
DSPC	789.6	15.60	111.0	Cancer biomarker

2.1.7 Development of LC-MS method

For the separation of the analytes, different columns were tested including PFP, C18, and ZIC-HILIC columns. Initially, two different chromatographic methods were developed for the analysis of polar and nonpolar analytes using ZIC-HILIC and PFP columns, respectively. However, both polar and nonpolar analytes could be reasonably separated using Merck SeQuant ZIC-HILIC (100 x 2.1 mm, 3.5 µm, 200 Å) LC column. During method development, different parameters were optimized

including mobile phase composition and gradient, and additives used in the mobile phase. The MS conditions used in the methods are given in Table 2.2.

Table 2.2. MS parameters used in LC-MS

Parameter	**Setting**
Ionization Mode	ESI
Polarity	Positive
Drying gas	Nitrogen
Fragmentor	70 V
Gas Temperature	350 °C
Drying Gas	12.0 L/min
Quadrupole Temperature	100 °C
Capillary Voltage	3000 V

In the final method, gradient elution with two solvent systems was performed for the separation. Solvent A was H_2O/ACN (90:10) (v:v) with 0.1% F.A. and 1.0 mM Am.Ac. while solvent B was ACN/H_2O (90:10) (v:v) with 0.1% F.A. and 1.0 mM Am.Ac. The optimized solvent gradient is given in Table 2.3.

Table 2.3. Solvent gradient used in chromatographic separation

Time (min)	**%B**
0.0	95
4.3	95
7.0	5
9.0	95
15.0	95

2.1.8 Extraction performance comparison of HLB/PTFE fiber with commercially available fibers

As mentioned before, HLB/PTFE SPME miniaturized fibers were produced and optimized with intention to use for small volume of samples (i.e., cell culture) and spatial resolution from tissue (i.e., tumor profiling) in untargeted metabolomics. For this reason, the developed extractive probes should have extraction ability to extract both polar and nonpolar molecules. However, nonpolar analytes have higher affinity toward the extractive phase when compared with the polar analytes. Therefore, nonpolar analytes will be extracted in higher amounts compared to polar analytes when they are present in same concentration. But, in biological samples, nonpolar molecules bind to proteins to be transported within the body, meaning that their free concentration is very low [55]. Contrary to nonpolar metabolites, polar analytes are not bound to binding matrix; therefore, their free concentration is relatively high. The difference in the affinity of polar and nonpolar analytes towards the extractive phase and the difference in their free concentrations results in balanced coverage of various compounds by SPME.

After optimization of the SPME coatings, the extraction performance of SPME fibers was evaluated by comparing the extraction capability of HLB/PTFE fibers with well-accepted HLB/PAN fibers and commercially available C18 fibers. All coatings had the same coating length and thickness. For this study, first, the analyte mixture was spiked to PBS to have 500.0 ng/mL final concentration for creatinine, leucine, glutamine, glutamic acid, guanine, arginine, tryptophan, 100.0 ng/mL for riboflavin, cholesterol and 200.0 ng/mL for DSPC and then extractions were performed. The experimental parameters used during the extraction were as follows; sample volume: 1.5 mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature:

20 °C. The experimental parameters used during the desorption were as follows; desorption solvent: ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A., desorption volume: 150.0 µL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

2.1.9 Optimization of extraction conditions

In a typical SPME method development, the effect of extraction time, sample pH, ionic strength, agitation conditions, and temperature are optimized. However, in the present study, the fibers will be used for *in-vivo* sampling. In *in-vivo* conditions, most of these parameters (pH, ionic strength, temperature) cannot be changed; therefore, were not evaluated. Consequently, only desorption solvent composition, desorption time, and extraction time were optimized. The details of these studies are given below.

2.1.9.1 Desorption solvent optimization

As a first optimization parameter, desorption solvent composition was examined. Because 10 different molecules with different physicochemical properties were used for the evaluation, the selection of the desorption solution was critical to ensure that all analytes can be desorbed with the selected solvent. For this study, analyte mixture was spiked to 10% synthetic serum to have 500.0 ng/mL final concentration of each analyte and then extractions were performed. The experimental parameters used during the extraction were as follows; sample volume: 1.5 mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The experimental parameters used during the desorption were as follows; desorption solvents tested: ACN/H_2O (80:20; v/v) with 0.1% F.A., MeOH/H_2O (80:20; v/v) with 0.1% F.A., ACN/H_2O (50:50; v/v) with 0.1% F.A. and ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. desorption volume: 150.0 µL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

2.1.9.2 Desorption time optimization

To provide the complete desorption of each analyte in the shortest possible time, desorption time was optimized.

For this study, the analyte mixture was spiked to 10% synthetic serum to have 500.0 ng/mL final concentration of each analyte and then extractions were performed. The experimental parameters used during the extraction were as follows; sample volume: 1.5 mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The experimental parameters used during the desorption were as follows; desorption solvent: ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A.; desorption volume: 150.0 µL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption times tested: 5, 15, 30, 60 and 120 min.

2.1.9.3 Extraction time optimization

Because each analyte has a different affinity toward the extractive phase, it will have different equilibrium time. Therefore, the optimum extraction time was optimized to find the time that provides sufficient sensitivity for the final method. Before the experiment, analyte mixture was spiked to 10% synthetic serum to have 500.0 ng/mL final concentration of each analyte and then extractions were performed. The experimental parameters used during the extraction were as follows; sample volume: 1.5 mL, extraction times tested: 5, 15, 30, and 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The experimental parameters used during the desorption were as follows; desorption solvent: ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. ; desorption volume: 150.0 µL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 120 min.

2.1.10 Sample volume investigation for 2.0 mm coated fibers

Considering that in routine cell cultures investigation the sample volume is only hundreds of microliters, downsizing the extractive phase of the SPME fiber to fit within such system is critical. However, when the extractive phase is miniaturized, the sensitivity of the final method may decrease. Besides, with such small sample volumes the criterion for non-depletive extraction may not be satisfied. Therefore, investigating the effect of sample volume for miniaturized fiber is critical. For this experiment, first, analyte mixture was spiked to 10% synthetic serum to have 500.0 ng/mL final concentration of each analyte and then extractions were performed. The experimental parameters used during the extraction were as follows; sample volumes tested: 1.5 mL, 200.0 µL and 50.0 µL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The experimental parameters used during the desorption were as follows; desorption solvent: ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. ; desorption volume: 150.0 µL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 120 min.

2.1.11 Validation of the developed SPME-LC-MS method

2.1.11.1 SPME calibration using 10% fetal bovine serum (FBS) in PBS

The developed SPME-LC-MS method was validated to show the reliability, reproducibility, and sensitivity of the analytical method using 10% fatal bovine serum (FBS) in PBS (a matrix used in cell culture). During validation, working range, the limit of quantitation (LOQ), accuracy, and reproducibility (intra- and inter-day precision), were determined using matrix-matched internal standard calibration.

For the determination of working range, 10% FBS in PBS was spiked with analytes to have 0.0, 100.0, 125.0, 150.0., 250.0, 500.0, 1000.0, 2500.0 and 5000.0 ng/mL of each analyte. The experimental parameters used during the extraction were as

follows; sample volume: 200 µL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The experimental parameters used during the desorption were as follows; desorption solvent: ACN/MeOH/H_2O (40:40:20; v/v/v) with 0.1% F.A. , desorption volume: 30.0 µL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 120 min. Nicotine and phenylalanine (100.0 ng/mL in final sample) were used as internal standard to normalize the differences of fibers, variations in injection volumes and instrumental drifts.

The LOQ of each analyte was calculated from the back calculation of nominal concentration using the linear regression equation of matrix-matched SPME calibration where LOQ was defined as the lowest concentration that provides maximum of 20% relative error (RE%) in back calculations of the nominal concentration.

2.1.11.2 Accuracy of the developed SPME-LC-MS method

The accuracy of the developed SPME-LC-MS method was shown by conducting a blind to analyst experiment. Three different concentrations of analytes that represent quality control (QC) points over working range of the matrix-matched calibration were spiked. The spike levels were:

Low-point (LOQ): 500.0 ng/mL of creatinine, and 150.0 ng/mL of cholesterol and L-leucine, 250.0 ng/mL of L(+)-glutamine, L(+)-glutamic acid, guanine, L(+)-arginine, L(-)-tryptophan, riboflavin.

Mid-point: 1000.0 ng/mL of creatinine, and 750.0 ng/mL of L-leucine, 250.0 ng/mL of L(+)-glutamine, L(+)-glutamic acid, guanine, L(+)-arginine, L(-)-tryptophan, cholesterol and riboflavin.

High-point: 3000.0 ng/mL of creatinine, L-leucine, 250.0 ng/mL of L(+)-glutamine, L(+)-glutamic acid, guanine, L(+)-arginine, L(-)-tryptophan, cholesterol and riboflavin.

Following the extraction/desorption conditions described in above sections, the unknown concentrations of analytes were determined by matrix-matched internal standard calibrations run in LC-MS. Finally, the RE% were calculated for each analyte.

2.1.11.3 Precision of the developed SPME-LC-MS method

The reproducibility of the method was validated by intra- and inter-day sampling. For intra-day precision, three different sets of extractions were performed three times per day. For inter-day precision, sampling was performed on three consecutive days. The same QC levels described for accuracy of the method were used in repeatability studies. Following the extraction/desorption conditions described above the unknown concentrations of analytes were determined by matrix-matched internal standard calibration and then the relative standard deviations (RSD%) were calculated for each analyte.

2.2 Results and discussion

2.2.1 Characterization of HLB particles

HLB is a copolymer made by divinylbenzene (nonpolar moiety) and N-vinylpyrrolidone (polar moiety). The repeating unit of HLB polymer is shown in Figure 2.4.

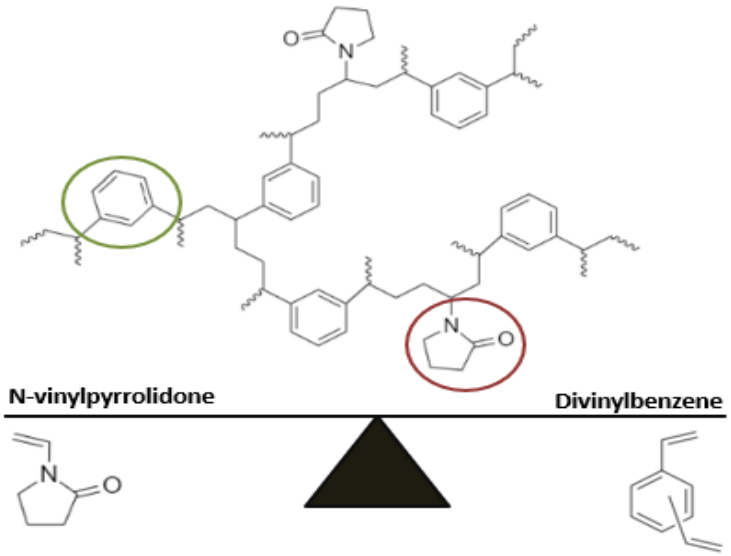


Figure 2.4. Hydrophilic lipophilic balanced (HLB) polymer

Due to the presence of both polar and nonpolar functional groups, it extracts a wide range of analytes. Analyte sorption is based on the interactions (Van der Waals, hydrogen bonding formation, π-π or electrostatic interactions), the sorption of analyte from its matrix is provided based on these interactions. Due to presence of nitrogen in polar moiety of HLB, polar analytes present in a sample can be extracted by enhancement of hydrogen bonding between polar moiety of extractive phase and analyte. On the other hand, presence of nonpolar aromatic ring provides a weak electrostatic interaction and allowing the extraction of nonpolar analytes [116].

In this thesis, the HLB extractive particles were synthesized based on the protocol explained by Lendor et al. [100]. Following the synthesis, to characterize the sorbent FTIR spectroscopy and SEM imaging techniques were used. As the first characterization study, the functional groups in the synthesized HLB particles were investigated by taking Fourier transform infrared (FTIR) spectra. The FTIR spectrum of synthesized HLB particles is given in Figure 2.5.

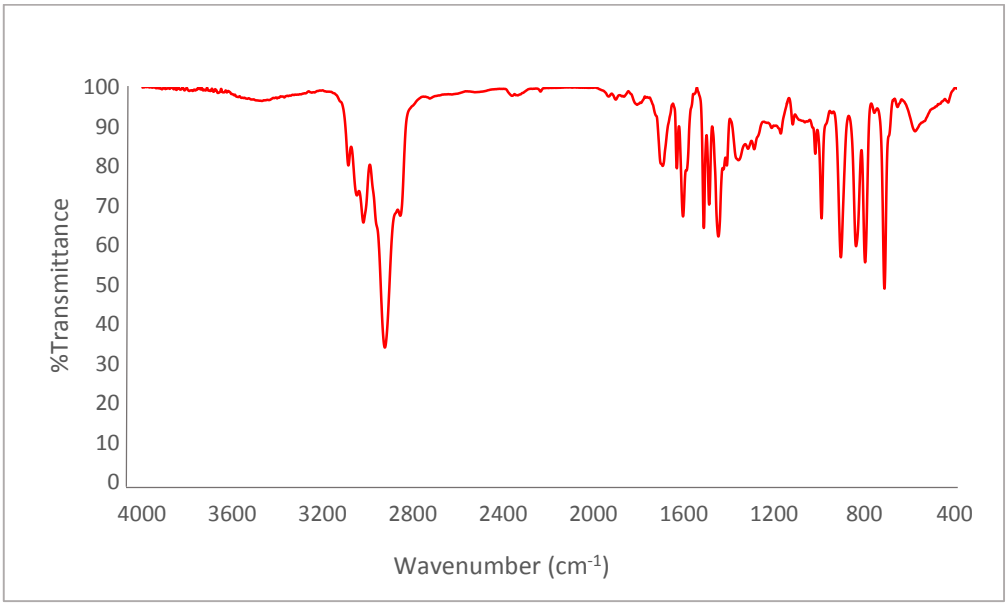


Figure 2.5. FTIR spectrum of synthesized HLB

FTIR spectrum of synthesized HLB particles was compared with the spectrum of commercial HLB [100] and similar peaks were obtained showing the successful synthesis of HLB particles. The peak at 1603 cm^{-1} indicated C-N stretching vibration by the presence of pyrrolidine moiety. Where the peak at 2929 cm^{-1} was caused by the stretching vibrations by aromatic rings by the presence of divinylbenzene moiety [117]. Also, the peaks at 710 to 904 cm^{-1} indicates the bending vibrations related to aromatic ring [118].

Scanning electron microscope (SEM) images of synthesized HLB particles were taken to observe the morphology and measure the diameter of particles. The SEM image of the particles is shown in Figure 2.6. Based on the figure, spherical particles were synthesized with particle diameters in a range of 1.3 μm to 4.0 μm where the mean diameter of the particles was calculated as 2.5 ±1.1 μm.

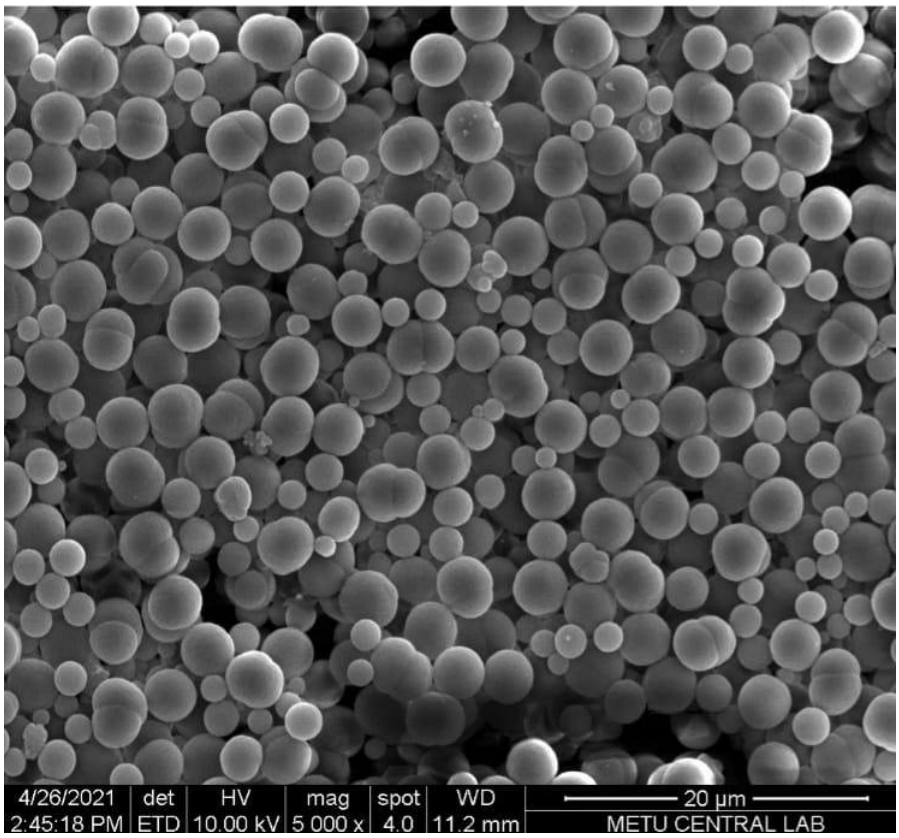


Figure 2.6. SEM image of synthesized HLB particles

2.2.2 Evaluation of the new SPME fibers

Optimization of the etching conditions for nitinol fibers: Before coating the surface of nitinol wire, coating conditions were optimized. As the first parameter, different etching conditions were tested, and the results were shown in Table 2.4.

Table 2.4. Optimization of nitinol fiber etching process

Condition	Etching procedure	Result
1	5 min in 12 M HCl	No groove was obtained
2	30 min in 12 M HCl	Groove was obtained but not etched homogeneously
3	60 min in 12 M HCl	Obvious etching was obtained (Figure 2.7b.)
4	120 min in 12 M HCl	The fiber was thickened too much and broken
5	180 min in12 M HCl	The fiber was thickened too much and broken
6	5 min in 8 M HCl	No groove was obtained
7	15 min in 8 M HCl	No homogeneous groove was obtained
8	30 min in 8 M HCl	No homogeneous groove was obtained

As seen from the descriptions given in Table 2.4, for 5 min etching no groves were obtained with both acid concentrations. In the case of 30 min etching with 12 M HCl, and 15 and 30 min etching with 8 M HCl, the formed grooves were not homogeneous. When relatively long etching conditions were used, 120 and 180 min, the fibers became too thin and were broken. A homogeneous etching with decent size of groove was obtained only when the fiber was etched using 12 M HCl for 60 min. Under this condition, before etching, the thickness of the nitinol wire was measured as 0.19 mm while the thickness within the groove was measured as 0.15 mm after etching process. In Figure 2.7, the pictures of nitinol wire before and after etching is shown.

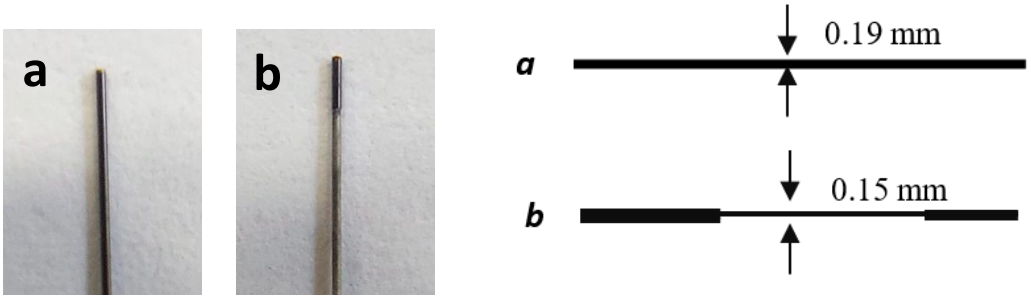
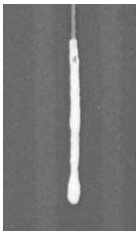
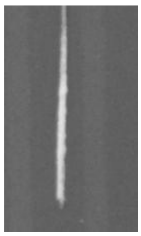
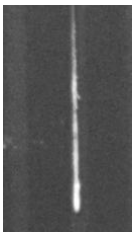
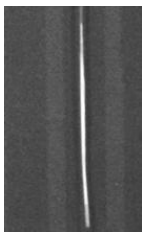


Figure 2.7. a) Nitinol wire b) Acid etched, nitinol wire

Optimization of fiber coating composition: Different ratios of HLB to PTFE-AF 2400 were evaluated to obtain the most homogeneous coating. Based on the obtained results, provided in Table 2.5, the coating condition 1, where the ratio of HLB/PTFE-AF 2400 was 3:2 (w:w), produced a coating that was stable under applied mechanical test conditions. However, as seen from the figure given as inset in the table, the coatings were not homogeneous. In the case of coating condition 2, where the amount of binder in sorbent solution is decreased to half compared to extractive particle, coatings were not mechanically stable for dipping to the agarose gel, which could be expected as less binder (PTFE-AF 2400) was present. In coating condition 3, where the solvent amount is doubled, the coatings were even less stable and heterogeneous. Also, this mixture resulted in thinner coatings compared to other coating compositions as both particles and glue were diluted. The best coating composition in terms of homogeneity and stability was acquired by adding HLB/PTFE-AF 2400 in a 1:1 (w:w) ratio shown in coating conditions 4.

Table 2.5. Optimization of composition of HLB and FC-72

Number	Sorbent Composition	Picture	Comment for applied mechanical tests
1	HLB/PTFE-AF 2400 (3:2) (w:w)		The coating seems heterogeneous but successful after mechanical tests.
2	HLB/PTFE-AF 2400 (2:1) (w:w)		The coating seems heterogeneous and not successful after immersion into the agarose gel.
3	HLB/PTFE-AF 2400 (1:1) (w:w) diluted		The coating seems heterogeneous and not successful after immersion into the agarose gel.
4	HLB/PTFE-AF 2400 (1:1) (w:w)		The coating seems homogeneous and successful after mechanical tests.

Evaluation of mechanical stability of fibers under different conditions: Because the fibers will be used *in-vivo* applications, they should be stable not only in aqueous media but also in solid media. Therefore, after choosing the optimum etching and coating conditions, fibers produced using different compositions of HLB/PTFE were

evaluated for their mechanical strengths. For that purpose, fibers were immersed separately into water and agarose. All of the coatings were stable for sampling from aqueous media (immersion into water), but HLB/PTFE-AF 2400 (2:1) (w:w) and HLB/PTFE-AF 2400 (1:1) (w:w) diluted coatings were not found stable after immersion into agarose gel. These fibers cannot be used for sampling from semi-solid samples. As the results of fiber coating optimization, HLB/PTFE-AF 2400 (1:1) (w:w) fibers used in the next experiment to check their mechanical stability after immersion into chicken tissue.

Since HLB/PTFE-AF 2400 (1:1) (w:w) fibers were found sufficiently stable in the mechanical tests, the fibers were finally checked in chicken tissue, which was chosen as solid tissue representative. The pictures of the application process are shown in Table 2.6. As can be seen from these results, the fibers were also stable through the entire process of puncturing and penetrating through the tissue.

Table 2.6. Immersion of HLB/PTFE fibers to chicken tissue

Before application	**After first application (15 min)**	**After second application (15 min)**	**During application for 4 h**	**After application**
				

2.2.3 Development of LC-MS Method

To show the extraction performance of the new SPME fibers, different molecules with a wide range of physicochemical properties in terms of their chemical structure, log P and pK_a values were chosen (Please refer to Table 2.1). Before quantification

of the selected analytes, a method for chromatographic separation was developed and analytes were detected by a single quadrupole mass analyzer. Different chromatographic columns including C18, PFP, and ZIC-HILIC columns were tested to separate the analytes. Optimum separation of all analytes could be achieved by the ZIC-HILIC column. The observed adducts and the selected ion for the monitoring of these adducts were given in Table 2.7. A typical chromatogram of each analyte is shown in Appendix A in Figure A.1 and mass spectra is shown in Figure A.2.

Table 2.7. Observed adducts and selected ions

Analyte	**Adduct**	**SIM Ion**
Creatinine	M+H	114.1
Leucine	M+H	132.1
Glutamine	M+H	147.1
Glutamic acid	M+H	1481.
Guanine	M+H	152.1
Arginine	M+H	175.1
Tryptophan	M+H	205.2
Riboflavin	M+H	377.2
Cholesterol	$M-H_2O+H$	369.4
DSPC	M+H	790.6

The instrumental working range of each analyte obtained with the LC-MS method was 1.0-500.0 ng/mL for creatinine, guanine, arginine, tryptophan, riboflavin, cholesterol and DSPC while 5.0-500.0 ng/mL for glutamine and glutamic acid. Typical calibration curves are shown in Figure 2.8.

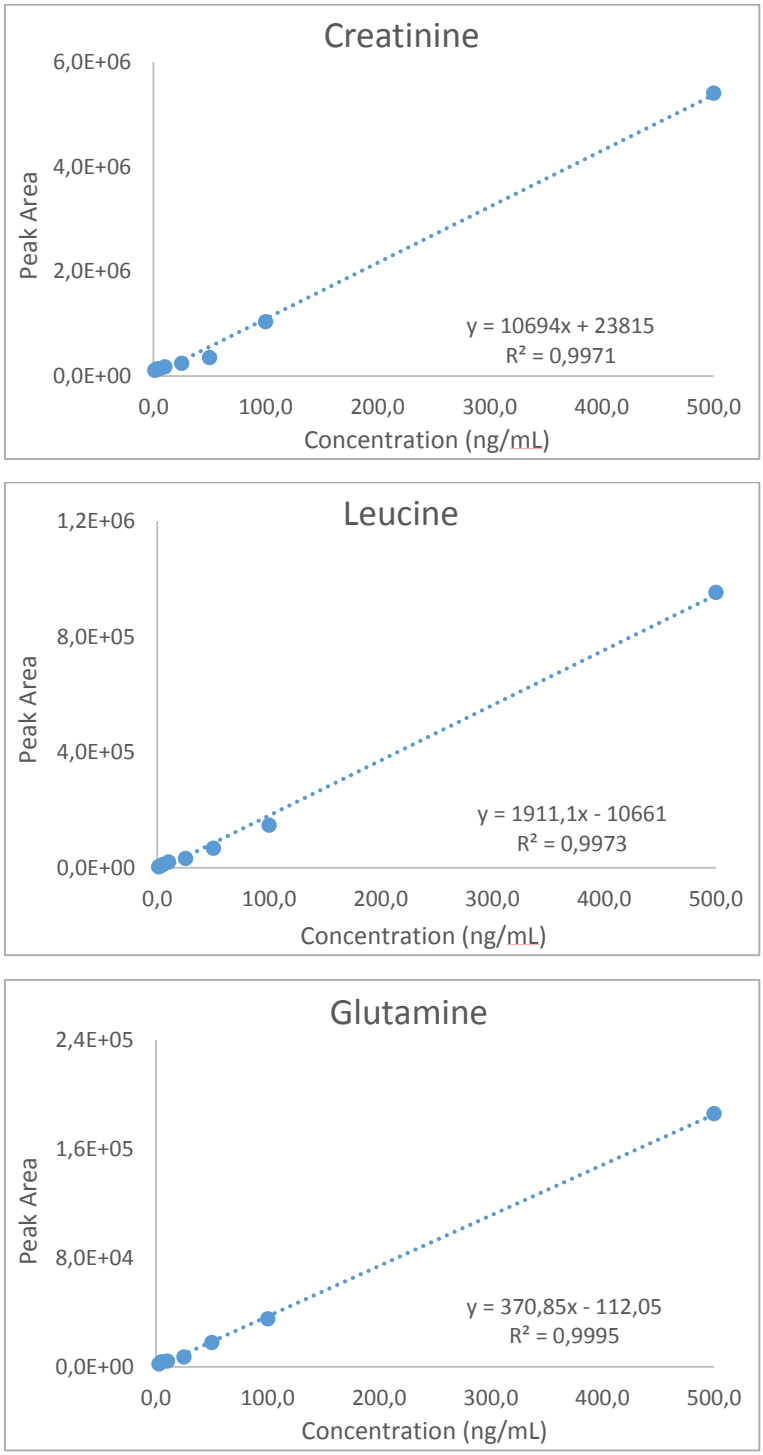
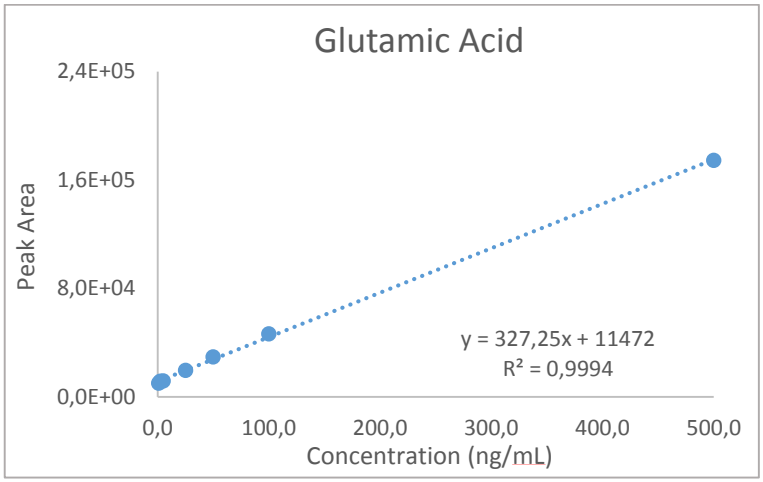
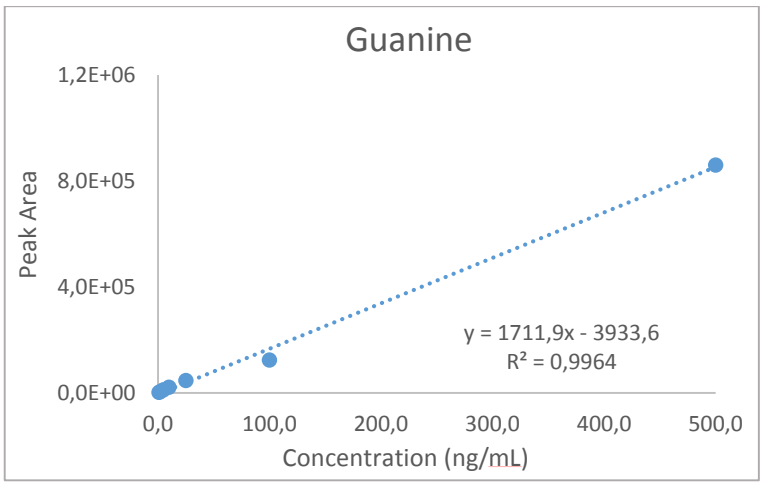


Figure 2.8. Typical calibration curves obtained in LC-MS





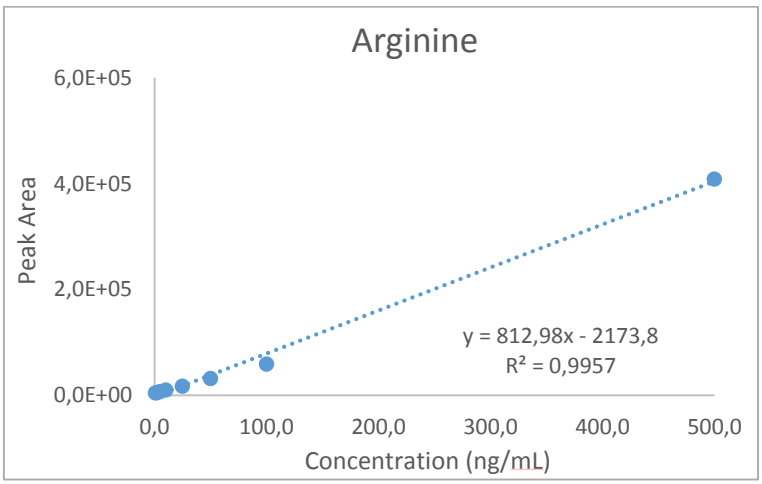


Figure 2.8. (cont'd.). Typical calibration curves ontained in LC-MS

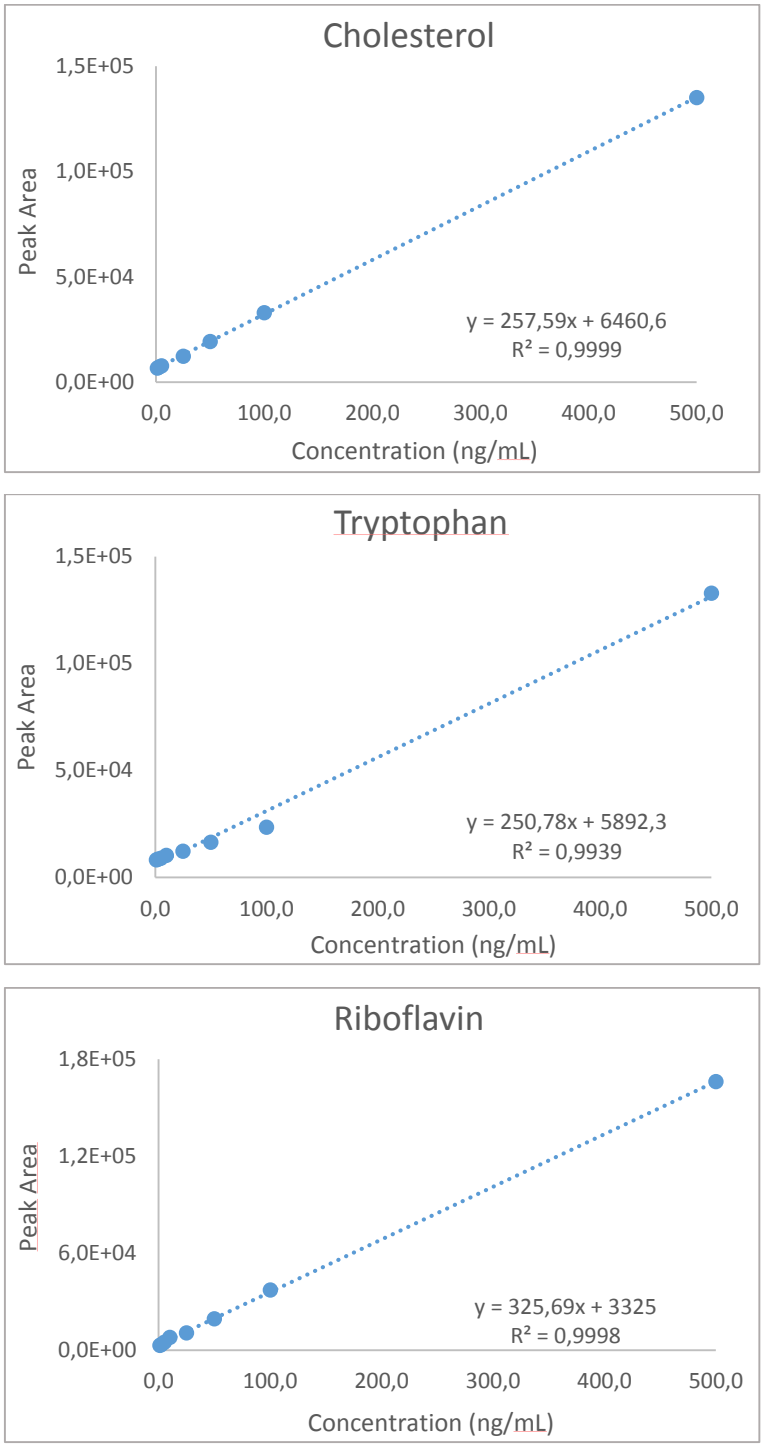


Figure 2.8. Typical calibration curves obtained in LC-MS

2.2.4 Extraction performance comparison of HLB/PTFE fibers with commercially available fibers

The extraction performance of home-made HLB/PTFE fibers was compared to the commercially available fibers found in the market (C18-PAN). Moreover, HLB/PTFE coated fibers were compared to HLB-PAN (well-accepted fiber with a wide range of analyte coverage). In this study, each fiber had the same coating length and thickness. The extraction results are shown in Figure 2.9.

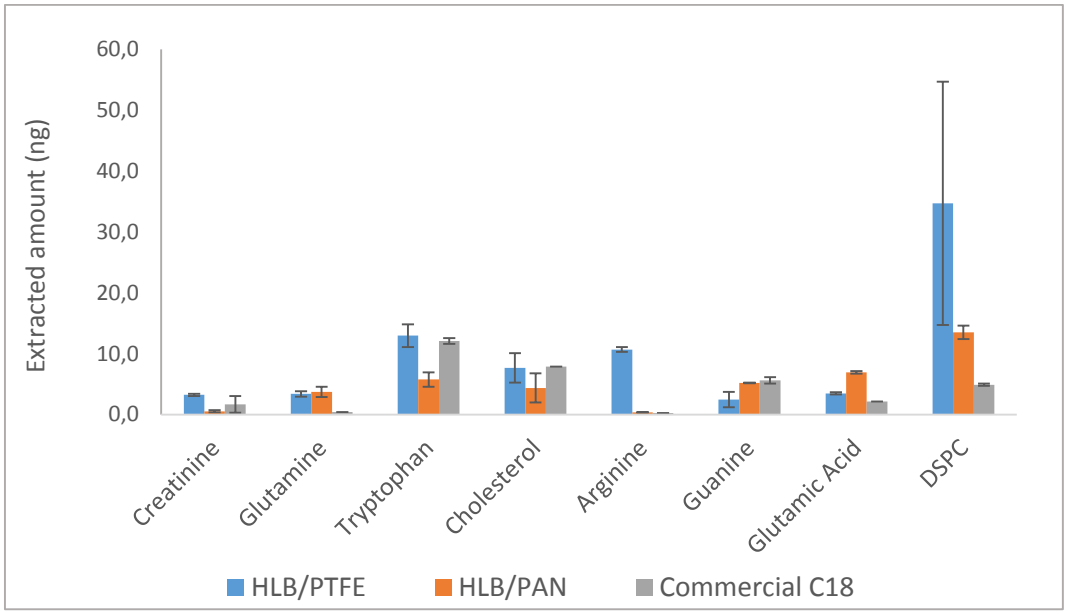


Figure 2.9. Extraction performance comparison of different fibers in PBS (sample volume: 1.50 mL, analyte concentration: 500.0 ng/mL mixture of creatinine, leucine, glutamine, glutamic acid, guanine, arginine, tryptophan, 100.0 ng/mL of cholesterol and riboflavin, 200.0 ng/mL of DSPC, extraction time: 60 min, agitation speed: 1000 rpm, desorption time: 60 min, desorption solvent: ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A., desorption volume: 0.15 mL, agitation speed: 1000 rpm)

Based on these results, it is clear that the extraction behavior of each coating is distinct for different analytes, and it is difficult to point to one of the coatings as the best. For instance, creatinine is extracted the most with new HLB/PTFE fiber while

it has been extracted the least by HLB/PAN fiber; on the other hand, C18 fibers provided high variations in extracted amounts of different analytes. In the case of glutamine, the best extraction was obtained with HLB-PAN, and C18 fibers showed the lowest extractions among all the tested coatings. For glutamic acid, the performance of HLB/PAN and HLB/PTFE were similar and superior to C18-coated fibers. For guanine, HLB/PTFE coated fibers showed the lowest extraction, while the other coatings had similar performances. Contrary to guanine, the best extraction recoveries for arginine were obtained with the new HLB/PTFE fibers. In the case of one of the most nonpolar analytes, cholesterol, HLB/PTFE was superior to HLB/PAN and similar in performance to C18 fiber, which is a well-accepted coating for extraction of the lipophilic analytes. The selected lipid, DSPC, again showed the best recoveries with the new coating but the lowest repeatability. As can be seen from the results, selecting one of the coatings as superior to the others is not possible. However, we can conclude that the new fibers provide reasonable performance comparable to the other SPME fibers with the advantage of being suitable for thermal and solvent desorption and can be candidate to be used for the extraction of wide range of analytes.

2.2.5 Preparation of 2.0 mm HLB/PTFE coated fibers

Initial extractions were performed using 10.0 mm in length HLB/PTFE coatings. But considering the final goal of the study, later 2.0 mm coated fibers were also prepared. These short-coated fibers are not only important for the sampling from the cell lines but also from tumors which are extremely heterogeneous in their chemical composition. To obtain chemical information from such heterogeneous systems, a spatial resolution of the chemical information is required. If sampling is performed from different substructures of tumor using short length coatings at short times, spatial resolution can be obtained. For preparation of short SPME samplers, the etching process described in Section 2.2.2 was applied by protecting 1.0 mm of the

wire from the tip from being etched. A picture of 10.0 mm coated SPME fiber and miniaturized SPME fiber with 2.0 mm coating length is shown in Figure 2.10.

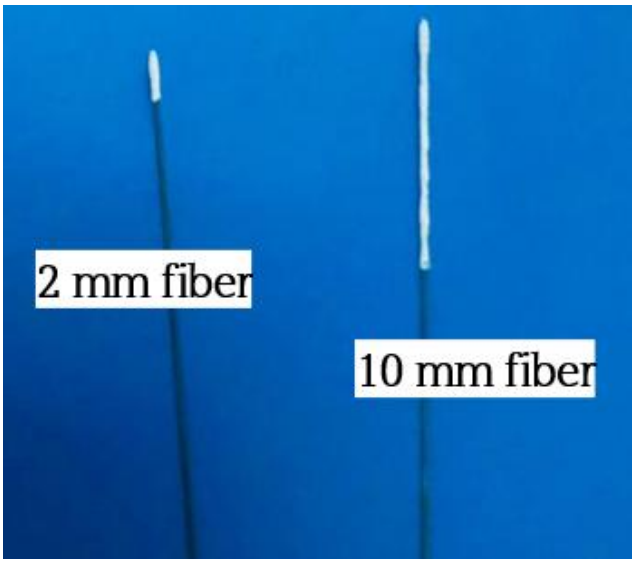


Figure 2.10. HLB/PTFE fibers with 2.0 mm and 10.0 mm coating lengths

2.2.6 Optimization of extraction conditions

After optimization of etching and coating conditions, extraction conditions were optimized. For this purpose, desorption solvent, desorption time, and extraction time were optimized. The obtained results are summarized below.

2.2.6.1 Desorption solvent optimization

Before optimization of extraction parameters, as a first step, desorption solvent composition, which is capable of quantitative desorption of each analyte, was investigated. SPME is a non-exhaustive extraction technique meaning that only a small portion of analytes is extracted from the matrix. However, all analytes

extracted by the extractive phase should be desorbed into a suitable solvent(s) to perform quantitative analysis. Therefore, the desorption solvent should be investigated to find a solvent composition that is capable of desorbing analytes with different physicochemical properties (structure, size, polarity) quantitatively. For this purpose, ACN/H_2O (80:20; v:v) with 0.1% F.A., MeOH/H_2O (80:20; v:v) with 0.1% F.A., ACN/H_2O (50:50; v:v) with 0.1% F.A. and ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. were tested as desorption solvents using both 10.0 mm and 2.0 mm coated SPME fibers. After completing the first desorption, using fresh solvents, a second desorption was performed with the same desorption conditions to investigate if there is carry over on the extractive phase. The desorbed amount of each analyte for 10.0 mm coating after first desorption is shown in Figure 2.11, and second desorption is shown in Figure 2.12.

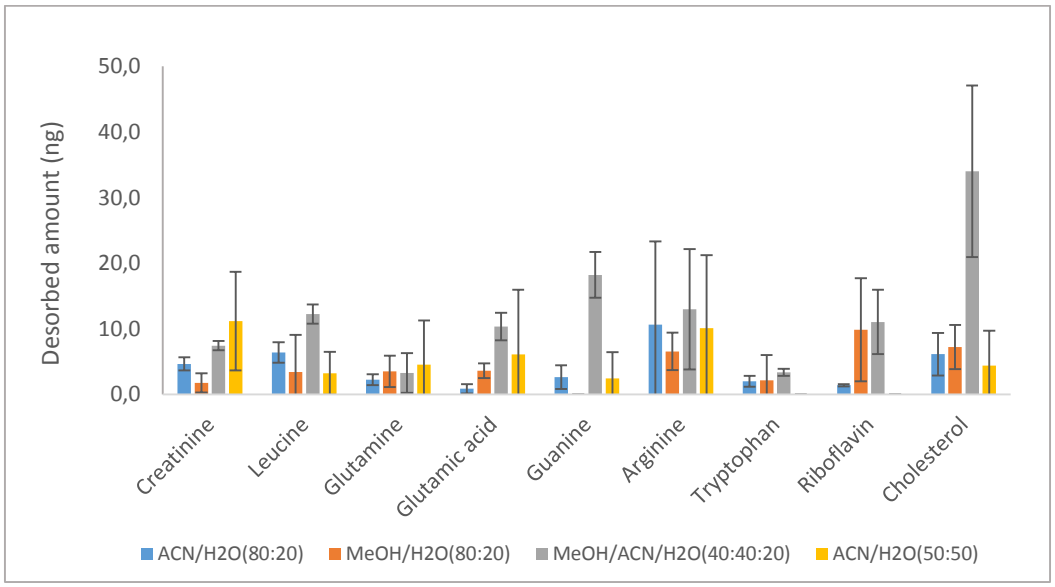


Figure 2.11. Effect of various solvents on desorbed amounts of analytes from 10.0 mm coated SPME fiber (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, sample volume: 1.50 mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption time: 60 min, desorption volume: 0.15 mL, agitation speed: 1000 rpm)

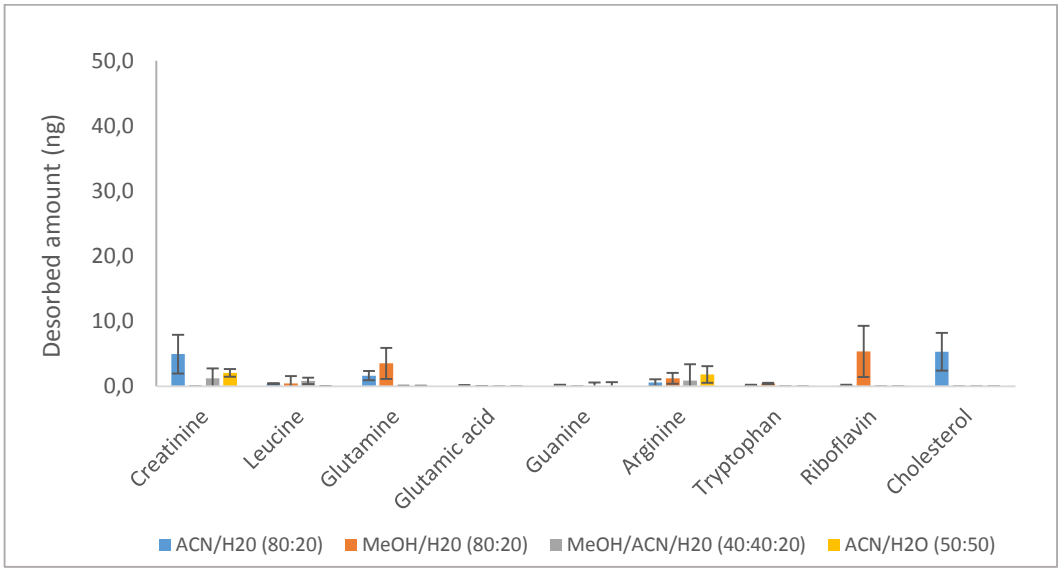


Figure 2.12. The carry over effect of the solvents (for 10.0 mm coated SPME fiber)

During this experiment, it was accepted that the complete desorption was achieved when 95% of the analytes were desorbed in the first desorption. Considering Figure 2.11 and 2.12, ACN/H_2O (80:20; v:v) with 0.1% F.A. provided complete desorption for glutamic acid, guanine, tryptophan and riboflavin (at least 95% of analytes were desorbed) while this solvent composition caused carry over for the rest of the analytes. ACN/H_2O (50:50; v:v) with 0.1% F.A. provided better desorption compared to ACN/H_2O (80:20; v:v) with 0.1% F.A. for desorption of leucine, glutamine, glutamic acid, guanine, tryptophan, riboflavin and cholesterol since polarity of the desorption solvent was increased. Also, MeOH/H_2O (80:20; v:v) with 0.1% F.A. was investigated as desorption solvent since methanol breaks the hydrogen bonding between the analyte and the extractive phase [119]. This solvent composition provided complete desorption except glutamine, arginine and riboflavin. Although big variations were seen within each tested point, still it was clear that ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. provided the highest recoveries and the lowest carryover. Therefore, this solvent was selected as the most suitable solvent for the desorption of analytes.

The solvent evaluation was also repeated for 2.0 mm coated SPME fibers as large variations were obtained for the 10.0 mm coated fibers. These results are shown in Figure 2.10. As expected, the same solvent composition was found successful for the quantitative desorption of the analytes. In the case of second desorption of analytes, each of the analytes were below detection limit, there was no carry over for any of the analytes, so only first desorption results were shown in Figure 2.13.

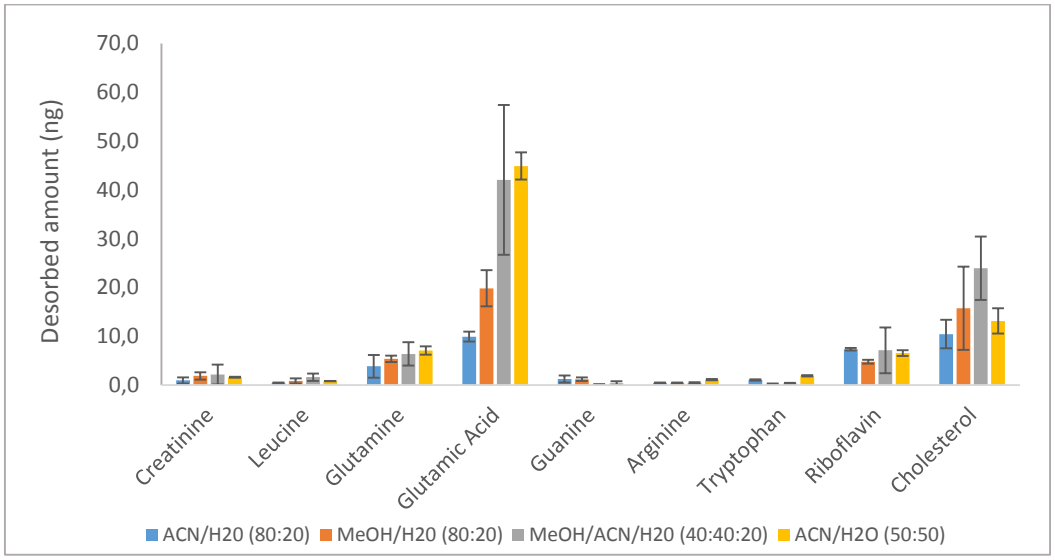


Figure 2.13. Effect of various solvents on desorbed amounts of analytes from 2.0 mm coated SPME fiber (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, sample volume: 1.50 mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption time: 60 min, desorption volume: 0.15 mL, agitation speed: 1000 rpm)

2.2.6.2 Optimization of desorption time

To have quantitative results, all of the analytes on the extractive phase, should be desorbed into the desorption solvent which was optimized as ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. For this reason, different desorption times were studied to determine the shortest time that provides quantitative desorption from the

fibers. The results of this investigation are shown in Figure 2.14 and 2.15 for 10.0 mm and 2.0 mm coated fibers, respectively.

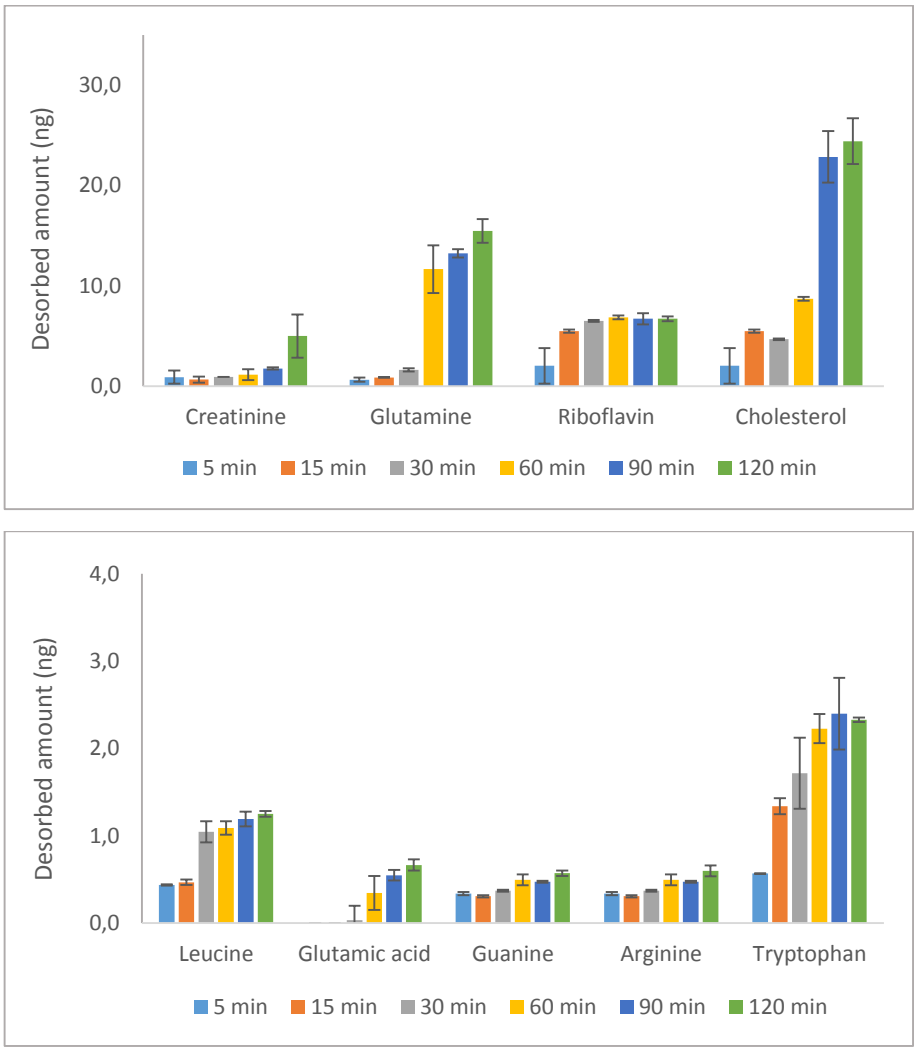


Figure 2.14. Desorption time profile of analytes from 10.0 mm coated SPME fibers (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, sample volume: 1.50 mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption solvent: ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. , desorption volume: 0.15 mL, agitation speed: 1000 rpm)

According to the results obtained using 10.0 mm coated fibers, complete desorption was achieved for, arginine in 15 min, leucine and riboflavin in 30 min, tryptophan,

glutamine, glutamic acid and guanine in 60 min, cholesterol in 90 min and for creatinine in 120 min (Student's t-test was applied for each point to compare is there any statistical difference between desorbed amount of analytes at 95% CL). For this reason, 120 min was chosen as optimum desorption time for desorption of all of the analytes.

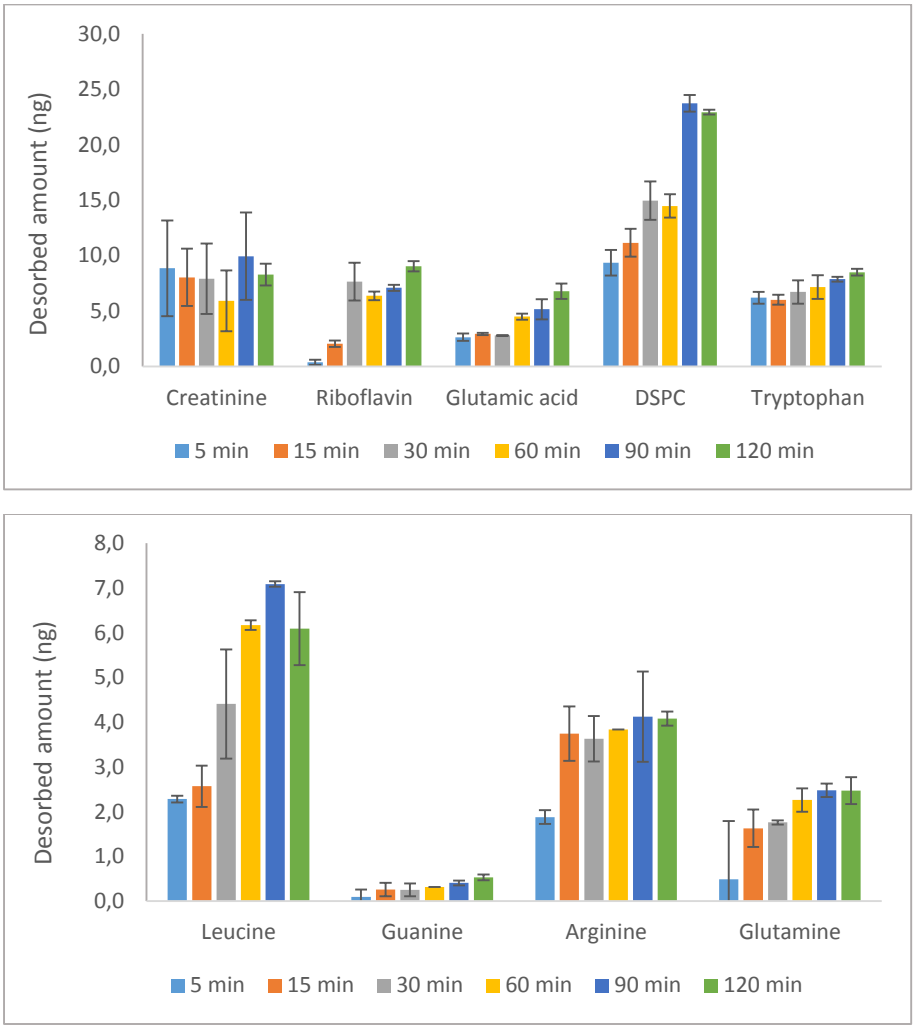


Figure 2.15. Desorption time profile of analytes from 2.0 mm coated SPME fibers (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, sample volume: 1.50 mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption solvent: ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. , desorption volume: 0.15 mL, agitation speed: 1000 rpm)

For 2.0 mm coated fibers, the required time for the complete desorption of arginine, leucine, riboflavin, glutamine, glutamic acid, guanine was found same as the previous experiment while the complete desorption of creatinine and tryptophan were 90 min and 30 min, respectively. For both of the coatings, desorption time optimized as 120 min in further experiments.

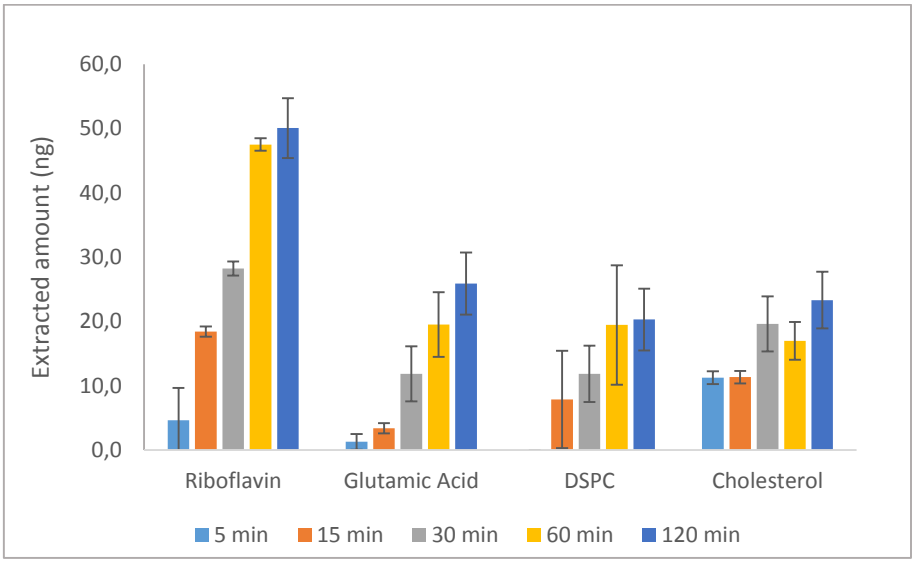
2.2.6.3 Extraction time optimization

Each analyte has a certain affinity toward the extractive phase. Therefore, the equilibrium extraction time differs from analyte to analyte depending upon their affinity, making it critical to investigate the extraction time profile to get the best sensitivity in shorter time. The sorption mechanism can vary depending upon molecular weight, molecular structure, presence of ion exchange moieties, hydrogen bonding ability, partition coefficient (Log P), and polar surface area of the analytes. In general, Log P value of analytes can be used to comment on the extraction since the coefficient is the measure of partition of analyte between octanol and aqueous phase. Depending upon this value, a comment can be made whether an analyte prefers to reside in its aqueous matrix or has tendency toward the extractive phase having nonpolar moiety[121]. Based on the obtained results from the study, it is clear that the absolute recovery for polar analytes (vary between 0.1% to 0.9%.) is less compared to nonpolar (vary between 1.2% to 13.4%) analytes selected for the study. Considering possible interaction between the analytes and HLB polymer in which vinylpyrrolidone moiety is a hydrogen acceptor, hydrogen bonding is expected to be the main contributor for the extraction of creatinine, leucine, glutamine, glutamic acid, arginine, molecules that bear amidine, carboxyl, and amine groups in their molecular structure (hydrogen donor groups). In case of guanine, which is a purine containing pyrimidine and imidazole groups, the extracted amount was found to be lower than other polar molecules. The presence of pyrimidine and imidazole groups

might affect the main contributing factor for the extraction of guanine and the π–π interactions may dominate rather than hydrogen bonding. Compared to some of the other polar analytes (guanine, arginine, glutamine), the extracted amount of tryptophan was found higher. Considering that this molecule can have both hydrogen bonding and π–π interactions with HLB particles the obtained results are reasonable. In case of riboflavin, the extracted amount was higher compared to other polar analytes. There are 5 hydrogen bond donor atoms in riboflavin and due to presence of aromatic diamino group, the sorption was driven both formation of multiple hydrogen bonds and π–π interactions. Moreover, the extraction of cholesterol and DSPC is expected to be driven mostly by van der Waals interactions. Although this interaction is weaker compared to hydrogen bond formation, nonpolar molecules have a higher tendency for extractive phase compared to aqueous sample, and thus higher amounts of these molecules were extracted from the sample.

The extraction time profiles for 10.0 mm and 2.0 mm coated fibers are shown in Figure 2.16 and 2.17, respectively. For both types of fibers, a similar trend of increase in extraction with increase of sorption time was observed as expected. Based on the results, it can be concluded that for most of the analytes, and for both fiber types, the equilibrium extraction was established approximately at 60 min. After equilibrium was achieved, no significant increase was observed in the extracted amount of analytes.

Moreover, it can be said that even with 5 min extraction, the extracted amount of analyte will allow to perform quantitative analysis. However, to increase the sensitivity of the final method longer times would be more suitable and for the rest of the studies 60 min of extraction time was selected.



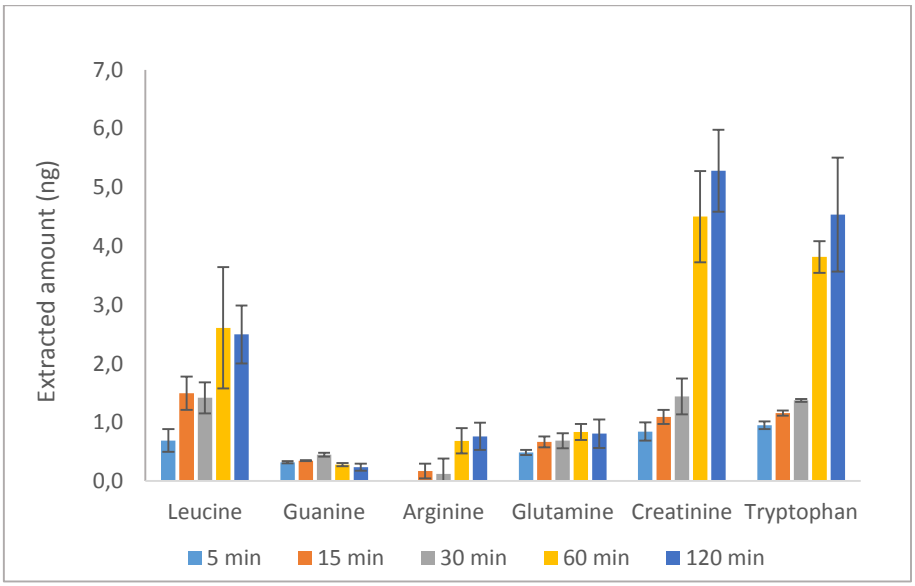


Figure 2.16. Extraction time profile of analytes with 10.0 mm coated SPME fibers (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, sample volume: 1.50 mL, agitation speed: 1000 rpm, desorption time: 60 min, desorption solvent: ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. , desorption volume: 0.15 mL, agitation speed: 1000 rpm)

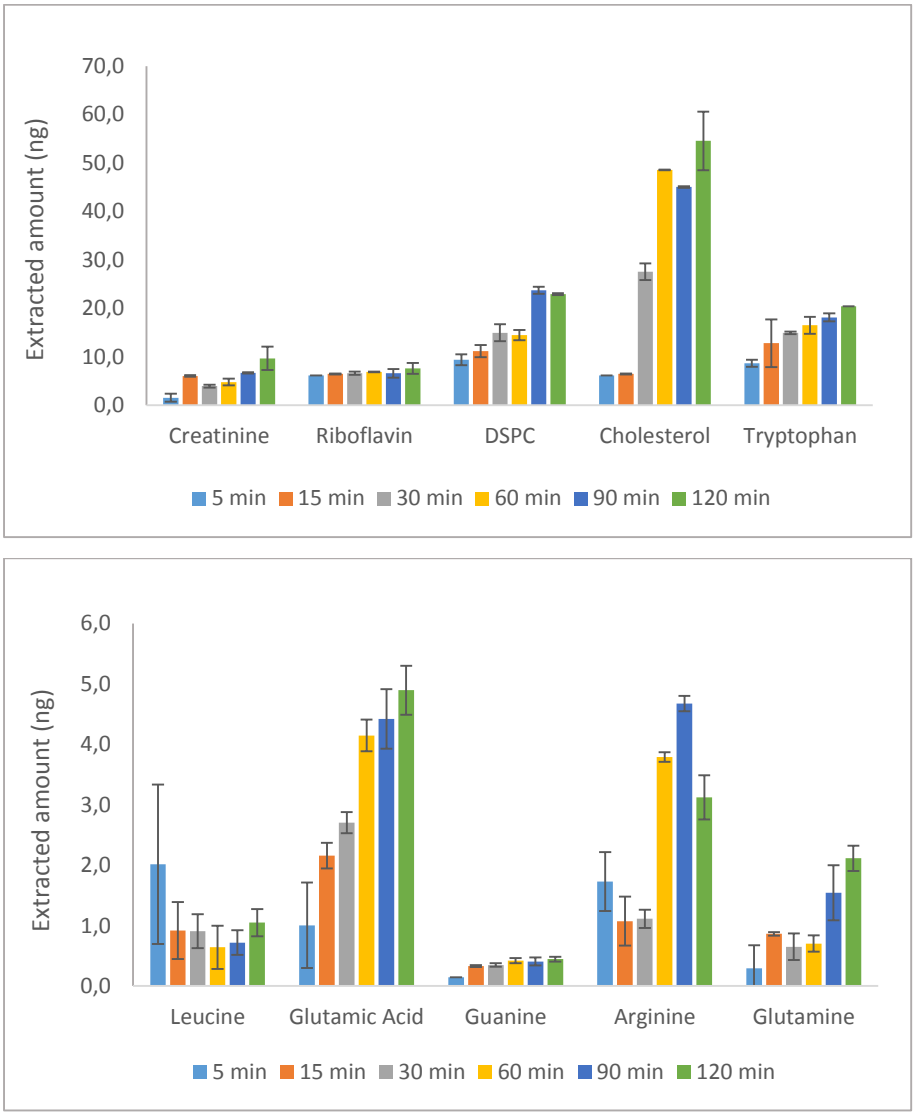


Figure 2.17. Extraction time profile of analytes with 2.0 mm coated SPME fibers (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, sample volume: 1.50 mL, agitation speed: 1000 rpm, desorption time: 60 min, desorption solvent: ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. , desorption volume: 0.15 mL, agitation speed: 1000 rpm)

2.2.6.4 Sample volume optimization

As described before, the main goal of the present study is to optimize the SPME coating for the sampling from cell line culture and provide spatial resolution through different substructures of a tumor. Therefore, there is a need for SPME probes with short extractive phases (2.0 mm coating length). It is worth reminding that since the study aims for IVIVE application, the sample cannot be manipulated in terms of its pH, ionic strength, temperature, or agitation. Consequently, for 2.0 mm coated fibers, the sensitivity of the final method would be lower than the sensitivity that can be obtained with the longer coated fibers. Another parameter that would affect the sensitivity of SPME fibers is the sample volume, if the used extraction conditions are not under non-depletive conditions. This was evaluated by changing the sample volume and observing the extracted analyte amount in each sample volume. The investigated sample volumes were shown below and the effect of this sample volume on extracted amount of each analyte is shown in Figure 2.18.

• Sorption from 1.5 mL sample and desorption to 50.0 µL solvent (Preconcentrated 30 times)

• Sorption from 200.0 µL sample and desorption to 50.0 µL solvent (Preconcentrated 4 times)

• Sorption from 50.0 µL sample and desorption to 50.0 µL solvent (No preconcentration)

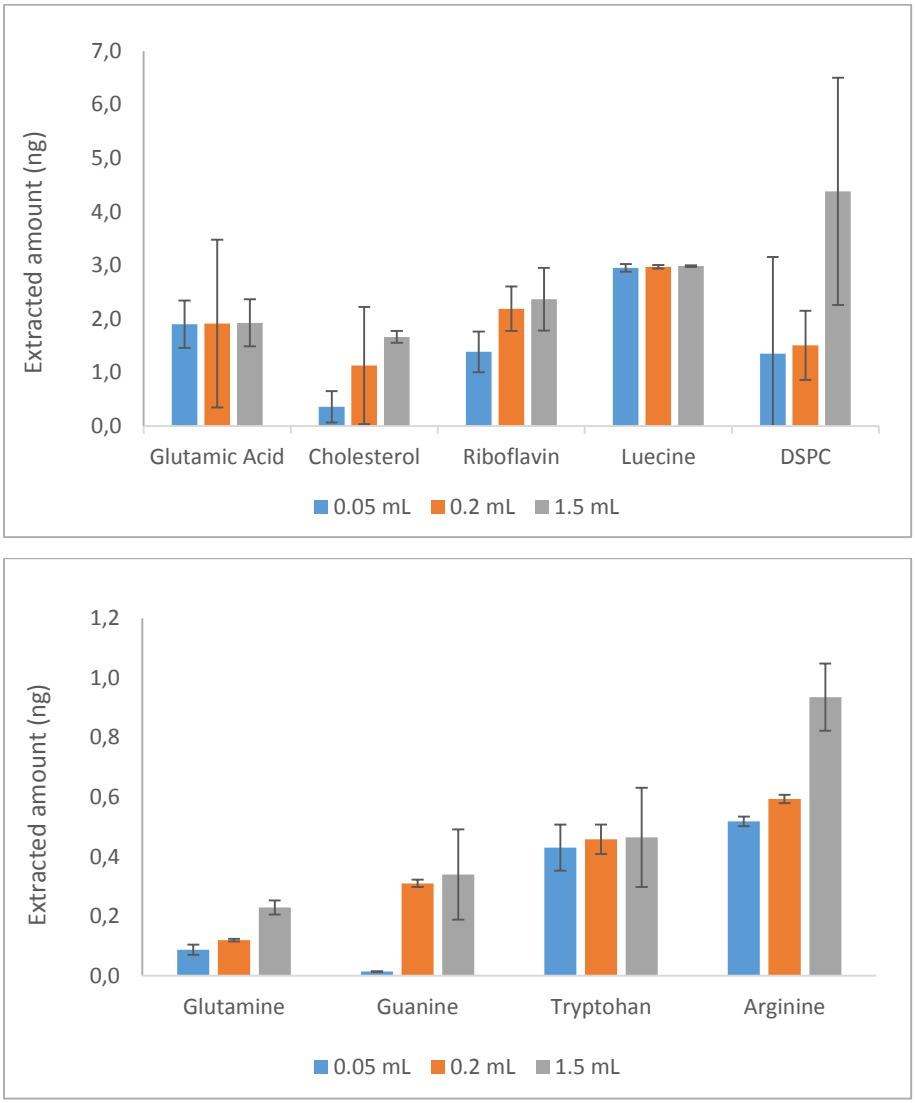


Figure 2.18. The effect of sample volume on extraction of analytes with 2.0 mm coated SPME fibers (Extraction conditions; sample matrix: 10% synthetic serum, analyte concentration: 500.0 ng/mL mixture, extraction time: 60 min, agitation speed: 1000 rpm, desorption time: 60 min, desorption solvent: ACN/MeOH/H_2O (40:40:20; v:v:v) with 0.1% F.A. , desorption volume: 0.15 mL, agitation speed: 1000 rpm)

As can be seen from the figure, only leucine, glutamic acid, and tryptophan satisfied non-depletive extraction conditions (assuming the extraction was approximately equilibrium at 60 min) since the same amount of analytes were extracted, and sample

volume will not affect the extracted amount of these analytes. However, for the rest of the compound, there was effect of volume change, suggesting that sample volume must be known in the investigated system.

In further applications, the developed HLB/PTFE fibers will be used for the sampling from cell culture media before conducting *in-vivo* animal experiments. The volume of cell culture in a 96-well plate will be 200.0 µL; therefore, the sample volume was selected as 200.0 µL for further experiments.

Optimized SPME parameters was given as below:

Extraction: 200.0 µL sample volume, 60 min extraction at 1000 rpm agitation speed.

Desorption: 30.0 µL of ACN/MeOH/H_2O (40:40:20, v:v:v) with 0.1% F.A., 120 min desorption at 1000 rpm agitation speed.

2.2.7 Validation of the developed SPME-LC-MS method

The developed SPME-LC-MS method was validated in terms of linearity, limit of quantification (LOQ), precision and accuracy based on the guidance of bioanalytical method validation for industry published by Food and Drug Administration (FDA) in 2018 [122].

2.2.7.1 SPME calibration using 10% fetal bovine serum (FBS) in PBS

As a first validation parameter, matrix-matched internal standard calibration curve was plotted to determine the working range of developed SPME-LC-MS method from 10% FBS in PBS. Phenylalanine and nicotine were used as internal standard to normalize the differences of fibers, variations in injection volumes and instrumental drifts. Using each plot, limit of quantitation (LOQ) was calculated for each analyte by back calculation of nominal concentration with lower than ±20% RE%. Because analytes were already present in serum samples, blank extractions (n=3) were

performed and the found response was subtracted from the response of each calibration point. Matrix-matched internal standard calibration plot of each analyte is shown in Figure 2.19. LOQ was calculated as 500.0 ng/mL for creatinine, 150.0 ng/mL for leucine and cholesterol and 250.0 ng/mL for glutamine, glutamic acid, guanine, arginine, tryptophan and riboflavin.

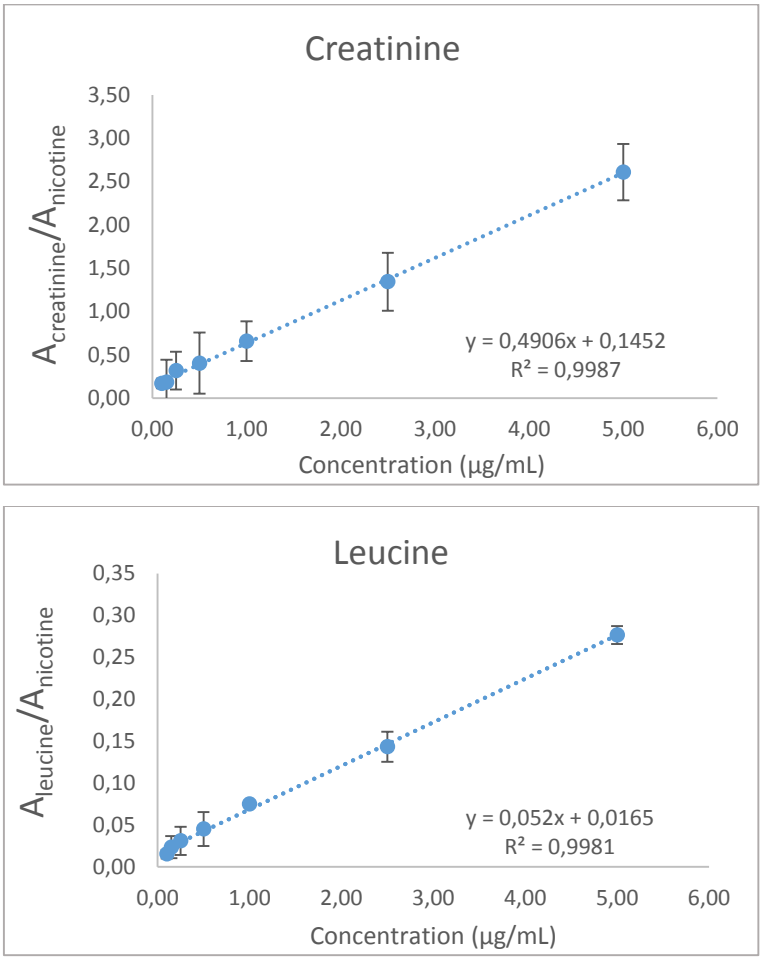


Figure 2.19. Matrix-matched internal standard calibration curves obtained using 10% FBS in PBS

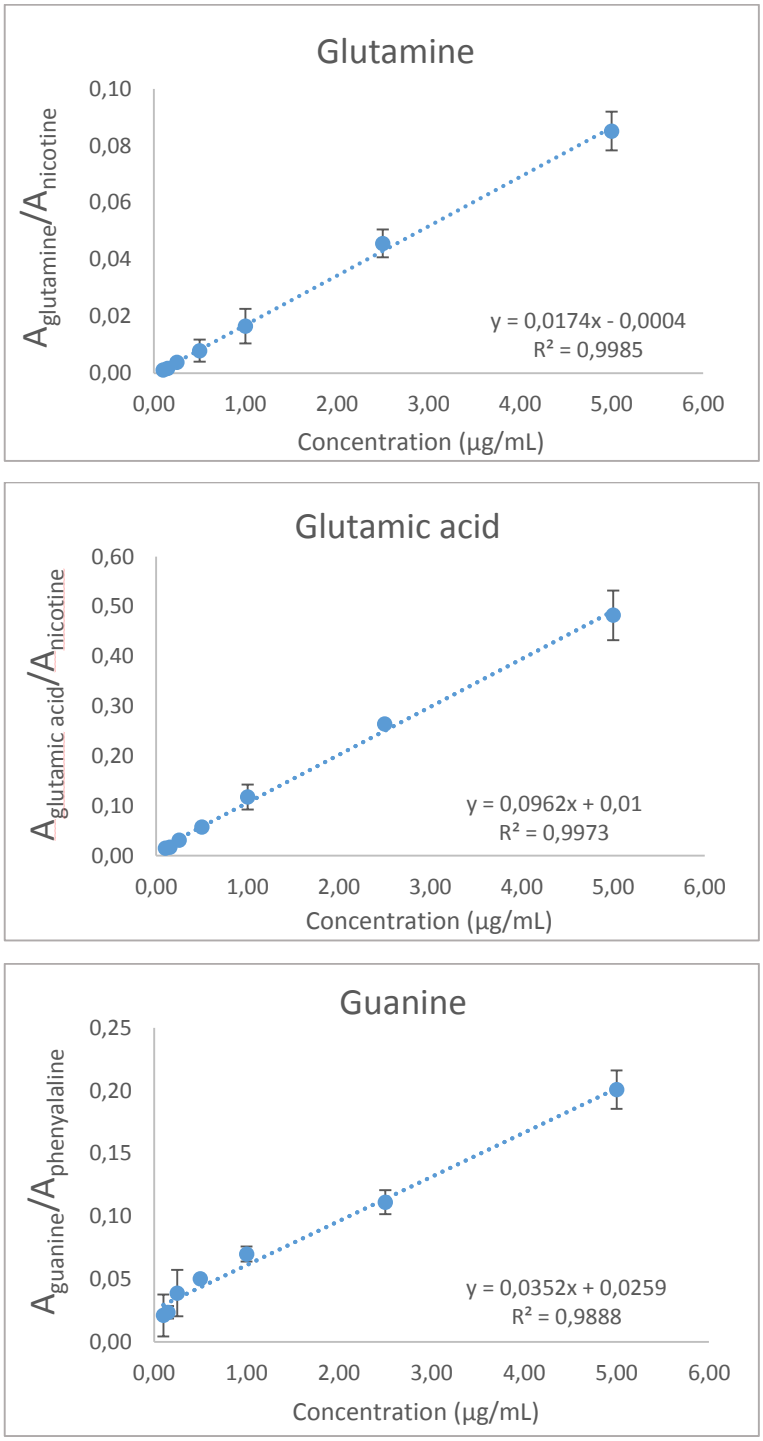
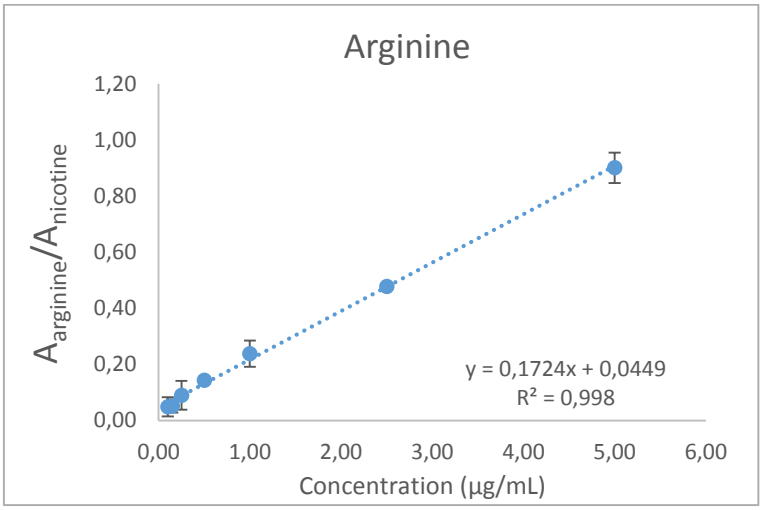


Figure 2.19 (cont'd.). Matrix-matched internal standard calibration curves obtained using 10% FBS in PBS



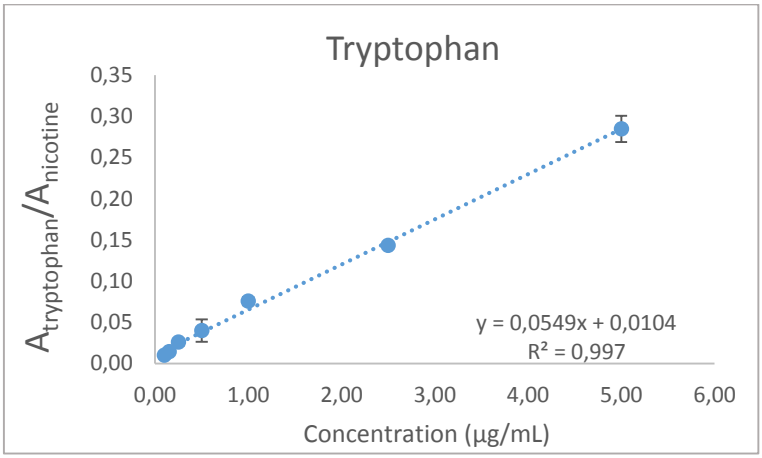


Figure 2.19 (cont'd.). Matrix-matched internal standard calibration curves obtained using 10% FBS in PBS

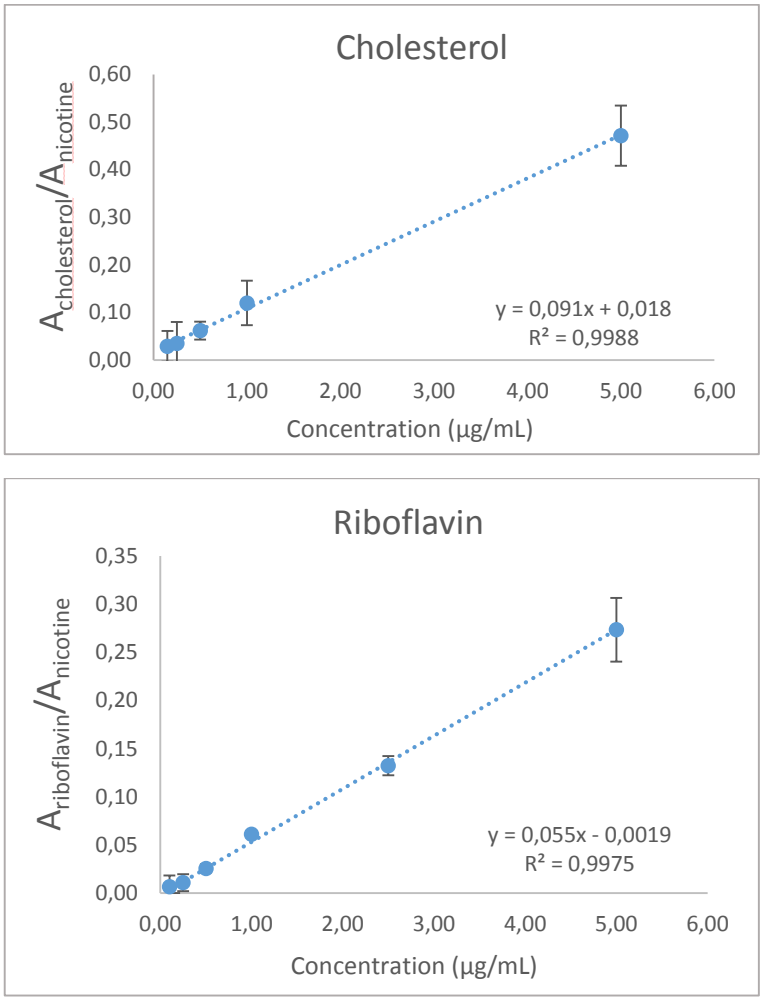


Figure 2.19. Matrix-matched internal standard calibration curves obtained using 10% FBS in PBS

2.2.7.2 Accuracy of the developed SPME-LC-MS method

To show the accuracy of the developed SPME-LC-MS method, a blind to analyst experiment was conducted. Three different concentrations of working range were chosen containing low- (LOQ), medium, and high-points of working range. Spiked concentrations of each analyte:

Low-point (LOQ): 500.0 ng/mL of creatinine, and 150.0 ng/mL of cholesterol and L-leucine, 250.0 ng/mL of L(+)-glutamine, L(+)-glutamic acid, guanine, L(+)-arginine, L(-)-tryptophan, riboflavin.

Mid-point: 1000.0 ng/mL of creatinine, and 750.0 ng/mL of L-leucine, 250.0 ng/mL of L(+)-glutamine, L(+)-glutamic acid, guanine, L(+)-arginine, L(-)-tryptophan, cholesterol and riboflavin.

High-point: 3000.0 ng/mL mixture of each analyte.

Matrix-matched internal standard calibrations were plotted, and the unknown concentrations of analytes were calculated using the regression equation of each analyte from 10% FBS in PBS. The RE% for calculated concentrations are given in Table 2.8.

Table 2.8. SPME-LC-MS method accuracy

Analyte	LOQ		Mid-point		High-point	
	Mean Concentration (ng/mL)	RE%	Mean Concentration (ng/mL)	RE%	Mean Concentration (ng/mL)	RE%
Creatinine	477.6	-4.5	844.5	-15.6	2500.1	16.6
Leucine	172.7	15.1	695.6	-7.4	3227.1	7.6
Glutamic acid	266.3	6.5	783.0	4.4	2444.5	-18.5
Glutamine	274.4	9.5	666.8	-11.1	3136.8	4.6
Guanine	277.3	10.9	794.3	5.9	3426.2	14.2
Arginine	293.0	17.2	785.1	4.7	2656.0	-11.5
Tryptophan	230.2	-8.0	667.6	-11.0	3498.2	16.6
Cholesterol	169.1	12.7	692.6	-7.7	3346.3	11.5
Riboflavin	250.5	0.2	766.8	2.2	2658.0	-11.4

As seen from Table 2.10, the method provided accurate results for the determination of each analyte in 10% FBS in PBS. According to the guidance of FDA, ± 20% RE is accepted for LOQ point while for other points within working range ± 15% RE is

accepted. For LOQ and mid-points, the %RE for each of the analytes was found acceptable. However, for high-point concentration (3000.0 ng/mL), %RE was found in higher percentage than accepted and these were 16.6, -18.5 and 16.6 for creatinine, glutamic acid and tryptophan respectively. In the case of using isotopologues of the analytes as internal standards, the standard deviations between sampling would be lower.

2.2.7.3 Precision of the developed SPME-LC-MS method

The intra-day reproducibility of the developed SPME-LC-MS method was evaluated by repeating three times in a day the protocol in spiked 10% FBS in PBS with low, mid, and high concentrations. For inter-day reproducibility of the method was evaluated for three consecutive days with the same spike levels used in intra-day reproducibility evaluations. The percent relative standard deviation (RSD%) for intra-day and inter-day reproducibility are shown in Table 2.11 and Table 2.12, respectively. The reproducibility of the method varied between 4-17% RSD and 5-12% RSD for intra-day and intra-day studies, respectively.

Table 2.9. Intra-day precision (RSD%) (n=3)

Analyte	**RSD%**		
	LOQ	**Mid-point**	**High-point**
Creatinine	9.9	9.1	12.3
Leucine	8.9	7.1	10.2
Glutamic acid	13.1	4.6	11.0
Glutamine	7.7	5.6	16.6
Guanine	10.9	5.9	14.2
Arginine	10.2	5.0	9.1
Tryptophan	12.5	5.2	4.1
Riboflavin	9.3	10.3	7.6
Cholesterol	3.7	7.1	11.0

Table 2.10. Inter-day precision (RSD%) (n=3)

Analyte	RSD%		
	LOQ	Mid-point	High-point
Creatinine	7.5	7.9	9.1
Leucine	5.2	9.0	6.7
Glutamic acid	6.1	8.0	11.0
Glutamine	6.3	10.1	11.1
Guanine	8.8	11.8	11.9
Arginine	10.0	7.8	8.4
Tryptophan	8.0	4.8	8.2
Riboflavin	9.0	11.0	7.5
Cholesterol	7.6	8.6	9.9

In a general point of view, the developed SPME-LC-MS method show good accuracy and reproducibility for the determination of both polar and nonpolar molecules in 10% FBS in PBS. During validation experiments, nicotine and phenylalaline were used as internal standards. In the case of using isotopologues of the analytes as internal standards, the standard deviations between sampling would be lower.

2.3 Summary and conclusion

Cancer research is paramount in curing disease in its early stages because it is one of the most deathful diseases worldwide. The development of anti-cancer drugs takes years, and in most cases, the candidate drugs do not show sufficient effect in clinical phases. During their pre-clinical evaluation, animal experiments become important to obtain informative results. However, animal experiments bring concerns about animal welfare. For this reason, the 3Rs rule (replacement, reduction, and refinement) was launched for conducting animal experiments. IVIVE experiments are in concordance with 3Rs rule and meets the requirements of this principle especially when IVIVE experiments are combined with SPME technique. Although there are several biocompatible SPME phases available in the market, there is still a need for the development of novel phases that have properties closer to be ideal. The ideal extractive phase of SPME should provide extraction of volatile, semi-volatile,

and nonvolatile compounds with a wide range of physicochemical properties and should be suitable for thermal and solvent-assisted desorption to be combined with LC and GC instruments. In this study, to prepare SPME fibers that cover wide range of analytes, HLB extractive phase was synthesized. To have SPME device suitable for both solvent and thermal desorption, the HLB particles were immobilized within thermally stable and inert PTFE-AF polymer.

To show the suitability of the SPME microprobes for untargeted analysis, representative molecules were chosen with a wide range of physicochemical properties. For this purpose, two different lengths of coatings were prepared; 10.0 mm, to be used in collected and relatively large volume samples, and 2.0 mm to be used in *in-vivo* and cell-culture media sampling. The mechanical stability of the fibers, (HLB/PTFE-AF 2400 prepared in 3:2 (w:w), 2:1 (w:w), diluted 1:1 (w:w), and 1:1 (w:w) ratio), was evaluated in terms of their suitability for immersion into solid samples and microprobes were found stable after immersion into chicken tissue. HLB/PTFE-AF 2400 prepared in 1:1 (w:w) ratio found as the optimum composition to obtain homogeneous coating and provided mechanical stability. After proving the fibers' mechanical stability, their extraction performance was compared with the commercially available fibers (C18 and HLB/PAN). HLB/PTFE-based fibers provided reasonable performance for the extraction of each analyte compared to other SPME fibers. SPME method optimization was performed using 10% synthetic serum in PBS to represent the cell culture medium. Desorption time was optimized as 120 min using ACN:MeOH: H_2O (40:40:20, v/v/v) as desorption solvent. Extraction time was optimized as 60 min to obtain optimum sensitivity for all of the analytes. The developed SPME-LC-MS method was validated using 2.0 coated HLB/PTFE AF microprobes in 10% fetal bovine serum (FBS) in PBS. The working range was determined by matrix-matched internal standard calibration and determined as 500.0-5000.0 ng/mL for creatinine, 150.0-5000.0 ng/mL for leucine and cholesterol, and 250.0-5000.0 ng/mL for glutamine, glutamic acid, guanine, arginine, tryptophan, riboflavin. The method was found accurate with ≤19% relative

error (RE%). The reproducibility of the method was shown by intra- and inter-day sampling and found acceptable with ≤17% RSD and 12% RSD respectively. The found RSD % and RE % can be improved by the use of isotopologues of each analyte. In summary, the extraction ability of 2.0 mm coated HLB/PTFE AF microprobes provided extraction of both polar and nonpolar molecules and can be used for untargeted screening of molecules using small volume of samples.

CHAPTER 3

DEVELOPMENT OF THIN FILM MICROEXTRACTION METHOD FOR DETERMINATION OF PESTICIDES IN AGRICULTURAL PRODUCTS

3.1 Experimental

3.1.1 Reagents and materials

Different classes of pesticides were selected for the study. These were trifluralin, parathion-methyl, carbaryl, chlorpyrifos-methyl, malathion, and diazinon, and all were purchased from Sigma-Aldrich. Helium was used as a carrier gas during chromatographic separation and purchased from Koyuncu. PTFE-AF 2400 which was used as a polymeric binder for the preparation of TFME devices was obtained from Sigma-Aldrich. For the dissolution of PFTE AF 2400, perfluorohexane (FC-72) was purchased from ABCR GmbH. LC-grade methanol was obtained from Merck. A stock solution of each pesticide (1.0 mg/mL) was prepared in methanol and stored at 4°C in the fridge. Working and calibration solutions were prepared before each analysis freshly by dilution of stock solution of each pesticide. pH 7.4 phosphate-buffered saline (PBS) salts; KCl, NaCl, and KH_2PO_4, were purchased from Isolab, while Na_2HPO_4 was purchased from Sigma-Aldrich. Agarose was used to prepare 2% agarose gel which mimics the semi-solid samples and purchased from Sigma-Aldrich. Two different brands of apple juices were obtained from a local market, namely, apple drink (which contains at least 10% apple juice) and 100% apple juice.

3.1.2 Instruments

For the quantification of analytes, Agilent 6890A gas chromatograph equipped with an Agilent 5973 quadrupole mass selective detector was used. The fragments of analytes were obtained using an electron impact (EI) ion source with 70 eV fragmentor voltage. For the separation of six pesticides, an ultra-inert (5%-phenyl)-methylpolysiloxane (Agilent Technologies, HP-5MS) column with 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness was used. Helium was used as a carrier gas in the GC. Extractions were performed using mechanical shaker (CAT AEK-SH10). The pH of the buffer solutions was measured using HANNA HI 2002 Edge pH meter. The ionic strength of the buffer solutions was measured using AZ8361 pen type LCD conductivity/TDS meter. Thermogravimetric analysis was performed to show the thermal stability of HLB and PTFE using Perkin Elmer, The Simultaneous Thermal Analyzer (STA) 6000.

3.1.3 Preparation of HLB/PTFE thin films

TFME samplers were prepared by a thin film applicator. The preparation of the slurry used for preparation of TFME samplers was explained in Section 2.4. The same slurry described in Section 2.4 was spread on the surface of the carbon mesh as a film with a thickness of 30 μm. The schematic representation of coating procedure using a thin film applicator is shown in Figure 3.1.

Figure 3.1. Schematic representation of the preparation of TFME sampler using thin film applicator

The resulting material was dried at 80 °C in an oven overnight and then cut with bistoury to have TFME samplers with 1.5 cm length and 0.5 cm width. The picture of the HLB/PTFE TFME samplers is shown in Figure 3.2. Another set of TFME samplers was prepared with 0.5 cm x 0.5 cm dimensions.

Figure 3.2. 1.5 cm x 0.5 cm HLB/PTFE thin films

3.1.4 Thermogravimetric analysis of HLB and PTFE-AF 2400

Thermogravimetric analysis (TGA) of HLB and PTFE-AF 2400 was performed to show the thermal stability and the suitability of the resulting material for direct

thermal desorption in GC. During the analysis, the sample was kept at 25 °C for 1 min and heated from 25 °C to 600°C at 10 °C/min for HLB. The total analysis time was 1 hour 47 min. In case of PTFE-AF, the sample again was kept at 25 °C for 1 min and then heated from 25 °C to 800 °C at 10 °C/min.

3.1.5 Development of GC-MS method

For the separation and quantification of the pesticides, GC-MS was used with an ultra-inert (5%-phenyl)-methylpolysiloxane column with 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness. Helium was used as a carrier gas at a flow rate of 1.2 mL/min. The injector port was kept at 250 °C during the injection. The injection volume was 1.0 μL with a split ratio of 1:1. The temperature gradient used for the chromatographic separation was as follows.

For the first 5 minutes the column was kept at 60 °C and heated up to 200 °C at 80 °C/min rate and kept at 200 °C for 2 min. Then, it was increased to 220 °C at 20 °C/min and kept for 1 min at 220 °C. Finally, it was increased to 240 °C at 20 °C/min and kept for 1 min. The total analysis time was 10.75 min. A selected ion monitoring (SIM) method was used for the quantification of each pesticide. A typical chromatogram of each analyte is shown in Appendix B in Figure B.1. and mass spectra are given in Appendix C in Figure C.1 to Figure C.6.

3.1.6 Optimization of TFME parameters

Several TFME parameters were optimized to obtain sensitive and reliable methods. For this purpose, desorption time, extraction time, and the effect of pH and ionic strength were investigated. In a typical study, before the extraction, TFME samplers were preconditioned in methanol to wet the pores and prepare the extractive phase

for the extraction. Then samplers were dipped into water for 3 s to remove the excess methanol from the surface of the coating. Then the excess water on the surface of coating was removed gently with a paper towel. After precondition and washing steps, extraction of analytes was performed from aqueous/solid samples. After extraction, TFME samplers were washed quickly with water to remove the matrix components. The excess water on the surface of coating was removed gently with paper towel. After extraction, the analytes were desorbed into methanol and extracts were analyzed in GC-MS. The details of each experimental parameter are given in further sections.

3.1.6.1 Desorption time optimization

To provide the complete desorption of pesticides from TFME samplers, as the first parameter, the desorption time was investigated. For this experiment, pesticide mixture of chlorpyrifos-methyl, malathion, carbaryl, diazinon, trifluralin, and parathion-methyl was spiked to PBS (pH 7.4) to have 250.0 ng/mL final concentration of each pesticide and extractions were performed from this matrix using 0.5 cm x 0.5 cm TFME samplers. Extraction conditions were as follows; sample volume: 4.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 1.5 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 5, 15, 30, 60, and 120 min.

3.1.6.2 Extraction time profile of pesticides

After providing complete desorption of analytes, as a second TFME optimization parameter, the extraction time profile of each pesticide was investigated. For this experiment, pesticide mixture was spiked to PBS (pH 7.4) to have 250.0 ng/mL final concentration of each pesticide and extractions were performed from this matrix using 0.5 cm x 0.5 cm TFME samplers. The extraction conditions were as follows; sample volume: 4.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 5, 15, 30 and 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 1.5 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

3.1.6.3 Effect of sample pH on extraction of pesticides

The importance of sample pH was explained in Section 1.2.4.6. To show the effect of sample pH for the extraction of pesticides, several pH points in a range between 3.0 and 12.0 was investigated. For this experiment, pesticide mixture was spiked to buffer solutions with pH of 3.0, 5.0, 7.0, 10.0 and 12.0 to have 250.0 ng/mL final concentration of each pesticide and then equilibrated for 1 hour. Extractions were performed from this matrix using 0.5 cm x 0.5 cm TFME samplers. The extraction conditions were as follows; sample volume: 4.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 1.5 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

3.1.7 Extraction from real samples

After optimization of TFME parameters, sampling was performed using apple juice. At this point, the effect of ionic strength was also studied using apple juice which is more complex compared to water due to presence of dissolved solids, ions, binding components.

3.1.7.1 1.0 ng/mL pesticide-spiked 10% apple juice

For the evaluation of the extraction performance of developed HLB/PTFE TFME samplers, extractions were performed from pesticide-spiked 10% apple juice as an initial experiment of real samples. For this purpose, apple drink that contains at least 10% apple juice was purchased from a local market. Extractions were performed using 1.5 cm x 0.5 cm TFME samplers. Blank matrix extractions were also performed. The extraction conditions were as follows; sample volume: 40.0 mL, analyte concentration: 1.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 0.600 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

3.1.7.2 Optimization of added NaCl to apple juice

As a first matrix, 10% apple juice was used. The sample was spiked with pesticides to contain 250.0 ng/mL of each pesticide in the final samples and equilibrated for 1 hour. Then, NaCl was added to have 0%, 5%, 10%, and 20% (w/v) NaCl concentration in the final samples. Extractions were performed using 1.5 cm x 0.5 cm TFME samplers. The extraction conditions were as follows; sample volume: 1.5 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed:

1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 1.0 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

As the next trial, pesticide mixture was spiked in 100% apple juice to have 250.0 ng/mL of each pesticide and equilibrated for 3 hours. Then, NaCl was added to have 0%, 5.0%, 10.0%, and 20.0% NaCl (w/v) concentration in the final samples. Extractions were performed using 1.5 cm x 0.5 cm TFME samplers. The extraction conditions were as follows; sample volume: 40.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 0.600 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

As the last trial, the salt effect was investigated in 100% apple juice which was diluted with water in half, to decrease the viscosity of the matrix. The pesticide mixture was spiked in 100% apple juice to have 250.0 ng/mL of each pesticide and equilibrated for 3 hours. Then, this sample was diluted in half. Finally, NaCl was added to each sample to have 0%, 5.0%, 10.0%, and 20.0% NaCl (w/v) concentration in the diluted samples. Extractions were performed using 1.5 cm x 0.5 cm TFME samplers. The extraction conditions were as follows; sample volume: 40.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 0.600 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min.

3.1.8 Validation of the developed TFME-GC-MS method

The developed TFME-GC-MS method was validated in terms of its linearity, limit of quantification (LOQ), precision and accuracy based on the guidance of bioanalytical method validation for industry published by Food and Drug Administration (FDA) in 2018 [122].

3.1.8.1 SPME calibration using 100% apple juice

As the first validation procedure, samples that will be used for matrix-matched external calibration was prepared by spiking 100% apple juice with pesticides to have 0.0, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0 50.0, 100.0, 250.0 and 500.0 ng/mL concentration and equilibrated for 3 hours. Then samples were diluted with water in half and NaCl was added to have 10% NaCl (w/v) in final samples. In this part of the study, 0.5 cm x 0.5 cm TFME samplers were used. The extraction conditions were as follows; sample volume: 40.0 mL, analyte concentration: 0.10, 0.25, 0.50. 1.0, 5.0, 10.0, 50.0, 100.0, 250.0, and 500.0 ng/mL, extraction time: 60 min, agitation: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 0.600 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min. The desorption solutions were injected into the port of GC for the determination of pesticides.

The LOQ of each pesticide was calculated from the back calculation of nominal concentration using the linear regression equation of matrix-matched TFME calibration. In these studies, the LOQ was defined as the lowest concentration that provides maximum of 20% relative error in back calculations.

3.1.8.2 Accuracy of the developed TFME-GC-MS method

The accuracy of the developed TFME-GC-MS method was shown by a blind analyst experiment. For this purpose, three different concentrations of pesticides were spiked to 100% apple juice containing low- (5.0 ng/mL), mid-(30.0 ng/mL) and high-point (300.0 ng/mL) of working range and equilibrated for 3 hours. Then samples were diluted with water in half and NaCl was added to have 10% NaCl (w/v) in final samples. The samplers were preconditioned and washed as described in Section 2.4. Extractions were performed in normalized samples using 0.5 cm x 0.5 cm TFME samplers. The extraction conditions were as follows; sample: apple juice:water 1:1 (v/v), sample volume: 40.0 mL, salt concentration 10% NaCl (w/v), analyte concentration: 5.0, 30.0, and 300.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 0.600 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min. The desorption solutions were injected into the port of GC for the determination of pesticides.

The unknown concentrations to analyst were calculated using matrix-matched external standard calibration and for each point RE % was calculated after releasing the information about the concentrations to the analyst.

3.1.8.3 Precision of the developed TFME-GC-MS method

The precision of the developed TFME-GC-MS method was shown by performing intra- and inter-day sampling. Three different concentrations of pesticides were spiked to 100% apple juice containing low- (5.0 ng/mL), mid- (30.0 ng/mL) and high-point (300.0 ng/mL) from working range and then the matrix normalization (dilution and salt addition) described before was applied. For intra-day

reproducibility, three different sets of extractions were performed three times in a day while for inter-day reproducibility, three different sets of extractions were performed on three consecutive days. The concentration of each pesticide was calculated using matrix-matched external standard calibration and for each point RE% was calculated.

3.1.9 Extraction from solid samples

In this part of the study, the TFME samplers were evaluated for potential use on field directly on the surface of the fruits or vegetables. For this reason, sampling was performed using 2% agarose gel spiked with pesticides as model matrix.

3.1.9.1 Extraction time profile

The extraction time profile of each pesticide was also monitored for the solid samples using agarose gel as a model. 2% agarose mixture was prepared and heated until boiling where it transformed to a transparent solution. Once the solution temperature dropped to 60 °C, pesticide mixture was spiked into the agarose solution at 250.0 ng/mL final concentration. The solution was stirred for the homogenous distribution of pesticides. Before solidification, 50.0 mL portion of the solution was transferred to a Petry dish and then cooled to room temperature to solidify to a gel. The sampling performed under static conditions from the gel surface is shown in Figure 3.3.

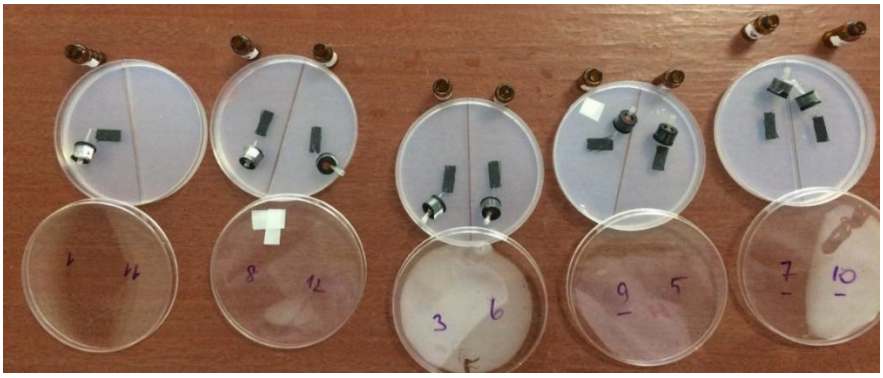


Figure 3.3. Extraction from agarose gel

3.1.9.2 Pesticide distribution analysis from the surface of 2% agarose gel

As another preliminary study for on-site analysis of pesticides, pesticide distribution analysis was conducted. To represent the solid samples, 2% agarose gel was prepared as explained in Section 2.1.5. For this experiment, 0.0, 10.0, 25.0 and 50.0, 100.0, 250.0, 500.0 and 750.0 ng/mL mixture of pesticides were spiked to 2% agarose gel and homogenized using a magnetic stirrer without allowing the solution to solidify. Then 2.0 mL of gels with different concentrations of pesticides was poured randomly into the wells of a 96-well plate. To obtain spatial resolution, a set of smaller size TFME samplers were used (0.5 cm x 0.5 cm). The extraction conditions were as follows; sample volume: 2.0 mL, analyte concentration: 0.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0, and 750.0 ng/mL, extraction time: 60 min, agitation: static condition, extraction temperature: 20 °C. The desorption conditions were as follows; desorption solvent: methanol, desorption volume: 0.600 mL, agitation speed: 1000 rpm, desorption temperature: 20 °C, desorption time: 60 min. Spiked concentrations of pesticides as a heat map was illustrated in Figure 3.4. The color abundances represent the concentrations.

A	1	2	3	4	5	6	7	8	9	10	11	12
B		0		50		25		100		10		
C	250					500					750	
D		10					0					
E		750		100		50				250		25
F	25											
G				750			250		500		0	
H		500	100		10			50				

Figure 3.4. Sampling from agarose gel using 0.5 cm x 0.5 cm HLB/PTFE thin films (color abundances are indicating the pesticide concentration levels)

3.2 Results and discussion

3.2.1 Thermal stability of HLB and PTFE-AF 2400

The thermal gravimetric analysis of HLB and PTFE-AF polymers were investigated for potential use of the fibers with direct thermal desorption to GC-MS. Obtained results are shown in Figure 3.5 and Figure 3.6 for HLB and PTFE-AF, respectively. As can be seen from these figures, HLB particles are thermally at least up to 300 °C while PTFE-AF is stable up to 450 °C (5% of the polymers were degraded at these temperatures). The thermal stability of particles were found coincident with the literature and reported as 300 °C for HLB and 360 °C for PTFE-AF [7], [123].

In general, the temperature of the injection port during direct thermal desorption is at 250 to 275 °C proving that the resulting SPME and TFME samplers are suitable for thermal desorption of analytes.

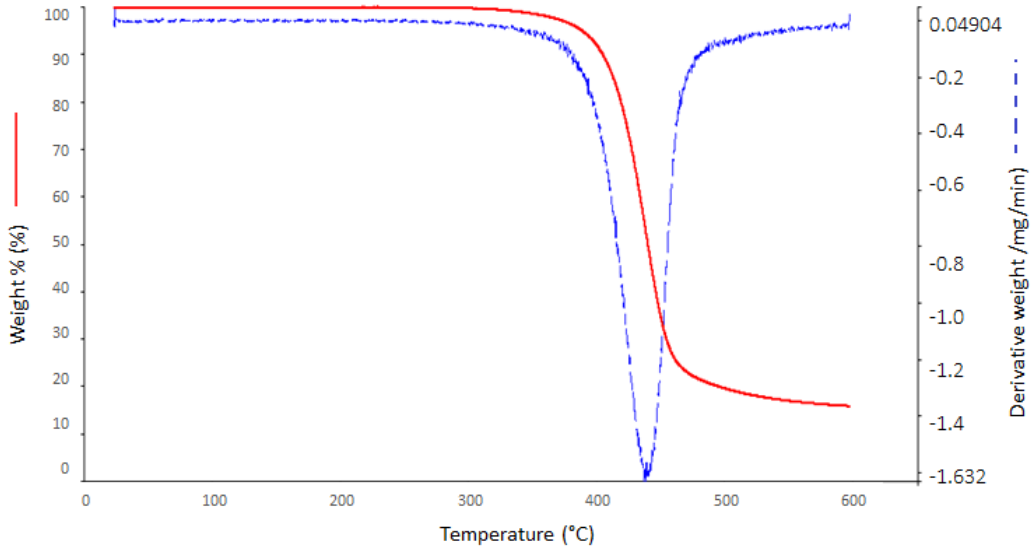


Figure 3.5. Thermogravimetric analysis of HLB

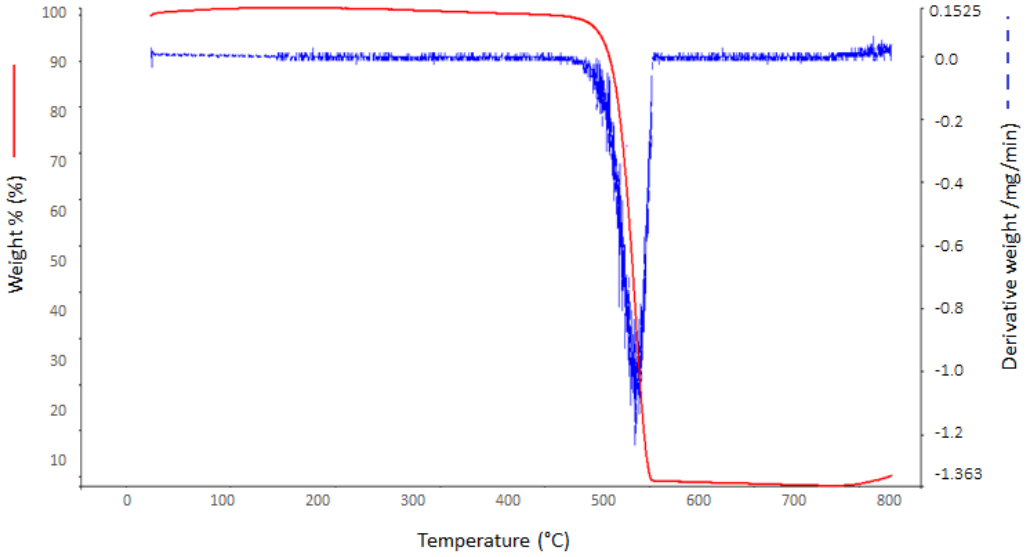


Figure 3.6. Thermogravimetric analysis of PTFE-AF 2400

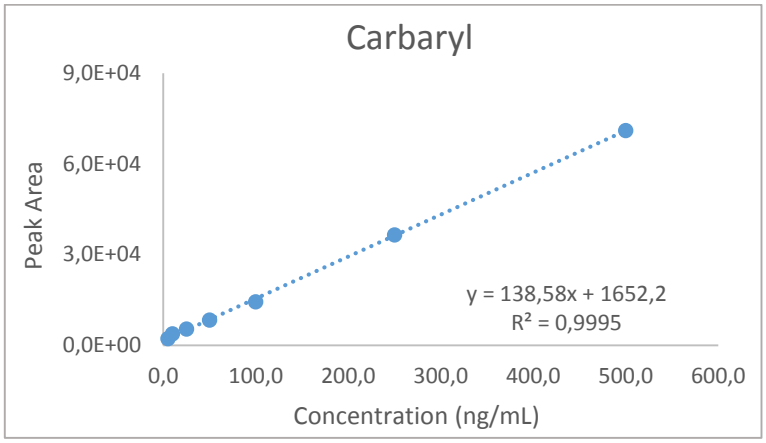
3.2.2 Development of GC-MS method

For the separation and quantitation of pesticides, the GC-MS method was developed. The quantitation ions and physicochemical properties of analytes are shown in Table 3.1. After the development and optimization of the chromatographic method, the working range of the instrument for each analyte was determined and found to be between 1.0 to 500.0 ng/mL for trifluralin, carbaryl, and chlorpyrifos-methyl while for diazinon, malathion and parathion was between 5.0 to 500.0 ng/mL.

Table 3.1. Physicochemical properties of analytes and their GC-MS parameters

Analyte	Molecular Weight (g/mol)	Log P	Quantitation ion (m/z)	Retention time (min)	Working Range (ng/mL)
Trifluralin	335.28	5.3	306	8.0	1.0-500.0
Carbaryl	201.22	2.4	144	7.4	1.0-500.0
Chlorpyrifos-methyl	322.53	4.3	286	9.6	1.0-500.0
Malathion	330.36	2.4	93	10.1	5.0-500.0
Methyl parathion	263.21	2.9	109	9.6	5.0-500.0
Diazinon	304.35	3.8	137	8.8	5.0-500.0

Typical external calibration curves generated after injection of various concentrations of standard solutions of analytes to GC-MS are shown in Figure 3.7.



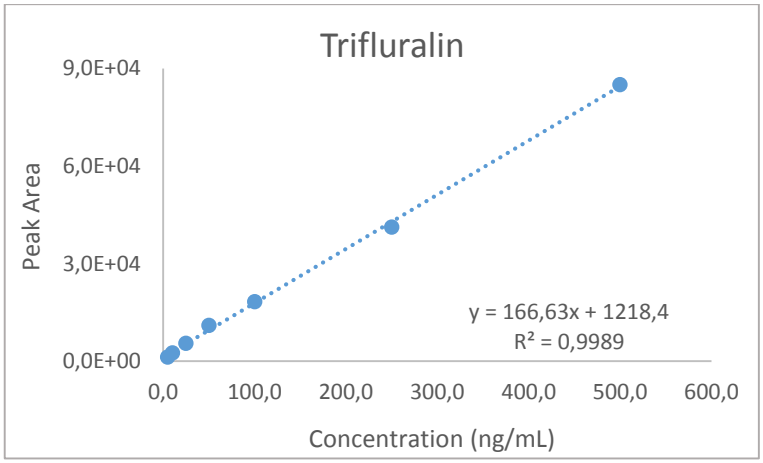
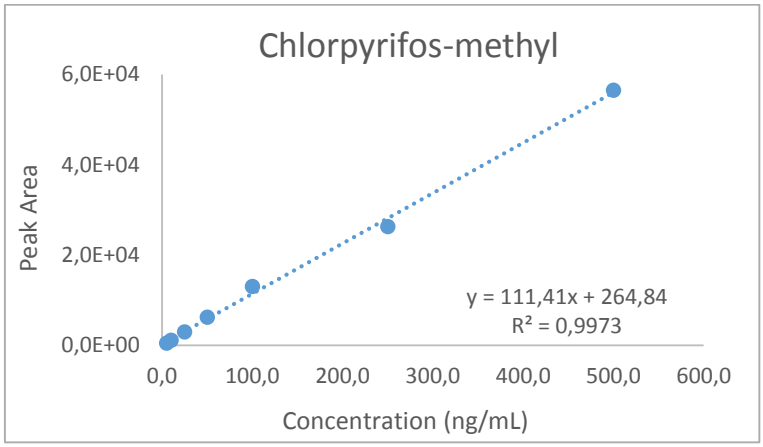


Figure 3.7. Typical calibration curves obtained with developed GC-MS method



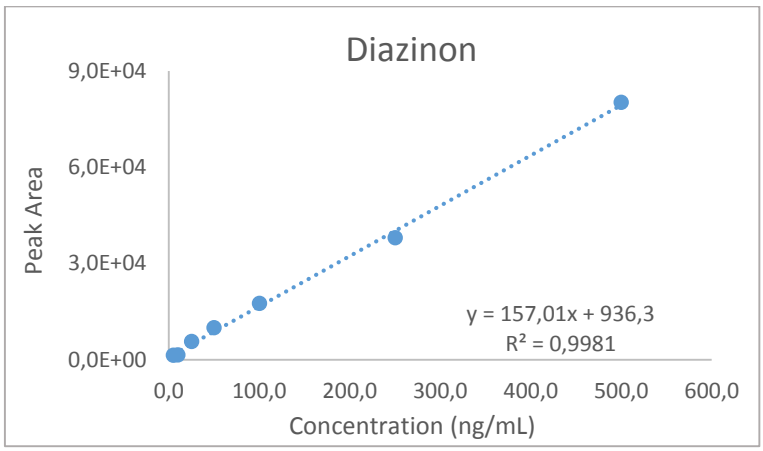


Figure 3.7 (cont'd.). Typical calibration curves obtained with developed GC-MS method

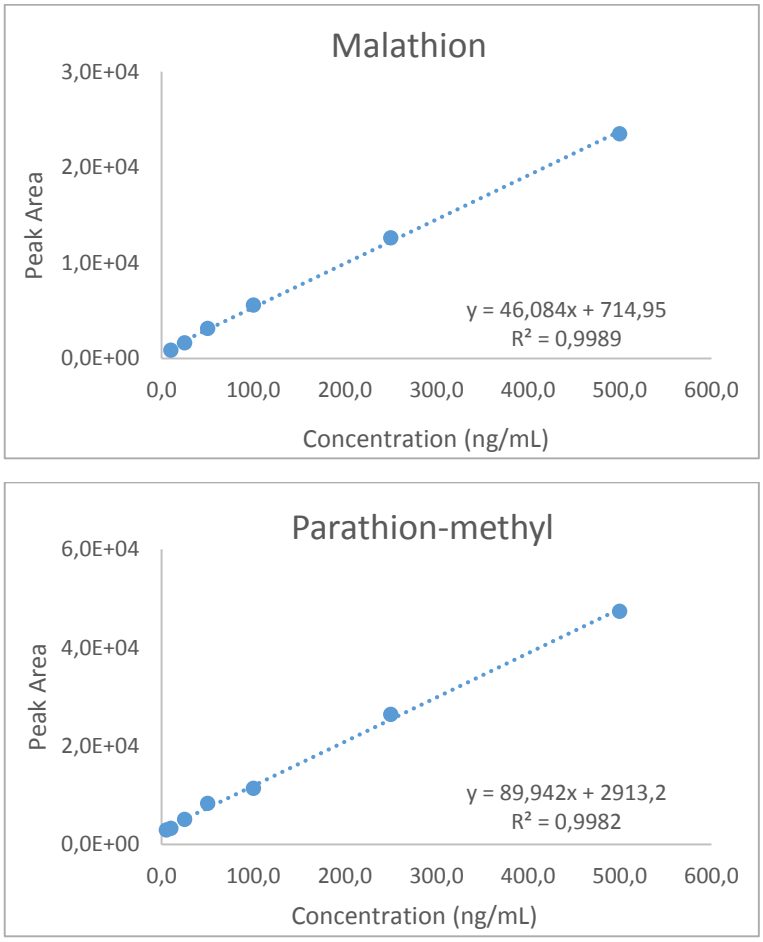


Figure 3.7. Typical calibration curves obtained with developed GC-MS method

3.2.3 Optimization of TFME parameters

Several TFME parameters were optimized to obtain sensitive and reliable method. Each optimization parameter was explained in detail in below sections.

3.2.3.1 Desorption time optimization

For the complete desorption of all analytes, the desorption time profiles of each analyte was investigated. As seen from Figure 3.8, complete desorption was obtained within 15 min for all of the analytes except chlorpyrifos-methyl that requires 30 min of desorption. To investigate whether there is carryover issue of analytes, a second desorption was performed with the same TFME samplers under the same extraction and desorption conditions using fresh solvent. There was no carry over of analytes indicating the complete desorption when methanol was used as desorption solvent. For this reason, the composition of the desorption solvent was not optimized further.

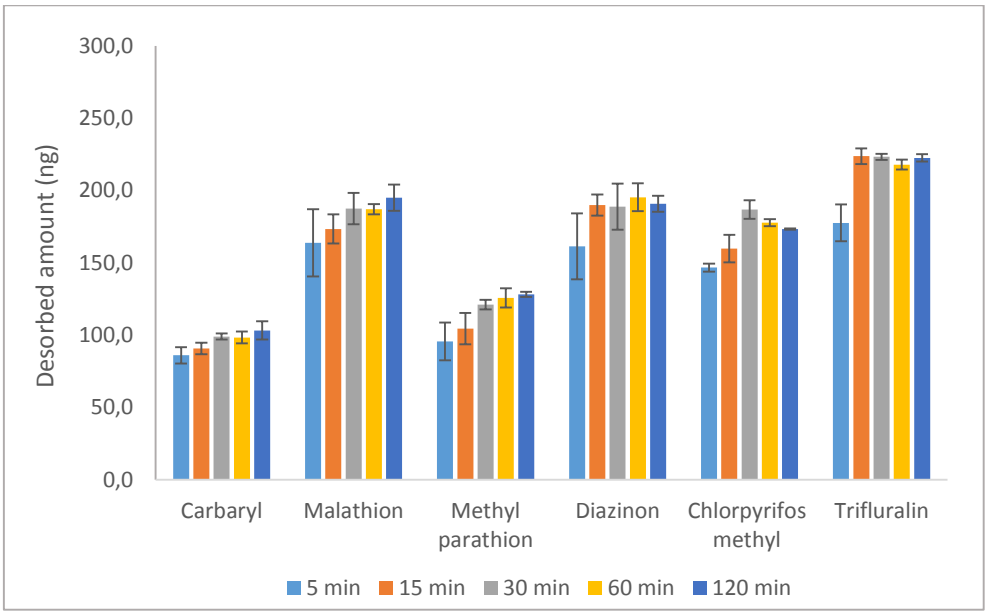


Figure 3.8. Desorption time profile of pesticides. (Sample volume: 4.0 mL PBS, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption solvent: methanol, desorption volume: 1.5 mL, agitation speed: 1000 rpm)

3.2.3.2 Extraction time profile of pesticides

For each pesticide, extraction time profile was investigated to achieve the best sensitivity in the shortest time. The results are shown in Figure 3.9. An obvious increase in the extracted amount of each pesticide was observed when 5 min sampling was compared to 15 min sampling. Under the studied conditions, all of the analytes reached equilibrium within 30 min except methyl-parathion and chlorpyrifos-methyl. When Student's t-test was applied at 95% confidence level (CL) to the means of the extracted amount of methyl-parathion, extracted amount in 120 min sampling was found still significantly different than the amount extracted in 60 min. In further studies, extraction time was optimized as 60 min to provide a reasonable balance for extraction of pesticides and time that will be spent for the analysis.

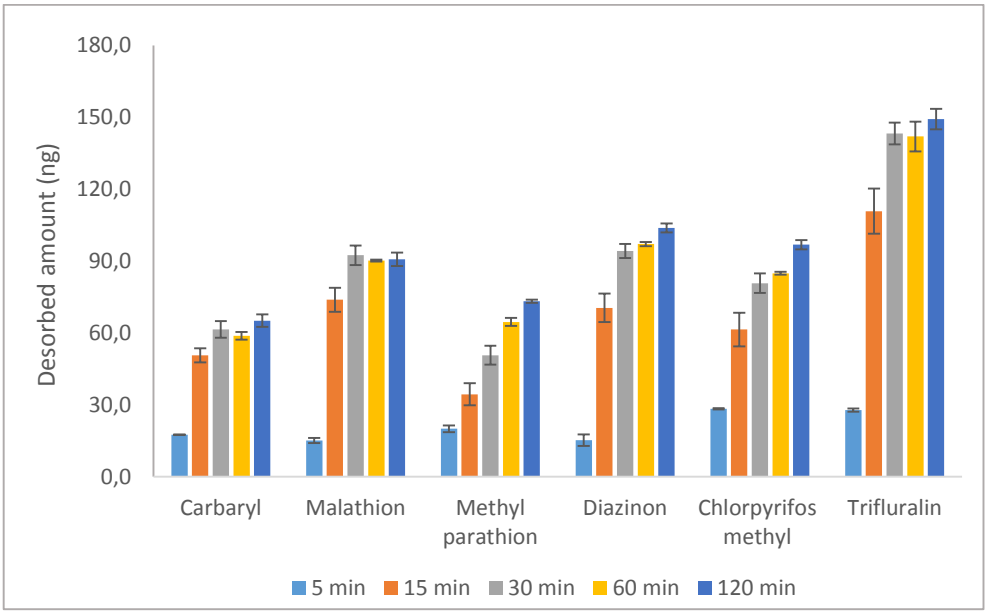


Figure 3.9. Extraction time profile of pesticides. (Sample volume: 4.0 mL PBS, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000

rpm, desorption solvent: methanol, desorption volume: 1.5 mL, desorption time: 60 min, agitation speed: 1000 rpm)

3.2.3.3 Effect of sample pH on extraction of pesticides

Vegetable/fruits and their juices can be acidic (such as apple, lemon and orange) or basic in nature (such as savoy cabbage, spinach, bananas and kiwi) with a pH range between 2.0 to 8.0.Thus, the effect of pH becomes critical for the extraction of pesticides from different vegetable/fruits and their juices. Therefore, the effect of pH on the extraction of pesticides was studied using a pH range between 3.0 to 12.0. Extracted amounts of analytes from samples with different pH are shown in Figure 3.10.

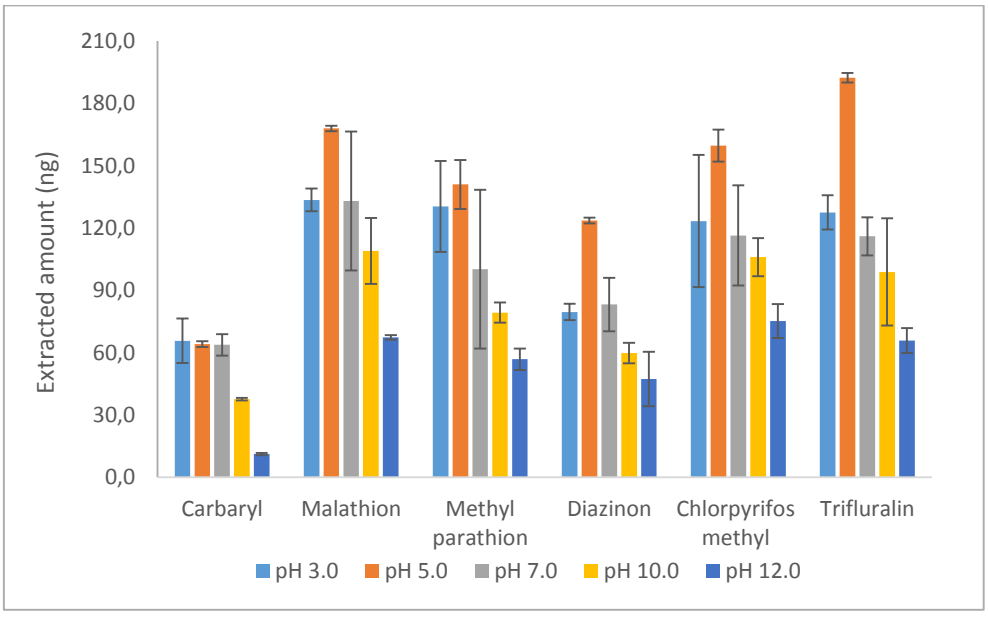


Figure 3.10. Effect of pH for on extraction of pesticides. (Sample volume: 4.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000

rpm, desorption solvent: methanol, desorption volume: 1.5 mL, desorption time: 60 min, agitation speed: 1000 rpm)

According to the figure, it is clear that the sample pH affects the extraction of selected pesticides. The extracted amounts decreased as sample pH increases up to 12.0 while the extracted amount of pesticides was increased under slightly acidic conditions. Moreover, for pH 5.0 statistically significant differences (at 95% CL) in the extracted amounts of malathion, diazinon and, trifluralin were observed when the results were compared to pH 3.0 and 7.0.

Based on the results obtained from ionic strength studies, it is known that there is a significant effect of ionic strength in the extraction of pesticides. For this purpose, ionic strength of the solution should also be considered in the pH evaluations. In Table 3.2, ionic strength of the studied pH buffers is given to fairly evaluate the effect of sample pH on the extraction of pesticides.

Table 3.2. Ionic strength of buffers used in evaluation of effect of sample pH

pH	**Ionic strength (mS)**
3.0	4.6
5.0	11.0
7.0	9.7
10.0	12.7
12.0	11.7

As seen from Table 3.2, pH 5.0 has a higher ionic strength compared to pH 3.0 and 7.0 buffers. Since the ionic strength is higher for pH 5.0 solution, it might also affect

the extraction of pesticides. The increase is significantly different only for diazinon and trifluralin at pH 5.0 and associated to higher ionic strength of this buffer.

Prediction about the sorption mechanism can be made based on the molecular structure of sorbent (HLB) and sorbates (pesticides). For extraction of malathion methyl, the extraction was carried out by weak electrostatic forces (London dispersion forces) between the analyte and extractive phase due to the charge distribution within the molecule. On the other hand, the extraction of trifluralin was decreased in pH 3.0 solution. In acidic pH, this analyte was protonated and has positive charges on the two nitro groups. The extractive phase is also protonated in acidic media due to presence of 5-membered lactam ring. As acidity increased from pH 5.0 to pH 3.0, the extracted amount of trifluralin decreased since both analyte and extractive phase have positive charge on their functional groups. However, still there is effect of π-π interactions for the extraction of it. Where the extraction of chlorpyrifos-methyl was carried out by formation of π-π interactions between pyridine part of chlorpyrifos methyl and divinylbenzene moiety of the HLB polymer as in the case of extraction of malathion, carbaryl and diazinon.

On the other hand, the extracted amount of pesticides shows a decreasing trend in alkaline solutions. There might also be effect of hydrolysis in the decreasing trend because all of the pesticides were hydrolyzed under alkaline conditions as stated in several studies. Trifluralin is hydrolyzed in to 2,6-dinitro-4-trifluoromethylaniline and isopropanol [124], carbaryl is hydrolyzed in to 1-napthol [125], malathion is hydrolyzed in to malate [126], parathion is hydrolyzed in to p-nirophenol [127], while diazinon is hydrolyzed in to 2-isopropyl-4-methyl-6-pyrimidinol [128]. The pH effect should be evaluated critically for the real samples because, each sample can have different pH as well as ionic strength and thus sorption mechanism of pesticides alter from sample to sample. In further studies, apple juice was used to evaluate the extraction performance of pesticides using a real sample and the pH was

measured as 4.5. No pH adjustment was performed in further experiments as this value was very close to the pH where the best sensitivity was obtained.

3.2.4 Extraction from real samples

3.2.4.1 1.0 ng/mL pesticide-spiked 10% apple juice

To show the extraction performance of developed HLB/PTFE TFME samplers, 1.0 ng/mL of pesticide mixture was spiked to 10% apple juice containing drink. The extraction results are summarized in Figure 3.11. All the pesticides were extracted from a sample that contained only 1.0 ng/mL concentration. According to EU, MRL for trifluralin, carbaryl, chlorpyrifos-methyl, methyl-parathion and diazinon is specified as 10 ng/mL, and for malathion is 20 ng/mL [126–130]. Using these samplers, much lower concentrations of pesticides than their MRL values can be determined.

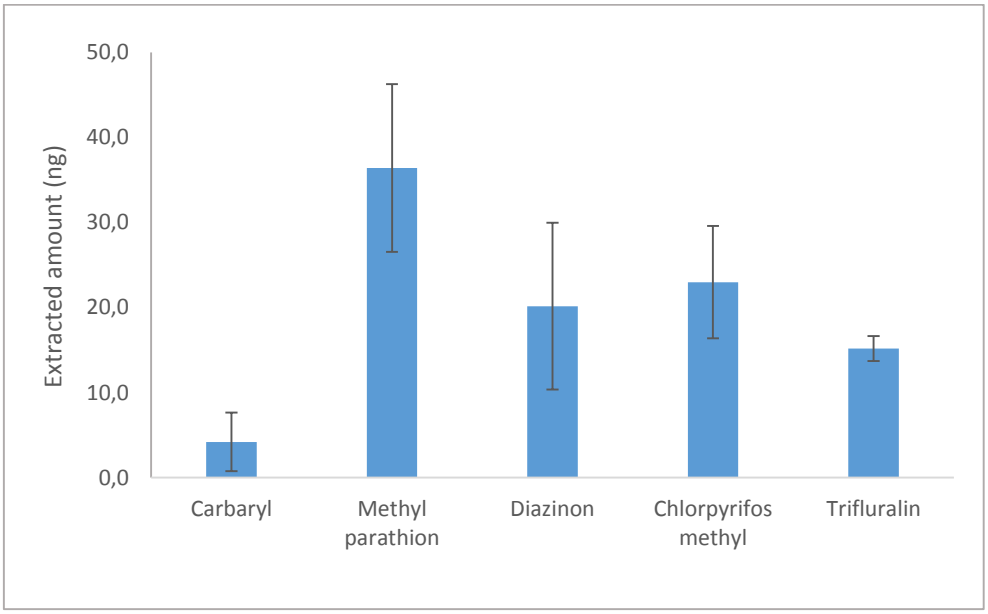


Figure 3.11. Extracted amount of pesticides from spiked apple juice. (Sample volume: 40.0 mL, analyte concentration: 1.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption solvent: methanol, desorption volume: 0.600 mL, desorption time: 60 min, agitation speed: 1000 rpm)

3.2.4.2 Optimization of NaCl added to apple juice

Ions present in solution may affect the sorption of analytes. Therefore, if the ionic strength of sample and matrix-matched calibration are differing erroneous conclusion can be derived. For this reason, the effect of ionic strength should be investigated and if it is needed must be normalized by addition of high amounts of salt to both sample and matrix-matched calibration. Furthermore, when high concentrations of salt is added to the sample, the extraction of polar analytes can increase due to salting-out effect. The opposite phenomenon, which is salting-in effect, can be also observed for nonpolar analytes because their solubility increases in the aqueous media when salt is added to the sample.

To investigate the effect of salt addition in real samples, the first sampling trial was performed using apple drink which contains at least 10% apple juice. The effect of added NaCl on the extraction of pesticides is shown in Figure 3.12. Salt addition remarkably increased the sorption of pesticides, the extracted amount of them increased by 3 to 8 times compared to no salt added juice. The extracted amount of pesticides reached their maximum value when 5% NaCl (w:v) was added, as a result of the salting-out effect. In other words, the solubility of the pesticides decreased in their aqueous matrices and their affinity to extractive phase had shown an increase as a consequence of salting-out effect.

However, as the salt concentration increased further, the extracted amount of pesticides started to show a decreasing trend which can be explained by the decreasing of diffusion coefficients due to saturation of the sample [134]. In a study, this decrease was explained by the formation of a salt layer that preventing the diffusion of analytes through the extractive phase [135].

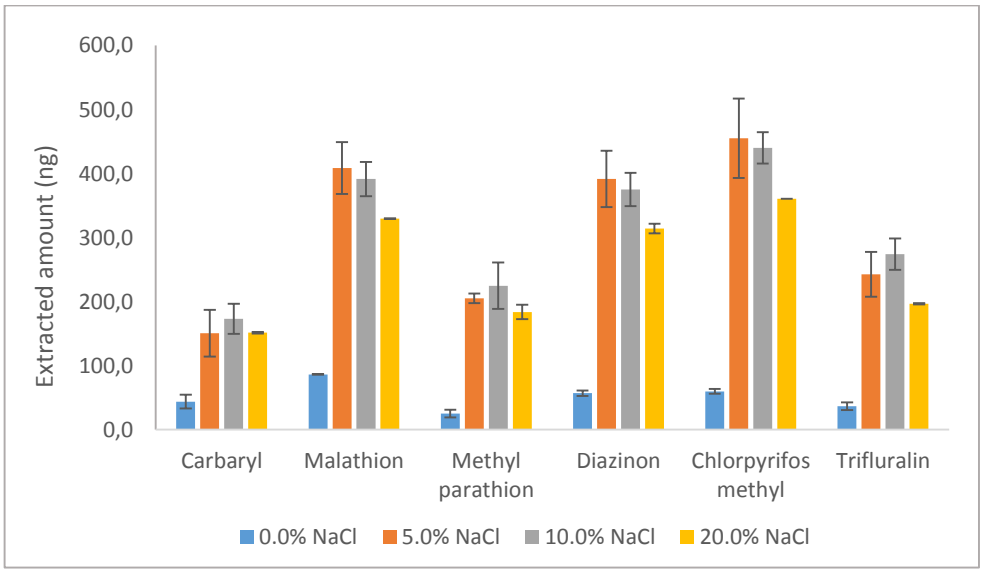


Figure 3.12. Effect of salt addition on extraction of pesticides from apple drink. (Sample volume: 1.5 mL, analyte concentration: 250.0 ng/mL, extraction time: 60

min, agitation speed: 1000 rpm, desorption solvent: methanol, desorption volume: 1.0 mL, desorption time: 60 min, agitation speed: 1000 rpm

Later, the salt effect was investigated in 100% apple juice. As can be seen from Figure 3.13, the extracted amount of pesticides decreased in 100% apple juice compared to apple drink (containing only 10% apple juice). Besides, a gradual increase in the extracted amount of pesticides was observed with an increase in salt concentration. Differently from 10% apple juice for which 5% NaCl was optimum for all compounds, in 100% apple juice the maximum extraction was achieved for carbaryl and diazinon at 10% NaCl while for malathion it was at 5% NaCl. In literature, it was reported by Giordano et al. that the addition of salt is critical for compounds with Log P value lower than 3 due to salting-out effect [136]. As could be expected based on these comments, no significant effect of salt addition was observed for the extraction of trifluralin (Log P 5.3) and chlorpyrifos-methyl (Log P 4.3) in the present study.

However, the extraction trends obtained from apple drink and 100% apple juice was found to be different, suggesting that other reasons could be contributing as well. One of the explanations for this observation could be associated with the presence of a higher amount of binding components in 100% apple juice. It can be speculated that, the binding components present in the matrix decreases the free form of relatively lipophilic compounds, and thus decreases the extracted amounts of these compounds. Another explanation for this situation could be the precipitation of relatively lipophilic pesticides from the solution as sample reaches saturation level.

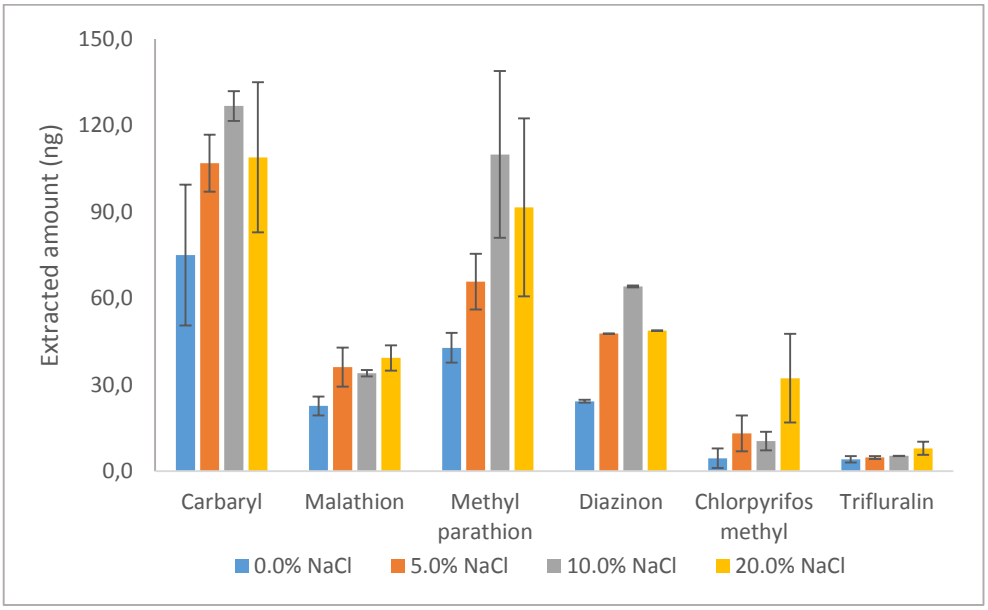


Figure 3.13. Effect of salt addition on extraction of pesticides from 100% apple juice. (Sample volume: 40.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption solvent: methanol, desorption volume: 0.600 mL, desorption time: 60 min, agitation speed: 1000 rpm

To further investigate the effect of matrix on the extraction, apple juice was diluted with water in half and then NaCl was added to observe the salt effect. The obtained results from the study are shown in Figure 3.14. The best extraction amounts were achieved by 10% NaCl addition. By this experiment, the importance of sample dilution to decrease the effect of matrix components was also shown. For instance, the extracted amount of relatively lipophilic pesticides which are trifluralin and chlorpyrifos-methyl was doubled in diluted apple juice samples compared to 100% apple juice.

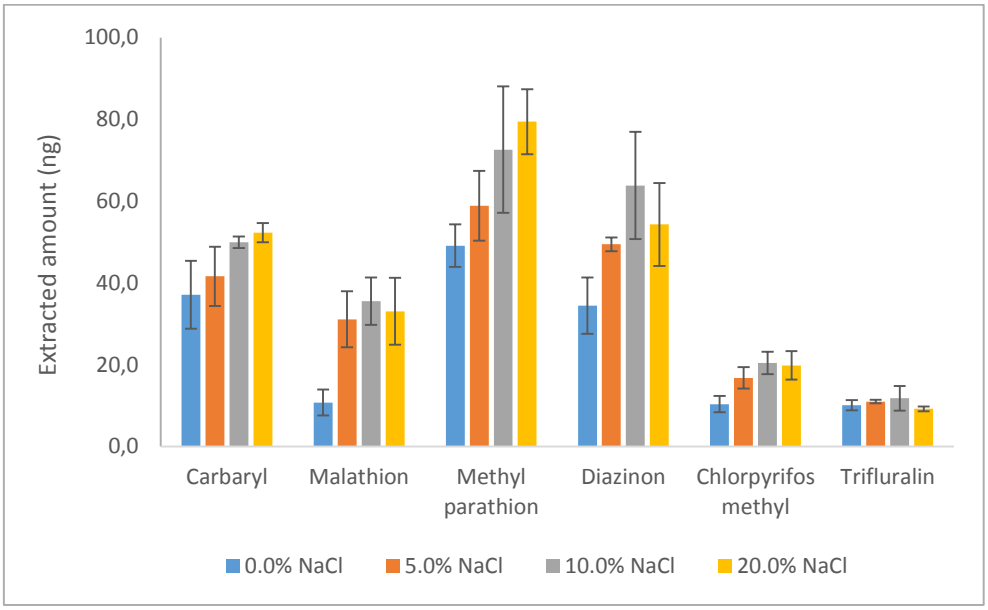


Figure 3.14. Effect of salt addition on extraction of pesticides from diluted apple juice. (Sample volume: 40.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation speed: 1000 rpm, desorption solvent: methanol, desorption volume: 0.600 mL, desorption time: 60 min, agitation speed: 1000 rpm

In a summary, it can be concluded that the salt addition increases the extraction recovery of analytes with a Log P value lower than 3 in complex matrices due to salting out effect. However, when matrix is diluted, the salt addition increases the extraction recoveries for compounds with Log P values both higher and lower than 3, suggesting that salting out and sample dilution must be evaluated at the same time to get the best sensitivity in the studied system. In further experiments, apple juice was diluted with water in half and 10% NaCl (w:v) was added in final samples to have a balanced sensitivity for each pesticide.

3.2.5 Validation of the developed TFME-GC-MS method

The developed TFME-GC-MS method was validated in terms of its accuracy and precision to show the suitability of the method for the analysis of real samples. During validation process, 100% apple juice was used as real samples. The samples were diluted with water in half and 10% NaCl (w:v) was added to final samples.

3.2.5.1 Validation of the developed TFME-GC-MS method

Real samples (fruit/vegetable juices) are complex samples; for this reason, to eliminate the effect of matrix components, matrix-matched calibration method is important. The matrix-matched external calibration curves were obtained for 0.0, 0.10, 0.25, 0.50. 1.0, 5.0, 10.0, 50.0, 100.0, 250.0 and 500.0 ng/mL analyte concentrations. 3 replicates of each calibration point were studied. The working range and LOQ was determined from back calculation of nominal concentration with acceptance for maximum ±20% relative error for limit of quantitation (LOQ) and ±15% for remaining points. The matrix-matched calibration curve for each analyte is shown in Figure 3.15. Based on these curves, LOQ was found to be 1.0 ng/mL for trifluralin and malathion while for carbaryl, diazinon, chlorpyrifos-methyl, and methyl-parathion were found as 5.0 ng/mL. The MRL values for selected pesticides vary between 10.0 to 20.0 ng/mL. The developed TFME-GC-MS method allows the determination of pesticides starting from 1.0 ng/mL using apple juice. LOQ is lower than the MRL values of pesticides, thus the selected pesticides can be determined sensitively to evaluate whether the juice is contaminated by pesticides or not.

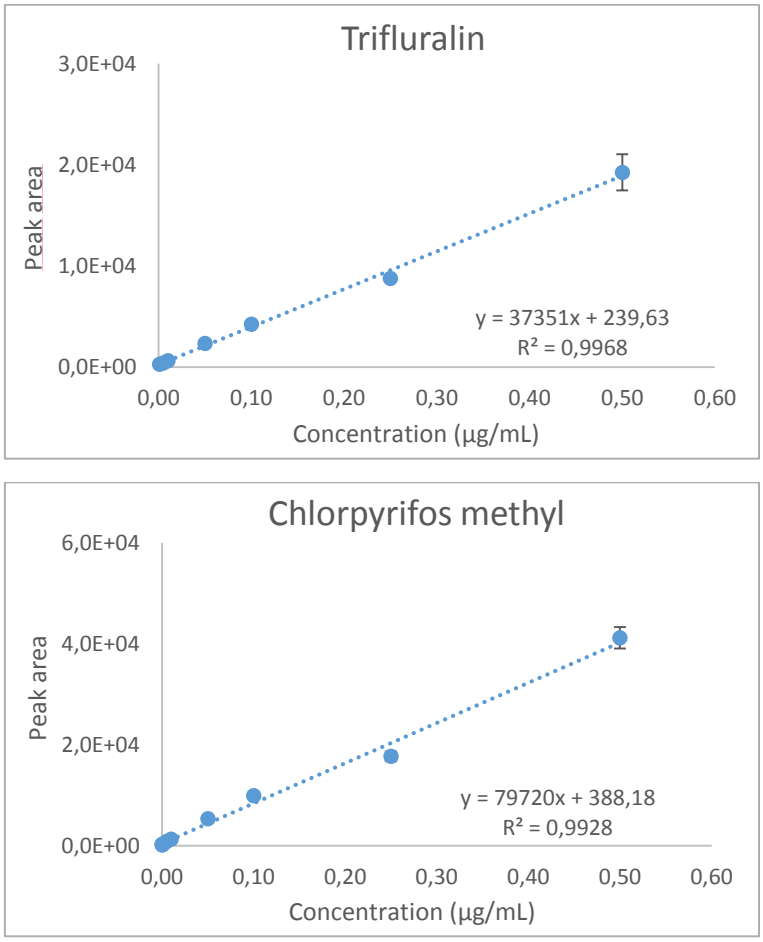


Figure 3.15. Matrix-matched external calibration curves obtained using apple juice:water (50:50 v:v) with 10% NaCl (w:v)

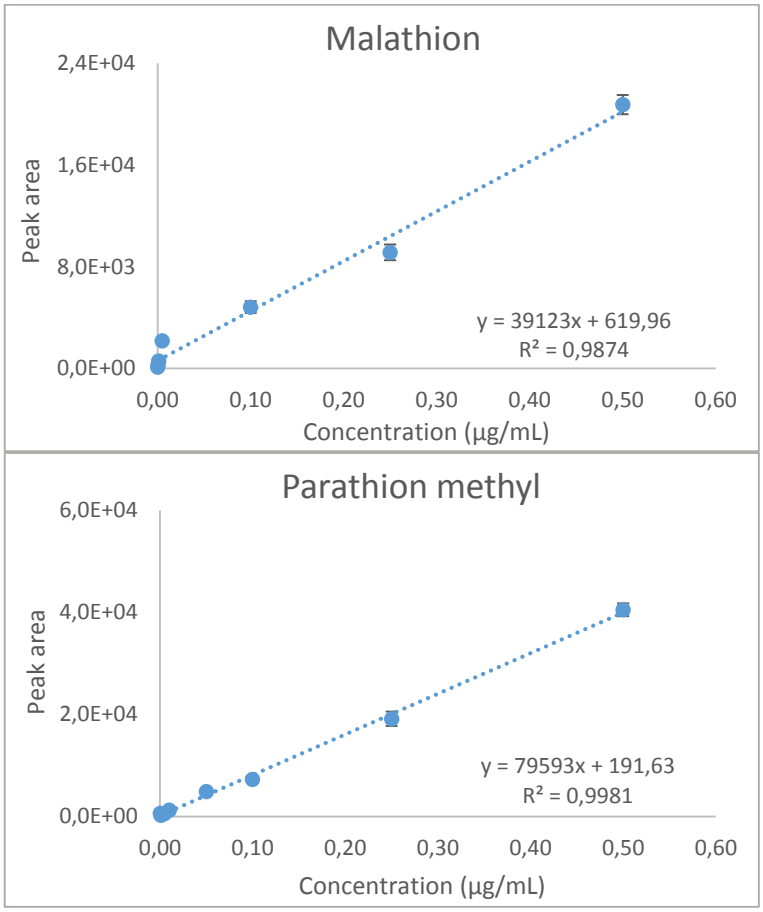


Figure 3.15 (cont'd.). Matrix-matched external calibration curves obtained using apple juice:water (50:50 v:v) with 10% NaCl (w:v)

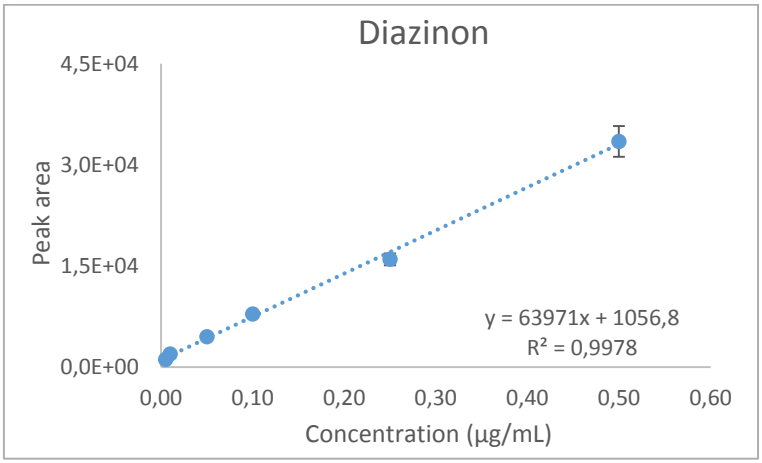


Figure 3.15. Matrix-matched external calibration curves obtained using apple juice:water (50:50 v:v) with 10% NaCl (w:v)

3.2.5.2 Accuracy of the developed TFME-GC-MS method

To show the accuracy of the developed TFME-GC-MS method a blind analyst experiment was conducted. For this purpose, three concentrations in working range were chosen including low-(LOQ), mid- and high-points of concentrations. The unknown concentrations of pesticides were determined using the regression equation obtained from the matrix-matched external standard calibration plot. The relative error for each point is given in Table 3.3. The method was found accurate with a RE% lower than 20%. Only for trifluralin 24.5% RE was found at 300.0 ng/mL. Analytes were spiked to apple juices from their stock solutions prepared in methanol. Higher volumes of addition of organic solvent might affect the extraction, and thus resulted in higher relative error.

Table 3.3. Accuracy of the TFME-GC-MS method in apple juice (n=3)

	Low-point		Mid-point		High-point	
	5.0 ng/mL		30.0 ng/mL		300.0 ng/mL	
Analyte	Calculated (ng/mL)	RE%	Calculated (ng/mL)	RE%	Calculated (ng/mL)	RE%
Trifluralin	5.0	0.3	34.1	13.8	373.6	**24.5**
Chlorpyrifos-methyl	4.9	1.4	30.9	3.0	324.4	8.2
Malathion	4.7	5.7	32.0	6.2	302.6	0.9
Parathion	5.3	6.0	26.1	12.9	283.9	5.4
Diazinon	5.1	1.4	30.0	0.3	287.8	4.1

3.2.5.3 Precision of the developed TFME-GC-MS method

The precision of the developed TFME-GC-MS method was shown by intra- and inter-day reproducibility. For intra-day repeatability, extractions were repeated three times in a day the protocol in spiked samples with low (5.0 ng/mL), mid (30.0 ng/mL), and high (300.0 ng/mL) concentrations. For inter-day repeatability, the developed TFME-GC-MS method was evaluated by repeating the extractions for three consecutive days with the same protocol in intra-day evaluations. The RSD% for intra-day and inter-day reproducibility is given in Table 3.4 and 3.5, respectively. The intra-day reproducibility of the TFME-GC-MS method varied between 6-21% RSD while inter-day reproducibility of the method varied between 3-20% RSD. Only for 300.0 ng/mL trifluralin higher than 20% RSD were found. In the present study, matrix matched external standard addition calibration was used. The variations could be lowered with the use of isotopologues of the analytes.

Table 3.4. Intra-day precision (RSD%) (n=3)

	Low-point	Mid-point	High-point

Analyte	5.0 ng/mL	30.0 ng/mL	300.0 ng/mL
Trifluralin	18.6	18.4	20.7
Chlorpyrifos-methyl	17.6	13.6	17.5
Malathion	17.9	9.5	7.9
Parathion-methyl	14.6	8.3	10.0
Diazinon	17.0	12.3	6.3

Table 3.5. Inter-day precision (RSD%) (n=3)

	Low-point	**Mid-point**	**High-point**
Analyte	5.0 ng/mL	30.0 ng/mL	300.0 ng/mL
Trifluralin	13.3	19.3	18.8
Chlorpyrifos-methyl	17.0	16.1	19.4
Malathion	15.4	2.6	11.9
Parathion-methyl	20.1	9.0	10.6
Diazinon	17.1	10.2	7.1

In overall validation studies, the accuracy, reproducibility and sensitivity of the method was found acceptable. The developed TFME-GC-MS method can safely be used for the multi-residual determination of pesticides in apple juice.

3.2.6 Extraction from solid samples

The main purpose of this part of the study is to show the applicability of the TFME samplers for on-site sampling of pesticides from agricultural areas using agarose gel as a model matrix.

3.2.6.1 Extraction time profile

After revealing the extraction time profile of analytes from aqueous samples, the extraction time profile was also investigated for the solid samples. To mimic the solid samples, 2% agarose gel was prepared, and sampling was performed by placing the membranes on the surface of the agarose gel. According to the results shown in Figure 3.15, the extraction time profile of each analyte is quite different than the extraction time profiles obtained in aqueous samples. None of the analytes reached equilibrium even at 60 min of sampling. As explained by Equation 1 given in Introduction Section, the extraction time required to reach the equilibrium is directly proportional to the boundary layer thickness, coating thickness, distribution coefficient of sorbent for the analyte, and it is inversely proportional to diffusion coefficient of the analyte. In this study, the sorbent thickness was same for both solid and liquid samples, as well as distribution constant did not change.

For small molecules D (the diffusion coefficient) is assumed to be 5.8×10^{-6} cm^2/s in aqueous medium and it decreases 5% in 2% agarose gel [137], which could be also considered as constant. Therefore, the only parameter that changed was the boundary layer thickness surrounding the sorbent which decreases with agitation speed. Since the driving force for the extraction is chemical potential difference between the sorbent surface and bulk of the sample, analyte is extracted via passive diffusion through the boundary layer, and the thickness of the boundary layer plays critical role in extraction kinetics. Therefore, faster kinetic extraction in agitated sample compared to static conditions is reasonable.

In fact, the mean squared displacement ($< x >^2$) can be calculated by Fick's diffusion law and can be used to predict at given extraction time from how far a molecule can reach the sorbent surface. In this formula d is the dimensionality of the system; D is the rate of the molecular diffusion and t is time of sampling:

$$< x^2 >= 2dDt$$

By assuming that the average value for diffusion coefficient in agarose gel is 5.51× 10^{-6} cm^2/s, and sampling was performed for 60 min, it can be calculated that only analytes that are present within 2.0 mm distance from the extractive phase can be extracted by the coating. This further suggests that equilibrium is not possible for relatively short extraction times from the gel.

Although at longer times, more analytes will be extracted from the gel, for the sampling from solid samples, extraction time was chosen as 60 min which is sufficient to get quantitative results.

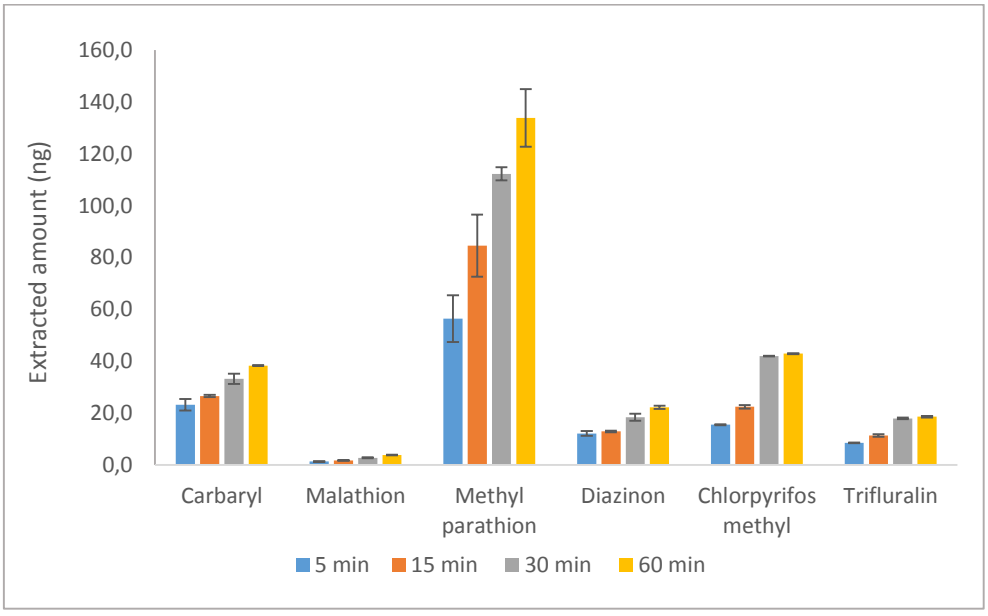


Figure 3.16. Extraction time profile of pesticides in 2% agarose gel (Gel volume: 50.0 mL, analyte concentration: 250.0 ng/mL, extraction time: 60 min, agitation:

static, desorption solvent: methanol, desorption volume: 0.600 mL, desorption time: 60 min, agitation speed: 1000 rpm)

3.2.6.2 Pesticide distribution analysis

To further show the suitability of the samplers for the on-site analysis, a pesticide profiling study was conducted. 2% agarose gel spiked to contain 0.0, 10.0, 25.0, 50.0, 100.0, 250.0, 500.0 and 750.0 ng/mL pesticide mixture was used to mimic the nature of vegetables/fruits. The extracted amount of each pesticide is shown in Figure 3.16. All the pesticides could be detected in agarose gel in all tested concentrations, while methyl-parathion could be detected at 10.0 ng/mL as the lowest concentration.

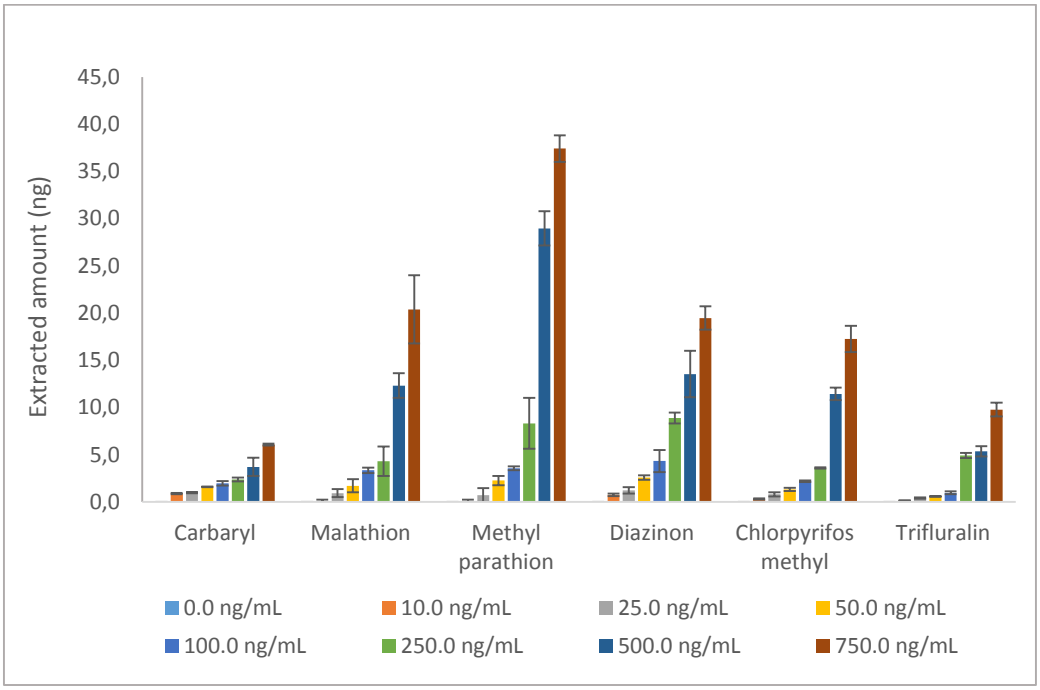


Figure 3.17. Pesticide distribution analysis in 2% agarose gel (gel volume: 2.0 mL, extraction time: 60 min, agitation: static, desorption solvent: methanol, desorption volume: 0.600 mL, desorption time: 60 min, agitation speed: 1000 rpm)

Sampling from solid samples for the determination of pesticides is quite important to evaluate whether a crop can be consumed safely or not. To have a healthy crop, farmers may mix the pesticides and use them as pesticide cocktails. Besides that, they also may apply pesticides on the surface of crops to for multiple times before harvesting them. The concentration on the front side of the crop can be higher while the back side does not contain pesticides. In such cases, to show whether a crop is contaminated by pesticides or not, sampling from different positions can be performed. TFME samplers were used to show their ability to provide spatial resolution, different concentrations of pesticides were spiked to 2% agarose gel, and they were poured in the wells of 96-well plate randomly. Spiked concentrations of pesticides in conditional format were illustrated in Figure 3.17. The color

abundances represent the concentrations of the pesticide mixture at the specific point. The obtained results matched well with spiked concentrations, showing the potential of the samplers for detection of distribution of pesticides through the solid surfaces (a chemical mapping of the surface). The MRL values for the selected pesticides vary between 10 and 20 ng/mL, the samplers were capable to differentiate the different concentrations. According to the preliminary investigation, on-site sampling can be performed allowing the sampling without plucking the crop.

Representative heat map:

A	1	2	3	4	5	6	7	8	9	10	11	12
B		0		50		25		100		10		
C	250					500					750	
D		10					0					
E		750		100		50				250		25
F	25											
G				750			250		500		0	
H		500	100		10			50				

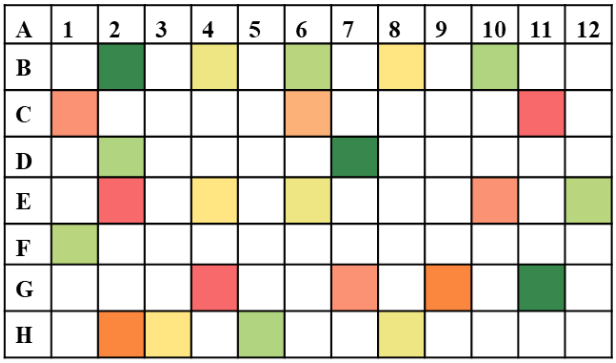
Heat map for trifluralin:

A	1	2	3	4	5	6	7	8	9	10	11	12
B												
C												
D												
E												
F												
G												
H												

Figure 3.18. Pesticide distribution heat map for 2 mL of 2% agarose gel with different concentrations (gel volume: 2.0 mL, extraction time: 60 min, agitation:

static, desorption solvent: methanol, desorption volume: 0.600 mL, desorption time: 60 min, agitation speed: 1000 rpm)

Heat map for carbaryl:



Heat map for chlorpyrifos-methyl:

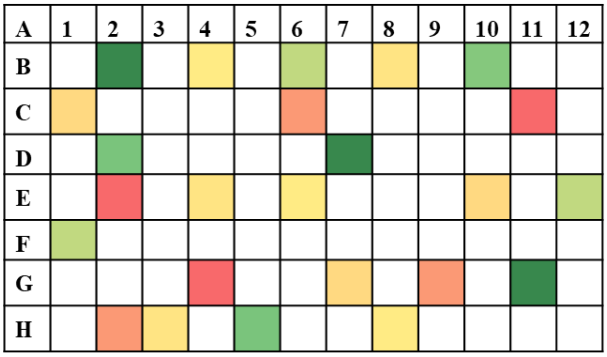
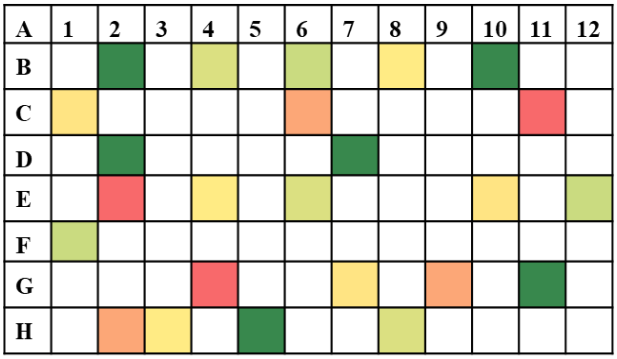
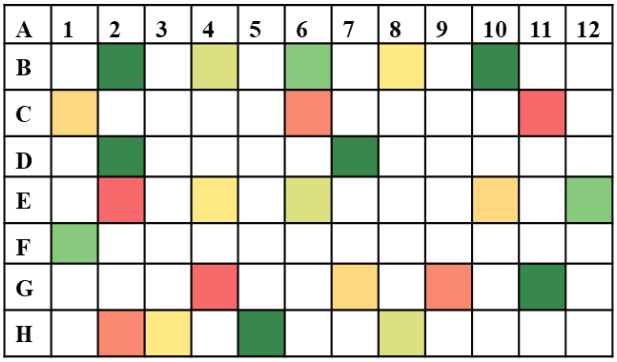


Figure 3.18 (cont'd.). Pesticide distribution heat map for 2 mL of 2% agarose gel with different concentrations

Heat map for malathion:



Heat map for methyl-parathion:



Heat map for diazinon:

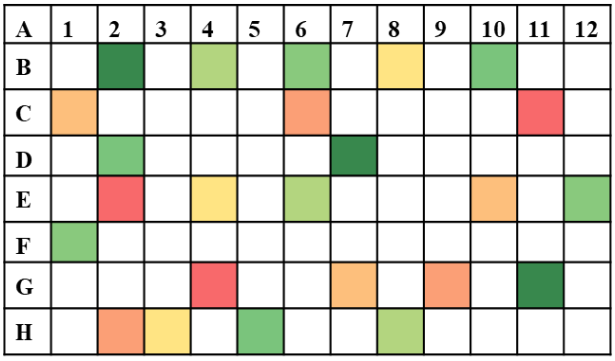


Figure 3.18. Pesticide distribution heat map for 2 mL of 2% agarose gel with different concentrations

3.3 Summary and conclusion

One of the concerns around the world is the uncontrollable use of agrochemicals. Controlling pesticides in the field is important for public and environmental health. For this reason, a maximum tolerable concentration is defined for each pesticide, to define the safety threshold for human health. To not exceed these levels, farmers may apply pesticides as pesticide cocktails, but the overall synergetic effect of these cocktails on human health is not fully investigated yet. At this point, there is a need for methods that are suitable for the multi-residual determination of pesticides even below their threshold levels. Therefore, the second microextraction application in the thesis was focused to develop HLB/PTFE TFME samplers and a new, reliable TFME-GC-MS method for the simultaneous analysis of six pesticides, including trifluralin, chlorpyrifos-methyl, carbaryl, diazinon, and malathion. As a first step, TFME samplers were prepared by coating the surface of the carbon fabric with HLB particles immobilized in thermal and solvent stable polyfluorinated polymeric binder. The particles were homogeneously coated (30 μm coating thickness) on the surface of the fabric using a thin film applicator. Following the preparation of the TFME devices general sorption characteristics of the coating for selected pesticides was investigated. In these studies, it has been found that 30 min desorption with methanol is sufficient to quantitatively desorb all pesticides from the extractive phase, 60 min extraction time provides sufficient sensitivity in a reasonable time (although equilibrium is not reached for some of the pesticides), and sample pH has significant effect on the adsorption (with a maximum at pH 5.0) of pesticides and increases under slightly acidic conditions.

For real matrix application, apple juice was selected as matrix and the effect of ionic strength specifically was studies together with sample dilution. Our results suggested that adding salt to the sample enhances the extraction of the pesticides to a maximum

value, and with further increasing NaCl amount results in sorption decrease. It has been also found that the optimum salt concentration depends on dilution of the apple juice. This value was found as 10% NaCl for pure apple juice, varied between 5 to 10% NaCl for half diluted apple juice, and 5% NaCl for 10% apple juice. Dilution of the sample to half with 10%NaCl (w/v) in final samples was found as the optimum matrix normalization before the extraction.

Moreover, method validation was performed to investigate the accuracy, reproducibility, and sensitivity of the method. The LOQ for trifluralin and malathion was 1.0 ng/mL while for carbaryl, diazinon, chlorpyrifos-methyl, and methyl-parathion was 5.0 ng/mL, showing sufficient sensitivity for all pesticides to meet their MRL values. Besides, the final method showed acceptable precision for intra- and inter-day reproducibility with ≤20% relative standard deviation (RSD%) and accuracy of ≤15% relative error (RE%), except for trifluralin at 300.0 ng/mL level, suggesting that the method can be used safely for the analysis of pesticides in apple juice samples.

Also, because the TFME samplers can be used for the multi-residual determination of pesticides in the field, sampling was performed under static conditions using 2% agarose gel to represent an on-site sampling from the fruit surface. The extraction time profile of each pesticide investigated in static conditions showed that for none of the analyte equilibrium is reached, but even from 5 min sampling, quantitative results can be obtained. Moreover, for 60 min sampling, a heat map for each pesticide was generated to show the ability of TFME samplers to differentiate the pesticide concentrations in solid samples. All the concentrations of pesticides were successfully differentiated. The TFME samplers fit for the on-site sampling by enabling spatial resolution of chemical changes on a surface.

In overall, it can be concluded that the HLB/PTFE-based extractive phases will gain attention in further SPME and TFME applications due to their solvent and thermal stability. Although it was not used in direct thermal applications in this study due to the absence of large volume thermal desorber, the sampler can provide information about both volatile, semi-volatile, and nonvolatile compounds in the system.

CHAPTER 4

CONCLUSION

SPME can be considered the chameleon of sample preparation as has numerous advantages including, flexibility in design, *in vivo* applicability, on-site sampling, etc. Although the technology has many advantages, not all of these advantages are present in a single device. Still, based on the nature of the study, different extractive phases need to be considered, which is troublesome, especially for new users. In this regard, the combination of HLB polymeric particles with PTFE AF polymer offer a universal extractive phase with unique properties. Because the HLB particles contain both polar and nonpolar moieties in their polymeric chain, the HLB/PTFE AF-based extractive devices are suitable for extraction of molecules with a wide range of physicochemical properties. PTFE AF is inert, biocompatible, solvent and thermal stable polymer with porous structure, making it suitable binder for extractive particles. Combining HLB and PTFE AF, both stable under temperatures typically used for thermal desorption in GC (250- 275 °C) and typical solvents, results in samplers which can be coupled with GC and LC. These features make the resulting SPME based tools suitable for a wide range of applications (clinical, environmental, pharmacokinetic, exposome, etc.). In the scope of this thesis, HLB/PTFE AF-based extractive devices were prepared in two different geometries and used for the first time in two critical applications.

In the first part of the thesis, SPME fibers were prepared with 10.0 mm and 2.0 mm coating lengths. The regular SPME fibers (10.0 mm coating length) were optimized for studies in which relatively large volume of samples are available while miniaturized SPME fibers (2.0 mm coating length) were optimized for studies in

which spatial resolution through tissue/tumor, small sample volumes and studies in which non depletive sampling are needed. Miniaturized fibers, except for the most nonpolar analyte, DSPC, provided non-depletive extraction (less than 5% analyte was extracted) which makes them suitable for time course analyses. Moreover, the validation studies showed that the fibers provide sufficient accuracy and reproducibility for further applications. In the future studies, the miniaturized SPME fibers will be used for the untargeted screening of molecules present in 2D and 3D cell cultures before and after the administration of the anti-cancer drug, and *in-vivo* animal sampling.

In the second part of the thesis, an extractive device with the same HLB/PTFE AF combination was prepared in the thin film geometry with primary aim to increase the sensitivity of the device. Because the resultant thin films have a larger surface area, more analytes can be extracted from the sample within several minutes (kinetic regime of extraction). This feature allows for performing sensitive analysis even for on-site sampling which is critical for environmental studies. Thus, the capability of the TFME devices for collected samples were shown by developing a method for analysis of pesticides in apple juice and for on-site analysis using agarose gel to represent semi-solid samples. The method developed for apple juice analysis showed quantitation limits (1.0 to 5.0 ng/mL) sufficient to detect selected pesticides even below their maximum allowable limits. Furthermore, to show the suitability of the TFME samplers for on-site sampling, a pesticide distribution analysis was performed, and a heat map was generated for each pesticide. The developed thin film samplers differentiated the pesticide concentrations on a semi-solid surface in a range of 10.0 and 750.0 ng/mL. These results suggest that using these samplers, a crop can be investigated whether it is contaminated by pesticide(s) or not. The promising results summarized here show that the samplers can provide a high spatial

resolution in terms of different concentrations and can be implemented in future for on-site applications.

In conclusion, it can be said that the novel HLB/PTFE AF extractive phase, as expected, has potential to be considered as universal extractive phase for SPME based applications. The two successful studies shown in this thesis are only representative applications showing its capability in two areas which require completely different needs.

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Anal. Chem., vol. 91, no. 15, pp. 10141–10148, 2019, doi: 10.1021/acs.analchem.9b02157.

APPENDICES

A. LC-MS chromatograms of analytes

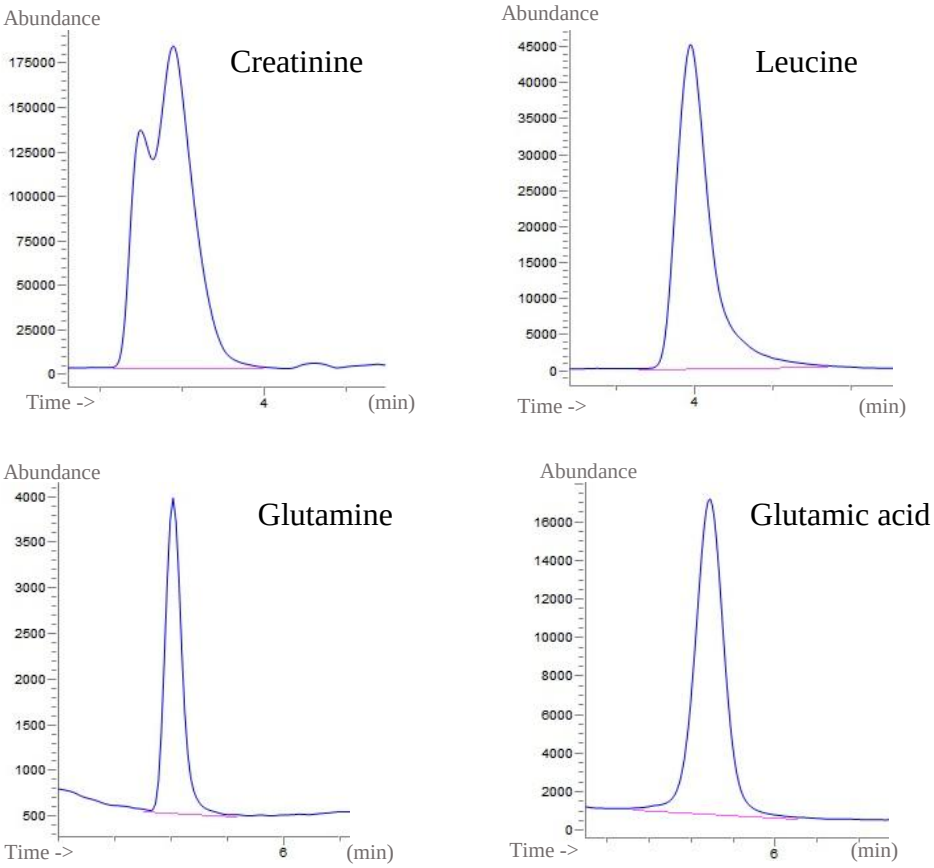

Figure A.1. LC-MS chromatograms of analytes obtained in SIM mode (concentration: 500.0 ng/mL).

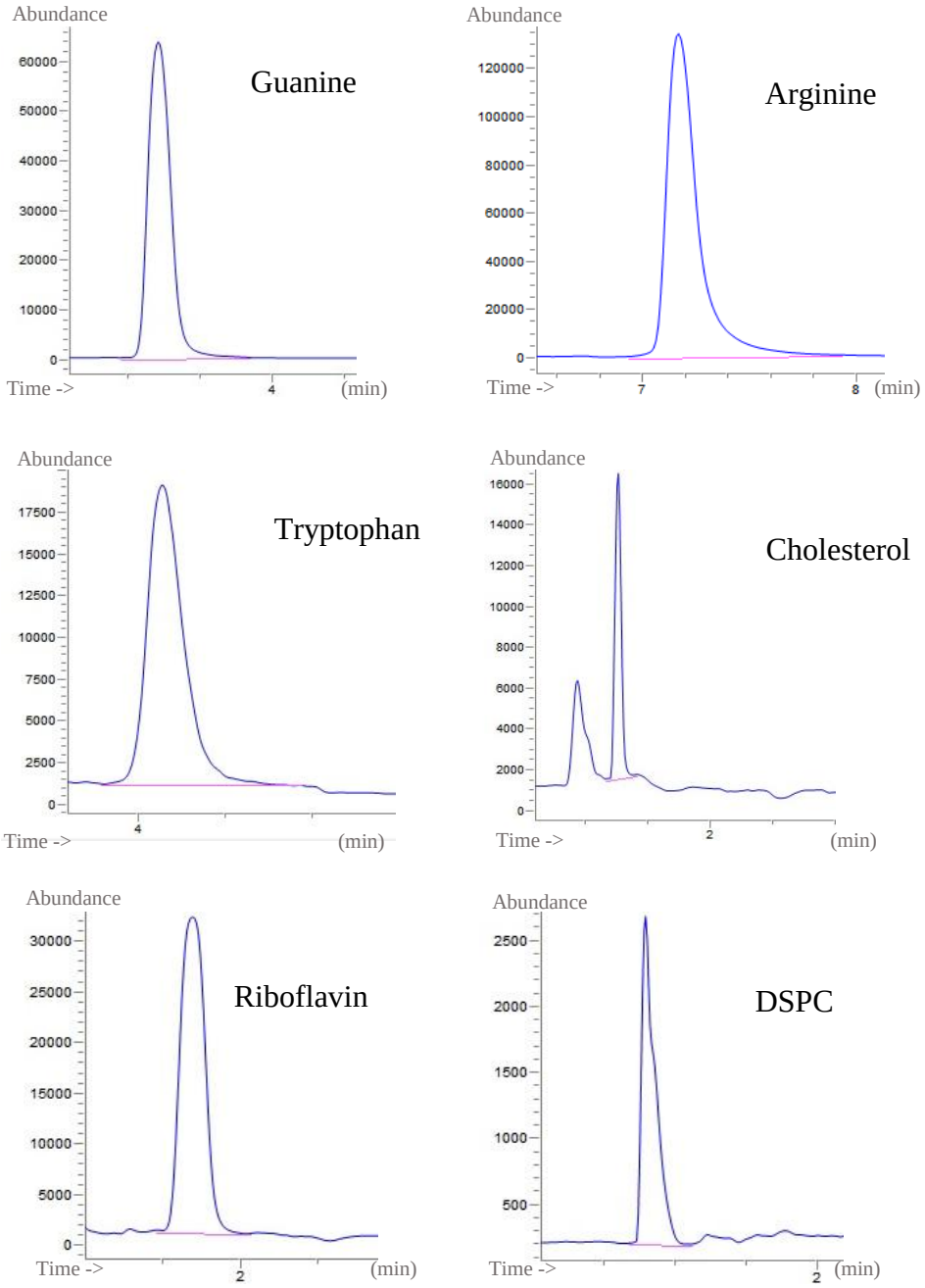

Figure A.1. LC-MS chromatograms of analytes obtained in SIM mode (concentration: 500.0 ng/mL).

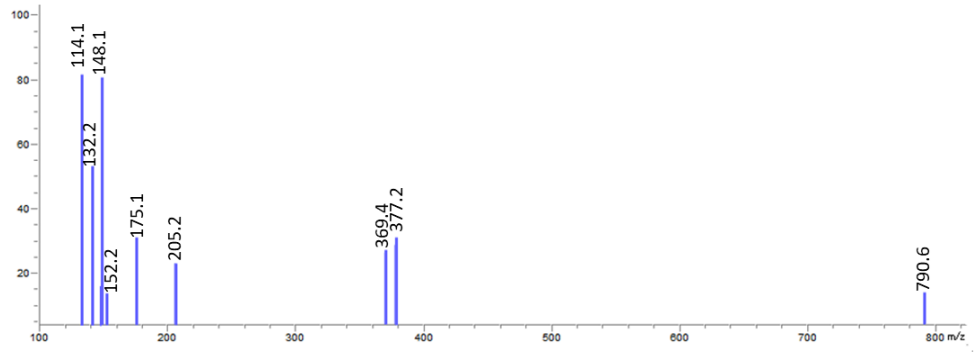

Figure A. 2. Mass spectrum of creatinine (m/z=114.1), leucine (m/z=132.1), glutamine (m/z=147.1), glutamic acid (m/z=148.1), guanine (m/z=152.2), tryptophan (205.2)7 cholesterol (m/z=369.4), riboflavin (m/z=377.2), DSPC (m/z=790.6)

B. GC-MS chromatograms of pesticides used in the study

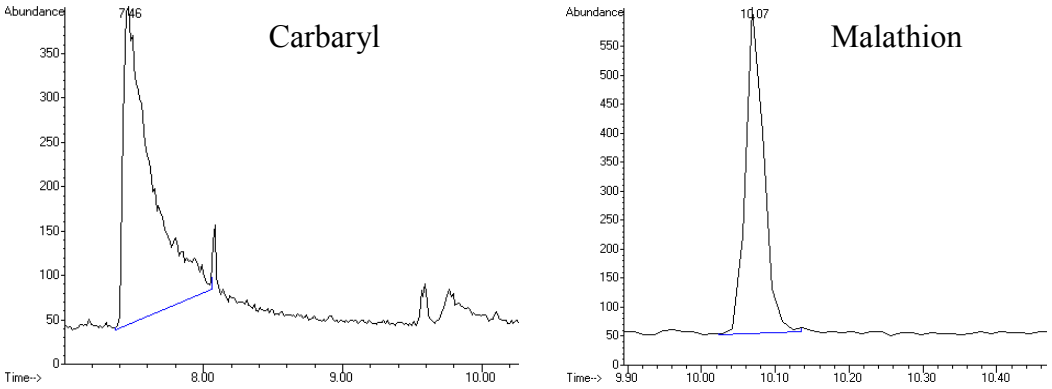

Figure B.1. GC-MS chromatograms of pesticides obtained in SIM mode (concentration: 500.0 ng/mL).

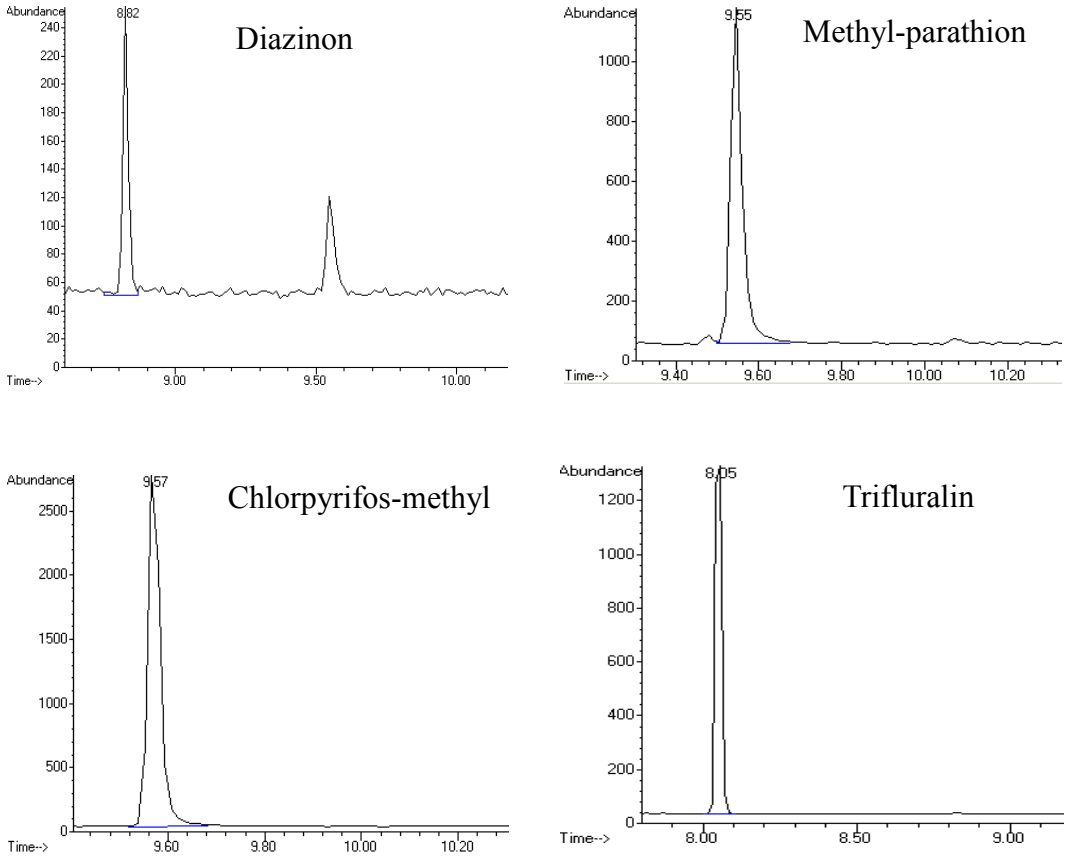

Figure B.1. GC-MS chromatograms of pesticides obtained in SIM mode (concentration: 500.0 ng/mL).

C. Mass spectra of pesticides (electron impact ionization)

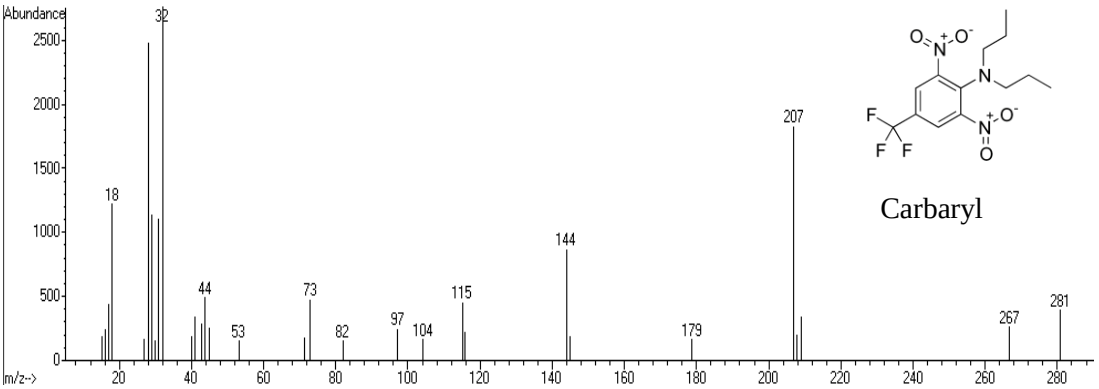

Figure C. 1. Mass spectrum of carbaryl obtained in GC-MS full scan mode.

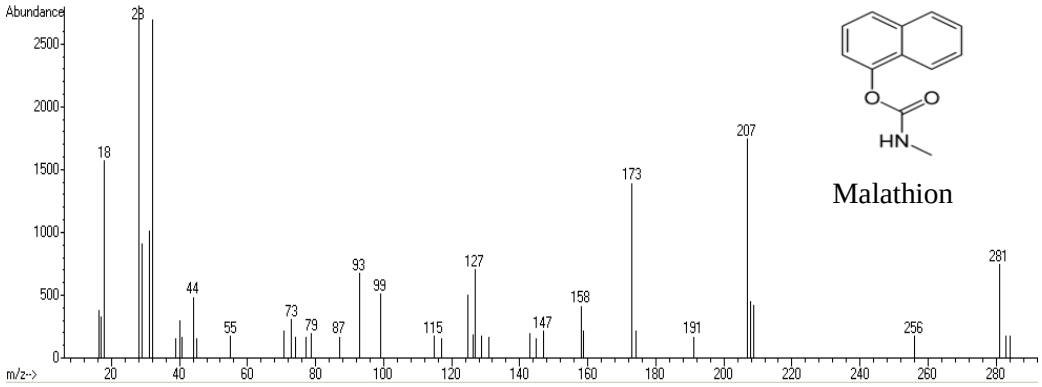

Figure C. 2. Mass spectrum of malathion obtained in GC-MS full scan mode.

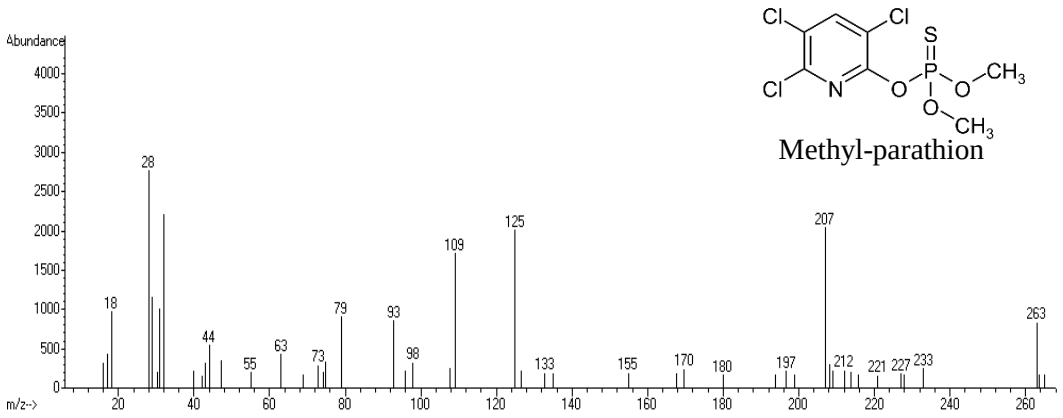

Figure C. 3. Mass spectrum of methyl-parathion obtained in GC-MS full scan mode.

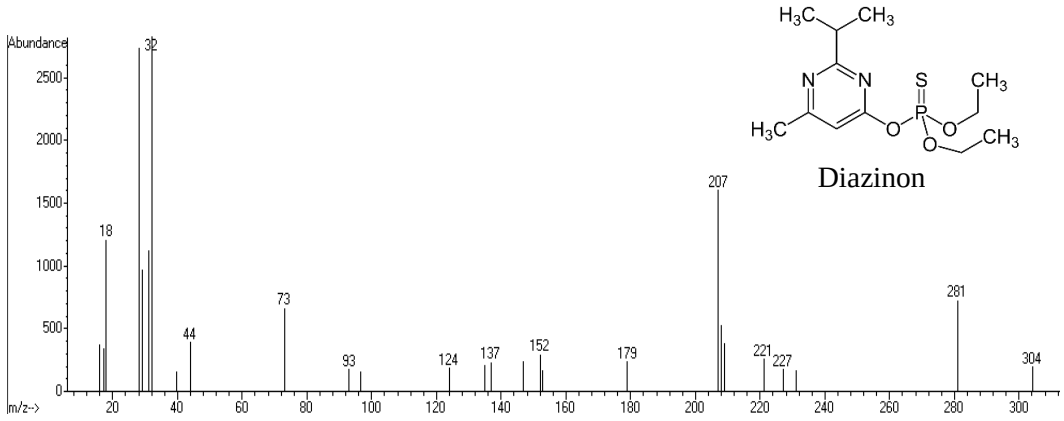

Figure C. 4. Mass spectrum of diazinon obtained in GC-MS full scan mode.

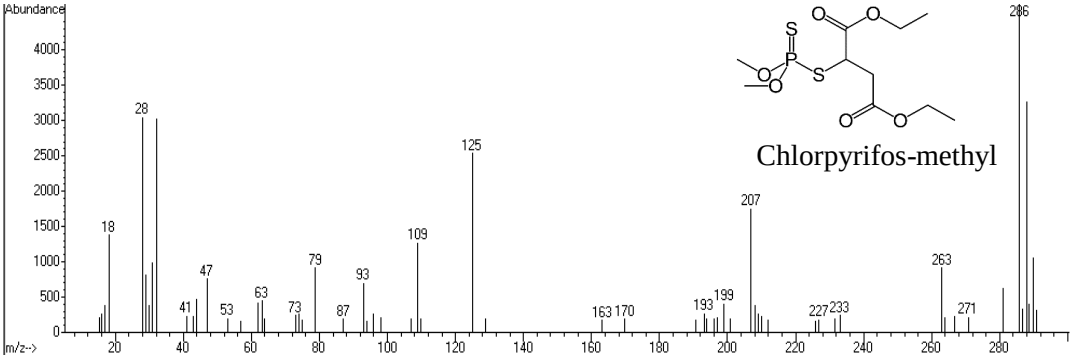

Figure C. 5. Mass spectrum of chlorpyrifos-methyl obtained in GC-MS full scan mode.

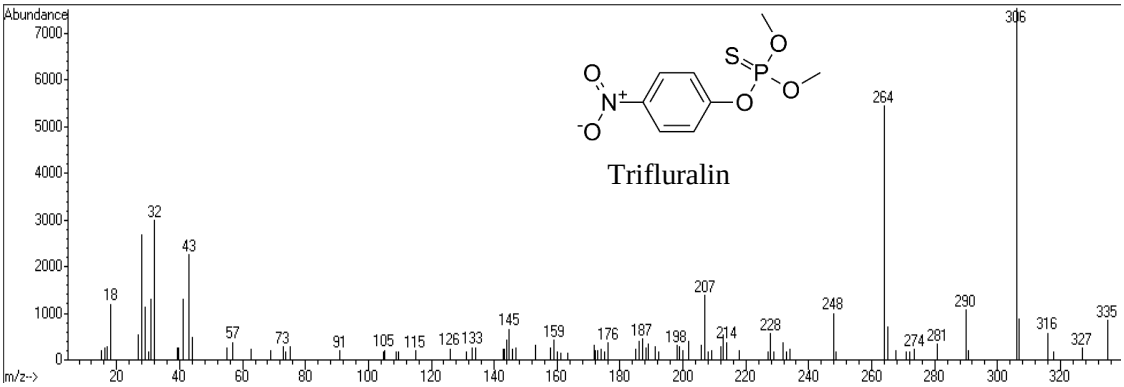

Figure C. 6. Mass spectrum of trifluralin obtained in GC-MS full scan mode.