UNIVERSITÀ DELLA CALABRIA

UNIVERSITY OF CALABRIA

Department of Pharmacy, Health and Nutritional Science

Department of Chemistry and Chemical Technologies

Ph.D. in

Translational Medicine

CYCLE

XXXI

THESIS TITLE

Development and Optimization of analytical protocols based on microextraction techniques for clinical screening and environmental control

SSD Analytical Chemistry, CHIM/01

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To this sweet moment…

Abstract

The development of analytical protocols for the determination of analytes at trace levels in complex matrices (e.g. biological fluids or contaminated water) is a crucial point for the environmental assessment and monitoring as well as for scientific research in the field of disease biomarkers. An essential part of analytical method development is represented by sample preparation due to its significant impact on most of the subsequent steps and the data quality. In recent years, the application of pro-ecological, automated, solvent-free sample preparation approaches or techniques employing a minimal amount of solvents or safe and non-toxic extractants has become one of the most popular research topics in analytical chemistry. In this context, microextraction techniques represent a suitable choice for the extraction of analytes from complex matrices because these techniques use less organic solvent and allow to perform in a single step extraction and concentration of analytes. Moreover, the use of microextraction techniques for sample preparation reduces the number of errors that commonly result from multi-stage procedures, and limits the negative impact on the environment and the health of analytical chemists performing laboratory work.

The goal of this Ph.D project was the development and optimization of analytical methods based on the use of microextraction techniques for the assay of disease biomarkers and environmental contaminants in biological fluids and environmental matrices. The microextraction techniques employed in this thesis were solid phase microextraction (SPME) and microextraction by packed sorbent (MEPS). SPME was used to evaluate the applicability of a new fiber (PDMS/DVB/PDMS) as analytical sampling tool for investigation in raw human urine. The PDMS/DVB/PDMS fiber was exploited to develop a DI-SPME-GC-MS method for the assay of polycyclic aromatic hydrocarbons (PAHs) with 2-6 aromatic rings in untreated human urine samples. Moreover, in the light of the increasing demand of faster and easier protocols allowing the assessment of disease biomarkers, SPME was applied to develop a reliable and rapid GC-MS approach for the determination of polyamines in human urine. Indeed, polyamines are widely recognized as among the most important cancer biomarkers for early diagnosis and treatment. SPME was also applied for the extraction of nine phthalates monoesters in urine samples. These compounds are important metabolites of phthalates and their assay can reliably rank exposures to phthalates over a period. MEPS was used to extract organophosphate ester flame retardant in aqueous matrices and, again, monoesters phthalates in urine. In both methods, in order to improve method sensitivity, programmed temperature vaporization (PTV) was chosen as gas chromatographic injection technique. For polyamines and phthalates monoesters, a prior derivatization step with suitable reagents was carried out before gas chromatographic analysis so as to improve chromatographic elution and resolution by decreasing volatility and polarity of analytes. Derivatization reaction was performed directly in aqueous samples

using alkyl chloroformates. The combined use of alkyl chloroformate as derivatizing reagent and SPME for analyte extraction was chosen to develop a simple protocol involving minimal sample handling and no consumption of toxic organic solvents. The variables affecting the different steps of the proposed protocols were optimized by the multivariate approach of experimental design which has allowed for the simultaneous investigation of the different factors in the entire experimental domain and the possible synergic effects between variables. In this thesis, experimental design was used to optimize the parameters influencing SPME extraction, MEPS extraction, PTV process and derivatization reaction. Gas chromatographic analyses were carried out using a GC-QqQ-MS instrument in selected reaction monitoring (SRM) acquisition which has allowed to obtain reconstructed chromatograms with well-defined chromatographic peaks and to achieve high specificity through the selection of appropriate precursor-product ion couples, improving the capability in analyte identification. Finally, during the period as visiting Ph.D student at University Duisburg-Essen, Faculty of Chemistry, Instrumental Analytical Chemistry, the object of research activity, coordinated by Professor Torsten C. Schmidt, concerned the extraction of fatty acid methyl esters (FAMEs) in wastewater by solid phase microextraction arrow (SPME arrow).

Riassunto

Lo sviluppo di protocolli analitici per la determinazione di analiti in tracce in matrici complesse (ad esempio fluidi biologici o acque contaminate) è un punto cruciale per la valutazione e il monitoraggio ambientale, nonché per la ricerca nel campo dei biomarcatori di patologie. Una parte fondamentale nello sviluppo di un metodo analitico è rappresentata dalla preparazione del campione in quanto questa ha un impatto significativo sulla maggior parte delle fasi successive e sulla qualità del dato. Negli ultimi anni, l'utilizzo di approcci di preparazione del campione pro-ecologici, automatizzati e privi di solventi o di tecniche che impiegano una quantità minima di solventi o solventi sicuri e non tossici ha registrato un notevole impulso nella chimica analitica. In questo contesto, le tecniche di microestrazione rappresentano una scelta opportuna per l'estrazione di analiti da matrici complesse in quanto queste utilizzano meno solvente organico e consentono di eseguire in un'unica fase l'estrazione e la concentrazione degli analiti. Inoltre, l'uso delle tecniche di microestrazione riduce il numero di fonti di errori che comunemente derivano da procedure a più stadi e limita l'impatto ambientale e quello sulla salute dei chimici che svolgono l'attività di laboratorio.

L'obiettivo di questo progetto di dottorato è stato lo sviluppo e l'ottimizzazione di metodi analitici basati sull'uso di tecniche di microestrazione per la determinazione di biomarcatori di patologie e contaminanti ambientali in fluidi biologici e matrici ambientali. Le tecniche di microestrazione utilizzate in questa tesi sono state la microestrazione in fase solida (SPME) e la microestrazione con sorbente impaccato (MEPS). L'SPME è stato utilizzato per testare l'applicabilità di una nuova fibra (PDMS/DVB/PDMS) in analisi condotte direttamente in urina umana non trattata. Questa fibra è stata utilizzata per lo sviluppo di un metodo DI-SPME-GC-MS per l'analisi di idrocarburi policiclici aromatici (IPA) da 2 a 6 anelli aromatici in campioni di urina umana non trattata. Inoltre, alla luce della crescente domanda di protocolli rapidi e semplici per la determinazione di biomarcatori di patologie, l'SPME è stato applicato con l'obiettivo di sviluppare un metodo GC-MS affidabile e rapido per la determinazione di poliammine in urina umana. Infatti, le poliammine sono ampiamente riconosciute tra i più importanti biomarcatori del cancro per giungere a diagnosi e trattamento precoci. L'SPME è stato anche applicato per l'estrazione di nove ftalati monoesteri in campioni di urina. Questi composti sono importanti metaboliti degli ftalati e la loro presenza in urina può essere correlata in modo affidabile all'esposizione del soggetto agli ftalati stessi. MEPS è stato utilizzato per l'estrazione di ritardanti di fiamma organofosfati da matrici acquose e, di nuovo, di ftalati monoesteri da urine. Per entrambi i metodi, al fine di migliorare la sensibilità del metodo, è stata scelta la vaporizzazione a temperatura programmata (PTV) come tecnica di iniezione gascromatografica. Per poliammine e ftalati monoesteri, prima dell'analisi gascromatografica è stata effettuata una preliminare reazione di derivatizzazione in modo ottenere una migliore eluizione e risoluzione

cromatografica tramite la diminuzione della volatilità e della polarità degli analiti. La reazione di derivatizzazione è stata eseguita direttamente in matrice acquosa utilizzando alchil cloroformiati. La scelta dell'uso combinato dell'alchil cloroformiato come reagente derivatizzante e dell'SPME per l'estrazione degli analiti è stata fatta nel tentativo di sviluppare un protocollo semplice che coinvolgesse un trattamento minimo del campione e nessun consumo di solventi organici. Le variabili che influenzano le diverse fasi dei protocolli proposti sono state ottimizzate tramite l'approccio multivariato del disegno sperimentale che consente l'analisi simultanea dei diversi fattori nell'intero dominio sperimentale e i possibili effetti sinergici tra le variabili. In questa tesi, il disegno sperimentale è stato utilizzato per ottimizzare i parametri che influenzano l'estrazione SPME, l'estrazione MEPS, il processo PTV e la reazione di derivatizzazione. Le analisi gascromatografiche sono state eseguite utilizzando uno strumento GC-QqQ-MS in modalità "selected reaction monitoring" (SRM) che ha permesso di ottenere cromatogrammi ricostruiti con picchi cromatografici ben definiti e di raggiungere, attraverso la selezione di appropriate coppie di ioni precursore-prodotto, un'elevata specificità, migliorando così l'affidabilità nell'identificazione degli analiti. Infine, durante il periodo come "visiting Ph.D student" all'Università Duisburg-Essen, Facoltà di Chimica, Chimica Analitica Strumentale, l'oggetto dell'attività di ricerca, coordinata dal Prof. Torsten C. Schmidt, ha riguardato l'estrazione degli esteri metilici degli acidi grassi (FAMEs) da acque reflue mediante solid phase microextraction Arrow (SPME Arrow).

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Chapter 1

Microextraction techniques in sample preparation

1.1 Introduction

The development of an analytical method involves several steps, that can be summarized in sampling, sample preparation, instrumental analysis and data analysis. Each step can influence the analytical protocol and the other steps, but mistakes made during the sample preparation cannot be corrected in the following steps. The extraction and sample preparation represent the crucial step of the analytical process although the remarkable advances in chromatography and detection techniques. The main purposes of the preparation sample step are:

- The extraction of interest analytes and the removal of interfering species;
- The conversion of the analytes in another forms amenable to instrumental analysis;
- The improvement of the sensitivity by concentrating the analytes.

Sample preparation has always represented the potentially most time-consuming and error-prone part of the analytical protocol. Many of conventional sample preparation protocols, used today, are still based on classic liquid-liquid extraction (LLE), solid phase extraction (SPE) or Soxhlet extraction in the case of solid sample. For these techniques the extraction is carried out several times in order to improve the recovery and very large sample and organic solvent volumes are required [1]. The use of large solvent volumes involves next concentration and purification steps that can lead to the loss of analytes. On the other hand, organic solvents are toxic, expensive, harmful to the environment and lead to additional work and cost for the subsequent disposal. Due to these aspects, one of the main purposes in the development of new extraction technique is to minimize the use of organic solvents [2]. In this context, the principal request for the research is the development of a new sample preparation techniques with the main purpose to analyze a large number of samples in the shortest time minimizing the solvent use than conventional techniques. The development of a new sample preparation approach should involve:

- Improvement the recovery and reproducibility;
- Reduction extraction times and costs;

- Sample preparation on-line with analytical technique, automation of the process;
- Miniaturization of the extraction process.

In the light of this, new methods aim at developing sample preparation approach based on less solvent use, fewer steps during the extraction, the possibility to purify and concentrate the compounds in the sample during the extraction reducing the sources of error. In this context, the microextraction techniques (METs) represent a good choice for the extraction of analytes from complex matrices [3]. The term "microextraction" derives from the use of a smaller amount of stationary phase and therefore of less organic solvent. METs are user-friendliness and sensitive, minimize laborious applications and allow to process many samples in a shortest time with more reproducibility and less environmental impact. Moreover, METs opened new possibilities in several fields, such as analysis of biological samples and environmental waters. METs can be divided into two groups: liquid- liquid extraction or solid-phase extraction, as shown in the following scheme (***Figure 1.1***) [4].

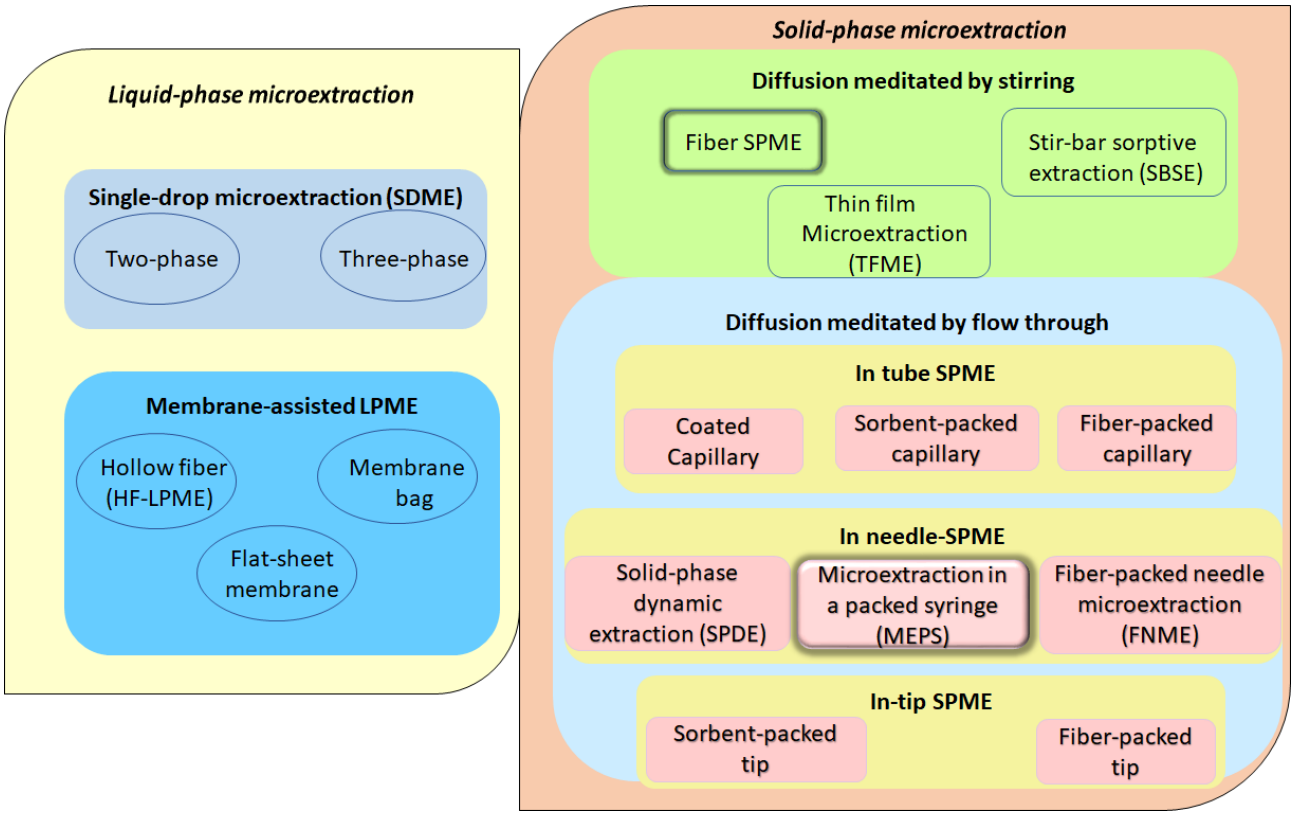

Figure 1.1: Classification of METs.

The classification of solid-phase microextraction techniques is based on the mechanism of dispersion of the analyte in the stationary phase [5, 6]. The diffusion of analyte can be mediated by stirring, as

in the case of fiber solid phase microextraction (SPME) or by flow-through as in the case of microextraction in packed sorbent (MEPS). Among the different microextraction techniques, SPME and MEPS are the most wide-spread and well-known microextraction technique. They are fast and simple and include automation, high-throughput performance, and greater sensitivity than other extraction techniques. The development of this techniques includes also their automation that allows reduction in analysis time and improvement in sample reproducibility and throughput [7].

1.2 Solid Phase Microextraction

1.2.1 Introduction

Solid phase microextraction was the first successful modern MET, invented by Pawliszyn and Arthur in 1990 [8]. The active element is a fused silica fiber covered with a stationary phase layer of different nature depending on the analytes to extract. The ability to perform the extraction and the concentration of analytes in a single step allows to avoid the losses of analyte and minimize the errors during the extraction. The advantages of SPME are:

- Possibility of extraction from liquid matrices and solid matrices;
- Possibility of sampling volatile and semi-volatile analytes;
- Extraction and analytes preconcentration in a single step;
- Fast and simple operations;
- Solvent-free extractions.

Solid phase microextraction involves the performance of two steps: partitioning of analytes between the extraction phase and the sample matrix and desorption of analytes from the stationary phase into analytical instrument. Initially, the first SPME experiments predicted that the coated fused silica fiber was exposed in the aqueous sample containing the analytes and subsequently placed in the GC injector for desorption, but this process involved the opening of the injector which resulted in loss of head pressure at the column [9]. The first SPME device provided that the coated fiber was incorporated into a microsyringe, as shown below (***Figure 1.2***):

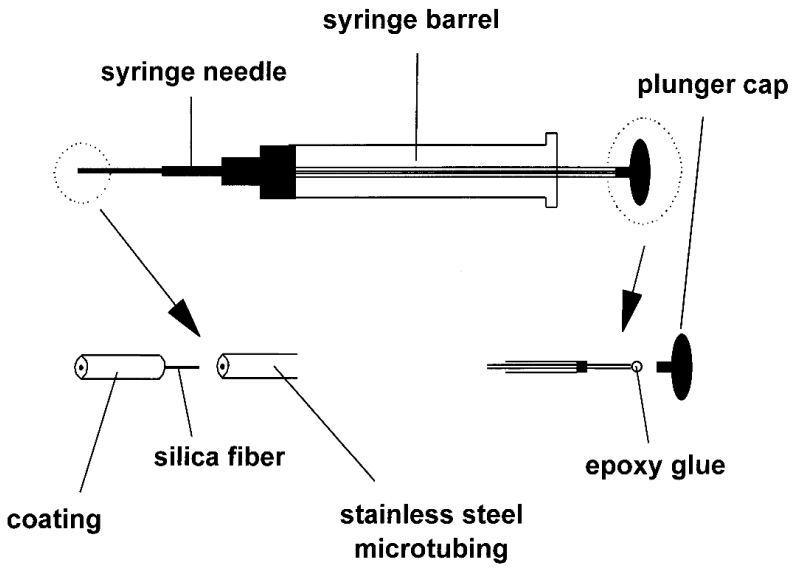

Figure 1.2: First SPME device based on Hamilton 7000 syringe.

In this case sample injection was then very much like standard syringe injection. The plunger allowed exposure of the fiber during extraction, desorption and its protection in the needle during storage and penetration of the septum. Considering this scheme the SPME devices currently available were developed (***Figure 1.2.1***).

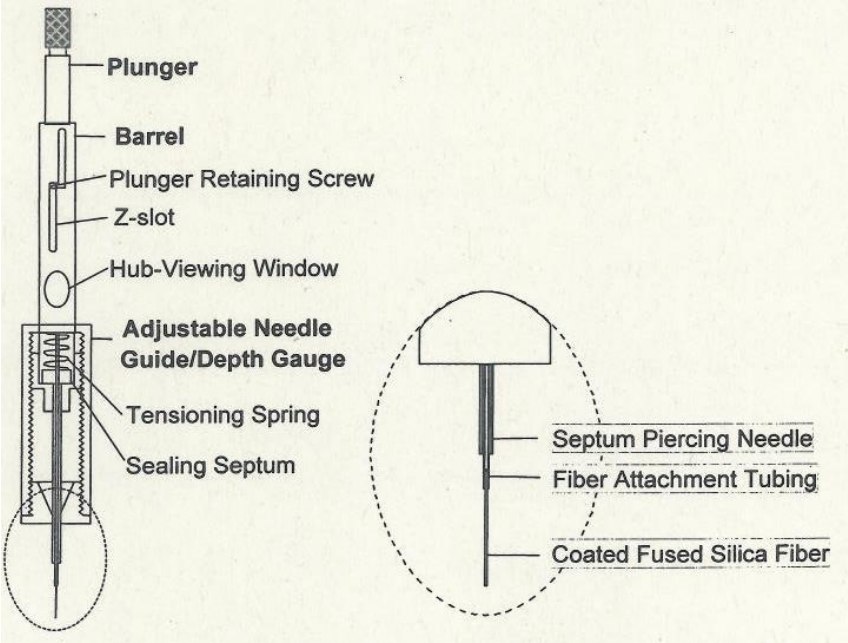

Figure 1.2.1: Example of a SPME device currently available.

1.1.1 SPME Fundamentals

In the SPME extraction the coated fiber is exposed in the sample matrix for a well-defined period of time. The transport of analytes from the sample matrix to the stationary phase of SPME fiber starts immediately when the fiber is inserted in the sample matrix, as shown below (***Figure 1.2.2***).

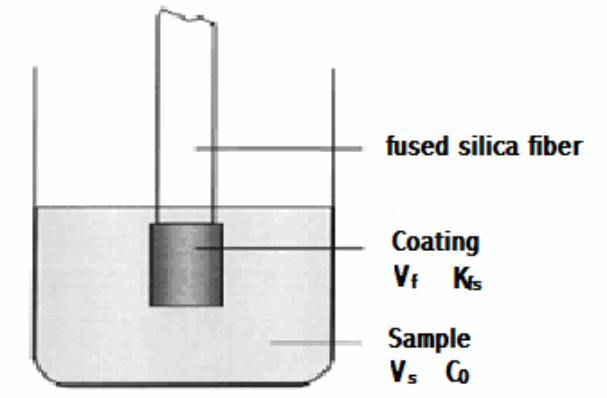

Figure 1.2.2: Sample extraction with SPME fiber. **V_f:** volume of fiber coating; **V_s:** volume of sample; **C_0:** initial concentration of analytes in the sample; **K_{fs}:** fiber/sample distribution coefficient.

Tipically, SPME extraction ends when the extracted analyte concentration reaches the equilibrium in the sample matrix and the stationary phase of fiber. When the equilibrium conditions are reached the exposure of the fiber in the sample matrix for a longer time does not allow to extract more analytes. In this way, SPME is different from other extraction techniques, such as SPE and its miniaturization, that allow an exhaustive extraction of analytes [10, 11]. If only the sample matrix and the fiber coating are considered the equilibrium conditions can be described, according to the law of mass conservation, by the following equation:

$$C_0 V_S = C_S^{\infty} V_S + C_f^{\infty} V_f \qquad \text{(Eq. 1.2)}$$

where C_0 is the initial analyte concentration, V_S is the sample volume and C_S^{∞}e C_f^{∞} are equilibrium concentration in the sample matrix and in the fiber coating. The distribution coefficient of analyte between the coated fiber and the sample matrix, indicated with K_{fs}, is described by:

$$K_{fs} = \frac{C_f^{\infty}}{C_S^{\infty}} \qquad \text{(Eq. 1.2.1)}$$

If the equations (1.2) and (1.2.1) are rearranged the following equation is obtained:

$$C_f^{\infty} = C_0 \frac{K_{fs} V_s}{K_{fs} V_f + V_s} \qquad \text{(Eq. 1.2.2)}$$

The number of moles of analyte (n) can be described can be obtained as shown below:

$$n = C_f^{\infty} V_f = C_0 \frac{K_{fs} V_s V_f}{K_{fs} V_f + V_s} \qquad \text{(Eq. 1.2.3)}$$

This latter equation indicates that there is a proportional relationship between sample concentration and the amount of analyte extracted onto the coating (n), which is the basis for analyte quantification with SPME. When the sample volume is very large, $Vs > K_{fs} V_f$, the equation (1.2.3) can be simplified to:

$$n = K_{fs} V_f C_0 \qquad \text{(Eq. 1.2.4)}$$

This means that the fiber can be exposed directly to the water, blood, ambient air and so on, because the amount of extracted analytes depends only on their concentration and not on the sample volume. This discussion is related to partitioning equilibrium involving liquid polymeric phases such as polydimethylsiloxane. In the case of solid sorbent coatings equation is analogous for low analyte concentration, considering that the total surface area available for adsorption is proportional to the coating volume if the porosity of the sorbent is constant. Moreover, equation (1.2.3) which is valid for sample matrix represented as a single homogeneous phase and no headspace in the system can be

modified considering the existence of other components in the matrix by considering the volumes of the individual phases and the appropriate distribution constants [10, 12].

1.1.2 Thermodynamics

The thermodynamic principle common to all chemical extraction techniques involves the distribution of the analyte between the sample matrix and the extraction phase. When a liquid is used as the extraction phase, the distribution constant, K_{es}, can be described as:

$$K_{es} = \frac{a_e}{a_s} = \frac{C_e}{C_s} \qquad \text{(Eq. 1.2.5)}$$

where a_e and a_s are the activities of analytes in the extraction phase and matrix, respectively, and can be approximated by the appropriate concentrations. For a solid extractant, adsorption equilibria can be explained using this equation:

$$K_{es}^{s} = \frac{S_e}{C_s} \qquad \text{(Eq. 1.2.6)}$$

where S_e is the solid extraction phase surface concentration of adsorbed analytes. The physicochemical constant reflects the chemical composition of the extraction phase and it determines the retention and selectivity of a separation column. SPME is very similar to chromatography concept for this it can be used to provide information about the thermodynamics of the partitioning process [13]. The distribution constants between the extraction phase and sample matrix are thermodynamic parameters and, as discussed below, they are influenced from several conditions including temperature, pH, salt and organic components in the sample matrix.

1.2.4 Kinetics

The kinetic concept is related to the extraction rate of SPME extraction. If the homogeneous water sample, perfectly agitated, is considered the follow extraction time profile can be obtained (***Figure 1.2.3***).

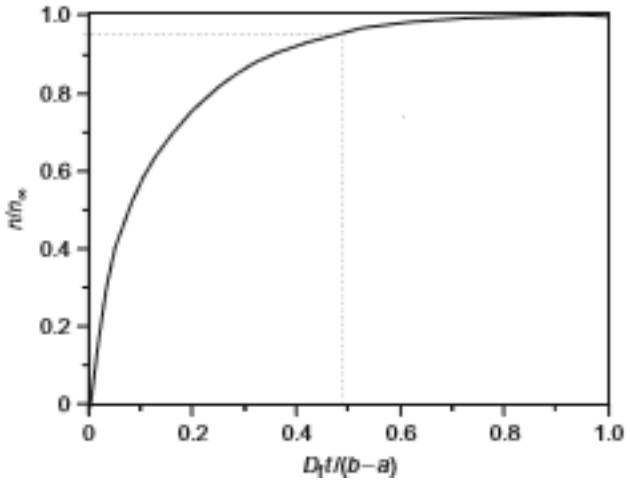

Figure 1.2.3: Mass absorbed versus time for a solution of infinite volume and for any K_{fs} value.

The extraction time profile shows that immediately after the immersion of the fibre in solution, there is a rapid increase in the mass absorbed by the fibre. The rate of increase then slows and eventually reaches equilibrium. Generally, the contact between the sample and the fiber is considered stationary and as the distance from the fiber surface increases, the fluid movement gradually increases until it corresponds to the bulk flow in the sample. The convection of molecules in the space surrounding the fiber surface can be described as a defined thickness static layer called Prandtl boundary layer, as described below (***Figure 1.2.4***).

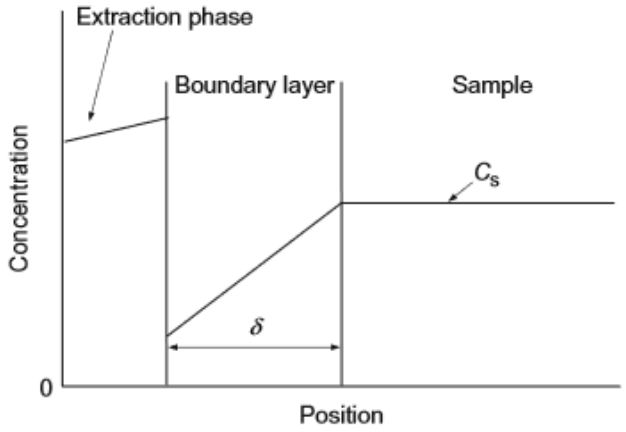

Figure 1.2.4: Boundary layer representation.

The thickness of the boundary layer (δ) is determined by both agitations in the sample and the diffusion coefficient of the analyte which means that the boundary layer thickness is different for different analytes in the same extraction process. At this point, the effects of different agitation modes

and therefore of different boundary layer sizes on the equilibration rate can be described in the follow graphs where mass absorbed versus extraction time is plotted (***Figure 1.2.5***):

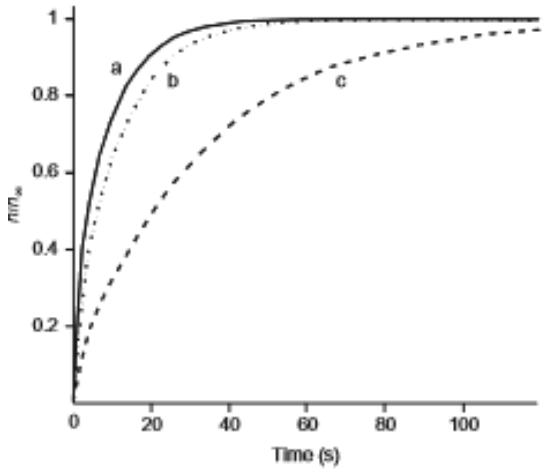

Figure 1.2.5: (a) Perfect agitations conditions; (b) Well agitated and thin boundary layer; (c) Poorly agitated and thick boundary layer.

When the extraction rate is determined by the presence of a boundary layer the analytes with a high K_{fs} have a long equilibration time even with a very thin boundary layer, which is characteristic of rapid agitation. Therefore, the agitation is important in terms of reducing the thickness of boundary layer increasing the transfer rate of analytes from sample matrix to coated fiber and thus decreasing the equilibration time [12, 14].

1.2.5 Parameters that can influence the extraction efficiency

The thermodynamic principles allow to evaluated the effects of extraction parameters on the partitioning coefficient. These extraction conditions can be optimized using a minimum number of experiments. In particular, the parameters that can influence the K_{fs} of analytes are: extraction temperature, agitation methods, pH, salting and organic solvent.

1.2.5.1 Extraction temperature

The effect of extraction temperature is described by the following equation:

$$K_{fs} = K_0 \exp\left[-\frac{\Delta H}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)\right] \qquad \text{(Eq. 1.2.7)}$$

where, K_0 is the distribution constant when both fiber and sample are at temperature T_0, ΔH is the molar change in enthalpy of the analyte when it moves from sample to fiber coating and R is the gas constant. When the K_{fs} value for an analyte is greater than 1, the analyte has a lower potential energy in the fiber coating than in the sample and the analyte partitioning into the fiber must be an exothermic process with ΔH lower than 0. This means that increasing the temperature the distribution constant of analyte decreases. For this during the screening of the parameters that can influence the extraction process the temperature is a very important parameter to optimize. On the other hand, an increase in temperature during extraction enhances the diffusion of analyte towards the fiber coating and in the headspace SPME extraction mode the temperature helps transfer analytes to the headspace. To solve the problem of reduction of distribution constant during an increase of temperature, the coating can be cooled simultaneously with sample heating. A new device called, internally cooled fiber SPME (***Figure 1.2.6***) was created by Pawliszyn et al. [15, 16].

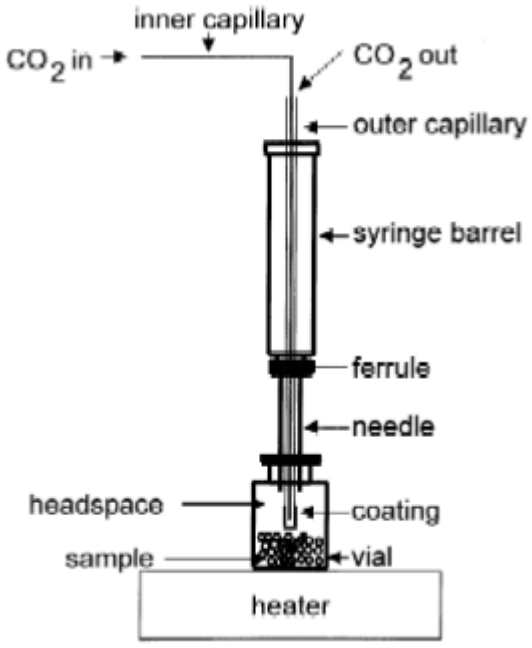

Figure 1.2.6: Representation of internally cooled SPME fiber.

In this device, a fused-silica tubing is sealed and coated at one end and liquid carbon dioxide is delivered via the inner capillary to the coated end of the outer capillary resulting in a coating temperature lower than that of the sample. This allows an accumulation of analytes at the tip of the fiber and in this way also the quantitative extraction of volatile analytes is possible [17].

1.2.5.2 Agitation Methods

The use of agitation methods increases the analytes transfer, from sample matrix towards the fiber coating, decreasing the extraction time. There are several agitation methods such as fast sample flow, rapid fiber movement, vial movement, stirring and sonication [18, 19]. The problem associated with the sonication method is the presence of thermally instable analytes, because the use of sonication produces the heating of the sample. Another problem of sonication as agitation is associated with the lifetime of fibers [20, 21].

1.2.5.3 pH

In SPME, only the neutral species are extracted by fiber coating and, therefore, the conversion of the analytes into neutral forms by pH adjustment can significantly improve method sensitivity. Accordingly, low pH values improve the extraction of acidic compounds and high pH improves that of basic compounds. The pH of an aqueous solution changes the constant for dissociable species, according to the following equation:

$$k = k_0 \frac{[H^+]}{k_a + [H^+]} \qquad \text{(Eq. 1.2.8)}$$

where k_0 is the distribution constant between the sample and the fiber of the undissociated form and k_a is the acidity constant of the dissociable analyte. Following this equation for example in ***figure 1.2.7***, when pH decreases, more acid is present in neutral forms which partition into the coating, resulting in higher sensitivity. To obtain the highest sensitivity, pH needs to be two units lower than the pK value corresponding to the acid.

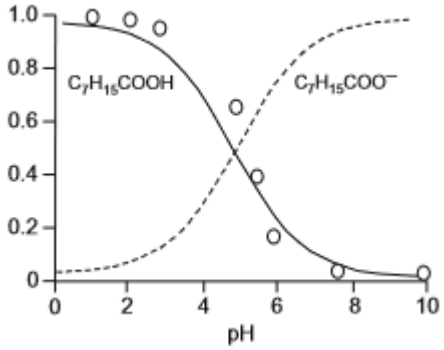

Figure 1.2.7: pH effect of acid compounds.

Moreover, it is very important to choose the right pH also because extreme value of pH can damage the fiber coating in direct immersion extraction mode [22].

1.2.5.4 Salting

The addition of salt can increase or decrease the diffusion coefficient of analytes, depending on analytes to extract. The salting effect generally increases with increasing compounds polarity, in these sense the addition of salt makes the organic compounds less soluble and the partition coefficients can improve. For example, in ***figure 1.2.8*** the extraction of benzene and toluene from aqueous sample is reported:

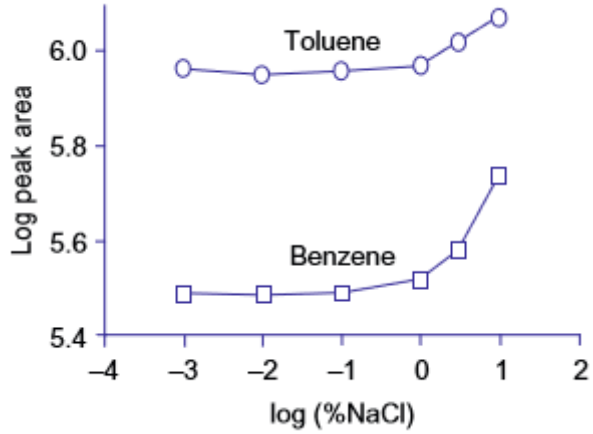

Figure 1.2.8: The effect of salt on SPME extraction of benzene and toluene.

An increase of analyte extraction occurs at salt concentrations above 1% and leads to improve the sensitivity of the extraction [12, 23].

1.2.5.5 Presence of organic solvent

The organic solvent in water matrix generally reduces the transfer of analyte from matrix to fiber coating. The diffusion coefficient, when an organic solvent is present, changes in according to the following equation:

$$K_{fs} = 2.303 K_{fw} exp\left(\frac{P_1 - P_2}{2}\right) \qquad \text{(Eq. 1.2.9)}$$

where K_{fw} is the distribution constant for the analyte between fiber and pure water; P_1 is equal to 10.2 and represents the polarity parameter for water; P_2= cPs + (1-c)P_1 is the water/solvent mixture polarity parameter for a solvent of concentration c and polarity parameter Ps. This means that the concentration of the solvent must be above 1% to change the properties of water and the distribution constant substantially [24]. For example, in ***figure 1.2.9*** the extraction of benzene, toluene, ethylbenzene and xylenes (BTEX), into PDMS coating, increasing methanol concentration in an aqueous matrix is reported:

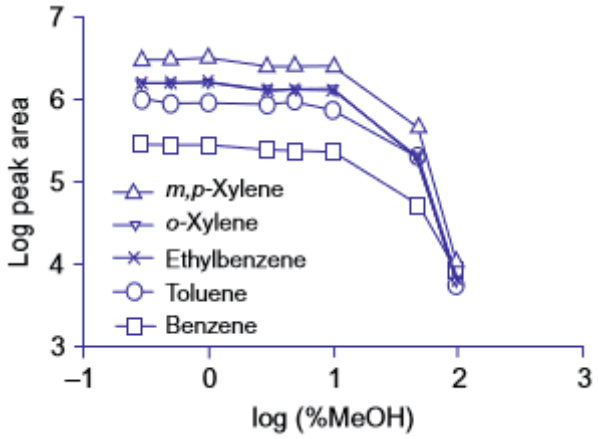

Figure 1.2.9: Solvent effect on SPME extraction.

1.2.6 Selection of the fiber coating

An important step in SPME method development is the selection of the fiber coating. The properties of the extraction phase should be carefully optimised because they determine the selectivity and reliability of the extraction method. The fiber used to extract the analytes from sample matrix presents a fused silica core with a length of 1 cm and a thickness of stationary phase included in the range of 7-100 µm. The efficiency of extraction process is depending on the distribution constant, $K_{fs,}$ of analytes between the fiber coating and sample matrix. Therefore, it is very useful to use the right coating for the given application. For example, the fiber polarity can provide selectivity by enhancing the affinity of the coating for polar analytes compared to a non-polar fiber coating. In ***figure 1.2.10*** the extraction of polar and non-polar analytes using two different coating, polydimethylsiloxane fiber (PDMS) and polyacrylate fiber (PA), is shown:

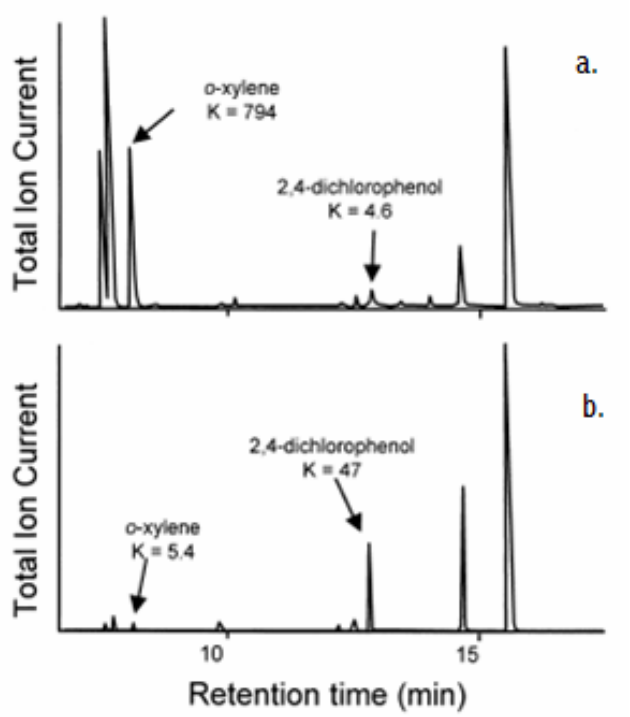

Figure 1.2.10: Total ion current GC–MS chromatogram of benzene, toluene, ethylbenzene, and *o, m, p*-xylenes (BTEX) and 2,4- dichlorophenol in water extracted with (a) PDMS coating and (b) PA coating.

The sensitivity during the extraction of non-polar analyte o-xylene changes when the PA coating (b) is replaced by PDMS coating (a). On the other hand, the opposite effect takes place in the case of polar analyte such as 2,4- dichlorophenol, for which the extraction improves when the PA coating is used. The SPME coatings commercially available can be classified by type of coating (absorbent or adsorbent), polarity and thickness, as shown in ***table 1.2***.

Table 1.2: Types of commercially available SPME fiber coatings.

Coating	Thickness	Extraction Mechanism	Polarity	Maximum Temperature (°C)	Recommended operating temperaures
PDMS	7 µm	Absorbent	Non-polar	320	200-320
PDMS	30 µm	Absorbent	Non-polar	300	200-300
PDMS	100 µm	Absorbent	Non-polar	300	200-300
PA	85 µm	Absorbent	Polar	320	220-320
PEG	60 µm	Absorbent	Polar	250	200-240
PDMS-DVB	65 µm	Adsorbent	Bipolar	270	200-270
DVB/CAR- PDMS	50/30 µm	Adsorbent	Bipolar	27	230-270
CAR-PDMS	85 µm	Adsorbent	Bipolar	320	250-320

The choose of the fiber coating is based on the affinity of stationary phase towards the analyte to extract. Therefore, polar coating such as polyacrylate (PA) coating have a major affinity with phenol, while bipolar coating such as PDMS-divinylbenzene (PDMS-DVB), Carboxen-PDMS (CAR-PDMS) can be used to extract analytes as alcohols, amines and ethers. On the other hand, non-polar coating such as polydimethylsiloxane (PDMS) is used to extract for example, BTEX and PAHs [25-29].

The coatings based on absorbent mechanism are usually liquid polymers, while the coatings based on adsorbent mechanism are solid materials. The mechanism of the two coatings is quite different as described in ***Figure 1.2.11***:

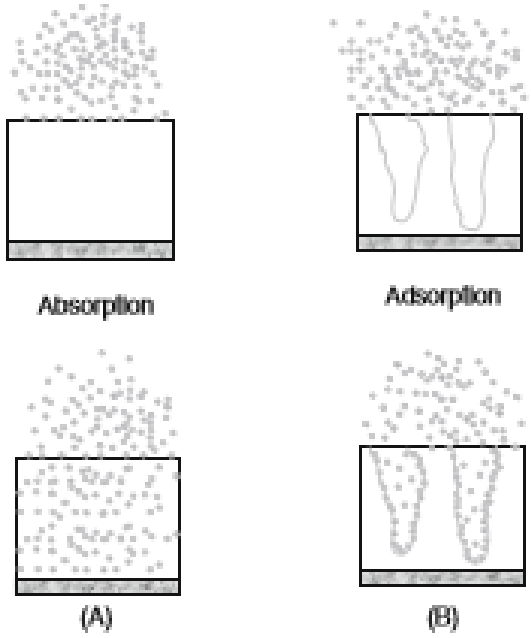

Figure 1.2.11: Schematic representation of Absorptive (A) vs Adsorptive (B) extraction.

In the case of liquid coatings, the analytes partition occurs onto the extraction phase where the molecules are solvated by the coating molecules. The diffusion coefficient in the liquid coating allows the molecules to penetrate the whole volume of the coating within a reasonable extraction time, if the coating is thin. The absorbent can be a gum or viscous oil that contains cross-linking agents and the polymer can be applied in various thicknesses over the fiber like in the case of PDMS. The retention of the analytes is based also on the thickness of the fiber coating. For example, it is difficult for absorbent phases to retain small analytes unless a thick coating is used. The three commercially available liquid coatings are polydimethylsiloxane (PDMS), polyacrilate (PA) and polyethylenglycole (PEG). In adsorbent coatings a solid material, usually a solid polymer, is suspended into a liquid polymer and coated on fiber. In this case, the interaction of analytes with a stationary phase is with solid particles. The adsorbent coating has a well-defined crystalline structure, which if dense, reduces the diffusion coefficient. Therefore, within the experimental time the extraction occurs only on the surface of coating. The retention of the analyte depends upon the size of the analyte and the pore diameter. The surface of an adsorbent can interact with an analyte, such as π-π bonding, hydrogen bonding or van der Waals interactions. The ability of an adsorbent to retain analytes is dependent by the total surface area, the amount of porosity and the size of the pores that are divided into macro- (openings with diameters of 500 Å), meso- (openings in the range of 20-500 Å) and micropores (openings in the range of 2-20 Å). In this case, the analytes can be extracted only on the surface of adsorbent coating. The surface area and the number of sites available for the

adsorption limits the amount of analytes extract. This is a crucial point in the extraction from complex matrix where the interfering species can replace the analytes on the adsorbed coating. To avoid this and the saturation problem a shorter extraction time is used. The commercially available adsorbent coatings are polydimethylsiloxane-Divinylbenzene (PDMS-DVB), Divinylbenzene/Carboxen-polydimethylsiloxane (DVB/Car-PDMS), Carboxen- polydimethylsiloxane (CAR-PDMS). The Carboxen-PDMS and DVB-PDMS fibers are prepared by suspending the particles in a high-molecular-weight proprietary PDMS that serves as an adhesive to retain the particles (***Figure 1.2.12***):

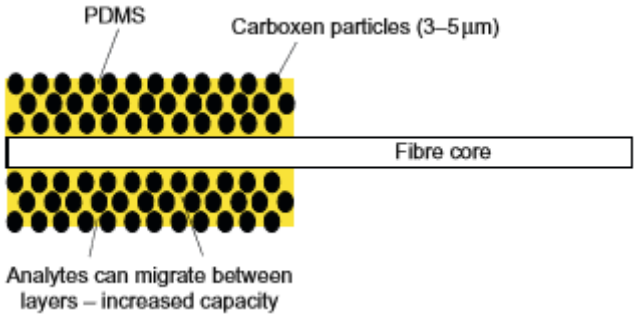

Figure 1.2.12: Representation of Carboxen-PDMS and DVB-PDMS fibers.

DVB is a porous polymer with the uniform and large micropores compared to Carboxen 1006. For this DVB is primarily used for the extraction of semivolatile analytes and larger volatile analytes. Carboxen 1006 /PDMS fiber belongs to the family of carbon molecular sieves SPME coating and allows to extract volatile and small analytes. The DVB/Carboxen-PDMS fiber contains both adsorbents that are layered to extend the molecular weight range of analytes extracted with one SPME fiber [30].

1.2.6.1 New coatings

The most recent researches are focused on development of new coatings materials to improve performances in terms of selectivity, robustness, carryover, swelling in solvent, operation temperatures and surface area. Some examples of new SPME coatings include carbon nanotubes, sol-gel coatings, metal organic frameworks, polymeric ionic liquids, aptamers and molecular imprinted polymers. These materials provide a high surface area and increase chemical interaction such as they can induce selectivity towards different compounds. Carbon nanotubes, CNTs, are allotropic forms of graphitic carbon and show high thermal, chemical and mechanical stability. They are hydrophobic and have excellent adsorption capacity but they can incorporate functional groups which increase

their solubility. CNTs functionalized can extract the analytes through hydrogen bonding, π–π stacking and electrostatic interactions. For this, they can be used to extract non-polar, polar and even ionic species. For example, CNTs were used to extract BTEX and phenols from water samples [31, 32]. The low operating temperatures, solvent instability and the lack of stationary phase of the commercial SPME fibers is overcome by sol-gel technology that improves the surface area as well. In different papers, it is possible to find the details on the sol-gel process [33, 34]. The sol-gel coatings were used to extract different analytes from food samples and methamphetamines from urine sample [35-37]. Metal organic frameworks, MOFs, are polymeric materials with different porous size with a metal ions and organic ligand as bridging unit. They have a high surface area and different pore size that depends from coordination number of the used transition metal ions. Moreover, they can work at high temperature depending from metal ions considered and from organic ligand. Coatings based on MOFs were used for different application such as the extraction of organic contaminants from soil, water and food samples [38-40]. Polymeric ionic liquids are organic salts, in their liquid form at room temperature composed of a cationic organic part and organics or inorganics anions. Due to their tunable physico-chemical properties, high termal stability, long lifetime, good conductivity, variable miscibility in several solvents they were useful for different applications. Among their applications, polymeric ionic liquids were used for the extraction of endocrine disrupting from water and urine samples such as to extract contaminants in food matrices and water pollutants [41-44]. Aptamers are a class of single stranded DNA/RNA molecules with high specificity and selectivity, good stability, low cost. Moreover, they are very ease to synthesize and allow to extract compounds with high polar properties. Their applications regard analysis in food matrix and biological fluid by liquid chromatography technique [45, 46]. Molecularly imprinted polymers (MIPs) are synthetic polymers obtained by polymerization of a monomer with a cross-linking agent in the presence of the template molecule. When the template is removed after polymerization, the coatings have very specific binding sites that are complementary in size and chemical properties to the template and provide high selectivity. The principle limitations of this coating is the template bleeding and thermal instability that can be improved optimizing each preparation steps. Their applications involve the extraction of cancer biomarkers in urine and environmental contaminants in agricultural soil, sea sediments and tap water [47-49].

1.2.6.1.1 PDMS-modified coatings

In complex matrices analysis the direct exposure of the fiber to the matrix could cause the damage and deterioration of the SPME fiber due to irreversible attachment of matrix components to the fiber

coating. For this, the analysis of complex matrices requires sample pre-treatment prior SPME extraction. To overcome these limitations and to analyze matrices such as food or biologic fluids without any preliminary sample-preparation step, a new SPME fiber modified with a PDMS layer was developed. In particular, a PDMS/DVB fiber, commercially available, was modified adding a thin PDMS layer. This new fiber keeps the original coating sensitivity and at the same time shows enhanced robustness when it is inserted directly into complex matrices [50, 51]. In the chapter 3 of this thesis, a work based on the use of PDMS-modified fiber and on the evaluation of its performance in human urine will be presented.

1.2.7 SPME extraction modes

The SPME extraction modes can be different, in particular the choice of extraction way depends on the type of analytes to extract and the complexity of the matrix. Three different extraction modes can be performed using SPME fiber as illustrated in ***figure 1.2.13***:

- Direct extraction;
- Headspace extraction;
- Extraction with membrane protection.

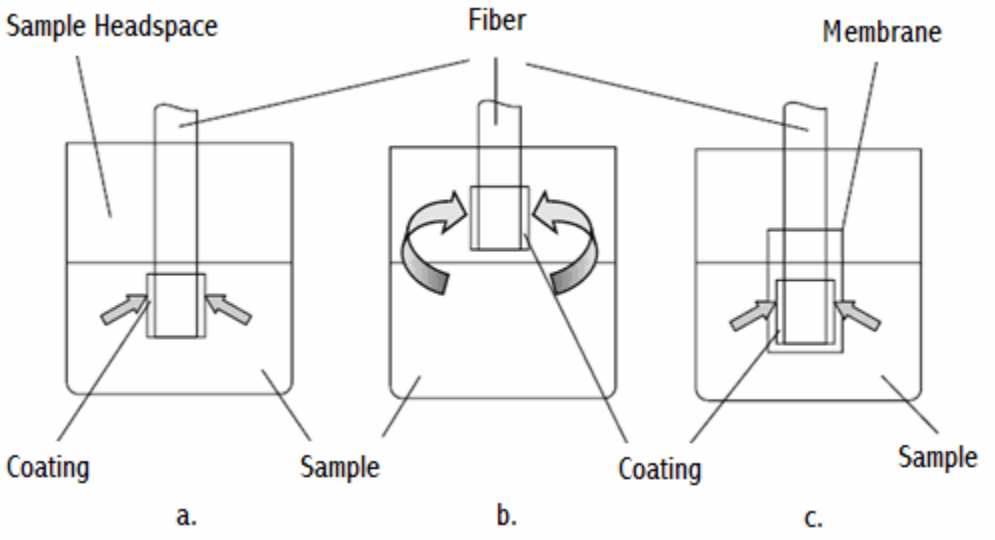

Figure 1.2.13: SPME extraction modes: (a) Direct immersion; (b) Headspace immersion; (c) Membrane-protected SPME.

1.2.7.1 Direct extraction (DI-SPME)

In the direct immersion the fiber covered with stationary phase is inserted in the sample and the analytes are transferred from the sample matrix to the coated fiber. In this case, the use of agitation is important to facilitate the transport of analytes from the bulk of solution to the vicinity of fiber. As discuss above, in the case of aqueous matrix efficient agitations such as fast sample flow, stirring or sonication are required. The use of agitation allows to reduce the effect caused by the depletion zone produced near the fiber as a result of fluid shielding and slow diffusion coefficients of analytes [19, 52]. The direct sampling is not indicated for the extraction of analytes in aqueous matrix with more than 1% of organic solvent or other matrices that could lead to a fiber deterioration.

1.2.7.2 Headspace extraction (HS-SPME)

In headspace extraction the fiber is exposed in the headspace of the sample. This approach is very useful for analytes characterised by high Henry's law constants. In the headspace mode the fiber coating is protected from damage by high molecular weight compounds and sample modification such as a changing of the pH without damaging for the fibers is allowed. The mass transfer to the fiber is limited by mass transfer rates from the sample to the headspace. The volatile analytes migrate to the headspace faster than semivolatiles analytes and for this they are at a higher concentration in the headspace that contributes to faster mass transport rates through the headspace. In the headspace extraction the equilibration times of volatile analytes are shorter than direct extraction in the same agitation conditions. The presence of analytes in the headspace prior to extraction and the diffusion coefficients in gaseous phase is higher than in liquid matrix. Temperature has a great effect on the kinetics of the extraction. Indeed, since the concentration of semivolatiles in headspace at room temperature is small, an increase of temperature can improve their extraction and reduce extraction times [53].

1.2.7.3 Membrane extraction

The principal aim of the use of membrane is to protect the fiber from damage or deterioration when the analytes to extract are in a very complex matrix. Moreover, the analytes with low volatility can be extract selectively by membrane-protected SPME. Indeed, in this case the kinetic of the membrane extraction is slower than direct extraction because the analytes must diffuse the membrane before they can reach to the fiber coating [54].

1.2.8 Derivatization reaction in SPME

The derivatization reaction is a particular way to modify the behaviour of the interest analytes. For example, if the analytes are not volatile enough to be extracted from the headspace, they can be derivatised to improve their volatility. In particular, the derivatization is very useful in SPME-GC applications when the analytes are polar and non-volatile. The derivatization reaction increases the volatility and decreases the polarity of polar group. The derivatization is usually used to convert acidic groups in ester groups [55, 56], aldehydes and ketones can be derivatized with hydrazine [57], several amines and aliphatic amines are transformed into amides [58], the silylation reaction can be carried out for alcohol groups and acylation reaction for phenolic groups [59, 60]. Derivatization reaction can be carried out in the sample matrix or before extraction (pre-extraction), on the SPME fiber or after extraction (post-extraction) and simultaneously with extraction [61, 62].

1.2.8.1 Pre-extraction derivatization

In pre-extraction derivatization the derivatizing agent is added to sample before to insert the fiber into the sample. This allows to convert the interest analytes, which can be successively extracted by SPME fiber and finally desorbed into GC injector port (***Figure 1.2.14***):

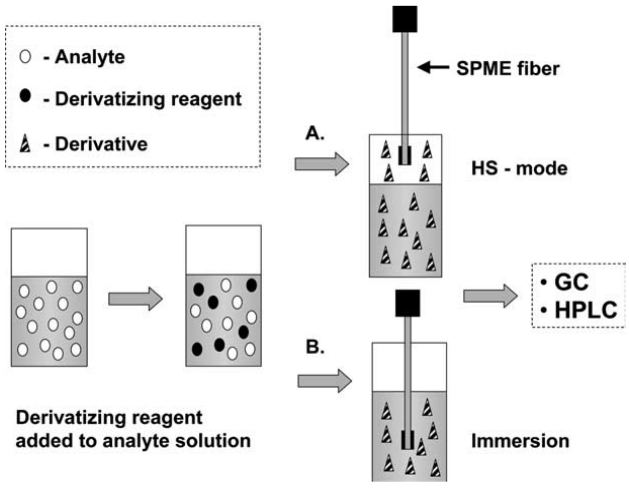

Figure 1.2.14: Derivatization in the sample matrix. (A) Sampling in headspace (HS) and (B) Sampling by immersion.

In this way, the extraction efficiency and chromatographic behaviour are improved since polar analytes are transformed in less polar derivatives. The derivatization reaction was used to convert several compounds in different matrices such as hair and aqueous sample [63-65]. In chapter 3 of this

thesis a derivatization reaction based on use of alkyl chloroformate [66] was directly carried out in aqueous matrix for the quantification of polyamines and phthalates monoesters in urine.

1.2.8.2 On-fiber derivatization

On-fiber derivatization takes place after the extraction of analytes when the fiber, loaded with analytes, is exposed in headspace mode in a vial containing the derivatizing reagent. Generally, the analyte molecules are less volatile than those of the derivatizing reagent (***Figure 1.2.15***):

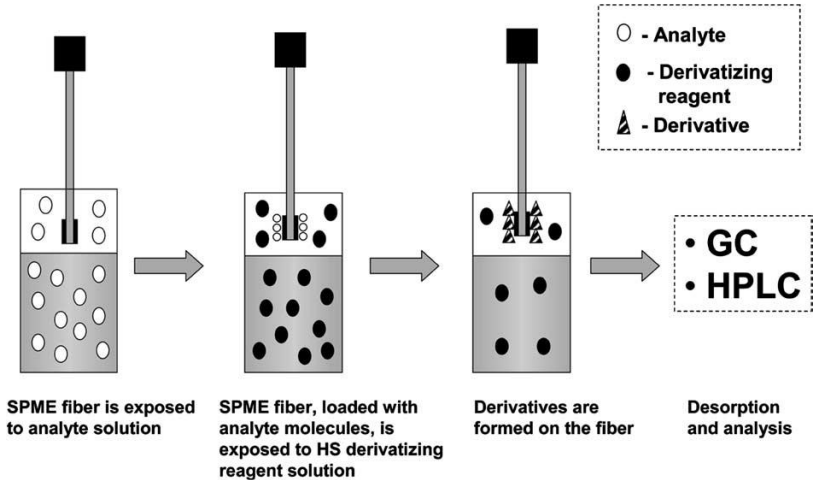

Figure 1.2.15: Derivatization on the SPME fiber after analytes extraction.

The on-fiber derivatization is usually used when there is a good extraction efficiency of interest analytes whereas the chromatographic behaviour and detection properties need to improve. This kind of derivatization was adopted, for example, for the extraction of short-chain aliphatic amines and chlorophenols in aqueous solution [67, 68] and the sampling of carbonyl compounds and amines in air matrix [69, 70].

1.2.8.3 Simultaneous sampling and on-fiber derivatization

The fiber is loaded with derivatizing reagent and then it is exposed in the vial with analytes. In this way, the analytes that have more affinity towards the fiber are extracted and converted into their corresponding derivatives. This requires that the derivatizing reagent is not too volatile to leave the fiber before the analytes extraction is completed (***Figure 1.2.16***):

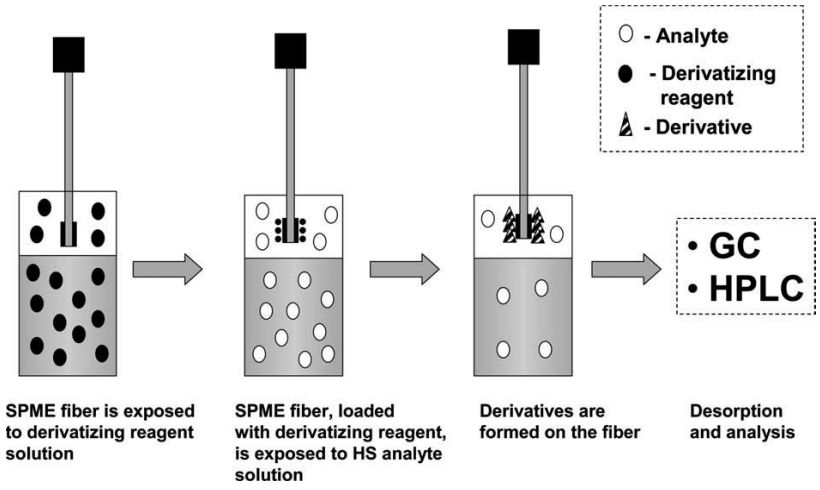

Figure 1.2.16: Simultaneous sampling and on-fiber derivatization.

The simultaneous extraction and on-fiber derivatization was used to determine the presence of aldehydes in beer and in water [71, 72], in environmental applications [73, 74] and in the evaluation of antioxidant activity of essential oil [75, 76].

1.2.9 Desorption of extracted analytes from SPME fiber

The two phase of SPME process are the partitioning of analytes on covered fiber and the introduction of the same fiber in the analytical instrument (***Figure 1.2.17***). During the desorption process the analytes diffuse from the fiber coating into the stream of carrier fluid for this it is very advantageous that high flow rate is used to ensure that the desorbed analytes are removed from the vicinity of the coating and to avoid the slowdown of desorption process.

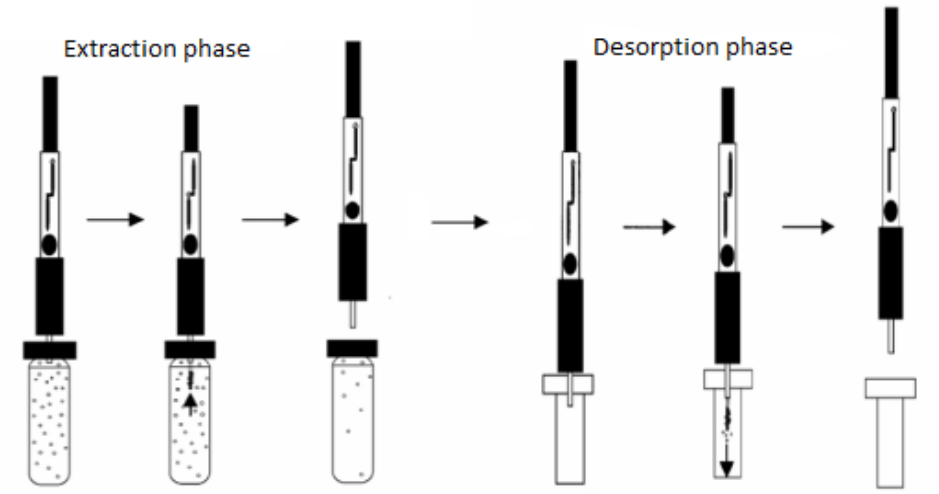

Figure 1.2.17: SPME process phases.

Due to its solvent-free nature and the small size of the fiber, SPME can be interfaced conveniently to several analytical instruments. The analytic instruments used in combination with SPME has been the gas chromatograph or the HPLC system via a special interface. A classical split/splitless GC injector can be used with an inner diameter of the narrow insert close to the outside diameter of the needle because this allows to increase the linear flow around the fiber. The desorption of analytes from the fiber in the GC injector is very rapid because the high temperatures of the injector lead to a dramatic decrease in the coating/gas distribution constant and, at the same time, they produce an increase in the diffusion coefficients. The desorption temperature used in the GC injector are in accord with the experimental working range temperatures determined for each fiber coating, as reported in table 1.2. The speed of desorption can be limited by the time required to introduce the fiber into the heated zone. The autosampler can be useful to obtained faster separation times and facilitate the injection. An alternative solution is the use of a dedicated injector that is cold during needle introduction but which heats up very rapidly after exposure of the fiber to the carrier gas [77]. The fiber can also contain the heating element and in this case no injector is necessary. In this case the fiber can be introduced directly into the front of the column and the analytes can be desorbed by heating with a capacitive discharge current after the fiber has been exposed from the needle [78, 79]. A flash desorption injector can be used by passing a current directly through the fiber when the rod is made of conductive material [80].

1.2.10 SPME applications

Due to its versatility, solvent-free nature, the development of new cotings, easy and fast sample preparation and the possibility to connect SPME online with sensitive instrument such as GC-MS instrument, solid phase microextraction is used in the development of many analytical protocols during the extraction procedure. It allows to realise fast and simple protocols to apply in routine analysis of different matrices. SPME applications cover several fields in which analytical chemistry may be used, from food and environment to bioanalysis.

In food analysis SPME can be used for different purposes including determination of contaminants for safety and quality of food, aroma profiling, traceability purpose and metabolomics investigations [81-86]. At the beginning, SPME was used for the analysis of food matrices in headspace mode because the fiber could be damaged due to the complexity of the matrix [87]. In this context the development of new coatings such as PDMS-overcoated fiber allows also the extraction of analytes with medium or low vapour pressure in DI-SPME approach, without matrix pre-treatment [88-90]. In environmental analysis SPME can be used in the extraction of contaminates from several matrices

including water, air, soil and sediments. SPME was used, both in direct and in headspace mode, to determine contaminants such as hydrazine, VOCs, PAHs, insecticides, perfluorocarboxylic acids, benzothiazoles, benzotriazoles and benzosulfonamides in different type of acqueous matrices, with derivatization step when it is necessary [63, 91-95]. To improve SPME robustness and its efficiency and sensitive new devices such as needle trap device (NTD) and thin-film microextraction (TFME) were developed. This latter is used, for example, for on-site sampling from aqueous matrices [96]. In air analysis the presence of particles can be a problem because the target analytes can be bonded to the surface of the aerosol particles. In this context, NTDs devices are able to capture both the free-form and bound fractions of organic molecules [97, 98]. In soil and sediments analysis SPME can be used in headspace or in direct immersion with a sample pre-treatment. An alternative method is the heating of the sample while cooling the fiber, improving the efficiency of the release of analytes from the matrix and facilitating the mass transfer into the headspace [99, 100]. SPME fiber technique is also applied for the assay of several compounds in biological samples such as urine, saliva, blood, plasma and tissue samples. In tissue samples such as liver, lung and brain, SPME shows several advantage due to its low invasiveness and non-destructive character minimizing the risk of compromising the organ through biopsy. In some investigations it was used to monitor the concentration of neurotransmitters in brain tissue and also to monitor organ function during organ transplantation in pigs [101, 102]. SPME is very useful for clinical screening, the identification of particular diseases and drug monitoring. In HS approach fiber SPME was used for the determination of illicit drugs from several biological samples [103, 104]. The HS-SPME was also used to analyze volatile compounds in blood, urine and fecal samples as biomarkers of lung cancer and for the diagnosis of gastrointestinal disorders [105-108]. Several DI-SPME protocols were described for the assay of biogenic amines, neuroendocrine tumor markers and markers in prostate cancer, after derivatization with alkyl chloroformates, in human urine [109-111]. The new coatings, carbon and sol-gel coatings, were also used in many works to facilitate the extraction from different biological fluids in direct immersion.

1.3 Microextraction by packed sorbent (MEPS)

1.3.1 Introduction

Microextraction by packed sorbent is a miniaturized version of conventional solid phase extraction (SPE) with different advantages compared to SPE. The principle of SPE is based on exhaustive and non-equilibrium extraction. In particular, an aqueous sample containing analytes passes through a packed sorption phase containing functional groups that are able to retain the analytes, in selective way, while the other components of matrix are unretained. Residual matrix is removed from the cartridges as exhaustive as possible, before the retained analytes are eluted by a volume of an appropriate organic solvent. The eluted analytes can be directly injected into the chromatographic system or be subject to further purification and concentration steps. Generally, the cartridge used in SPE contain from 10 mg to 10 g of sorbent materials and the volume of elution solvents used is higher [112, 113]. MEPS was introduced in 2004, from Abdel-Rehim and co-workers, as a new microextraction technique based on the same principle of SPE [114, 115]. MEPS and SPE use the same sorbents but the MEPS sorbent is integrated onto a liquid syringe. This allows to MEPS the possibility to work on-line with the analytical instruments. One of the important differences between SPE and MEPS, is not only the possibility to use the sorbent multiple times after washing it, but also the direction of sample and solvent flow. In SPE the flow is in one direction whereas in MEPS the flow is in two directions, up and down. MEPS results very promising for several reasons: it is a simple technique, fully automated, reduces the solvent and sample volume, facilitates the concentration of the analytes and the analysis cost is less than conventional SPE.

1.3.2 MEPS procedure

In MEPS the sorbent, that contain 1-4 mg of solid material, is packed inside a syringe between the needle and the barrel (called BIN) as shown in ***figure 1.3***. This technique allows to achieve sample extraction, pre-concentration and clean-up in a single step and device.

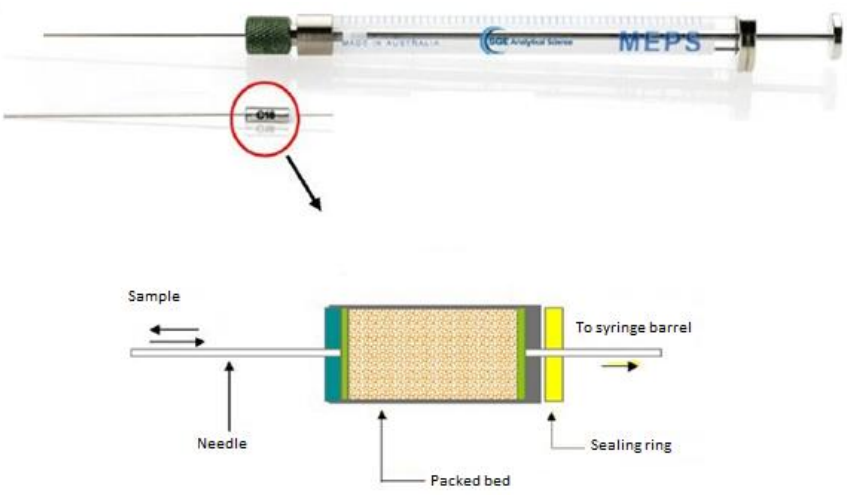

Figure 1.3: Representation of SGE syringe with packing sorbent.

Due to the small amount of sorbent used, only a small volume of elution solvent can be used for the elution of analytes from the adsorbent. The MEPS syringe can be used in different way: manually, semi-automated and fully automated. In particular, in semi-automated mode the use of electronic pipette, called eVol®, is very common (***Figure 1.3.1***).

Figure 1.3.1: Electronic Pipette eVol® for semi-automated process.

In this way, the work of analyst is minimal and this allows to minimize error sources. The schematic steps involve that the sample passes through the sorbent and the analytes are adsorbed to the solid phase. The solid phase is washed with water to remove the other components of matrix and then the analytes are eluated with an organic solvent. The loading of the sample should be done many times, in this way the recovery increases as described elsewhere [116, 117]. The MEPS extraction involves five steps (***Figure 1.3.2***):

- Conditioning of solid sorbent;
- Sample loading;
- Sorbent drying or washing;
- Elution solvent loading;
- Washing of the sorbent for another extraction.

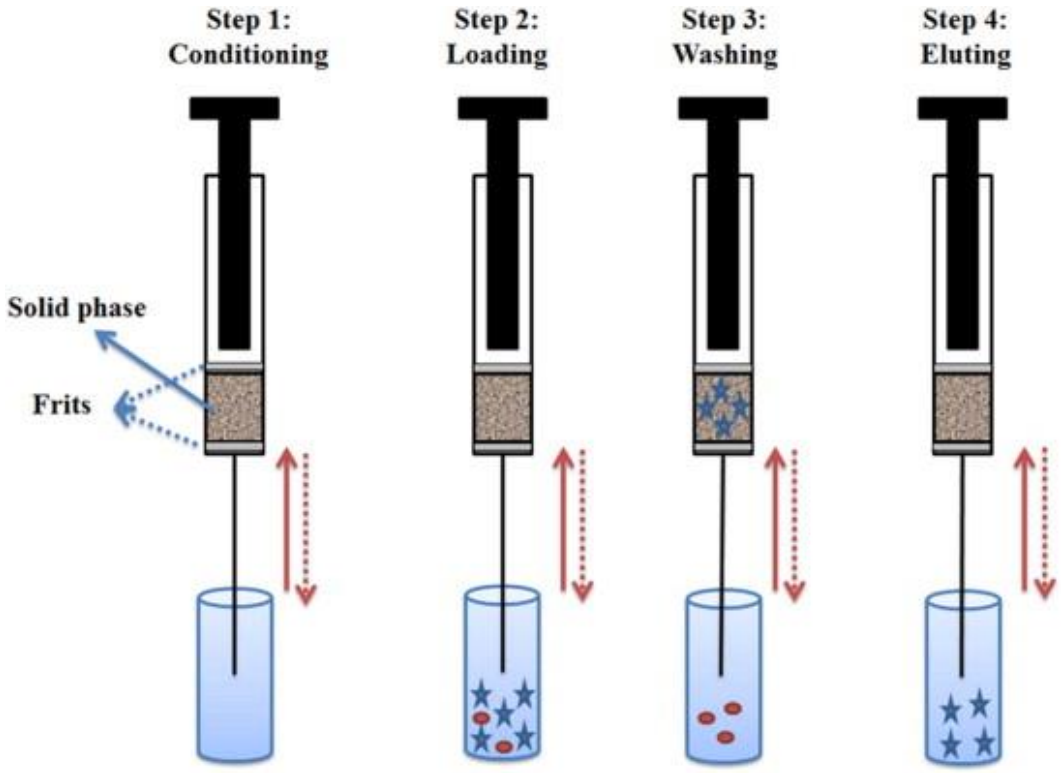

Figure 1.3.2: MEPS extraction steps.

As described in the figure 1.3.2, the conditioning step allows the sorbent activation when it is used for the first time or its clean up and regeneration for a new extraction. During a MEPS extraction, the sample is loaded in the solid sorbent and the analytes are adsorbed. If is necessary, the washing step

with an organic solvent or a drying step to delete the interfering species is required. Finally, the analytes are eluted with a proper organic solvent and the cartridge can be wash to avoid carry-over effect [118].

1.3.3 MEPS Sorbents

Many sorbents used for MEPS extraction are commercially available, covering a numerous range of applications. Several types of extraction can be carried out and the choice depends on the type of sample and analyte to be extracted. The reversed phase extraction, carried out by C_{18}, C_8 and C_2 sorbents, involves the extraction of hydrophobic analytes from water sample. The normal phase extraction involves the extraction of polar analytes from non-polar organic solvent and the sorbent that can be used is unmodified silica (SIL). During the mixed mode and ion exchange extraction, the extraction of charged analytes from aqueous or non-polar organic samples is carried out. In this case, the sorbents used are based on mixed mode such as C_8/SCX or cation exchange using sulfonic acid bonded silica (SCX). Other sorbents available include polystyrene-divinylbenzene (PS-DVB), porous graphitic carbon, molecular imprinted polymers (MIP) based on different templates, metal organic framework (MOF) based MIPs and restricted access material (RAM) [119-121]. The presence of all these sorbents and the possibility to use several elution solvents allow the retention of target analytes to be modulated in order to remove the interfering species that can influence the analysis.

1.3.4 Critical parameters in MEPS extraction

Many parameters can influence the extraction efficiency of a MEPS process. Firstly, the sorbent selection represents a crucial point of MEPS extraction because the affinity of analytes depends on the strength of the interaction between the target analytes and the sorbent used. The speed of sample loading can influence the interaction between the sorbent and the analytes. Indeed, a suitable speed of sample loading allows to improve this interaction and the analytes will be adsorbed onto the solid sorbent in the BIN. Another important variable is the sample volume and the number of cycles to load the sample. The choice of sample volume is based on a compromise between analytical performance and extraction efficiency. In this context, another parameter to optimize is the way to load the sample. Indeed, the multiple cycles of extraction can be performed in two different ways: draw-eject, where the sample and the waste of sample are in the same vial and extract-discard, where the waste of the sample is in a different vial than that of sample [119, 122]. The way to load the sample depends on

the complexity of matrix nature, as demonstrated in many studies [123, 124]. As regard the washing step, the number of washing cycles and the type of solvent have also to be optimized in order to remove unwanted species. In the elution step the elution solvent and its speed are critical parameters because in this phase the interactions between the analytes adsorbed and the sorbent should be broken [125]. Finally, the pH of the sample is a parameter to consider because most sorbents at extreme pH may be damaged and, moreover, it is convenient to adjust the solution pH to force the analyte to exist in the ionized state or in the nonionized state as completely as possible.

1.3.5 MEPS applications

Due to its simplicity, speed, sensitivity and selectivity during the sample preparation, MEPS extraction can be used in several fields from clinical, for the identification of drugs and disease biomarkers, to environmental field, for the identification of organic contaminants. Moreover, the possibility of automation allows to interface the microextraction by packed sorbent with several chromatographic instruments, such as liquid or gas chromatography. In biological samples, MEPS extraction was used for different purposes such as monitoring of a particular disease or cancer, and identifying drugs or other metabolites presence in matrix [126-129]. In environmental matrices (water, wastewater and sludge samples) MEPS coupled with gas chromatography was used to determine many organic pollutants such as PAHs, aromatic amines and estrogenic compounds [130-135].

In the chapter 4 of this thesis two applications based on the development and optimization of a method based on MEPS extraction of organophosphate flame retardant and phthalates monoesters coupled with programmed temperature vaporization-gas chromatography tandem mass spectrometry analysis will be described.

1.4 An overview on the other microextraction techniques

Solid phase microextraction techniques include, in addition to SPME and MEPS (in-needle SPME), the stir-bar sorptive extraction (SBSE), the thin film microextraction (TFME), in-tube SPME (IT-SPME) and in-tip SPME. Liquid phase microextraction technique (LPMEs) is a new sample preparation technique that uses minimal amounts of solvent and is inexpensive and rapid. LPME is divided into single-drop microextraction (SDME) and membrane assisted LPME by using a hollow fiber (HF), membrane bag or flat-sheet membrane module.

1.4.1 In-tube SPME (IT-SPME) and In-tip SPME

In In-tube SPME and in-tip SPME the diffusion of the analytes is mediated by flow-through. In-tube solid phase microextraction (IT-SPME) was introduced in 1997 by Eisert and Pawliszyn [136]. IT-SPME is a new sample preparation that involve an open tubular capillary column as an SPME device. Compared with the conventional SPME fiber, this SPME approach is a fully automated analytical technique that provides higher analytical efficiency. IT-SPME allows also to overcome some problems related to the use of conventional fiber SPME such as fragility. In IT-SPME organic compounds in aqueous samples are extracted from the sample into the internally coated stationary phase of a capillary. The compounds extracted are then desorbed by introducing a stream of the mobile phase or by using a static desorption solvent and then the desorbed compounds are injected into the LC column for analysis. The principal advantage of IT-SPME is the automation of the SPME/HPLC process, allowing extraction, desorption and injection to be performed continuously using an autosampler. IT-SPME can be used with all GC commercial columns thus increasing the number of stationary phase. This technique requires lower sample volumes and is versatile according a wide range of available coatings [137, 138]. The main disadvantages of the IT-SPME is the requirement of samples very clean because the capillary can be blocked. In-tip SPME is another recent approach of SPME. This technique uses a procedure similar to MEPS in which a solid packing material is inserted into pipette tips and sample preparation takes place on the packed bed. This allows a simple and fast utilization and lower cost per sample. The extraction is done off-line and only part of the sample is injected into the chromatograph, therefore the sensitivity is not high as with online MEPS. The relevant disadvantages of In-tip SPME extraction is the requirement of sample pretreatment such as filtration or dilution of complex matrix [139, 140]. Silica and monolith particles relatively large through pores are used as sorbents but in terms of stationary phases their number and properties are growing.

1.4.2 Stir-bar sorptive extraction (SBSE) and thin-film microextraction (TFME)

The extraction techniques based on diffusion of the analytes mediated by stirring include stir-bar sorptive (SBSE) and thin film microextraction (TFME). Stir-bar sorptive extraction is a new sample preparation technique developed by Pat Sandra and coworkers with the aim of increasing the extraction sensitivity of SPME by incorporating higher sorbent loading compared to SPME [141]. SBSE uses a magnetic stir bar coated with a PDMS phase, similar to SPME but in a thicker layer and thus the phase is 50-25 times thicker than in SPME. PDMS-coated stir bars, commercially available, have a length of 10 and 40 mm coated by a different volume of PDMS liquid phase. Extraction and

preconcentration of the analyte is carried out by introducing the SBSE device directly into the aqueous sample. For direct immersion stir-bar sorptive (DI-SBSE) the stir bar is placed in an amount of liquid sample and the sample is stirred until the partition equilibrium time is reached. The extraction time is determined by several parameters such as sample volume, stirring rate, temperature and stir bar dimensions and should be optimized for a given application. After extraction the stir bar is removed from the sample rinsed with purified water to remove matrix components and dried to remove residual water droplets. For headspace stir-bar sorptive extraction (HS-SBSE) the stir bar is placed in a liquid or solid sample with special devices to hold the stir bar in the sample. Desorption from the bar can be carried out by either heating or extraction with a small volume of a liquid solvent. The sample can be analyzed with a gas or liquid chromatographic system. When SBSE is combined with GC, thermal desorption is the main method once the bar has been inserted in the heated GC injection port and the analytes have been desorbed to the column. Thermal desorption unit consist of two programmable temperature vaporization injectors in series. Liquid desorption can be conducted with both GC and LC, in this case the stir bar is placed in a small vial and the desorption can be performed by adding a few microliters of the appropriate solvent or mobile phase. The main disadvantages of SBSE is that the process is manual in most cases. The life time of a single stir bar is 20 to more than 50 extractions depending on the matrix. In addition to PDMS several coatings were developed to cover a wide range of polarities [142-144]. Thin-film microextraction (TFME) is a sampling SPME device developed to improve sensitivity and achieve higher extraction efficiency. A thin film of PDMS is cut into a house-shape and mounted on a stainless steel wire for support. It combines the advantages of both SPE and SPME as the high surface area of porous SPE sorbents and robustness [145, 146]. The extraction selectivity in TFME provides a high degree of clean-up of interfering species from samples which minimizes the possibility of the matrix effects. TFME compared to conventional methods results in significant time and cost savings.

1.4.3 Single-drop microextraction (SDME) and Hollow-fiber membrane liquid-phase microextraction (HF-LPME)

Single-drop microextraction (SDME) is based on the suspension of a single droplet of organic solvent from the end of a mycrosyringe needle in an aqueous solution. The analytes are extracted by passive diffusion and then the droplet containing the analytes is injected directly into the GC or HPLC system. The microsyringe served as both the solvent holder and sample injector, this is a convenience of the microextraction operation. SDME involves a two-phase extraction system where analytes are extracted from an aqueous sample to an organic phase. It is possible to have three-phase extraction

when analytes are extracted from an aqueous sample into an organic sample and are then extracted again into a separate aqueous phase. The use of the two-phase SDME presents a high enrichment factors and significant sample cleanup. The main disadvantages are the instability of the droplet and the choice of solvent. In particular, the volatility of the solvent is crucial because solvents with high volatility are not suitable whereas those with too high a boiling point limit the applicability range [147, 148]. Hollow-fiber membrane liquid-phase microextraction (HF-LPME) allows to overcome the limitations of SDME by introducing a polymeric membrane to act as a support for the extracting solvent. This not only enables the use of larger volumes but acts as a physical barrier between the phases. The membrane is in polypropylene or other hydrophobic materials that are compatible with a broad range of organic solvents. The advantage of the use of a membrane is that owing the pore structure the concentration of high molecular mass compounds in the sample extract is reduced. Analytes are extracted into the intermediary organic phase, represented by the supported liquid membrane, and then into the aqueous phase. The extraction process depends on the partition coefficients of the analytes. In two-phase extraction the aqueous sample and organic solvent, donor and acceptor respectively, make contact with each other through the membrane pores. When the pores are prefilled with an organic solvent the two phase system changes to a three-phase system where the donor and acceptor (both aqueous phases) are separated by the organic-solvent-filled hydrophobic membrane. The advantages of HF-LPME represent a very low consumption of solvent and no problem of droplet stability, a notable cleanup efficiency, high enrichment factors and the capability of online coupling to chromatography system [149, 150].

1.5 References

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Chapter 2

Experimental design

2.1 Introduction

The experimental design is a multivariate approach that allows to organize the experiments to evaluate the significant variables of a system. The aim of the use of experimental design is to obtain the better information about the variables effect on the system studied with a minimum number of experiments, saving time and cost. The experimental design can be used for optimization, quantitative study and screening:

- Optimization: the goal of optimization is to research the better value of each variable to obtain the better results in terms of response;
- Quantitative study: can be used to explore the experimental range and to decide the relationship between factors and responses and then to choose the mathematical model;
- During the screening, the identification of the most important factors of the system is achieved.

Generally, the first step of an experimental design is the screening that means to identify the principal variables of the system which can be successively studied with other models to obtain the optimized values. The traditional approach "one factor at a time" (OFAT) could be used but it provides the true result only in some cases. When the factors to study are independent this strategy with a right levels number could identify the best response. When the factors effects are dependent, there is an interaction between them, and the result obtained with the use of a one factor at a time strategy can lead to false optima. The OFAT strategy can request more experiments compared with experimental design, explorers only a piece of experimental range and does not consider the interaction between factors [151, 152].

Many papers describe the use of experimental design to optimize several variables. For example, it was used in the optimization of MEPS and SPME critical parameters, to improve the extraction efficiency [153-156].

In the chapter 3 and 4 of this thesis both screening application and optimization design will be used to optimize the extraction method variables.

2.2 Design Matrix

An experimental design can be described by different experiments conducted in different condition. A model of three factors can be represented from the following equation (Eq. 2.1):

$y =$ (response)

$b_0 +$ (an intercept or average)

$b_1x_1 + b_2x_2 + b_3x_3 +$ (linear terms depending on each of the three factors)

$b_{11}x_1^2 + b_{22}x_2^2 + b_{32}x_3^2 +$ (quadratic terms depending on each of the three factors)

$b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$ (interaction terms between the factors)

The intercept is referred to the average experimental value, the linear terms establish a direct relationship between the response and the factors. The quadratic terms allow to visualize the curvature and to obtain the maximum and minimum of the response. The interaction terms arise because the influence of two factors on the response is rarely independent. The experimental matrix is a matrix where each column represents one of the 10 parameters involved in the eq. 2.1 whereas each row is an experiment (***Figure 2.1***)

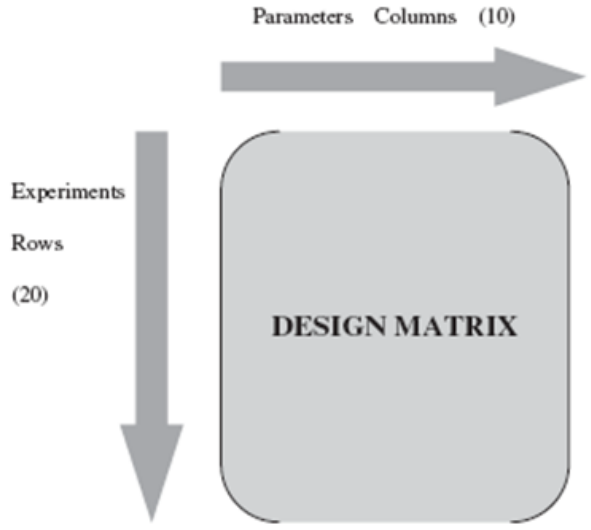

Figure 2.1: Example of experimental matrix.

The experimental matrix depends on the number and arrangement of experiments and on the mathematical model to evaluate. For an experimental design with 10 parameters and twenty

experiments, the twenty responses form a column vector with twenty rows, called *y;* the design matrix, called *D,* is represented from twenty rows and ten columns, and finally the ten coefficients form a column vector with twenty rows called *b.* The relationship between responses, coefficients, and experimental conditions is explain from the following matrix relationship: $y = D.b$ and is a good approximation within the limits of experimental error (***Figure 2.1.1***).

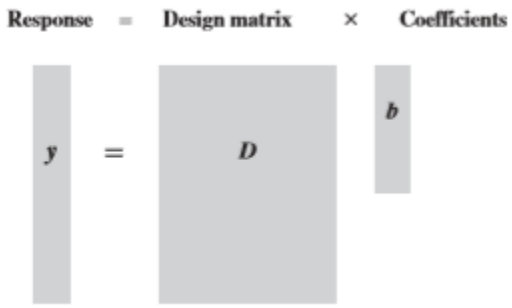

Figure 2.1.1: Representation of matrix relationship.

2.3 Screening design

In the case of many variables to optimize the first step is to identify the more important variables through a screening design. This kind of design give less information than optimization design but with a small number of experiments they allow to determine more significance variables to study later. The factorial and Plackett-Burman designs, described belowe are used to this purpose.

2.3.1 Factorial Design

The factorial design is the most common screening design. The number of experiments in a factorial design derives from $N = l^f$ where *l* is a levels number and *f* is a factors number. The first step during the planning of experiments is the selection of a maximum level and a minimum level for each factors based on the type of system to study. The next step is the planning of experiments based on the combination of the maximum level (+) and minimum level (-) chosen above. This representation of levels is called coded values or levels. The following table is an example of two levels and two factors factorial design where the experiments to perform are 4 and they are a combination of the two levels, maximum and minimum:

Table 2.1: Experiments to perform in a two levels and two factors factorial design.

Experiment	Factor 1	Factor 2
1	-	-
2	-	+
3	+	-
4	+	+

After the execution of the experiments the response, i. e. the dependent variable, for each experiment is obtained. A graphic representation of the dependent variable, called response surface, is used as a function of the factors to visualize the results obtained. An example is the surface obtained evaluating the effect of pH and reagent concentration on the yield of a generic reaction (***Figure 2.1.2***):

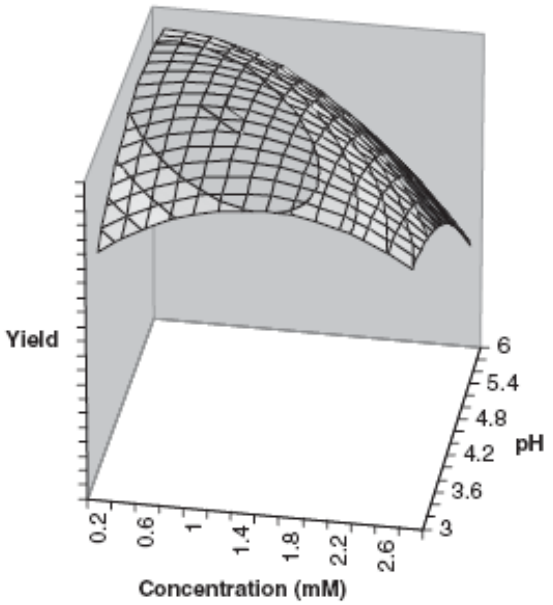

Figure 2.1.2: Response surface for a generic reaction evaluating the effect of pH and reagent concentration.

The last step is the evaluating of the results through a design matrix obtained from the combination of the factor levels used and from their interaction. In this way a design matrix with different columns is obtained, as shown below:

Table 2.1.1: Design Matrix for two factors and two levels factorial design.

Intercept	Factor 1 (x_1)	Factor 2 (x_2)	Factors interaction (x_1x_2)
+	-	-	-
+	-	+	-
+	+	-	+
+	+	+	+

The coefficients should be calculate considering the following matrix equation: $y = D.b$ where b represents the four coefficients. During the interpretation of the results it is possibly to compare them only if the coded coefficients are considered, and not real values, because in this way the same scale is used. The two factors and two levels factorial designs can be used for the screening of some important variables in a determined system, but they present some drawbacks. In particular, they provide an approximation within the experimental region; they do not consider the quadratic terms because the experiments involve only two levels and they cannot give information about a system reproducibility. If the number of factors increase the planning of experiments provides the development of a factorial design with more factors that means more experiments to perform. For example, for a two levels and ten factors designs the number of experiments to perform will be 1024 (2^{10}). In the same way, in a multilevel design where for each factors more levels are considered the planning of several experiments should be take into account. For example, for a two factors and three levels design the number of experiments to perform is nine (3^2), as shown below (***figure 2.1.3***), where the axes represent the studied factors and the black circles are the experiments.

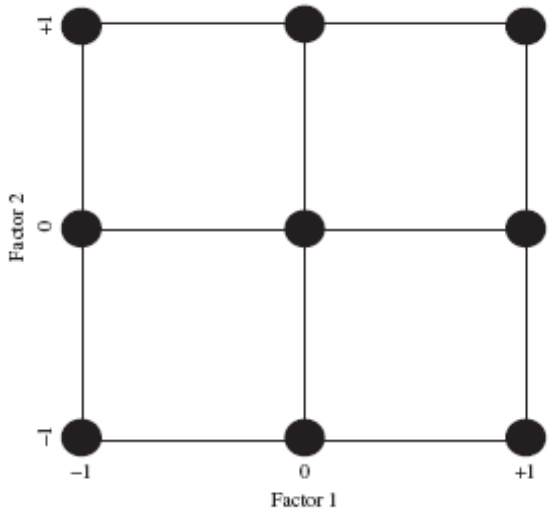

Figure 2.1.3: Representation of a two factors and three levels designs.

In a three levels designs the quadratic terms can be considered, however as the size of the experiments increases it becomes impracticable to perform the design for this there are different way to reduce the volume of experimentation [157].

2.3.2 Fractional factorial designs

The fractional factorial designs allow to obtain the information but with a lower number of experiments. If a two levels and three factors factorial design is considered the number of experiments to perform will be 8 (2^3), as shown in the experimental matrix reported in ***table 2.1.2***.

Table 2.1.2: Representation of two levels and three factors factorial design.

Experiments			Design matrix							
Factor 1	Factor 2	Factor 3	1	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
1	1	1	1	1	1	1	1	1	1	1
1	1	-1	1	1	1	-1	1	-1	-1	-1
1	-1	1	1	1	-1	1	-1	1	-1	-1
1	-1	-1	1	1	-1	-1	-1	-1	1	1
-1	1	1	1	-1	1	1	-1	-1	1	-1
-1	1	-1	1	-1	1	-1	-1	1	-1	1
-1	-1	1	1	-1	-1	1	1	-1	-1	1
-1	-1	-1	1	-1	-1	-1	1	1	1	-1

If a correct subset with some properties is considered a fractional factorial design can be achieved, as shown in the ***table 2.1.3***.

Table 2.1.3: Two levels and three factors fractional factorial design.

Experiments			Design matrix							
Factor 1	Factor 2	Factor 3	1	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
1	1	1	1	1	1	1	1	1	1	1
1	-1	-1	1	1	-1	-1	-1	-1	1	1
-1	-1	1	1	-1	-1	1	1	-1	-1	1
-1	1	-1	1	-1	1	-1	-1	1	-1	1

The fractional factorial design is characterized by the following properties: i) each column in the experimental matrix is different; ii) in each column the two levels are represented by the same values (+1 and -1); iii) for each experiment at level '+1' for factor 1, there are an equal number of experiments for factors 2 and 3 which are at levels '+1' and '−1', and so on for every combination of factors. This property is called orthogonality that means that each factors is independent. In this matrix the first columns are different but the last four correspond each to one of the first four columns. As consequence, since the number of experiments is reduced also the amount of information is

reduced and only four effects can be studied. A fractional factorial design and its properties can be matched by a cube, where each face represents two rather than four experiments, and every alternate corner corresponds to an experiment (***Figure 2.1.4***).

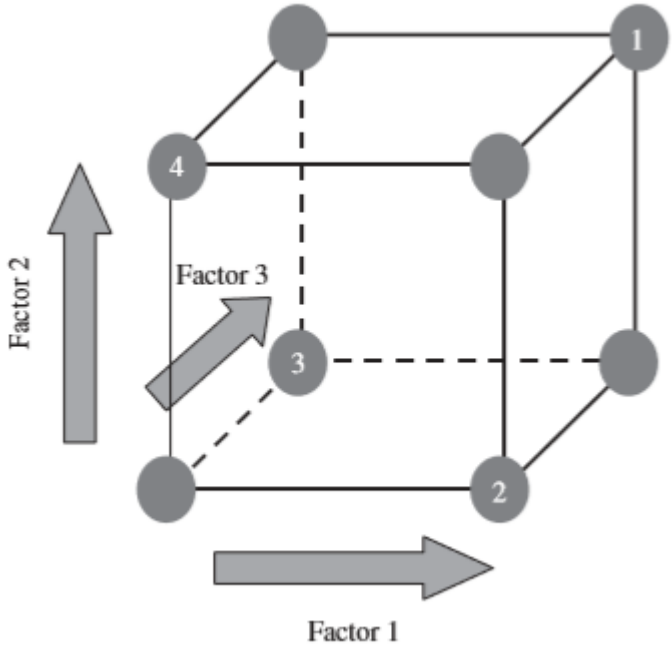

Figure 2.1.4: Fractional factorial design representation.

Among the different advantages of two levels fractional factorial design, it does not consider quadratic terms because the experiments are performed at two levels, among the experiments there are not replicates and this influences the reproducibility information and finally the number of experiment must be a power of two [157].

2.3.3 Plackett–Burman design

To overcome the limitations of fractional factorial designs, Plackett and Burman proposed a two levels factorial design where the number of experiments is a multiple of four and the number of factors is one less the number of experiments. In this way the number of experiments is reduced compared to factorial designs. For example, in the following table a Plackett-Burman design for 11 factors and 12 experiments is reported.

Table 2.1.4: Plackett-Burman design for 11 factors and 12 experiments.

Experiments	Factors										
	1	2	3	4	5	6	7	8	9	10	11
1	−	−	−	−	−	−	−	−	−	−	−
2	+	−	+	−	−	−	+	+	+	−	+
3	+	+	−	+	−	−	−	+	+	+	−
4	−	+	+	−	+	−	−	−	+	+	+
5	+	−	+	+	−	+	−	−	−	+	+
6	+	+	−	+	+	−	+	−	−	−	+
7	+	+	+	−	+	+	−	+	−	−	−
8	−	+	+	+	−	+	+	−	+	−	−
9	−	−	+	+	+	−	+	+	−	+	−
10	−	−	−	+	+	+	−	+	+	−	+
11	+	−	−	−	+	+	+	−	+	+	−
12	−	+	−	−	−	+	+	+	−	+	+

The construction of this table is very interesting, because the first row is represented from the experiments at a single level, the other rows are correlated diagonally. The most important property is the orthogonality then the factors are independent. Despite in the Plackett-Burman design the number of experiments is a multiple of four some of this kind of designs exist for 3, 7, 11, 15 factors. This is possible when the experimental factors are less than the number in a standard design, the final factors can be set to dummy factors. The dummy factors are variables that has not influence in the system and it could be everything [158].

2.4 Optimization design

The optimization designs are used after the evaluating of the system with a screening design when a number of variables is high. The optimization designs allow to identify the best variables responses and how this response is correlated with the factors through the quantitative models. For example, the central composite design is an optimization model.

2.4.1 Central composite Design (CCD)

The screening designs do not provide information on system reproducibility, quadratic terms and the degrees of freedom for the lack-of-fit for the model (D) are often zero. The models with a lower factors number can give more information. A three factors and two levels factorial design provide four experiments to identify linear terms and intercept but it does not give information on the

interaction, quadratic terms and replicates. If the number of experiments is eight, with a fully factorial design, the information on interaction terms are provided but to estimate the quadratic terms a star design should be used where three levels for each factors are necessary, +1, -1 and 0, where 0 represents the central point replicates. To evaluate the correspond error the central experiment can be repeated 5 times. Finally, the planning of fully factorial design, a star design and five central point replicates provide the implementation of twenty experiments. This kind of design is called central composite design, CCD, and for three factors it realises twenty experiments, 10 parameters, five-degree freedom to determine the experimental error and the model quality (***Figure 2.1.5***).

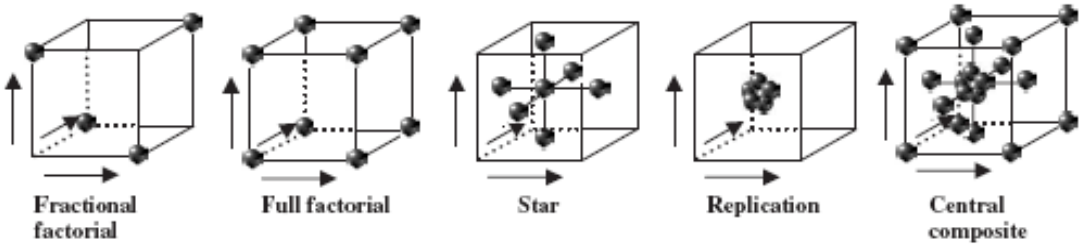

Figure 2.1.5: Representation of three levels central composite design.

When the design is performed the values of the different terms can be calculated and evaluated through design matrix or statistical models. In a central composite design, the number of experiments is high when there are many factors to study. For example, if five factors should be studied, before to perform the CCD design, the screening design can be applied to identify the significance of each factors [157].

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Chapter 3

Solid-phase microextraction coupled with gas chromatography triple quadrupole mass spectrometry analysis in environmental and clinical applications

3.1 Introduction

In recent years many scientific contributes were based on the development of methods for early diagnosis of different diseases through analysis of specific markers in biological fluids. Many disorders on human health present a particular pathway that produces compounds or biomarkers characteristic of that illness. A part of the research efforts concerning human health were focused on the development of methods that can be used to identify and quantify the biomarkers of several pathologies. The biological fluids are the most important matrices for the evaluation of the pathology and its evolution because they represent the principal ways out of many biomarkers. In particular, among the several biological fluids urine is the simplest matrix to collect and its sampling is not invasive. In general, sample preparation is a very important step during the development of an analytical method. When the matrix is a biological fluid particular interest is addressed towards the treatment of the sample. Among the different sample preparation approaches microextraction techniques represent a suitable tool for the analytes extraction, also in clinical application. In particular, SPME has some characteristics that are particularly suitable for applications in clinical analytical chemistry. Indeed, SPME allows to perform the extraction and the concentration of analytes in a single step and to complete sample preparation without the use of organic solvents, can be interfaced with different separation and identification systems, and offers the possibility of automation. Another important feature of an analytical method, as well as the sample preparation, is the use of instruments highly sensitive and selective. A high efficient separation system such as gas chromatography coupled with the capability of tandem mass spectrometry for the unequivocal confirmation and accurate quantification represents the major tool to develop versatile and specific analytical methods in clinical application.

In this chapter, the development and the optimization of SPME-GC-MS/MS methods for the assay of biomarkers in urine are presented. In particular, in the first work a new SPME matrix compatible fiber was investigated in raw urine for the coating performance and for the extraction capability of polycyclic aromatic hydrocarbons. The other two studies presented regard the determination of polyamines in urine as biomarkers of several diseases and the assay of phthalates monoesters in urine as markers of human exposition to phthalates.

Investigating the robustness and extraction performance of a matrix-compatible solid-phase microextraction coating in human urine and its application to assess 2-6-ring polycyclic aromatic hydrocarbons using GC-MS/MS [159]

3.2 Introduction

In the last years, the medical research led the development of new clinical protocols for monitoring of markers in biofluids involved in human diseases. This research was based on the development of cost effective and environmentally friendly analytical protocols. SPME is the most wide-spread microextraction technique for sample preparation. From its introduction has experienced a rapid growth in several fields, especially in clinical and pharmaceutical analysis [160-163]. The development of new coatings capable of dealing with new analytical challenges is among the most attractive areas of investigations. Pawliszyn and coworkers developed a new matrix compatible fiber by modification of the SPME fiber solid coating available commercially polydimethylsiloxane-divinylbenzene (PDMS/DVB), with a thin layer of polydimethylsiloxane (PDMS) [164]. The outer layer protects the solid coating avoiding fouling and the fiber results suitable for sampling in complex matrices maintaining the extraction capability of the original solid coating [164, 165]. This overcoated fiber (PDMS/DVB/PDMS) has shown good results for the extraction of different contaminants in complex food matrices [164, 166-169]. Polycyclic aromatic hydrocarbons, PAHs, are environmental pollutants formed by incomplete combustion of organic matter and some of them are probable carcinogens [170]. The exposure takes place generally by inhalation of polluted air, tobacco, smoke or by ingestion of contaminated food and water. Generally, PAHs are analysed using LC or GC with fluorescence detector or MS [171]. In order to evaluate the PAHs exposition, the urinary hydroxylated PAH metabolites and unmetabolized were analyzed [172-174]. The use of unmetabolized PAHs as markers is based on the lower effect of metabolic intraindividual variability and the high specificity due to the sure association with exposure. Urine includes several compounds with different abundance and physiochemical properties. PAHs quantitation is very delicate due to the presence in small concentration of unmetabolized analytes and the complexity of the urine [172, 175]. For this reason, the analysis of these analytes in urine requests a robust sample preparation to avoid the extraction of interfering species. SPME is a very promising extraction technique in urine before gas chromatographic assay. PDMS fiber in direct immersion was used after dilution of the sample [172, 176-180] influencing the sensitivity of the method. Waidyanatha *et al*. and later Campo proposed a method based on headspace SPME extraction (HS-SPME) and gas chromatographic analysis for the sampling of urinary PAHs up to four aromatic rings [173, 181]. Recently, PDMS fiber in direct

immersion (DI-SPME) was used to monitor PAHs with 4-6 aromatic rings [172]. The headspace extraction results the best choice because the direct contact between the fiber and the matrix causes a reduction of fiber performance [173, 182]. The use of PDMS fiber in direct immersion mode in complex matrices is justified by fiber matrix-compatibility rather than its affinity for the target analytes. The solid coating PDMS/DVB is the best choice for the extraction of PAHs in complex matrices due the presence of π-π interaction with target analytes [183]. The disadvantage of the use of PDMS/DVB coating is the fouling caused by the adsorption of matrix components [183, 184]. For this reason, only few extractions can be carried out with the same fiber also if the interaction between the DVB and the aromatic rings of the analytes allowed a good extraction performance.

The goal of this work was the evaluation of the new PDMS/DVB/PDMS fiber as analytical sampling tool for the investigation in raw human urine. The target analytes considered to evaluate the PDMS/DVB/PDMS fiber performance were 16 urinary PAHs with 2–6 aromatic rings. The extraction under kinetic and thermodynamic conditions and lifetime experiments were performed for both PDMS/DVB/PDMS and PDMS/DVB fibers to compare their endurance and performance. After extraction the analyses were performed with a gas chromatograph interfaced with triple quadrupole (QqQ) MS in selected reaction monitoring (SRM) acquisition mode. Finally, the developed method was tested for the PAH quantification in undiluted urine samples of smoking and non-smoking volunteers.

3.2.1 Experimental section

3.2.1.1 Materials

PAHs calibration mix, containing the investigated analytes at 10 µg/mL in acetonitrile, was used. The investigated analytes are reported, with their physiochemical properties, in the following table (***Table 3.2***). The labeled internal standards used, naphthalene-d_8 (NAP-d_8), phenanthrene-d_{10} (PHE-d_{10}), pyrene-d_{10} (PY-d_{10}), chrysene-d_{12} (CHR-d_{12}) and benzo[a]pyrene-d_{12} (BaP-d_{12}) were purchased from Sigma-Aldrich (Milan, Italy) while dibenz[a,h]anthracene-d_{14} (DahA-d_{14}) was bought from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). The polydimethylsiloxane 100 µm (PDMS) and polydimethylsiloxane/divinylbenzene 65 µm (PDMS/DVB) fibers tested were purchased from Supelco (Bellefonte, PA, USA) and conditioned as recommended by the manufacturer. The overcoated fiber PDMS/DVB/PDMS was prepared according the procedure developed by Souza-Silva et al. [166]. Sylgard 184® (PDMS pre-polymer and curing agent) was purchased from Dow

Corning (Midland, MI, USA). The fiber was conditioned at 250 °C for 1 h prior use and visually inspected to verify the uniformity of the outer PDMS layer. Synthetic urine (negative urine control) was produced by Cerilliant and distributed Sigma-Aldrich (Milan, Italy).

Table 3.2: Investigated analytes. logP: partition coefficient, MW: molecular weight, b.p: boiling point.

Compound	Abbreviation	Number of aromatic rings	logP[a]	MW (Da)	b.p[a] (°C)
Naphthalene	NAP	2	3.45	128.17	218
Acenaphthylene	ACY	3	4.26	152.19	280
Acenaphthene	ACE	3	4.26	154.21	279
Fluorene	FLE	3	4.16	166.22	298
Phenanthrene	PHE	3	4.68	178.23	340
Anthracene	ANT	3	4.68	178.23	340
Fluoranthene	FLT	4	5.17	202.25	384
Pyrene	PY	4	5.17	202.25	393
Benz[a]anthracene	BaA	4	5.91	228.29	438
Chrysene	CHR	4	5.91	228.29	448
Benzo[k]fluoranthene	BkF	5	6.40	252.31	480
Benzo[b]fluoranthene	BbF	5	6.40	252.31	480
Benzo[a]pyrene	BaP	5	6.40	252.31	495
Indeno[1,2,3-cd]pyrene	IP	6	6.89	276.33	495
Dibenz[a,h]anthracene	DahA	5	7.14	278.35	524
Benzo[ghi]perylene	BghiP	6	6.89	276.33	500

(http://www.chemspider.com)

3.2.1.2 Instrumentation, apparatus and data processing

A TSQ Quantum GC (Thermo Fischer Scientific) with a triple quadrupole mass spectrometer (QqQ) Quantum and a TRACE GC Ultra equipped with a TriPlus autosampler were used for the analyte uptake evaluation and method development. The mass spectrometer was operated in electron ionization (EI) in selected ion monitoring (SIM) for the comprehensive evaluation of the

PDMS/DVB/PDMS coating and in selected reaction monitoring (SRM) modes for the analytes quantification. The transfer line and ionization source temperatures were set at 280 °C and 250 °C, respectively. The emission current was set at 50 µA. The scan width was set at 1.0 m/z for all segments. Peak width of Q1 was fixed at 0.7 amu. Argon of purity of 99.999% at a pressure of 1.0 mTorr was used as collision gas. A Thermo PTV straight Liner (0.7 mm × 2.75 mm × 105 mm) was used as GC inlet liner. The capillary column was a Phenomenex Zebron ZB-5ms (30 m × 0.25 mm i.d., 0.25 µm film thickness; 95% polydimethylsiloxane, 5% phenyl). The lifetime experiments were carried out with Pegasus 4D GC-ToF/MS system consisting of an Agilent 6890N gas chromatograph equipped with a split/splitless injector (Agilent Technologies, Palo Alto, CA, USA), an MPS2 autosampler for automated SPME (Gerstel, Mülheim and der Ruhr, Germany), and a high speed ToF mass spectrometer (LECO, St. Joseph, MI, USA). For SPME analysis a splitless Sky® liner (i.d. 0.75 mm) was used (Restek Corporation, Bellefonte, PA, USA). Chromatographic separation was achieved by using a Rxi®-5Sil MS capillary column, 30m, 0.25mm i.d., 0.25µm film thickness (Restek Corporation, Bellefonte, PA, USA). MS operational conditions were: electron ionization (EI) at 70 eV; ion source temperature: 210 °C; transfer line temperature: 250 °C; mass range: m/z 55–350; acquisition rate: 20 Hz; detector voltage: −1500 V. For both GC instruments, the injector temperature was set at 270 °C and the GC oven program consisted of the following steps: 70 °C for 10 min, then ramped at 20 °C/min to 200 °C and held at this temperature for 1.5 min, then increased at 20 °C/min to 260 °C and held 2 min and finally ramped at 20 °C/min to 340°C and held for 5 min. Helium of purity 99.999% was used as carrier gas at a flow of 1 mL/min. An optical stereomicroscope (Olympus SZX10, Olympus, Japan) was used to evaluate the morphology of the fibers. The comparison between the extraction time profiles and between the fiber constant values (fc) was made by the non-parametric Mann-Whitney U test, performed by Statistica 8.0 (StatSoft 2007 Edition, Tulsa, USA).

3.2.1.3 Samples and analytical procedure

For analyte uptake evaluation and coating lifetime experiments the urine from 10 non-smoker volunteers of age comprised between 22 and 31 years was collected. The urine used for the application of developed method to real samples was obtained as aliquots from spot urine specimens provided by 12 volunteers (i.e., 6 non-smokers and 6 smokers) between 23 and 27 years. The smoker subjects used to smoke 10-15 cigarettes per day. The urine samples were collected in an amber glass vial and frozen at -20°C until use and before its use the urine was centrifuged at 4000 rpm for 3 min. The optimized method gave the best conditions to use in the preparation and extraction of the sample. In

particular, 8 mL of centrifuged urine sample was placed in a glass vial and then 40 µL of the internal standard mixture, described above, at 200 ng/L in acetonitrile was added. The vial was crimped, and the solution was vortexed for few seconds. SPME extraction was performed with a PDMS/DVB/PDMS fiber in direct immersion mode for 40 min at 80°C under agitation with an incubation time of 10 min to ensure a uniform temperature throughout the whole sample. The extracted analytes were thermally desorbed by introducing the fiber into the injector set at 270 °C for 10 min. Under these desorption conditions a successive thermal blank of the coating did not show carryover of the analytes.

3.2.1.4 Lower limit of quantification, calibration procedure, matrix effect and figures of merit

The food and drug Administration (FDA) guidance were used to evaluate the lower limit of quantification (LLOQs), linearity, intraday and interday precision and accuracy [185]. LLOQs were chosen as the lowest concentration for each analyte that provides a response at least five times the signal compared to blank signal and that can be quantified with suitable accuracy (80-120%) and precision (coefficient of variation CV <20%). The determination of LLOQs was carried out analyzing synthetic urine samples spiked with a different and known concentration of PAHs and they were analyzed in quintuplicate. The obtained LLOqs values for each analyte were used as the lowest concentration of the standard curves. The linearity was evaluated using a matrix-matched calibration method [186]. The calibration curves were obtained in the range between the LLOQ of each analyte and 100 ng/L. The preparation of calibration standards involved the use of a blank sample (blank synthetic urine sample without internal standards), a zero sample (blank synthetic urine sample with internal standards) and seven non-zero samples (blank synthetic urine samples spiked with the internal standards and with a known amount of analytes covering a range descibed above). Internal standards used were NAP-d_8, PHE-d_{10}, CHR-d_{12}, and BaP-d_{12} and they were added at 1 ng/L to all the calibration standards and samples. Each calibration standard was analyzed in triplicate. To evaluate the matrix effect, intra-day, inter-day precision and accuracy, the quality control (QC) samples at three concentration levels representing the entire range of calibration curve were prepared by spiking a real urine sample obtained from non-smoking subject, in the following way: one at 3x the LLOQ (low QC sample), one near the center (middle QC sample), and one at 0.8x the upper boundary of the standard curve (high QC sample). Matrix effect was evaluated with a method proposed by Matuszewski [187]. Six urine samples of non-smoking donors were used to determine the matrix effect dividing each sample into two aliquots to prepare raw urine with 1 ng/L of internal

standards and the samples at QC concentration with internal standards at 1 ng/L. All samples were analyzed in triplicate and the matrix effect was calculated as the percentage ratio of analyte peak area for the spiked real sample minus the peak area for the unspiked blank real sample against the peak area for the spiked synthetic urine sample. The relative matrix effect was also evaluated for each QC concentration level by comparing the precision value (CV%) obtained by analyzing five aliquots from the same subject (i.e., intra-subject precision) with the precision value obtained by analyzing six urine samples from different subjects (i.e., inter-subject precision) spiked at the same concentration. Intra-day precision and inter-day precision were expressed as CV% whereas accuracy was calculated as percent ratio between the concentration estimated from the calibration curve and the spiked concentration.

3.2.2 Results and discussion

3.2.2.1 Assessment of analyte uptake under kinetic and thermodynamic conditions by PDMS/DVB/PDMS coating

To evaluate the behavior of the new coating an extraction time profile of the analytes in human urine spiked at 1.3 ng/mL was carried out. In the extraction time profile the linear portion (kinetic regime) was obtained by performing extractions from 5 to 40 min whereas extractions up to 25 h were carried out to study the equilibrium conditions. Generally, in nonequilibrium conditions the presence of analytes and their movement in the boundary layer surronding the fiber surface represents the rate-limiting step of the uptake process because in this region the flux of analytes is dependent on analyte diffusion rather than convection conditions. For the new coating, that contains an extra thin PDMS layer, the analytes will have to diffuse through the PDMS layer and then reaching the solid coating. To study the effect of analyte diffusion through the additional PDMS layer the agitation of 1000 rpm was used since this allowed to minimize the tickness of the boundary layer [188]. Under these conditions the analytes diffusion through the PDMS layer influences the uptake rate of the analytes on the coating. Indeed, the ***figure 3.2*** shows the linear trend between the slope of the linear portion of the extraction time profile and the diffusion coefficients in PDMS for the PAHs investigated suggested by Rusina et al. [189]. This demonstrates that the uptake rate of the analytes on the coating is faster for compounds with higher diffusion coefficients. Therefore, in urine matrix and under the convection conditions used, the diffusion in the outer PDMS layer of the coating influences the overall rate of the extraction.

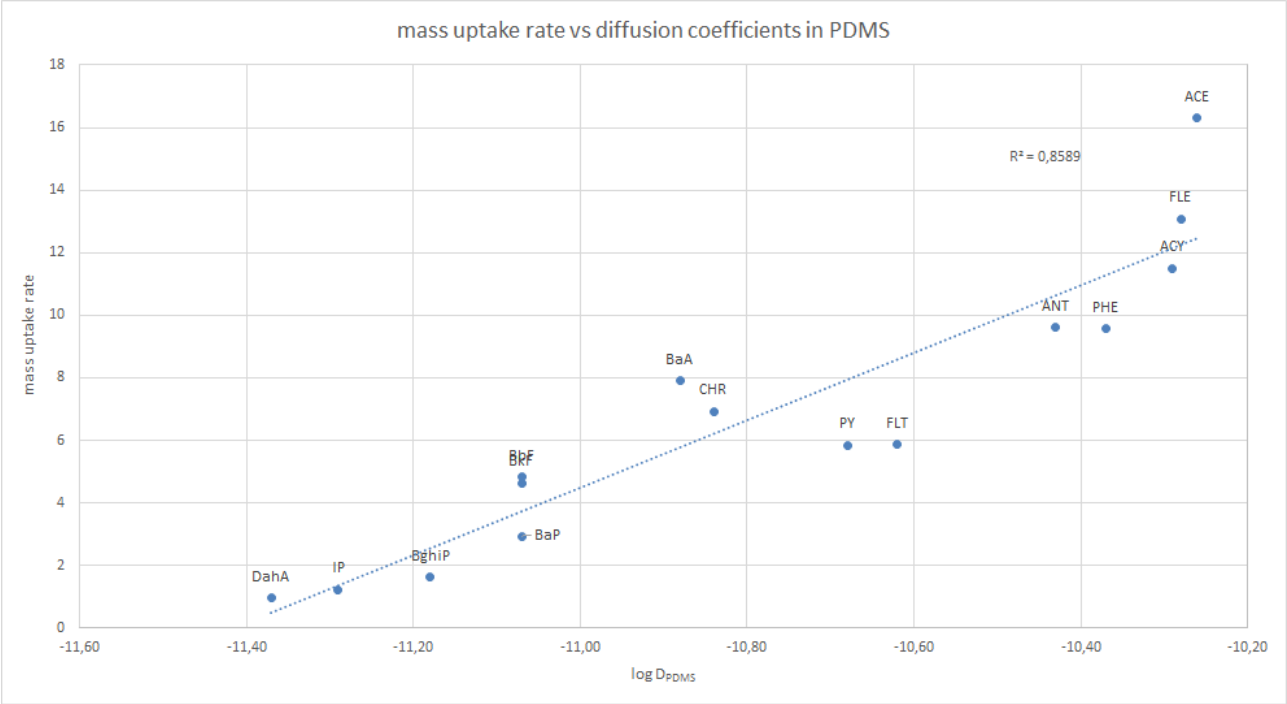

Figure 3.2: Uptake rate of analytes on the coating versus the logarithm of the diffusivities of the analytes in polydimethylsiloxane (PDMS).

If Fluorene is considered as a representative of medium hydrophobicity compounds, the uptake rate is faster for the PDMS/DVB/PDMS than the PDMS/DVB coating, as shown in ***figure 3.2.1*** in which the amount of analytes extracted at different time is reported.

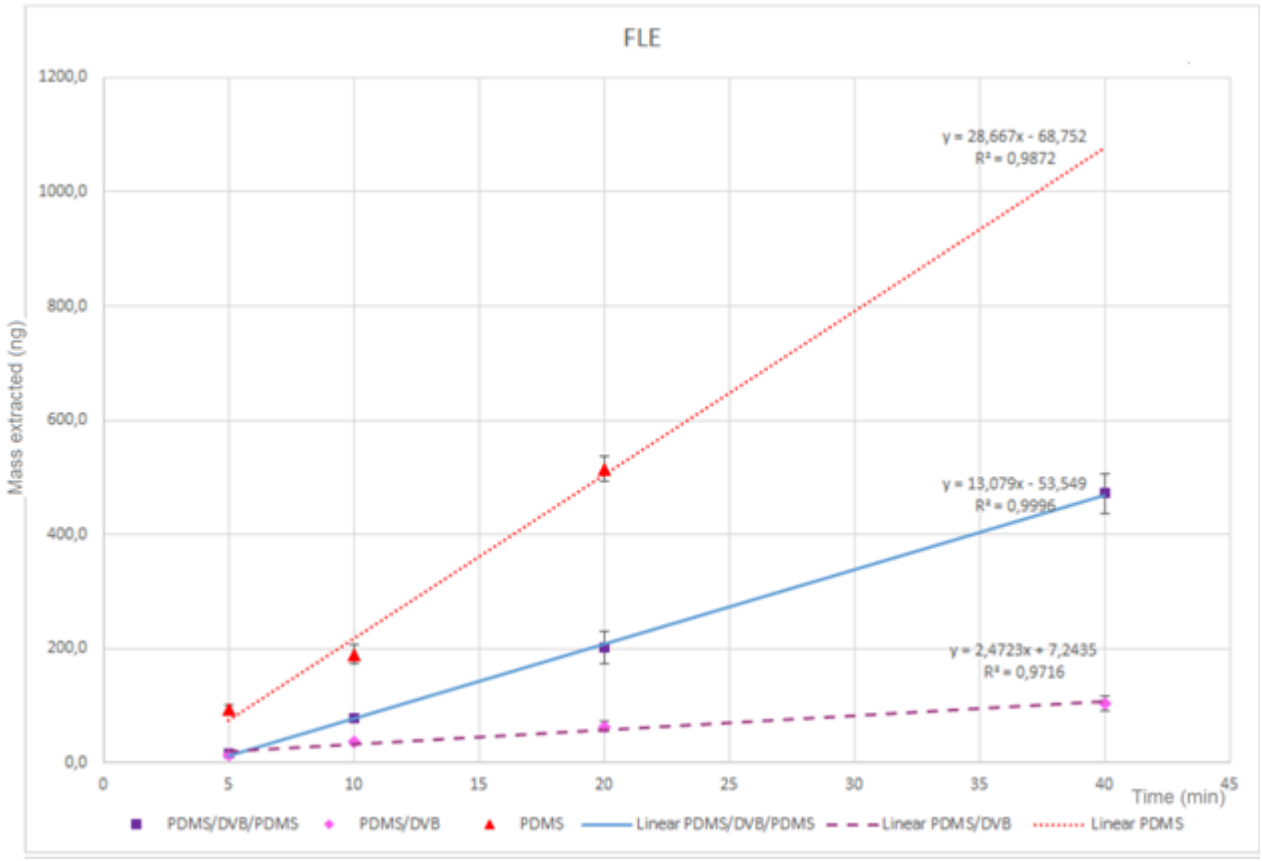

Figure 3.2.1: Extraction time profiles acquired from 5 to 40 min for fluorene (FLE) with polydimethylsiloxane/divinylbenzene (PDMS/DVB), polydimethylsiloxane (PDMS) and polydimethylsiloxane/divinylbenzene/ polydimethylsiloxane (PDMS/DVB/PDMS) fibers.

To verify the significant variation between the different coatings the Mann-Whitney U test to slope values ($p < 0.05$) was applied. This phenomenon could be caused by the higher surface area of the PDMS/DVB/PDMS coating than the PDMS/DVB and by the affinity of FLE toward the PDMS phase. On the other hand, the effect of boundary layer is more important in the case of analytes with higher hydrophobicity ($\log P > 5.91$) because they have a slower diffusion in this layer. If Benzo[b]fluoranthene is considered as representative of compounds with higher hydrophobicity the uptake rate is no more dependent on the coating type used, as shown in ***figure 3.2.2***.

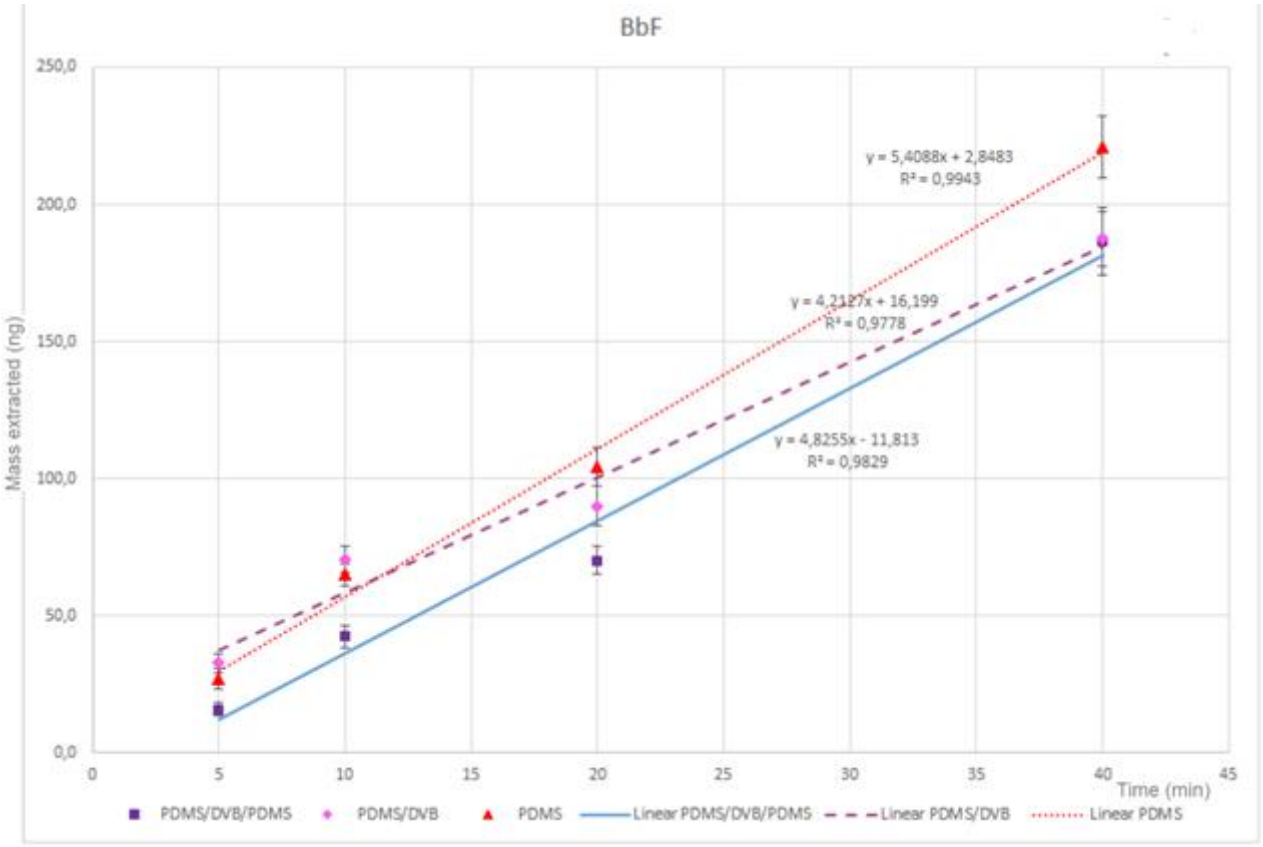

Figure 3.2.2: Extraction time profiles acquired from 5 to 40 min for Benzo[b]fluoranthene (BbF) with polydimethylsiloxane/divinylbenzene (PDMS/DVB), polydimethylsiloxane (PDMS) and polydimethylsiloxane/divinylbenzene/ polydimethylsiloxane (PDMS/DVB/PDMS) fibers.

The Mann-Whitney U test applied in the case of Benzo[b]fluoranthene indicates that the kinetic of the PDSM/DVB/PDMS and PDMS/DVB coatings is statistically equal. The obtained results are in according with recent studies developed on other class of compounds [190]. From the obtained results, the sensitivity for the analysis of less hydrophobic PAHs in pre-equilibrium conditions improves when the PDMS/DVB/PDMS coating is used respect to PDMS/DVB coating. At the same time, the application of the new coating does not influence the sensitivity of hydrophobic analytes. The complete extraction time profiles acquired from 5 min to 25 hours for FLE and BbF obtained with the three different coatings are reported in ***figure 3.2.3.***

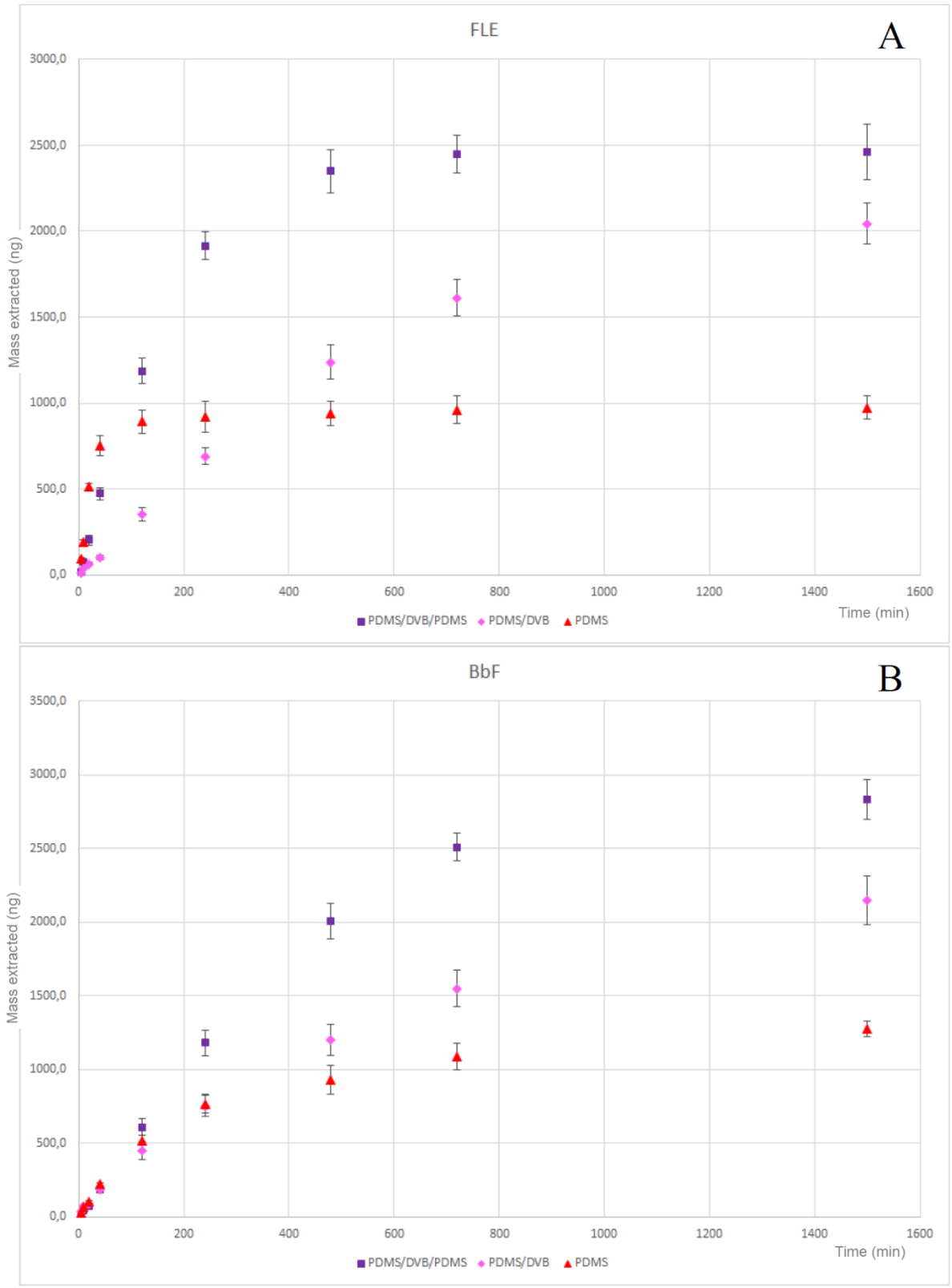

Figure 3.2.3: Extraction time profiles acquired from 5 min to 25 hours for: (A) fluorene (FLE) and (B) benzo[b]fluorenthene (BbF) by polydimethylsiloxane/divinylbenzene (PDMS/DVB), polydimethylsiloxane (PDMS) and polydimethylsiloxane/divinylbenzene/polydimethylsiloxane (PDMS/DVB/PDMS) fibers.

The amount of the analytes extracted at equilibrium conditions was used for the calculation of the fibers constants f_c by equation 3.2. It represents the coating capacity toward a given analyte and is taken into account when K_{fs} cannot be used because the active volume of sorption cannot be calculated such in the case of solid coatings.

$$f_c = \frac{n_e V_s}{(C_0 V_s) - n_e} \qquad \text{(Eq. 3.2)}$$

In equation 3.2 n_e indicates the analyte amount extracted at equilibrium condition, V_s represents sample volume (8 mL) and C_0 is the initial concentration of the analytes in the sample (1.3 ng/mL). The f_c calculated for each analyte in equilibrium conditions with PDMS, PDMS/DVB/PDMS and PDMS/DVB coatings are shown in ***figure 3.2.4.***

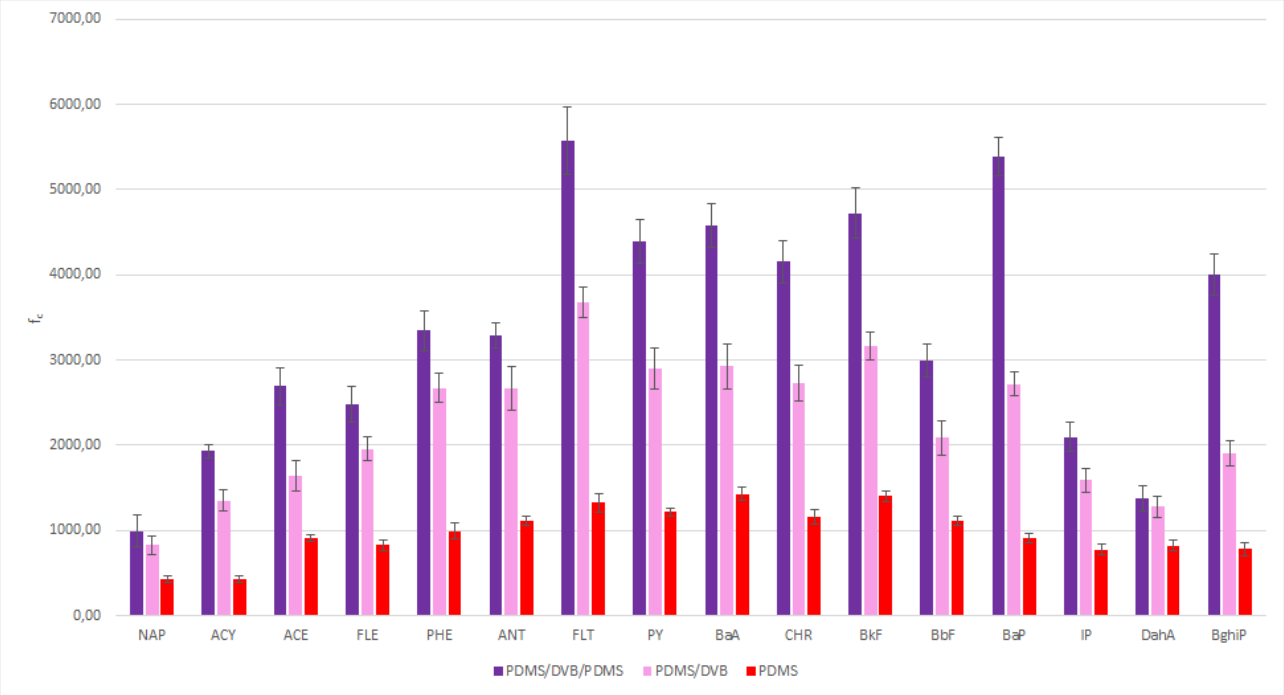

Figura 3.2.4: Fiber constants calculated for each analyte with different coatings in equilibrium conditions.

The Mann-Whitney U test applied in this case demonstrated that there are significant differences between the fiber constant values obtained with PDMS/DVB/PDMS and PDMS/DVB coatings except for NAP and DahA. The results obtained shows the enhanced partitioning of analytes with a high log*P* value (log*P*> 4.0) into the PDMS layer. The higher values obtained for PDMS/DVB/PDMS and PDMS/DVB coatings than PDMS coating demonstrate the greater affinity of PAHs with the DVB-based coating.

3.2.2.2 Coating lifetime evaluation for PDMS/DVB/PDMS and PDMS/DVB coatings in human urine

Quality control lifetime (QCL) analysis was performed to evaluate the lifetime of PDMS/DVB/PDMS and PDMS/DVB coatings. The QCL strategy consists in carrying out analysis of spiked ultrapure water samples before the first extraction of the coating in human urine and after each batch of 20 extractions in urine. The use of spiked ultrapure water samples is suitable to avoid that the signals were influenced by matrix components. Six samples constituted by 8 mL of water (4% acetone content) spiked at 1 µg/L of the targeted analytes were analyzed. Direct immersion sampling was carried out for 30 min at 40°C with agitation speed of 500 rpm. Following extraction, the fiber was rinsed for 30 seconds in ultrapure water. The adsorbed analytes were thermally desorbed by introducing the fiber for 15 min into the GC injector set at 270°C. After the desorption, the fiber was washed for 1 min in a water/methanol mixture (50:50, v/v). Afterwards, a batch of twenty extractions in pooled urine from non-smoker individuals was carried out and at the end of the sequence, the coating was inspected under microscope to evaluate its morphology. Subsequently, three QCL samples were analyzed prior the following batch of twenty extractions in urine. The same routine was followed for 80 urine extractions in case of PDMS/DVB, and 120 urine extractions for PDMS/DVB/PDMS. The ***figures 3.2.5*** and ***3.2.6*** report the images acquired under microscope after every set of 20 extractions for PDMS/DVB/PDMS and PDMS/DVB coatings, respectively.

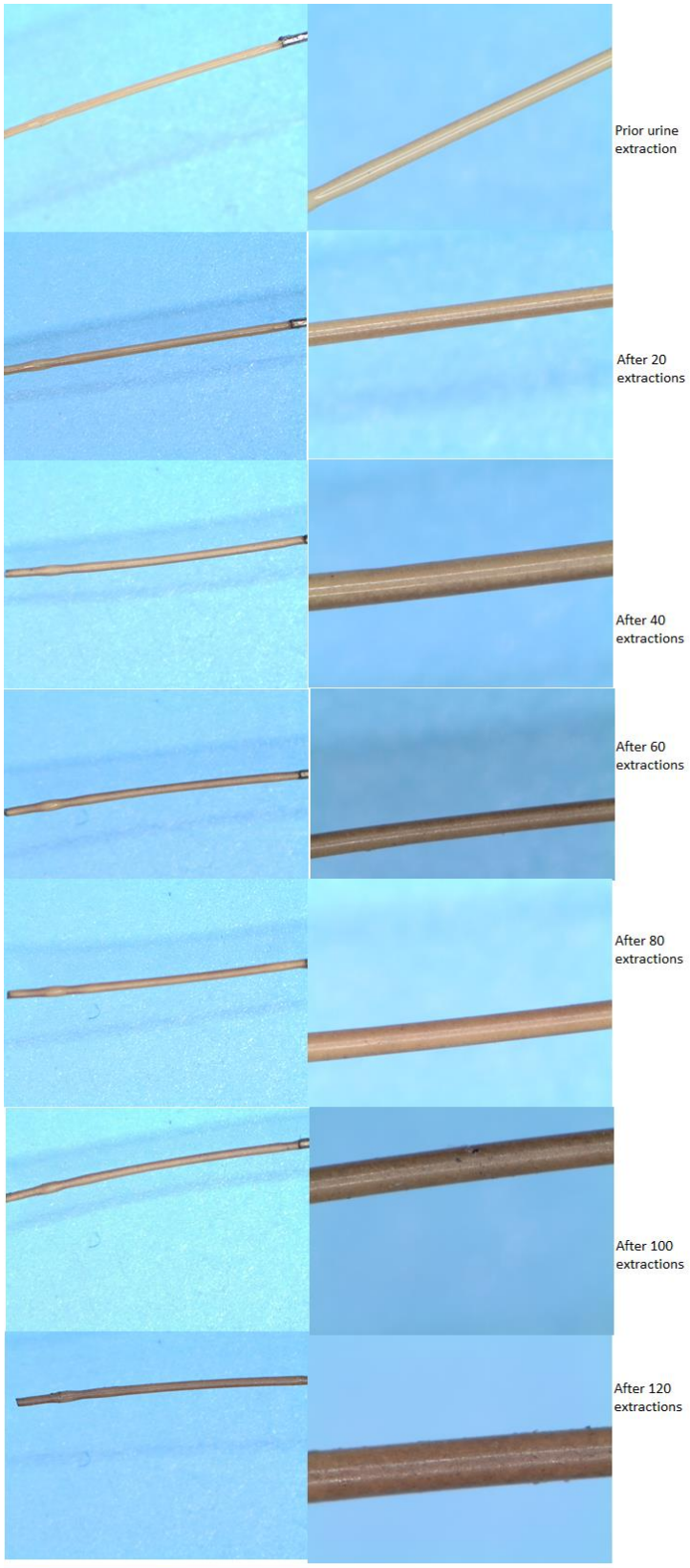

Figure 3.2.5: Stereomicroscope images of PDMS/DVB/PDMS fiber lifetime.

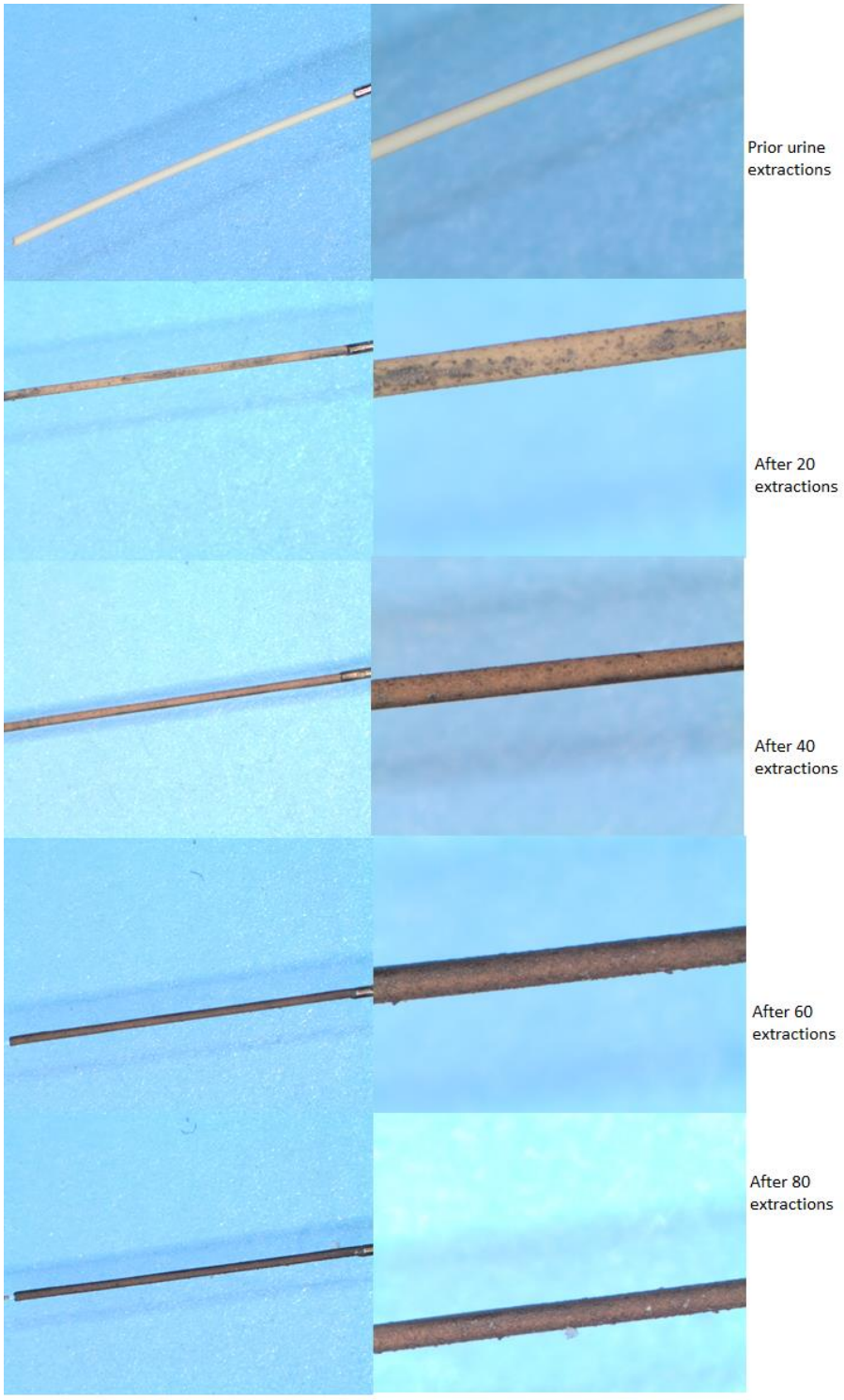

Figure 3.2.6: Stereomicroscope images of PDMS/DVB fiber lifetime.

The PDMS/DVB/PDMS coating demonstrates more robustness and enhanced extraction performance compared with a PDMS/DVB fiber in repetitive analyses in urine. In the case of PDMS/DVB coating a deterioration of the fiber after some urine extraction is visible. The additional PDMS layer involved

in the new coating avoids the adsorption of matrix components. This confirms the results obtained by other works present in literature that involve the evaluation of the PDMS/DVB/PDMS lifetime in food matrices [164, 166-169]. In terms of extraction efficiency (***figure 3.2.7***), the PDMS/DVB coatings shows an improvement in the extracted amount for the hydrophobic analytes after 20 analyses and a drastic decrease after only 40 analyses for all the considered analytes.

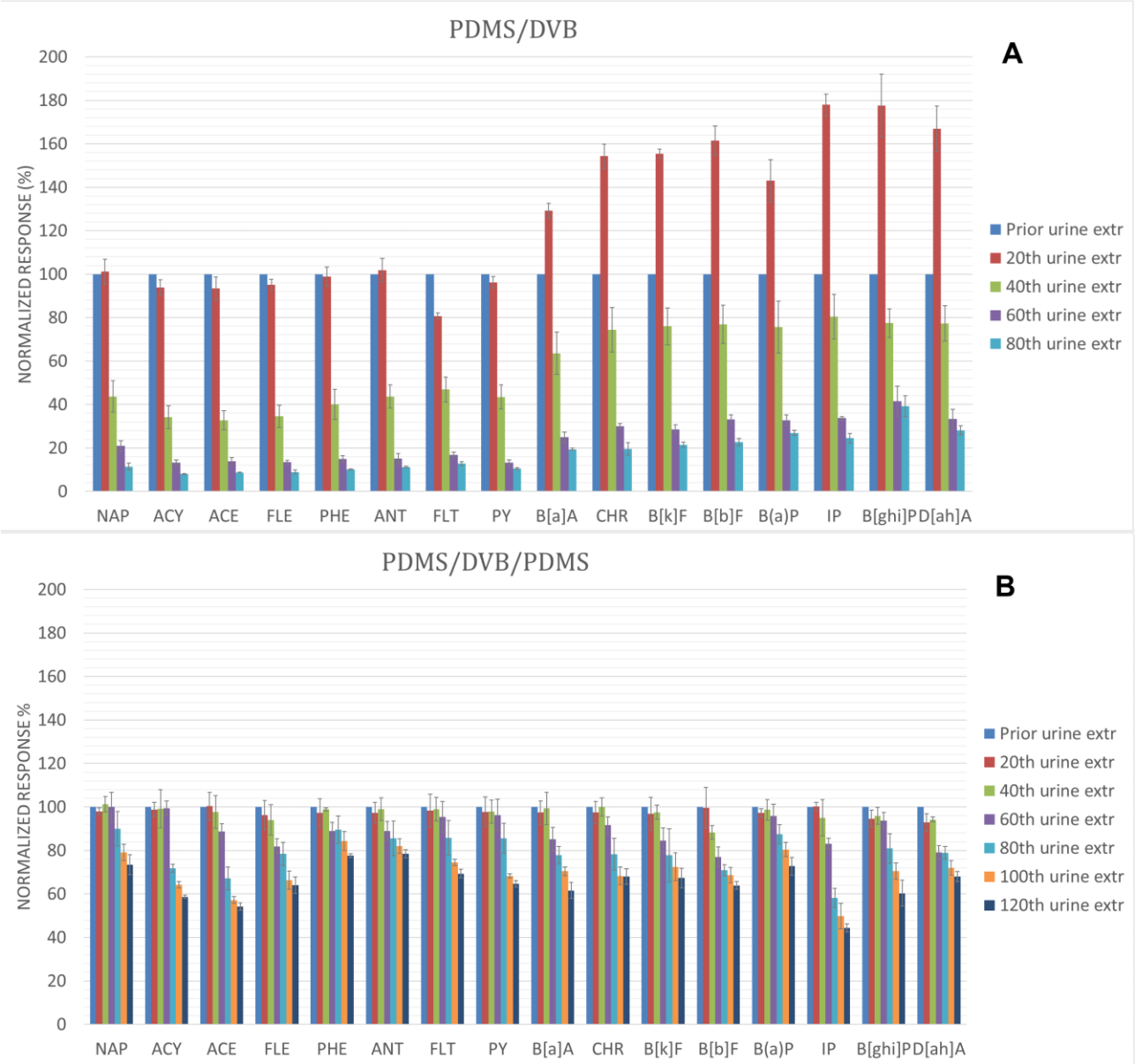

Figure 3.2.7: Lifetime evaluation of (A) polydimethylsiloxane/divinylbenzene (PDMS/DVB) and (B) polydimethylsiloxane/divinylbenzene/ polydimethylsiloxane (PDMS/DVB/PDMS) fibers. Response normalized taking extraction efficiency of fiber before the lifetime evaluation as 100% for each analyte.

The increase of signals after 20 analyses can be explained considering the adsorption of the hydrophobic PAHs onto carbon-containing residues of matrix components on the coating surface. On the other hand, the PDMS/DVB/PDMS fiber allows perform up to 120 extractions in raw urine retaining better extraction performance than the PDMS/DVB fiber. Therefore, the trends of the two extraction efficiency profiles confirmed the microscope images and demonstrated the clear enhancement in matrix compatibility and robustness achieved by the PDMS/DVB/PDMS coating.

3.2.2.3 Optimization GC-QqQ-MS/MS SPME conditions

The analyses were conducted with a gas chromatograph coupled with a triple quadrupole mass spectrometer operating in selected reaction monitoring (SRM). The SRM acquisition mode allows to achieve more sensitivity and ensures unequivocal identification of the analytes [191-194]. Two transitions were considered for each analyte. In particular, the transition that gave the best S/N ratio was selected for quantification and the second more sensitive transition was selected for identification and to avoid false positive determinations. This is in line with the European Commission Decision 2002/657/EC [195]. In ***table 3.2.1*** the optimized transitions with their collision energy and the retention time for each analyte are reported.

Table 3.2.1: Optimized transitions for each analyte. RT: Retention time; SIM: Single ion monitoring.

Compound	**RT (min)**	**SIM quantifying ion (m/z)**	**Scan time (s)**	**SRM transition, m/z (collision energy, V)**	
				Quantification	Identification
NAP	14.54	128	0.06	128→102 (19)	128→77 (19)
ACY	16.99	152	0.06	152→150 (27)	152→126 (20)
ACE	17.28	154	0.06	154→152 (28)	153→126 (25)
FLE	18.16	166	0.12	166→165 (24)	165→115 (19)
PHE	19.83	178	0.06	178→152 (22)	178→176 (30)
ANT	19.90	178	0.12	178→152 (22)	178→176 (30)
FLT	21.79	202	0.12	202→200 (35)	202→150 (45)
PY	22.20	202	0.06	202→200 (35)	202→152 (29)
BaA	24.71	228	0.06	228→226 (35)	228→224 (45)
CHR	24.79	228	0.06	228→226 (35)	228→224 (45)
BkF	26.52	252	0.06	252→250 (30)	250→248 (32)
BbF	26.56	252	0.06	252→250 (30)	250→248 (32)
BaP	26.99	252	0.06	252→250 (30)	250→248 (30)
IP	28.51	276	0.06	276→274 (38)	276→272 (55)
DahA	28.52	278	0.06	278→276 (44)	278→274 (44)
BghiP	28.89	276	0.06	276→274 (38)	274→272 (38)
NAP-d_8	14.43	-	0.06	136→108 (25)	-
PHE-d_{10}	19.79	-	0.06	188→160 (27)	-
PY-d_{10}	22.17	-	0.06	212→208 (33)	
CHR-d_{12}	24.74	-	0.06	240→236 (35)	-
BaP-d_{12}	26.92	-	0.06	264→260 (32)	-
DahA-d_{14}	28.45	-	0.06	292→288 (44)	-

The parameters that can influence the SPME extraction such as, extraction time, extraction temperature and addition of salt were evaluated acquiring signals in SRM mode. In particular, the SPME extraction time was chosen considering the results obtained from the thermodynamic and kinetic experiments. A pre-equilibrium extraction time of 40 min was considered as the best compromise between extraction efficiency and analysis run time. The extraction temperature is not only important in headspace SPME extraction but also in direct immersion mode because it can

influence the viscosity of the sample and then the diffusivity of the analytes. It was evaluated from 40 °C that it is the lower temperature of the heater/agitator module of the TriPlus autosampler used to 80 °C. In ***figure 3.2.8*** the signals for each analyte obtained at three different temperature values are reported as normalized values taking the signals at 40°C as 100%. As shown, higher value of temperature leads to an increase of the signal for all analytes except NAP.

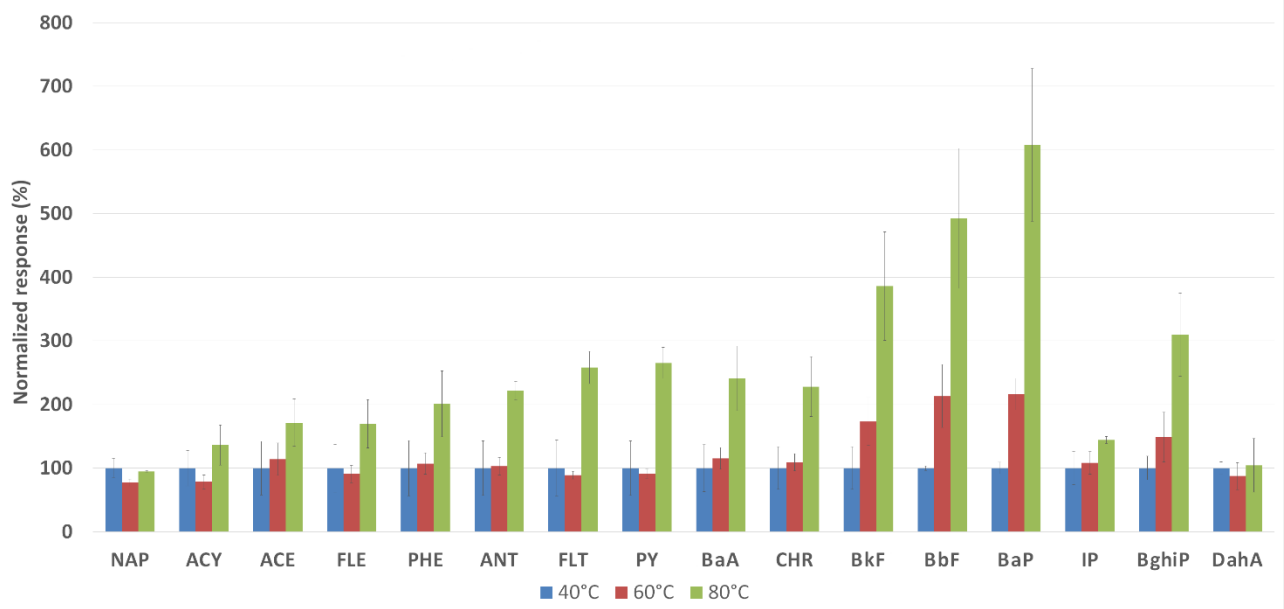

Figure 3.2.8: Responses obtained for each analyte at different extraction temperature by analyzing a sample at 50 ng/L.

This is in line with previous results for PAHs extraction by DI-SPME and HS-SPME [172, 173]. With an extraction temperature of 80°C an improvement was obtained for BbF, BkF and BaP. For this reason, 80 °C was selected for further investigations. As regards the salting out effect, it was evaluated by adding sodium chloride in a range between 0 and 10% (w/w) to a blank human urine samples spiked at 10 ng/L. In ***figure 3.2.9*** the normalized signals of each analyte, taking signal for no salt addition as 100%, obtained at 0%, 5% and 10% of salt are reported.

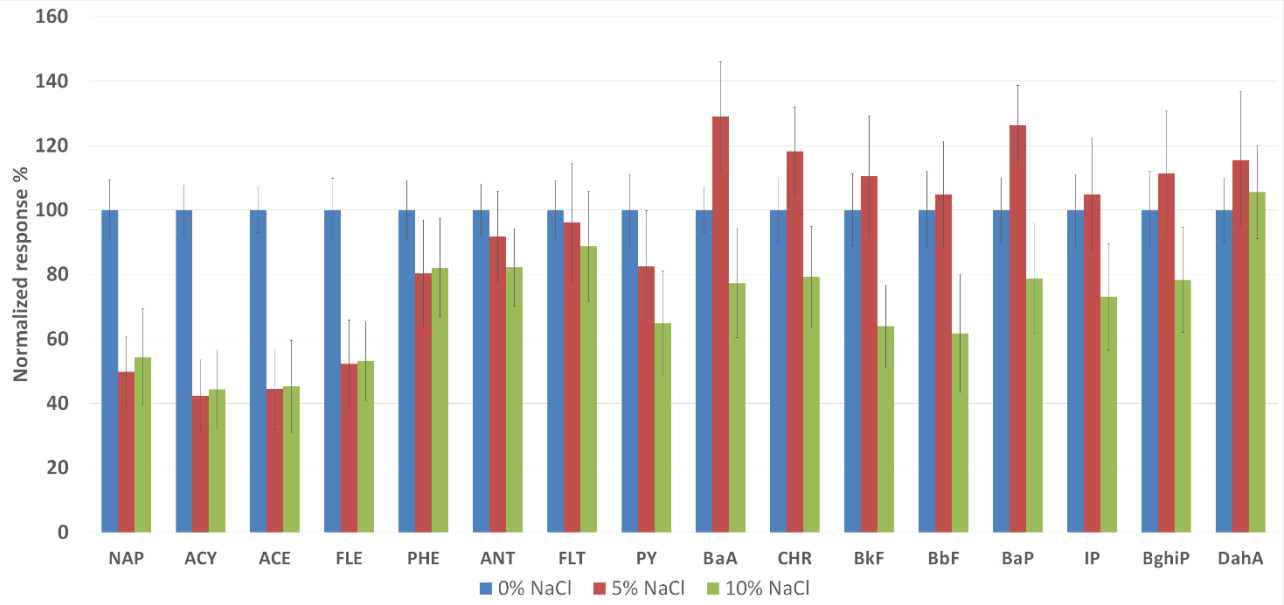

Figure 3.2.9: Responses obtained for each analyte at different salt concentrations by analyzing a sample at 50 ng/L.

When a concentration of 5% or 10% of salt is used a decrease in signal can be observed for the analytes with a log*P* ranging from 3.45 to 5.17. On the other hand the signals of analytes with a higher log*P* improve adding 5% of salt. The best compromise was attained with no addition of NaCl to the sample.

3.2.2.4 Lower limit of quantification and linearity

As described in the experimental section, to achieve a more reliable quantification of analytes matrix-matched calibration was used. Since this calibration approach requests a blank matrix with properties similar to sample matrix, synthetic urine was chosen. An alternative strategy for preparation of calibration samples could have involved the use of blank real sample matrix. However, this blank matrix is not always available for the presence of low levels of urinary PAHs even in nonsmoking subjects due to other sources [196]. The LLOQs, determined analyzing in quintuplicate synthetic urine samples spiked with different and known concentration of PAHs are reported in ***table 3.2.2***.

Table 3.2.2: Calibration parameters, internal standards, lower limits of quantitation (LLOQs) and LOQs obtained with SPME methods reported in literature.

Compound	LLOQ (ng/L)	Range (ng/L)	R^2	Internal standard	LOQ values (ng/L)	
					DI-SPME Ref. [172]	HS-SPME Ref. [173]
NAP	0.2	0.2-100	0.9995	NAP-d_8	-	22.8
ACY	0.5	0.5-100	0.9996	NAP-d_8	-	4.14
ACE	0.2	0.2-100	0.9936	NAP-d_8	-	6.02
FLE	0.05	0.05-100	0.9996	PHE-d_{10}	-	4.62
PHE	0.1	0.1-100	0.9993	PHE-d_{10}	-	5.10
ANT	0.2	0.2-100	0.9996	PHE-d_{10}	-	2.28
FLT	0.1	0.1-100	0.9978	PHE-d_{10}	2.2	4.26
PY	0.1	0.1-100	0.9987	CHR-d_{12}	1.9	3.72
BaA	0.1	0.1-100	0.9999	CHR-d_{12}	1.5	5.24
CHR	0.2	0.2-100	0.9997	CHR-d_{12}	0.6	4.79
BkF	0.5	0.5-100	0.9994	CHR-d_{12}	0.5	19.7
BbF	0.5	0.5-100	0.9996	CHR-d_{12}	0.5	16.2
BaP	0.5	0.5-100	0.9979	BaP-d_{12}	0.5	15
IP	1	1-100	0.9992	BaP-d_{12}	0.5	-
BghiP	1	1-100	0.9994	BaP-d_{12}	1.1	-
DahA	1	1-100	0.9995	BaP-d_{12}	1.1	-

The LLOQs obtained for each analyte were used as the lower concentration of the calibration curves. The range chosen to evaluate the linearity for each analyte is also reported in table 3.2.2. The comparison with other methods present in literature points out that the LLOQs obtained for 2-4 ring PAHs are lower than those achieved by HS and DI-SPME with PDMS fiber. Comparable LLOQs values were obtained for 5-6 ring PAHs in DI-SPME extraction [172, 173]. The table shows also the deuterated standards used for the method evaluation and for the correction of matrix effect and instrumental drift. Initially, DahA-d_{14} was used but it showed a poor chromatographic behaviour and low instrumental response. Indeed, it returns unsatisfactory results even in the correction of corresponding compound response. For all analytes a good linearity was obtained with a correlation coefficient value > 0.99 using the other deuterated compounds.

3.2.2.5 Matrix effect and analytical performance

When the matrix-matched calibration is used as quantitation method the similarity between the real matrix and that used to prepare the calibration standards should be evaluated during method validation [186]. The Matuszewski method was used to evaluate the matrix effect [187] as described in section 3.2.1.4, and the results are reported in ***table 3.2.3***.

Table 3.2.3: Matrix effect results at every QC levels.

Compound	Concentration (ng/L)	Matrix effect (%)	Intra-subject precision (CV%)	Inter-subject precision (CV%)
NAP	0.6	139	14.1	34.5
	10	81	9.5	10.0
	80	86	9.2	11.2
ACY	1.5	89	14.0	20.9
	10	84	8.5	11.4
	80	92	7.1	7.2
ACE	0.6	93	11.8	15.9
	10	78	10.1	10.7
	80	89	5.2	8.9
FLE	0.15	120	8.1	13.9
	10	100	10.9	14.5
	80	104	5.2	9.3
PHE	0.3	114	1.8	4.8
	10	102	3.9	7.9
	80	108	3.2	4.0
ANT	0.6	116	6.1	11.4
	10	101	4.7	7.4
	80	91	5.1	8.9
FLT	0.3	92	7.9	10.4
	10	85	4.7	8.1
	80	81	2.8	9.8
PY	0.3	131	4.6	11.5
	10	127	5.1	7.7
	80	117	5.1	7.1
BaA	0.3	106	3.6	5.5
	10	116	2.0	4.7
	80	94	3.5	6.0
CHR	0.6	103	3.9	4.2
	10	112	1.1	3.2
	80	105	1.5	2.3
BkF	1.5	95	14.1	21.4
	10	107	9.4	12.7
	80	106	10.6	11.4
BbF	1.5	86	6.3	18.5
	10	90	9.5	10.8
	80	91	9.1	12.8
BaP	1.5	109	6.0	10.6
	10	104	4.5	9.7
	80	101	2.3	3.9
IP	3	61	10.8	27.7
	10	73	9.4	15.6
	80	79	9.5	16.0
BghiP	3	116	11.4	19.6
	10	104	5.8	14.0
	80	110	6.9	9.4
DahA	3	57	18.1	30.8
	10	82	15.3	24.1
	80	84	12.4	18.6

In the calculation of matrix effect the ratio of analyte peak area to internal standard peak area was considered (see table 3.2.2 for the analyte/internal standards pairs). The matrix effect shows values between 79% and 127% at the lower concentration, for all analytes except for NAP, PY and DahA. The relative matrix effect calculated in terms of intra-subject and inter-subject precision, as described in experimental section, shows a similar intra and inter-subject precision. This means that the matrix does not present a significant effect on the method. The coefficients of variation calculated fall in the range 1-20%, as reported in the table, except for NAP, IP and DahA at the lowest concentration. The inter-subject precision is slightly higher to the intra-subject precision and therefore the matrix effect values obtained for all analytes can be considered acceptable according to the Matuszewski method [187]. The non-parametric Mann-Whitney U test was used to evaluate if the matrix effect was not dependent on the concentration level. The application of the test demonstrated that no significant difference ($p<0.05$) is present for the majority of cases. Contrariwise, statistical differences were observed only for NAP, CHR, ANT and BaA.

Accuracy, intraday precision and interday precision were evaluated by analyzing the QC samples, in quintuplicate for each concentration level, once a day on six consecutive days. The values obtained, reported in ***table 3.2.4*** are inside the range suggested by FDA (CV within 15% of nominal value and 20% at LLOQ level and accuracy within ±15% and ±20% at LLOQ level) for all analytes except for NAP and DahA.

Table 3.2.4: Accuracy and precision values.

Compound	Concentration (ng/L)	Intra-day accuracy (%)	Intra-day precision (CV%)	Inter-day accuracy (%)	Inter-day precision (CV%)
NAP	0.6	128	19.3	149	31.5
	10	121	18.0	126	23.5
	80	78	11.3	77	23.2
ACY	1.5	75	14.1	90	21.0
	10	91	11.5	91	23.9
	80	89	11.9	87	16.3
ACE	0.6	83	15.2	81	24.1
	10	84	12.0	80	19.8
	80	92	10.3	93	12.8
FLE	0.15	111	8.3	120	20.3
	10	107	11.0	112	15.3
	80	108	7.5	115	13.4
PHE	0.3	116	12.7	118	18.5
	10	111	5.4	123	9.0
	80	114	6.8	112	11.6
ANT	0.6	119	4.5	122	8.0
	10	114	7.6	118	14.9
	80	92	10.4	104	14.1
FLT	0.3	99	8.3	110	15.4
	10	105	8.2	109	16.5
	80	97	6.7	105	10.1
PY	0.3	83	17.1	90	22.3
	10	93	14.0	93	16.4
	80	90	11.6	89	17.4
BaA	0.3	107	6.3	108	7.1
	10	99	4.8	105	8.3
	80	106	4.5	105	9.0
CHR	0.6	95	4.6	103	7.5
	10	106	3.9	105	6.1
	80	102	2.1	103	4.5
BkF	1.5	101	9.3	99	20.2
	10	89	12.3	104	17.8
	80	95	7.5	94	12.1
BbF	1.5	105	8.2	110	16.4
	10	95	9.5	108	12.1
	80	90	7.5	91	13.1
BaP	1.5	103	12.8	108	13.9
	10	113	10.8	116	11.3
	80	107	5.9	111	9.8
IP	3	114	14.5	110	22.4
	10	99	10.5	117	16.5
	80	108	7.1	107	15.1
BghiP	3	86	19.0	90	22.9
	10	84	6.1	97	19.9
	80	91	6.5	90	13.7
DahA	3	96	17.5	127	33.0
	10	94	14.7	130	25.2
	80	104	11.9	114	20.8

The chromatogram obtained in SRM mode with optimized method for the QC sample at 10 ng/L is reported in ***figure 3.2.10.***

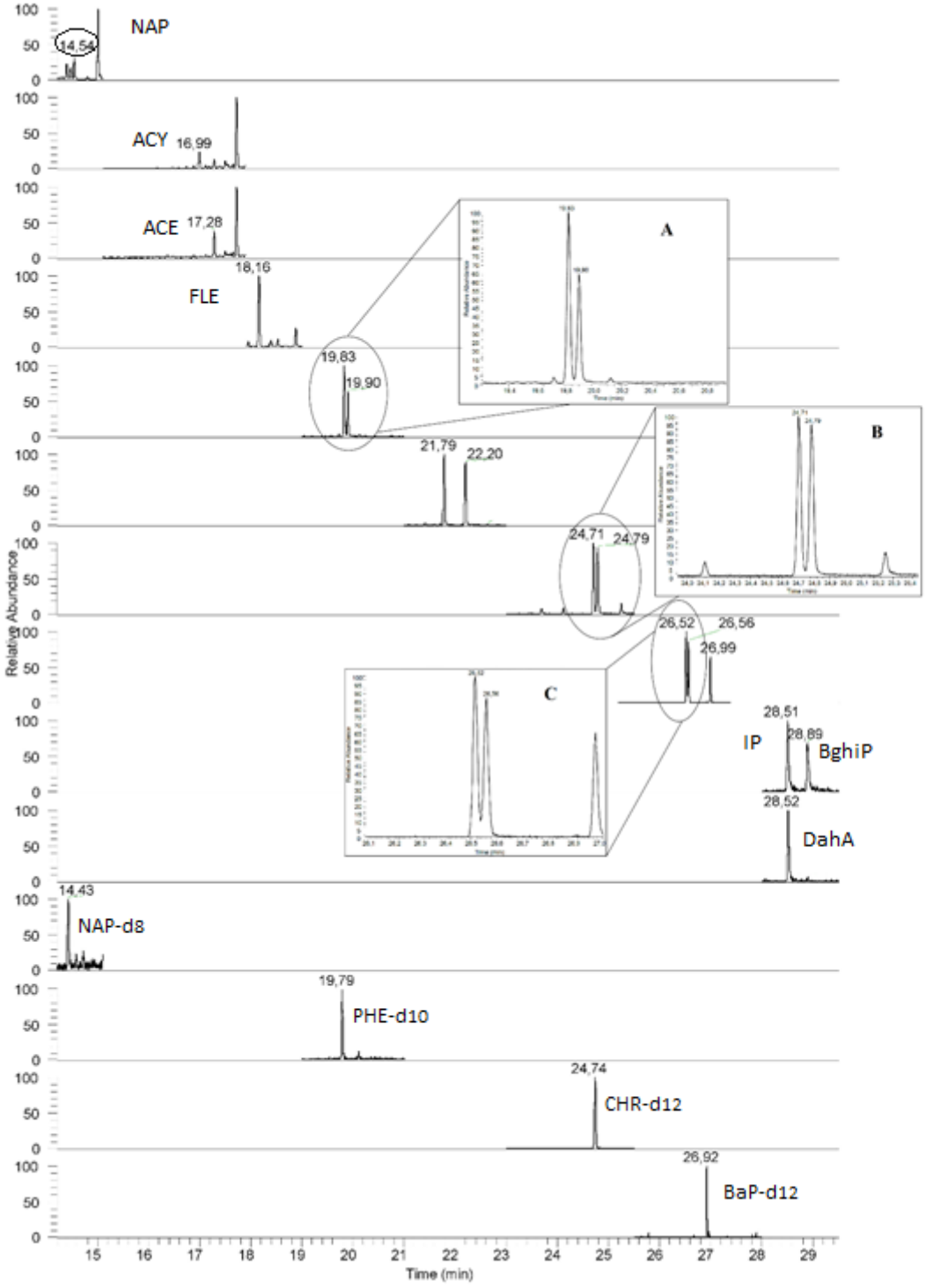

Figure 3.2.10: SRM chromatogram at 10 ng/L. The chromatogram in the zoomed window: A is referred to PHE and ANT separation, respectively; B is referred to BaA and CHR separation, respectively; C is referred to BkF and BbF separation, respectively.

3.2.2.6 Application to real samples

The developed protocol was applied to the PAHs screening in six urine of non-smoking subjects and six urine of smokers. The results are reported in ***table 3.2.5.***

Table 3.2.5: Median levels of urinary PAHs in real samples (min-max).

	NAP	**ACY**	**ACE**	**FLE**	**PHE**	**ANT**	**FLT**	**PY**
Non-smokers (ng/L)	25.2 (12.1-35.4)	0.78 (<0.5-1.70)	0.95 (0.44-1.56)	0.67 (0.06-1.80)	3.61 (1.45-4.61)	0.48 (<0.2-0.79)	0.91 (<0.1-1.61)	1.42 (0.58-1.94)
Smokers (ng/L)	37.4 (21.8-65.9)	0.75 (<0.5-1.58)	0.74 (0.22-1.17)	2.11 (1.49-3.27)	21.4 (9.49-31.9)	1.51 (0.94-2.02)	3.89 (1.94-7.09)	3.71 (2.75-5.42)
	BaA	**CHR**	**BkF**	**BbF**	**BaP**	**IP**	**BghiP**	**DahA**
Non-smokers (ng/L)	0.59 (0.37-0.70)	0.69 (<0.2-1.04)	1.14 (<0.5-1.95)	1.35 (<0.5-2.27)	<0.5 (<0.5-0.94)	<1 (<1-1.20)	<1 (<1-1.84)	<1 (<1-1.33)
Smokers (ng/L)	3.28 (1.79-4.11)	2.50 (1.84-3.45)	2.04 (<0.5-4.47)	1.48 (<0.5-3.53)	1.08 (<0.5-4.75)	<1 (<1-3.59)	1.47 (<1-6.71)	<1 (<1-<1)

All investigated PAHs were quantified in the real samples, except in smoker subjects for DahA. To our knowledge, this is the first time that the quantification of 16 PAHs (from two to six rings) in urine samples of smokers and nonsmokers has been carried out in a single method. Moreover, these results demonstrate that the proposed method can be used to monitor unmetabolized PAHs in raw urine at concentration levels of nanograms per liter level.

3.2.3 Conclusions

For the first time the new PDMS/DVB/PDMS coating was evaluated in bioclinical analysis. PAHs were considered as target analytes for the investigation of the analyte uptake under kinetic and thermodynamic conditions and for the fiber lifetime evaluation. The results showed the enhanced matrix compatibility obtained with the PDMS/DVB/PDMS coating respect to PDMS/DVB coating. From the evaluation of extraction properties, at equilibrium conditions the outer PDMS layer increased the coating capacity toward hydrophobic analytes. On the other hand, in preequilibrium conditions PDMS/DVB/PDMS coating showed an improved sensitivity in comparison with the PDMS/DVB coating for less hydrophobic PAHs. The additional PDMS layer protects the solid coating and improves also the extraction uptake of the fiber. The new coating was tested for the development of a new DI-SPME-GC-QqQ-MS method for the quantification of 2-6 aromatic rings PAHs in untreated human urine. The developed method is the only application for the quantification of unmetabolized PAHs with 2-6 aromatic rings in a single analytical run. This approach proposes a simplified sample preparation avoiding the solvent extraction and the solid-phase cleaning that are usually carried out before the instrumental analysis. Moreover, the combined use of tandem mass spectrometry and SPME leads to an improvement of the sensitivity. The LLOQs values achieved with this method are lower than those obtained with the PDMS fiber in headspace and in direct immersion mode. Finally, the results of this work open new perspective for SPME in the gas chromatographic analysis of biofluids. Indeed, since the PDMS/DVB/PDMS fiber combines a high matrix compatibility with the extraction capability of the PDMS/DVB coating it represents a valid alternative to other commercial SPME coatings for urine analysis.

Development of a fast and simple gas chromatographic protocol based on the combination use of alkyl chloroformate and solid phase microextraction for the assay of polyamines in human urine [197]

3.3 Introduction

One of the purposes of recent clinical research is the development of new and rapid cancer diagnostic techniques in order to obtain early diagnosis and therefore to start the treatment of disease. The presence or a high concentration of these biomarkers can provide information on type of cancer and its progression [198]. Polyamines are aliphatic amines with low molecular weight involved in normal growth and cellular differentiation. These compounds regulate the function of different enzymes bound to the membrane and some ion channels. The increase of polyamines biosynthesis indicates a rapid tumor growth and this causes the increase of their levels in biofluids such as urine and plasma [199, 200]. They are among the most important cancer biomarkers for early diagosis and treatment [201]. The polyamines that are present in all living organisms are putrescine, cadaverine, spermidine and spermine. Polyamines are present also in their acetylated forms, *N*-acetylputrescine, *N*-acetylspermine, and *N*-acetylspermidine that derive from the metabolic pathway of aliphatic polyamines catalyzed by acetyl-CoA and sperimidine/spermine *N*-acetyltransferase [202].

Many analytical protocols were developed for the assay of polyamines based on both high-performance liquid chromatography (HPLC) with optical [203-206] or mass spetrometric detection (MS) [207-213] and gas chromatography (GC) with MS [214-219] or traditional detectors [220-224]. Due to its robustness, high capability in peak separation, low running costs GC is the most suitable in analytical approach. For some analytes such as amines, the use of GC entails a derivatization step to improve chromatographic elution and resolution by decreasing volatility and polarity of the investigated analytes. Several derivatization reactions such as perfluoroacylation [200], alkylsilylation [216] and alkoxycarbonylation [214, 215, 218] were described. The perfluoroacylation and alkylsilylation reactions involve many time consuming steps and intense reaction conditions. The derivatization reaction based on the alkoxycarbonylation allows to carry out the reaction in aqueous matrices reducing the steps involved in the reaction [225]. The alkoxycarbonylation reaction as derivatization tool can be used also in combination with the solid phase microextraction (SPME), as demonstrated from our research group [193].

Urine is the most common matrix used in clinical screening because the collection is easy and non-invasive. Polyamines were extracted from urine with several sample techniques, such as solid phase extraction (SPE) [206, 222], liquid-liquid extraction (LLE) [200, 205, 214] single hollow fiber

supported liquid membrane extraction [203] and ion exchange resin procedure [207]. Most of these extraction techniques are long, and time consuming, involve many steps and the use of organic solvent, present low automation. For these reasons they are not optimal for routine controls in clinical analysis. To overcome these limitations the miniaturized tecniques such as solid phase microextraction (SPME) can be used. The use of SPME minimizes the errors source due to the possibility to execute in a single step extraction and concentration of the analytes. The use of SPME in combination with the gas chromatography allows to work without organic solvents and in automated way [161].

In this work a SPME-GC-QqQ-MS method for the assay of polyamines in urine after a derivatization reaction with alkyl chloroformate was developed. The use of derivatization reaction in combination with SPME allows to reduce the use of solvent, the sample preparation steps and to perform the reaction directly in urine. The variables that can influence the SPME extraction were optimized by the multivariate approach of experimental design. Furthermore, for the first time a GC-QqQ system in SRM acquisition was used for the assay of polyamines in order to achieve a high specific protocol capable of unequivocal identification.

3.3.1 Experimental section

3.3.1.1 Chemicals and materials

Putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm), and N^1-Acetylspermine trihydrochloride (N^1-AcSpm) were obtained from Fluka (Milan, Italy). N^8-Acetylspermidine dihydrochloride (N^8-AcSpd), and N^1-Acetylspermidine hydrochloride (N^1-AcSpd) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) and Cayman Chemical Company (Ann Arbor, Michigan, USA), respectively. The internal standards used namely, 1,6-diaminohexane dihydrochloride (1,6-DAH) and spermidine-(butyl-d_8) trihydrochloride (Spd-d_8), were obtained from Sigma Aldrich (Milan, Italy). Propyl chloroformate, sodium phosphate dibasic, and sodium phosphate tribasic dodecahydrate were purchased from Sigma-Aldrich (Milan, Italy). The SPME fibers tested, polyacrilate 85µm (PA), carboxen/polydimethylsiloxane 85 µm (Car/PDMS), divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm (DVB/Car/PDMS), polydimethylsiloxane/divinylbenzene 65 µm (PDMS/DVB) and polydimethylsiloxane 100µm (PDMS) were bought from Supelco (Bellefonte, PA, USA) and conditioned as recommended by the manufacturer. Aqueous solutions were prepared using ultrapure water obtained from a Milli-Q plus

system (Millipore, Bedford, MA). Synthetic urine (negative urine control) produced from Cerilliant (proprietary composition; Round Rock, TE, USA) was commercialized by Sigma-Aldrich.

3.3.1.2 Instrumentation and data processing

A TSQ Quantum GC (Thermo Fischer Scientific) constituted by a triple quadrupole mass spectrometer (QqQ) Quantum and a TRACE GC Ultra equipped with a Triplus autosampler was used to perform the analysis. The capillary column Restek Rxi-5MS (30 m×0.25 mm i.d., 0.25 µm film thickness, 95% polydimethylsiloxane, 5% polydiphenylsiloxane) was used for chromatographic separation. The GC oven temperature was initially held at 70 °C for 3 min, then ramped at 15 °C/min to 300 °C and held at this temperature for 10 min. The carrier gas was helium (purity 99.999%) at 1 mL/min, whereas argon (purity 99.999%) at a pressure of 2.3 mTorr was used as collision gas. The liner used in the GC injector was a Thermo PTV straight liner 0.75 × 2.75 ×105 mm. The injector was set at 270 °C in splitless mode. The QqQ mass spec-trometer was operated in electron ionization (EI). The preliminary analyses for the identification of the derivatized analytes were performed in full scan mode, whereas analyte quantification was carried out in selected reaction monitoring (SRM) mode. The ionization source was set at 250 °C whereas the transfer line temperatures was 280 °C. The emission current was set at 25 µA and the scan width and peak width of Q1 were set at m/z 1 and 0.7 amu for all segments.

Xcalibur software 2.0 was used for the instrument control and data processing. Excel (Microsoft, USA) was used to evaluate experimental data whereas Statistica 8.0 (StatSoft 2007 Edition, Tulsa, USA) was used to perform the experimental matrix design and the non-parametric Mann-Whitney *U* test.

3.3.1.3 Samples and analytical procedure

Early-morning urine samples were collected from three female and three male between the ages of 29 and 35 years and frozen at – 20 °C. 2 mL of urine was put in a 10 mL vial suitable to be used in autosampler and spiked with internal standards (1,6-DAH and Spd-d_8) at 0.5 µg/mL.Then the pH was adjusted to 12 with 760 µL of a 0.10 M solution of phosphate/hydrogen phosphate buffer. Afterwards, the derivatization was carried out by adding 100 µL of propyl chloroformate to this sample, keeping the solution under stirring for 15 min. Finally, 5 mL of ultrapure water was added to the vial and then crimped. A DVB/Car/PDMS 50/30 µm fiber was used to perform the SPME extraction in direct immersion for 15 min at 40 °C. After the adsorption the fiber was introduced into GC injector for 10

min for thermal desorption. The blank sample (ultrapure water spiked with the derivatizing mixture) was performed after the sample analysis to verify the analyte carryover.

3.3.1.4 Lower Limit of quantification and calibration procedure

Lower limits of quantification (LLOQs) and linearity were evaluated according to the Food and Drug Administration (FDA) guidance [185]. The LLOQs were calculated as the lowest concentration of each analyte that provides a response at least five times the signal compared to blank signal and that can be quantified with an accuracy of 80-120% and a precision in terms of RSD < 20% [185]. Syntethic urine samples spiked at different and known concentrations of polyamines were analyzed in quintuplicate and evaluated to determine the LLOQs values. The values obtained for each analyte were used as the lowest concentration point of each standard curve. The analytes were quantified by matrix-matched calibration method using synthethic urine, as matrix similar to the real matrix, for the construction of standard curves [186]. The calibration standards was the following: one blank sample, one zero sample and seven non-zero samples. The one blank sample is a blank synthetic urine sample without internal standard, the zero sample is a blank synthetic urine sample with internal standard and the non zero samples are blank synthetic urine samples spiked with the internal standards and with a known amount of polyamines in the range between their LLOQs and 3 µg/mL for Spm and 10 µg/mL for other polyamines. The internal standards used, 1,6-DAH and Spd-d_8, were added to each calibration standards at 0.5 µg/mL and each concentration level of calibration standards were analyzed in triplicates.

3.3.1.5 Matrix effect and figures of merit

Matrix effect was evaluated with the method proposed by Matuszewski [187] by comparing the signals of each analyte obtained in real urine and synthetic urine. Three quality control (QC) levels were considered in the range of calibration curves: the low QC level at 3 × the LLOQ, the middle QC level near the center and the high QC level at 0.8 × the upper level of the standard curve. The samples used for the evaluation of matrix effect were prepared in synthetic urine and real urine with the internal standard at 0.5 µg/mL. As regards the real urine samples, the specimens provided by six healthy donors were taken into account. Each sample was divided into two aliquots. One of this was used to prepare the blank real urine sample spiked only with internal standards at 0.5 µg/mL whereas the other one was used to prepare real samples spiked at the QC levels. All samples were derivatized as described in 3.3.1.3 and analyzed in triplicates. The relative matrix effect was also evaluated for

each QC level by comparing the precision value (CV%) obtained analyzing five aliquots of real urine from the same subject (intra-subject precision) with the precison value obtained by analyzing six urine samples from different subjects (inter-subject precision) spiked at the same concentration.
Intra-day and inter-day precision and accuracy were determined according to the Food and Drug Administration guidance [185]. They were calculated analyzing five replicates for each QC level once a day on six consecutive days by using synthetic urine. Precision values were expressed as percentage relative standard deviation (RSD%) whereas accuracies were calculated as percent ratio between the concentration estimated from the calibration curve and the spiked concentration.

3.3.2 Results and discussion

3.3.2.1 Derivatization and preliminary SPME analysis

Alkyl chloroformates can be directly added in aqueous solution to derivatize the aliphatic amino group of polyamines [225, 226]. The pH of the reaction and the amount of alkyl chloroformate can influence significantly the formation of alkoxycarbonyl derivatives. The pH control is crucial because an alkaline value is necessary to allow the reaction to proceed at an acceptable rate at room temperature. Polyamines derivatization was performed at pH 12, because at this value the amino groups of the analytes are in non-ionized forms. To verify the formation of derivatives a synthetic urine sample spiked at concentration of 20 µg/mL for N^1-AcSpd and N^8-AcSpd and 5 µg/mL for the other analytes was prepared, derivatized with propyl chloroformate and extracted by solid phase microextraction using a PDMS/DVB fiber. The sample was analyzed in full scan mode and mass spectra were evaluated to identify the derivatized analytes. All propoxycarbonylated polyamine, whose EI mass spectra are shown in ***figure 3.3***, was recognized and assigned to well-separated seven chromatographic peaks. The molecular ion peak was observed in all mass spectra except for Spm and N^1-AcSpm.

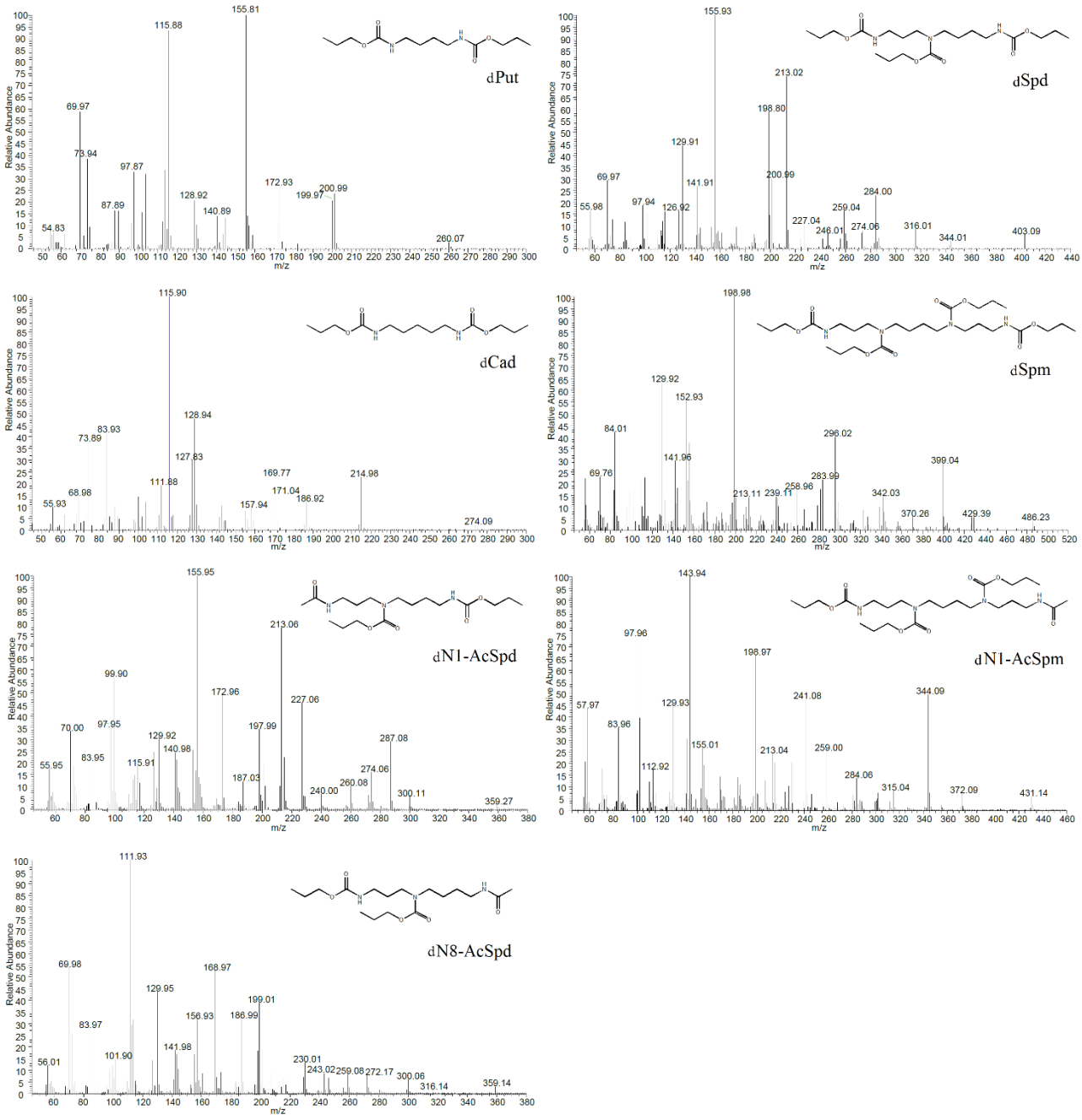

Figure 3.3: Mass spectra of the derivatized analytes acquired in full scan mode.

The tri-derivatized N^1-AcSpm contains in the spectrum the fragment at *m/z* 431 that derives from the loss of $CH_3CON{=}CH_2$ moiety due to the cleavage of C—C bond in β position to the nitrogen atom. In the mass spectra of tetra-derivatized spermine the fragment at *m/z* 486 represents the loss of propanol from the molecular ion. The fragment at *m/z* 156 is common to Put, Spd and N^1-AcSpd derivatives due to the presence of the $CH_3(CH_2)_2OCONH(CH_2)_4$ group in the molecular structure of these compounds. It can derive from the loss of the complementary part to this group and a formal

loss of H_2 from the molecular ion. In the mass spectra of N^8-AcSpd the base peak at *m/z* 112 is related to the loss of the $CH_3(CH_2)_2OOCNH(CH_2)_3NCOO(CH_2)_2CH_3$ group and a formal loss of H_2. The base peak at *m/z* 116 in the mass spectra of Cad corresponds to the $[CH_3(CH_2)_2OOCNHCH_2]^+$ ion, and that at *m/z* 144 in the mass spectra of N^1-AcSpmcorresponds to the $[CH_3(CH_2)_2OOCNH(CH_2)_3]^+$ ion. In the Spm (MW) mass spectra the base peak at *m/z* 199 is obtained by the loss of $CH_3(CH_2)_2OOCNH(CH_2)_3N(COO(CH_2)_2CH_3)CH_2$ and $HCOO(CH_2)_2CH_3$ with a proton transfer from the molecular ion. As regards the influence of the amount of alkyl chloroformate used as derivatizing reagent different volumes of propyl chloroformate were considered (10, 30, 50, 70, 100 and 130 µL) and the corresponding samples were analyzed in triplicate. The peak areas obtained for each propoxycarbonylated analytes are shown in ***figure 3.3.1.***

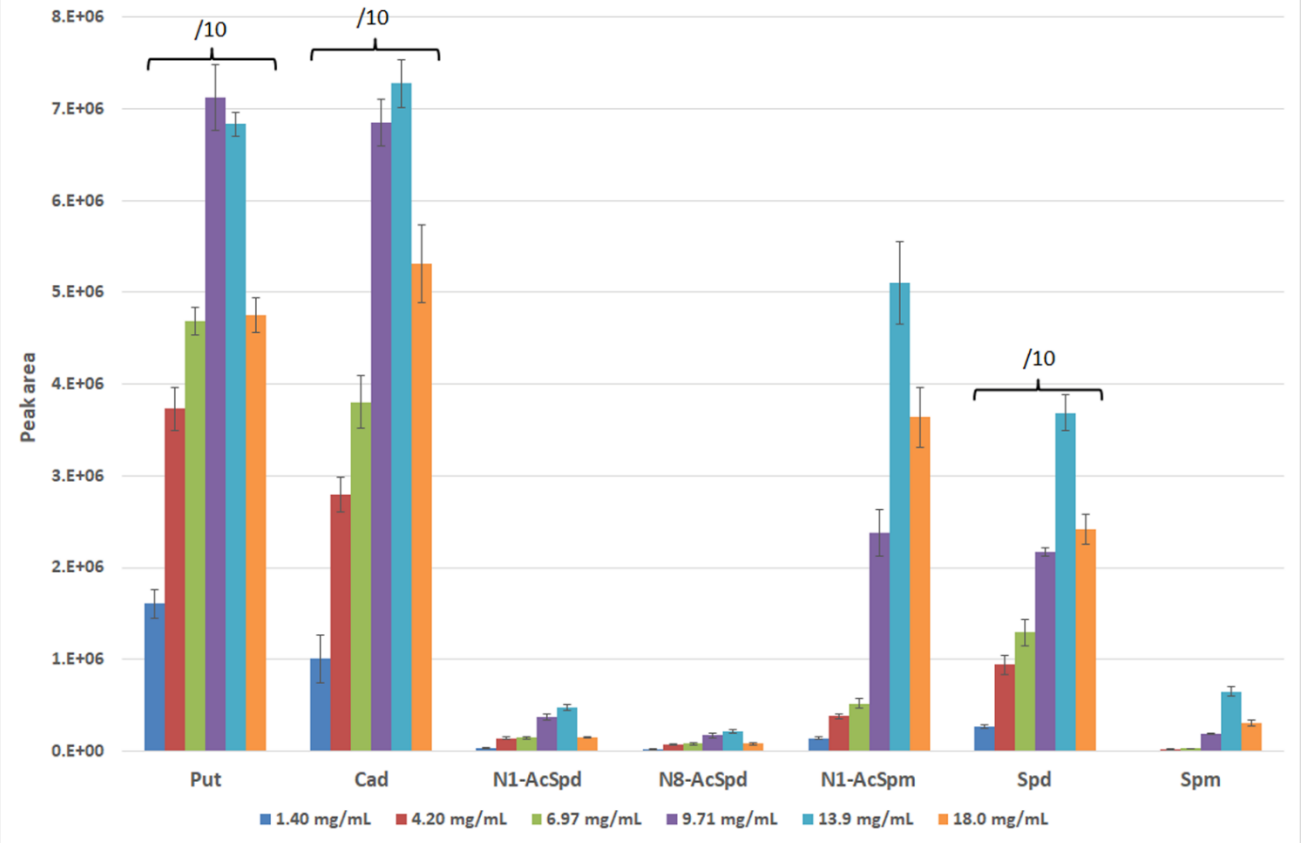

Figure 3.3.1: Peak areas obtained by derivatizing analytes at 1 µg/mL with different volumes of propyl chloroformate (n = 3 for each volume).

The volume of 100 µL of propyl chloroformate was used for further investigations because it gave the most intense signals for all analytes except putrescine. Choi et al. used the ethyl chloroformate as

derivatizing reagent and reported that Spd and Spm did not present appreciable peaks when a classic liquid injection of analytes was carried out in GC with the same column used in this work [226]. In our case the propoxycarbonylated derivatives desorbed in GC injector from the SPME fiber show a clear peak with an excellent peak shape for spermidine and a moderate tailing (asymmetry factor value of 2.39) for spermine, as shown in ***figure 3.3.2.***

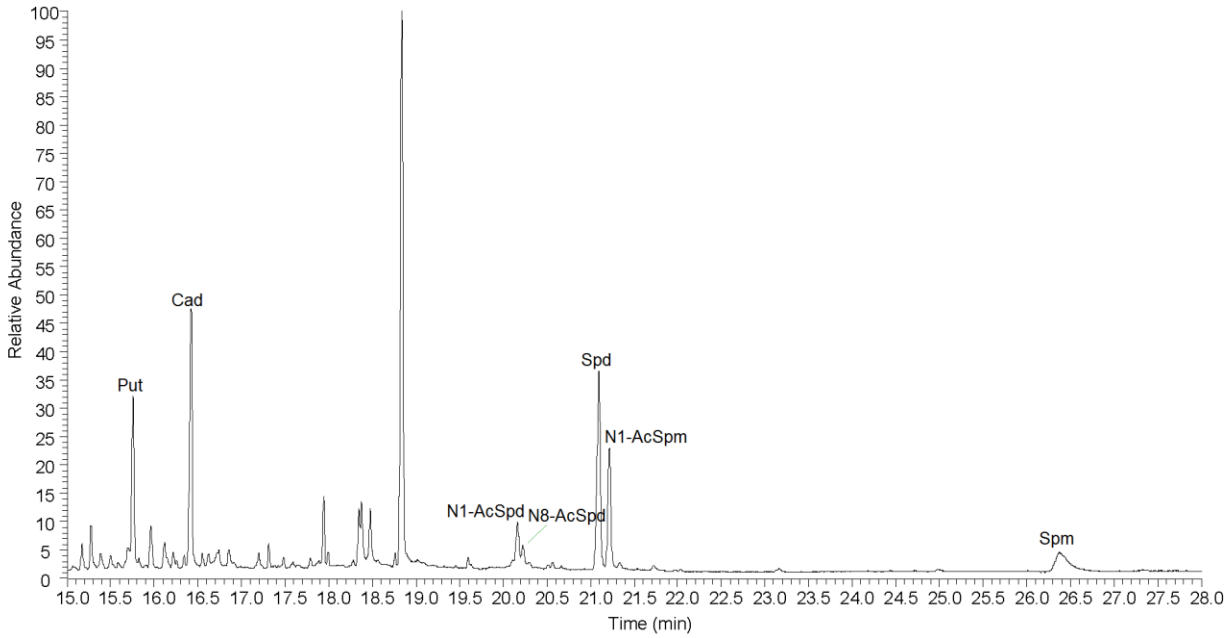

Figure 3.3.2: SPME-GC–MS chromatography (EI-full scan acquisition mode) for synthetic urine spiked at 20 µg/mL for N^1-AcSpd, N^8-AcSpd and Spm and at 5 µg/mL for Put, Cad,Spd and N^1-AcSpm.

The poor chromatographic behaviour in liquid injection can be attributed to the presence of organic solvent. Indeed, the expansion of solvent during the injection causes a large vapour cloud in the injector of GC, worsening the focusing of the analytes on the head of the chromatographic column. Contrariwise, since SPME is a solventless technique, there is no solvent expansion resulting in improved focusing of the analytes on the GC column and, therefore, in narrow chromatographic peaks. A similar improved chromatographic behavior obtained by using SPME compared to liquid injection were reported by Tagarelli *et al*. for benzotriazoles and benzosulfonamides analysis [176].

3.3.2.2 GC-MS/MS analysis and optimization of SPME parameters

The use of triple quadrupole coupled with a gas chromatograph allows to obtain sensitive and selective analytical protocols [227-229]. The signal acquisition in SRM mode entails the proper choice of parent/daughter ion pair to attain the best compromise between sensitivity and specificity. According to this criterion, the production spectra for several precursor ions were acquired by collision-induced dissociation (CID). For each analyte, the transition that presents the best signal noise ratio was selected for quantification and the second more sensitive was used for identification to avoid unambiguous recognition. The use of a second SRM transition is in line with the European Commission Decision 2002/657/EC [195]. The transitions optimized and their collision energy for each analyte with other parameters are shown in ***table 3.3***.

Table 3.3: Retention times (r.t.) and electron ionization tandem mass spectrometry (EI-MS/MS) parameters (collision energies are indicated in parenthesis).

Compound	**r.t. (min)**	**Scan time (s)**	**SRM transition, m/z (collision energy, V)**	
			Quantification	Identification
Put	15.75	0.1	156→114 (6)	156→170 (9)
Cad	16.42	0.05	170→128 (7)	116→74 (7)
N^1-AcSpd	20.19	0.05	227→141 (7)	227→98 (9)
N^8-AcSpd	20.24	0.05	169→114 (8)	199→130 (6)
Spd	21.10	0.07	156→70 (9)	156→114 (6)
N^1-AcSpm	21.22	0.07	144→102 (6)	199→130 (7)
Spm	26.41	0.5	199→130 (6)	153→84 (10)
1,6-DAH	17.07	0.05	130→74 (6)	-
Spd-d_8	21.05	0.07	162→76 (8)	-

Many variables can influence the SPME extraction efficiency. The most critical step of the entire SPME procedure is the choice of the suitable coating since efficacy of extraction strongly depends on affinity analyte and polymeric material [230]. Therefore the extraction efficiency of five fibers, namely polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB), Carboxen/polydimethylsiloxane (Car/PDMS), divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Car/PDMS), and polyacrilate (PA), was evaluated in direct immersion under the same experimental conditions. The synthetic urine samples were prepared at concentration of 1 µg/mL and

extracted at room temperature for 20 min. The peak areas obtained for each analyte with the five fibers were shown in ***figure 3.3.3.***

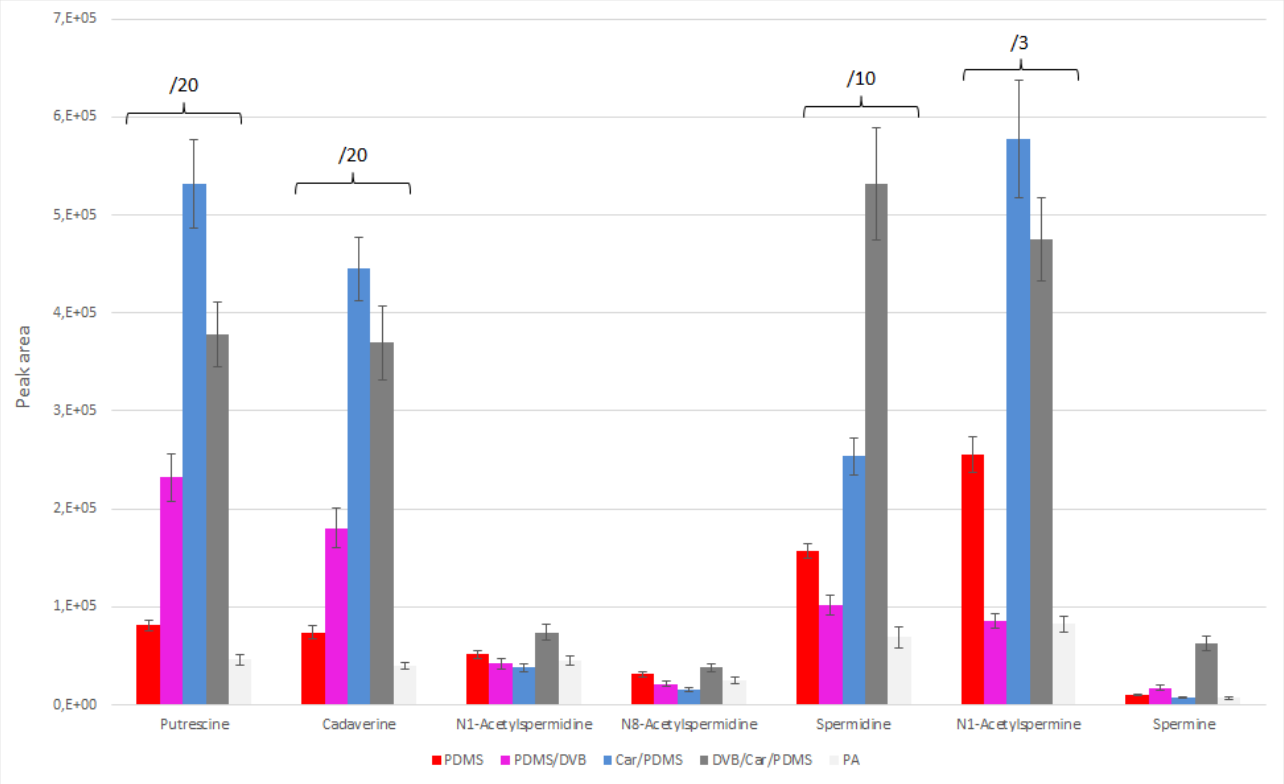

Figure 3.3.3: Peak areas obtained, for each analyte at 1 µg/mL, with five different SPME fibers.

The DVB/Car/PDMS fiber showed the higher peak area, especially for less sensitive analytes such as N^8-Acetylspermidine, N^1-Acetylspermidine and spermine. Therefore, despite this result was not obtained for putrescine, cadaverine and N^1-Acetylspermine, the DVB/Car/PDMS fiber was chosen for further investigations. The other critical factors of SPME extraction such as extraction time, extraction temperature and addition of sodium chloride were optimized by the use of experimental design [231-233]. This multivariate approach allows to evaluate the factors simultaneously and the interaction among them by performing few experiments. A central composite design (CCD) was chosen to optimize these variables and in particular a design consisting of 20 experiments (2^3 + (2 × 3) + 6), where 3 is the factors number and 6 is the number of star points, was carried out. The axial distance α was chosen with a value of 1.68 in order to fulfill the rotatability condition. The experimental domain for each variable, chosen on the basis of some preliminary experiments, was: NaCl 0-10%, extraction time 15-45 min and extraction temperature 40-60 °C. The samples were prepared in synthetic urine spiked at 5 µg/L and the experiments, shown in ***table 3.3.1***, were performed in SRM acquisition mode.

Table 3.3.1: Experiments performed to optimize the SPME variables. (C): Central point.

Exp	Extraction time	Extraction temperature	%NaCl
2	21.1	44.1	8.0
9	15.0	50.0	5.0
4	21.1	55.9	8.0
17 (C)	30.0	50.0	5.0
18 (C)	30.0	50.0	5.0
3	21.1	55.9	2.0
20 (C)	30.0	50.0	5.0
11	30.0	40.0	5.0
19 (C)	30.0	50.0	5.0
13	30.0	50.0	0.0
10	45.0	50.0	5.0
16 (C)	30.0	50.0	5.0
14	30.0	50.0	10.0
1	21.1	44.1	2.0
15 (C)	30.0	50.0	5.0
7	38.9	55.9	2.0
12	30.0	60.0	5.0
6	38.9	44.1	8.0
8	38.9	55.9	8.0
5	38.9	44.1	2.0

To evaluate the mathematical models, the lack of fit test was carried out for all responses. It resulted statistically acceptable (p_{lf} >0.05) for all analytes except for N^1-acetylspermine and sperimine and, therefore, in the evaluation of the optimal working conditions these analytes were not taken into account. The Pareto charts obtained from the CCD design show the influence of each variable and cross effect on the analytes responses. The absolute value of the estimated effects is represented by the bar length and the vertical line delimits the 95% of the confidence interval. Therefore, the factors that cross the line have a significant effect on the response. The CCD used to optimize the SPME parameters provided the Pareto charts shown in ***Figure 3.3.4***.

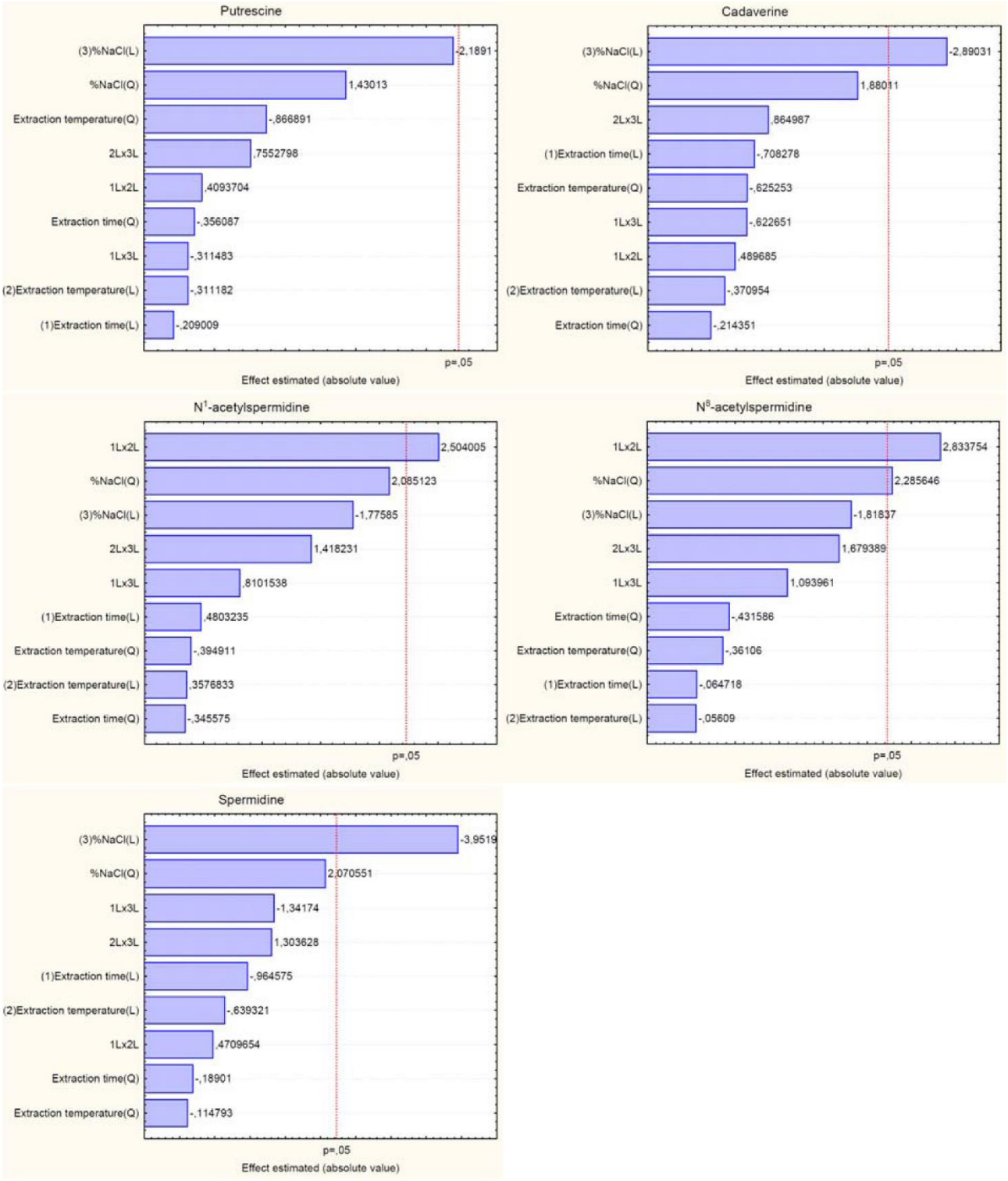

Figure 3.3.4: Pareto charts obtained from CCD of SPME parameters.

The signal of putrescine is not affected by any of the studied variables whereas for the cadaverine and sperimidine only the linear term of % NaCl has a significant impact ($p \leq 0.05$) on the response. The negative sign of these coefficients indicates that the analytes signals decrease with the increasing of ionic strength. This result has been already reported by Tagarelli *et al.* [193] and could be explained

by supposing that the addition of NaCl to sample determines a greater affinity of interfering species for the fiber giving rise to a stronger competition with the analytes. For N^1-acetylsperimidine and N^8-acetylsperimidine the interaction term between extraction time and extraction temperature has a significant effect ($p \leq 0.05$). From their response surfaces (***figure 3.3.5***) it is clear that the response decreases when extraction temperature is held at low values and extraction time is increased. These graphs show that the highest response is attained when both variables are either at the lowest or the highest level.

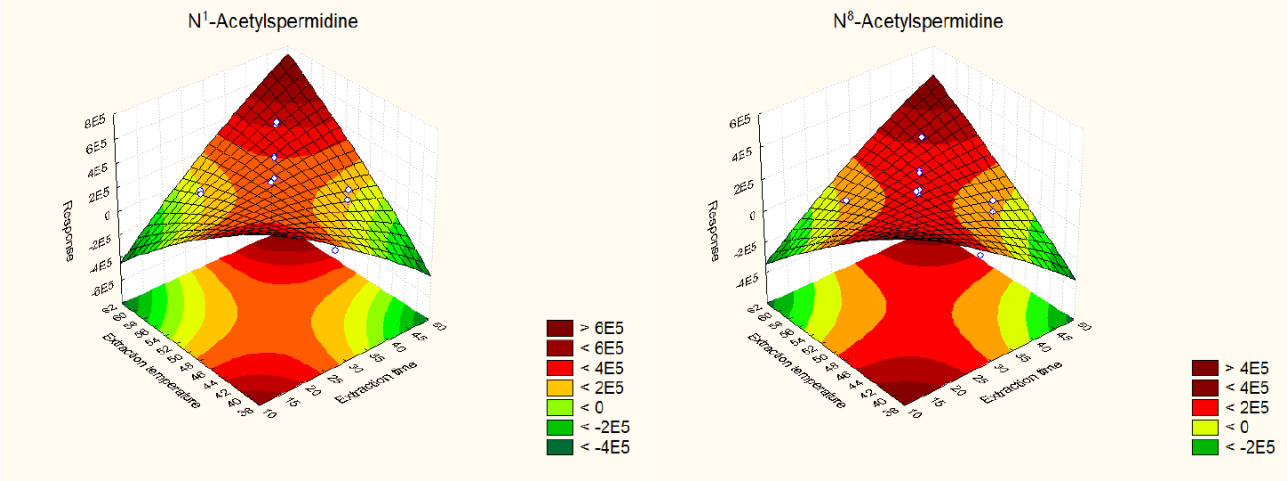

Figure 3.3.5: Response surfaces (extraction time versus extraction temperature) for N^1-acetylsperimidine and N^8-acetylsperimidine obtained from CCD.

Also in this case, the behavior of N^1-acetylsperimidine and N^8-acetylsperimidine may be justified by the stronger competition between the analyte and the matrix interfering species. On the other hand, an increase of response was detected at higher extraction temperature with the extension of the extraction time, indicating that competition between matrix components and analytes depends on extraction temperature. From the evaluation of these results, 40 °C was considered as extraction temperature also because lower values were hindered by technical limitation of the autosampler heater/agitator module. As regards extraction time its maximum response was found at the edge of the experimental domain. For this a further optimization steps were carried out by considering a synthetic urine sample spiked at 5 µg/mL and analyzed in triplicate at 5, 10 and 15 min (***figure 3.3.6***).

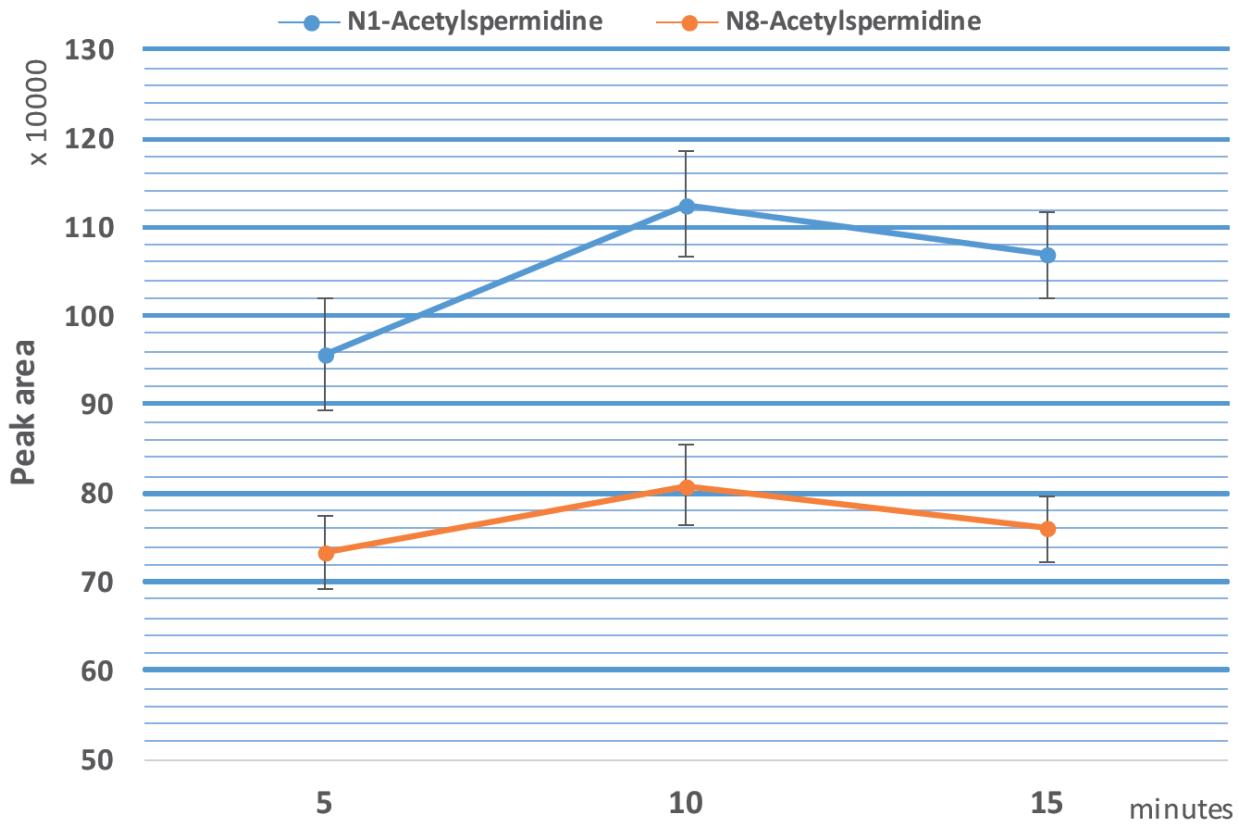

Figure 3.3.6: Peak areas of N^1-AcSpd and N^8-AcSpd obtained at different extraction times and at concentration of 5 µg/mL.

The highest response was obtained by considering an extraction time of 10 min for both analytes with the precision in terms of RSD value of 5.2% for N^1-AcSpd and 5.6% for N^8-AcSpd. The analyses performed with an extraction time of 15 min showed a better precision, in particular 4.5% for N^1-AcSpd and 4.8% for N^8-AcSpd. According to these results and considering the result of the Mann-Whitney *U* test performed on the responses obtained at 10 min and 15 min that showed a non-significant difference ($p < 0.05$), 15 min was selected as optimal operation value. In the light of these findings, the next investigations were conducted with a DVB/Car/PDMS fiber, an extraction time of 15 min at 40 °C in absence of sodium chloride.

3.3.2.3 Lower limit of quantification and linearity

The determination of LLOQs for each analyte was carried out in synthetic urine by analyzing samples spiked at different concentration of investigated analytes. The LLOQs determined were used as the lowest values of calibration range. The linearity was evaluated with the matrix-matched calibration

method, as described in experimental section, in a concentration range between the quantification limits and 3 µg/mL for Spm and 10 µg/mL for all other analytes. For each analyte seven point calibration curve was built adding 1,6-DAH and Spd-d_8 as internal standards at 0.5 µg/mL. The LLOQs, linearity and the analyte/internal standard pairs are reported in ***table 3.3.2***. Satisfactory results in terms of linearity were attained, with determination coefficient values >0.99 for all the analytes, except for Spm.

Table 3.3.2: LLOQs, calibration parameters and internal standards.

Compound	LLOQ (µg/mL)	Range (µg/mL)	Calibration curves	R^2	Internal standard
Put	0.01	0.01-10	y=0.2671x+0.0011	0.9963	1,6-DAH
Cad	0.03	0.03-10	y=0.3673x-0.0058	0.9985	1,6-DAH
N^1-AcSpd	0.1	0.1-10	y=0.0880x+0.0014	0.9971	Spd-d_8
N^8-AcSpd	0.1	0.1-10	y=0.0345x-0.0028	0.9962	Spd-d_8
Spd	0.01	0.01-10	y=5.8080x+0.0022	0.9956	Spd-d_8
N^1-AcSpm	0.01	0.01-10	y=1.1649x-0.0043	0.9971	Spd-d_8
Spm	0.1	0.1-3	y=0.0277x-0.0010	0.9887	Spd-d_8

3.3.2.4 Matrix effect and analytical performance

The matrix effect was calculated with the method proposed by Matuszewski [187] as described in experimental section. It was determined for each QC level as the percentage ratio of analyte mean peak area for the spiked real sample minus the mean peak area for the blank real sample against the mean peak area for the spiked synthetic urine sample. No matrix effect was observed for Cad, N1-AcSpd and N8-AcSpd, whereas low values were obtained for all the other analytes. In order to evaluate the capability of internal standards to balance the matrix effect, in the calculation of matrix effect the ratio of each analyte peak area to internal standard peak area was considered. As shown in ***table 3.3.3***, an appreciable correction of the matrix effect was achieved, with acceptable values for all analytes except for N^1-AcSpm at 8 µg/L.

Table 3.3.3: Results obtained from the matrix effect evaluation.

Compound	Concentration (µg/mL)	Matrix effect (%)	Intra-subject precision (CV%)	Inter-subject precision (CV%)
Put	0.03	109	12.9	14.6
	0.3	85	10.7	9.7
	8	93	7.5	11.3
Cad	0.09	75	15.9	22.6
	0.5	101	10.8	11.3
	8	102	8.6	6.8
N^1-AcSpd	0.3	85	18.7	18.1
	1	90	17.2	21.0
	8	103	10.8	14.6
N^8-AcSpd	0.3	115	19.5	24.6
	1	86	15.7	20.4
	8	105	16.4	19.8
Spd	0.03	120	14.0	19.4
	0.3	78	13.8	16.8
	8	71	9.1	8.1
N^1-AcSpm	0.03	97	17.2	19.2
	0.3	79	10.2	10.3
	8	68	9.6	11.0
Spm	0.3	81	10.7	12.3
	0.6	110	11.0	15.5
	2.4	121	19.5	26.1

As shown in the table, the relative matrix effect was also evaluated comparing the precision values obtained by analyzing the urine of a single volunteer (intra-subject precision) in quintuplicates with the precision obtained from the analysis of urine provided by six volunteers (inter-subject precision). The results show satisfactory and comparable CV% values. Only, the inter-subject precision for N^8-AcSpd at 0.3 µg/mL and Spm at 2.4 µg/mL are out of the normal accepted range. Despite this results that could be caused by a non-optimal behaviour of the internal standards the method is robust enough to endure the possible differences present in urine from different subjects.

Intra-day and inter-day precision and accuracy were evaluated at the QC levels, as described in experimental section, by analyzing five replicates of the spiked samples at different concentrations, once a day on six consecutive days. The results obtained are reported in ***table 3.3.4***.

Table 3.3.4: Inter-day and intra-day precision and accuracy obtained with the proposed method.

Compound	**Concentration (µg/mL)**	**Intra-day accuracy (%)**	**Intra-day precision (CV%)**	**Inter-day accuracy (%)**	**Inter-day precision (CV%)**
Put	0.03	9	10.9	14	10.5
	0.3	8	12.4	13	9.2
	8	-4	5.8	-2	5.7
Cad	0.09	11	8.7	9	9.1
	0.5	-9	4.2	-15	6.2
	8	9	1.8	6	3.3
N^1-AcSpd	0.3	-27	22.9	-23	23.7
	1	-17	12.4	16	24.8
	8	-5	17.1	-5	21.4
N^8-AcSpd	0.3	-14	18.7	-24	28.4
	1	-6	9.0	11	24.5
	8	11	10.8	-4	24.8
Spd	0.03	-19	7.8	-22	8.1
	0.3	11	10.1	7	12.7
	8	3	5.8	3	4.6
N^1-AcSpm	0.03	-3	9.4	0	10.2
	0.3	-13	7.7	-17	10.6
	8	-17	5.4	-16	4.4
Spm	0.3	6	19.1	9	30.4
	0.6	1	18.5	7	22.5
	2.4	-12	20.7	-11	25.0

The values of accuracy and precision obtained are in line with the Food and Drug Administration guidance [185]. In particular, the precision and accuracy values obtained for each analyte not exceed 15% of the RSD value except for the inter-day precision of N^1-AcSpd, N^8-AcSpd and Spm. This results could be explained, again, by an unsatisfactory behaviour of internal standards.

3.3.2.5 Application to real samples

The optimized method was applied to determine the levels of polyamines in six urine of healthy individuals. The results obtained by analyzing the samples in triplicates in accordance with the optimized method as described in experimental section (***table 3.3.5***) are in accordance with the values reported in literature [216, 234].

Table 3.3.5: Urinary polyamines in healthy individuals (min-max).

Compound	Put	Cad	N^1-AcSpd	N^8-AcSpd	Spd	N^1-AcSpm	Spm
Real samples (µg/L)	0.247 (0.107-0.389)	0.123 (0.094-0.186)	1.13 (0.249-1.83)	1.16 (0.261-1.98)	0.159 (0.070-0.205)	0.043 (0.033-0.059)	0.479 (0.312-0.924)

A typical chromatogram of a real urine sample, shown in ***figure 3.3.7***; confirms the capability of tandem mass spectrometry to minimize matrix interferences, and improve the signal/noise ratio. This feature allows achieving clean reconstructed chromatograms with well-shaped chromatographic peaks.

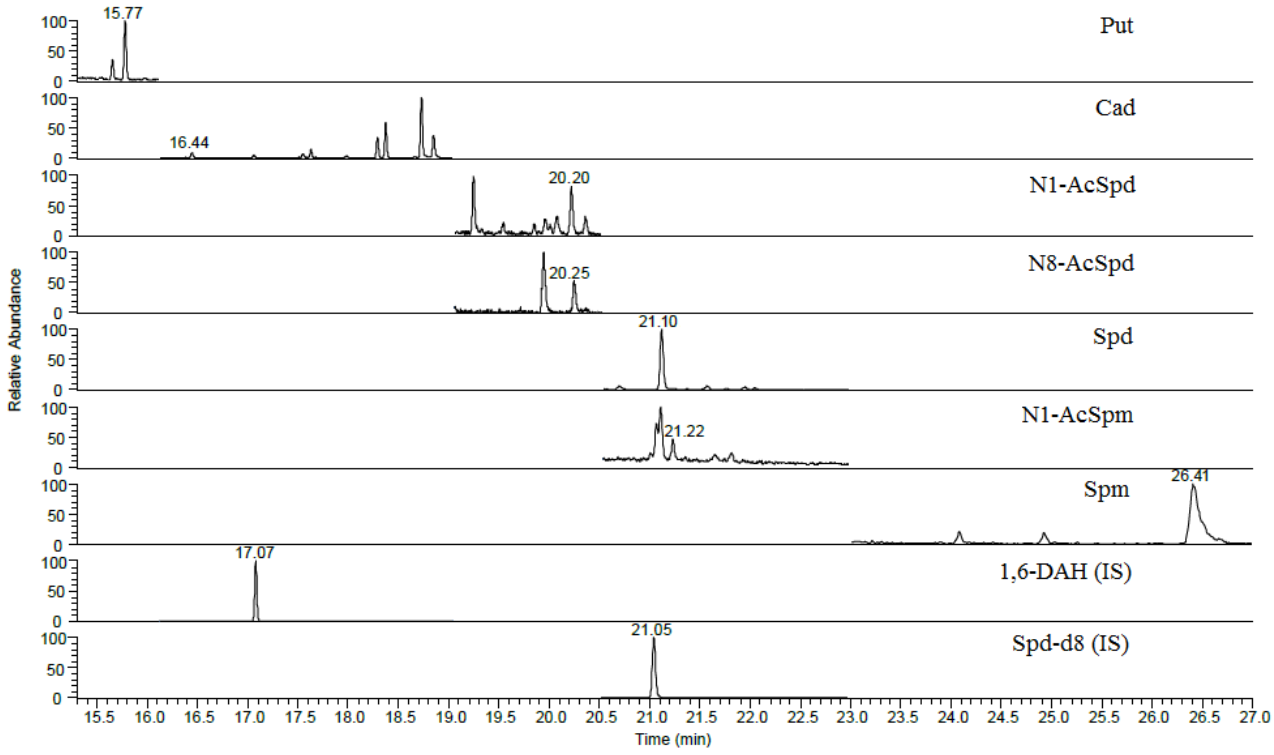

Figure 3.3.7: SPME-GC-MS/MS chromatogram.

Afterwards, the method was tested in a pathological case. The same real urine samples of a healthy volunteer were spiked with spermine at 2.4 µg/L and at 8 µg/L for other analytes. The values of accuracy obtained were satisfactory, in particular they presented a deviation from the true value between – 27% to +5% except for N^1-AcSpm that was -33%. These results demonstrate that, using some forethought for acetylated compounds and spermine, the proposed method can be used to monitor the levels of polyamines in cancer detection programs.

3.3.3 Conclusions

A new SPME-GC-MS/MS protocol for the assay of polyamines in human urine was developed and optimized. The method is based on the combined use of alkyl chloroformate as derivatization approach and solid phase microextraction as extraction technique. The final protocol allows to determine these import biomarkers by an easy and automated method involving a minimal handling of sample and no consumption of organic solvents. The SPME extraction was directly carried out by autosampler in the same vial in which urine was directly derivatized, thus reducing the handling error rate and promoting higher throughput. The SPME fiber was chosen by testing five different fiber in univariate mode whereas the most critical variables affecting the SPME extraction were optimized by the multivariate approach of experimental design. Finally, the acquisition in selected reaction monitoring allowed the achievement of high specificity and sensitive improving the capability in analyte identification and the signal/noise ratio. Finally, the satisfactory performances in terms of linearity, sensitivity, matrix effect, accuracy and precision demonstrate the possibility to use the proposed method for the assay of of free and acetylated polyamines in real clinical scenarios.

Development of a fast and simple method for the assay of urinary phthalate monoesters by Solid Phase Microextraction-Gas chromatography-triple quadrupole mass spectrometry

3.4 Introduction

Phthalates esters are industrial compounds that derive from phthalic acid. They are used as plasticizers or as solubilizing or stabilizing agents in a variety of products such as personal care products, medical devices, pharmaceutical, packing materials, toys etc. [235]. Over time, their presence in environment is increase because they are not chemically bounded to the products and, therefore, they can be easily released. Phthalates esters were detected in many different areas, especially in industrial countries, and have become one of the most popular ubiquitous environmental contaminants [236, 237]. The major exposure routes for phthalate esters are inhalation, ingestion and dermal contact, although some study suggested that food represents the principal source of exposure to these compounds [238-240]. When phthalate esters enter in the human body they are rapidly metabolized and hydrolyzed to their respective phthalates monoesters and excreted through urine in their free or glucuronide-conjugated forms ***(figure 3.4).***

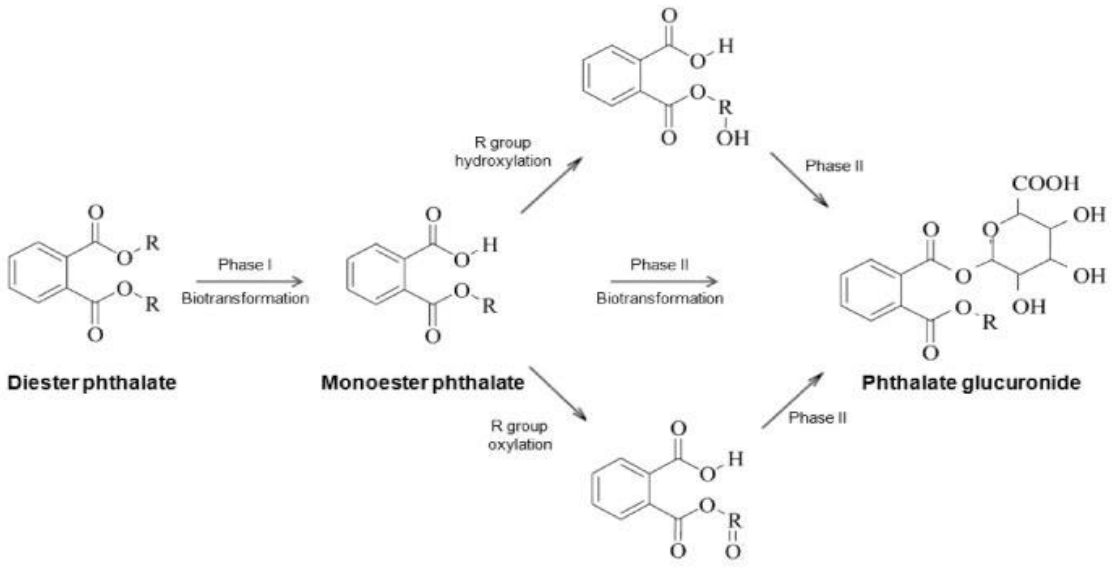

Figure 3.4: Metabolic pathway of phthalates.

The monoesters contain a free reactive carboxylic acid that can be conjugated with α-D-glucuronic acid to produce more hydrophilic compounds [241]. In order to detach glucuronic acid β-glucuronidase was used to perform enzymatic hydrolysis. The total concentration of phthalates monoesters metabolites in urine is normally used as biomarkers of individual intake of phthalate

esters. They are hazardous to human health, they affect development and reproductive functions because they act as endocrine disrupting agents. They have also carcinogenic, hepatotoxic effects and an excessive exposure to phthalate esters can increase the possibility of fetal death and malformations [242-245]. Most of the works present in literature for the determination of some phthalates monoesters are based on the use of LLE or SPE as sample preparation resulting with a high environmental impact and time consuming due to the several steps to perform [246-249].

The main purpose of the present work was the development of a fast and simple method for the assay of monomethyl phthalate (MMP), monoethyl phyhalate (MEP), monoisobutyl phthalate (MiBP), monobutyl phthalate (MBP), monocyclohexyl phthalate(McHP), monoethylhexyl phthalate (MEHP), monoisononyl phthalate (MiNP), monooctyl phthalate (MOP) and monobenzyl phthalate (MBzP) in human urine ***(Figure 3.4.1).***

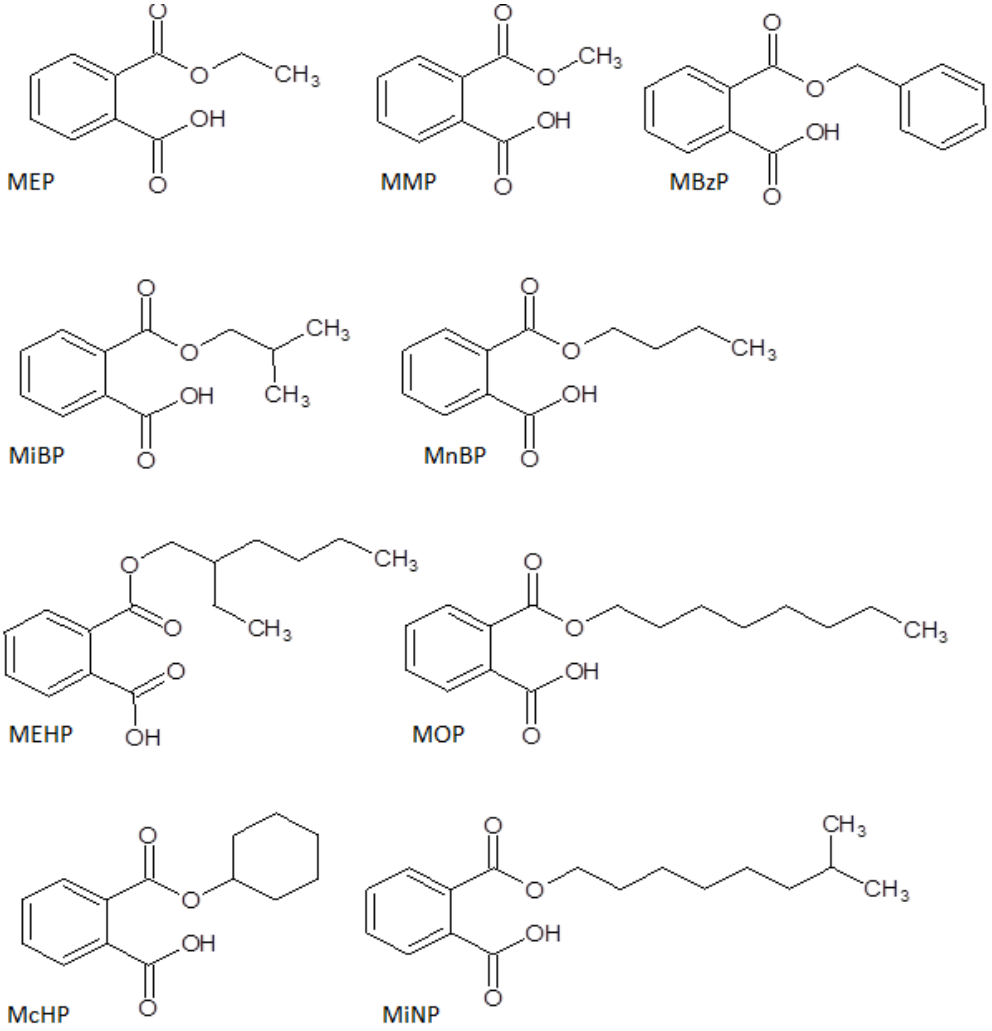

Figure 3.4.1: Chemical structures of investigated analytes.

The method provided for aqueous derivatization based on alkyl chloroformate, followed by a solid phase microextraction-gas chromatography-triple quadrupole mass spectrometry (SPME-GC-QqQ-MS) analysis. The goal is to achieve an analytical protocol that involves a minimal handling of sample, no consumption of organic solvents and, after the addition of derivatization reagents, the complete automation of process. Moreover, this work proposes the use of GC-QqQ-MS for the determination of phthalates monoesters. to obtain high specificity by selecting appropriate precursor-product ion couples. Finally, the variables affecting the derivatization reaction and the SPME analysis were optimized by the multivariate approach of "Experimental design" (DoE) [250].

3.4.1 Experimental Section

3.4.1.1 Chemicals and Materials

Monoethyl phthalate (MEP), monomethyl phthalate (MMP), monobenzyl phthalate (MBzP), mono-isobutyl phthalate (MiBp), mono-*n*-butyl phthalate (MnBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-*n*-octyl phthalate (MnOP), monocyclohexyl phthalate (McHP), mono-isononyl phthalate (MiNP) were purchased from Chemical Reasearch 2000 (Rome, Italy). Acetonitrile (ACN), pyridine, sodium chloride, propyl chloroformate and propanol were bought from Sigma Aldrich (Milan, Italy). The five tested fibers, commercially available, namely polyacrylate 85 µm (PA), carboxen/polydimethylsiloxane 85 µm (Car/PDMS), divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm (DVB/Car/PDMS), polydimethylsiloxane/divinylbenzene 65 µm (PDMS/DVB), and polydimethylsiloxane 100 µm (PDMS), were obtained from Supelco (Bellefonte, PA, USA) and conditioned as recommended by the manufacturer. β-glucuronidase from *E. coli* K12 was bought from Sigma-Aldrich (Milan, Italy). Synthetic urine (negative urine control) produced from Cerilliant was commercialized by Sigm-Aldrich (Milan, Italy). The Ultrapure water was obtained from a Milli-Q plus system (Millipore, Bedford, MA).

3.4.1.2 Instrumentation

GC-MS analysis was performed with a TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a triple quadrupole mass spectrometer (QqQ) Quantum and a Trace GC Ultra equipped with a Triplus autosampler. The capillary column was Restek Rxi-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness, 95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC

oven temperature was initially held at 70 °C for 5 min, then ramped at 20 °C/min to 250 °C, then ramped at 10 °C/min to 320 °C and held at this temperature for 10 min. The carrier gas was helium (purity 99.999%) at 1 mL/min, whereas argon (purity 99.999%) at a pressure of 2.3 mTorr was used as collision gas. A Thermo PTV straight Liner 0.75×2.75×105 mm was used in the GC injector. Analyses were performed in splitless mode with the injector temperature set at 280 °C. The triple quadrupole was operated in electron ionization (EI) and was used in full scan mode to identify the derivatized analytes and then in selected reaction monitoring. The emission current was set at 25 µA and the transfer line and ion source were set at 280 °C and 250 °C respectively. The scan width was set at 1.2 m/z whereas the peak width of Q1 was 0.7 amu for all segments. Xcalibur software was used as instrumentall control whereas Excel (Microsoft, USA) and Statistica 8.0 (StatSoft 2007 Edition, Tulsa USA) were used to perform and evaluate experimental data and experimental design matrix.

3.4.1.3 Optimized analytical procedure

Firstly, synthetic urine was treated in the same way that the real urine has to be treated in order to enzimatically hydrolyze the glucuronide-conjugated forms of phthalate monoesters. In particular, an amount of urine was treated with ammonium acetate 1 M to adjust the pH to 7, a value at which the enzyme is active. Afterwards β-glucuronidase was added and hydrolysis was carried out at 37 °C for 2 hours. Finally, the mixture was acidified with sulforic acid 10% to pH 2. pH needs to be adjusted to neutral values so as to allow the investigated analytes to derivatize. Therefore, the mixture was treated with NaOH 5 M to adjust the pH to 7. The optimized analytical procedure consists in weighing directly in the vial used for autosampler 600 mg of NaCl and 500 µL of synthetic urine, previously treated with β-glucuronidase, spiked with the considered analytes. Afterwards, 238 µL of pyridine (py) and 10 µL of propanol (PrOH) were added and the mixture was stirred for 10 min. Finally, 223 µL of propyl chloroformate (PCF) was added and the mixture was stirred for 2 min in order to carry out the derivatization of analytes. Afterwards, an appropiate volume of ultrapure water was added to achieved a final volume of 6 mL and then the vial was crimped. SPME extraction was performed by autosampler with a divinylbenzene/carboxen/polydimethylsiloxane 50/30µm (DVB/Car/PDMS) in direct immersion mode for 40 min at 80 °C. The extracted analytes were thermally desorbed by introducing the fiber into the injector set at 280 °C for 10 min.

3.4.2 Results and Discussions

3.4.2.1 Gas chromatography-tandem mass spectrometry analysis

A derivatization reaction was carried out for the investigated analytes otherwise not amenable to gas chromatographic analysis. The esterification of the acidic moiety was carried out directly in synthetic urine in according to the method proposed by Husek [251]. An example of the derivatization products obtained is shown in ***figure 3.4.2***.

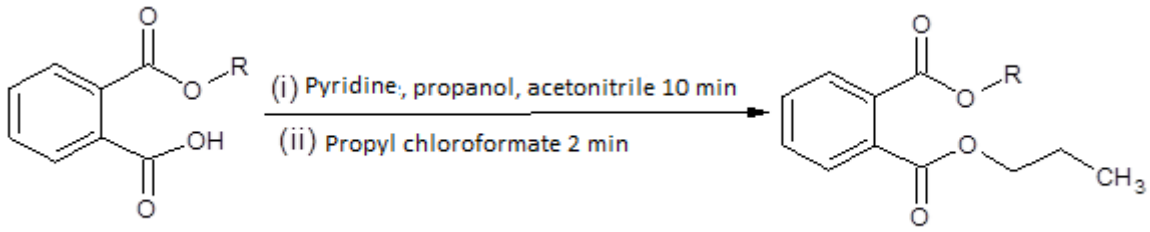

Figure 3.4.2: Scheme of derivatization reaction for a generic phthalate monoester.

A preliminary derivatization with 250 µL of pyridine and propanol and 100 µL of propyl chloroformate was carried out for verifying the successful esterification reaction. The mixture was extracted with ethyl acetate and analysed in GC-MS. The analysis was acquired in full scan mode to identify the derivatized analytes and to evaluate the chromatographic conditions. The best chromatographic separation was achieved by using the temperature programme described in experimental section (***Figure 3.4.3***).

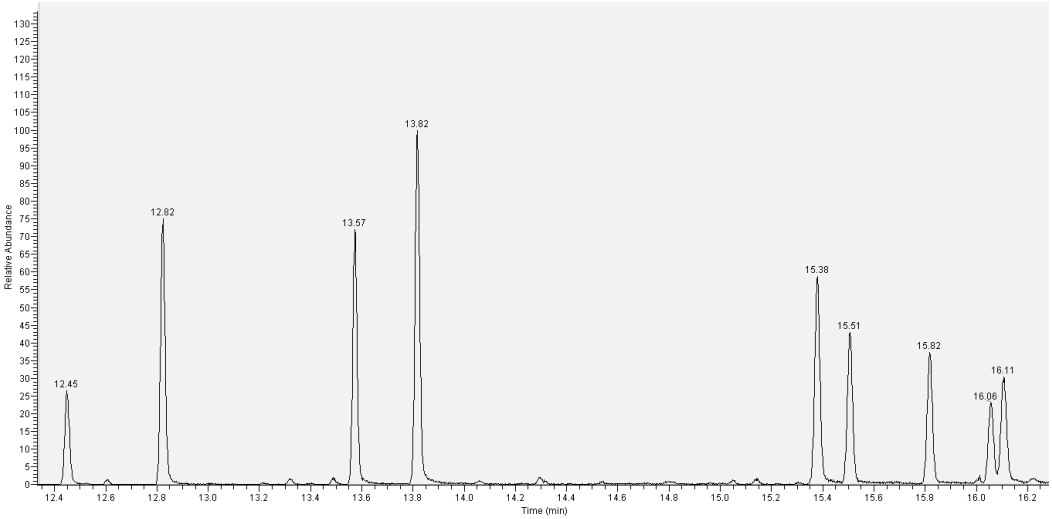

Figure 3.4.3: GC-MS chromatogram obtained in full scan mode with each analyte at 10 µg/mL.

The use of GC-QqQ-MS system represents a powerful analytical tool due to the high separation efficiency of chromatographic system and the great sensitivity and specificity of the triple quadrupole in selected reaction monitoring (SRM) acquisition mode. Two SRM transitions were chosen in according with the European Commission decision 2002/657/EC [195]. The transition with the best S/N ratio was selected for quantification of analytes whereas the second more sensitive was used to identify the analytes. The selected transitions with the corresponding collision energy and other instrumental parameters are reported in ***table 3.4.***

Table 3.4: Retention times and MS/MS transitions (collision energy are indicated in parenthesis) for the investigates analytes.

Compound	**Ritention time (min)**	**Scan time (s)**	**SRM transition, m/z (collision energy, V)**	
			Quantification	Identification
MMP	12.45	0.01	163→77 (21)	163→92 (23)
MEP	12.82	0.01	177→149 (12)	195→149 (11)
MiBP	13.57	0.01	149→65 (22)	191→149 (7)
MBP	13.82	0.01	149→65 (22)	191→149 (7)
McHP	15.38	0.01	149→65 (22)	209→149 (9)
MEHP	15.51	0.01	149→65 (22)	209→149 (7)
MiNP	15.82	0.01	149→65 (22)	191→149 (7)
MnOP	16.06	0.01	149→65 (22)	209→149 (11)
MBzP	16.11	0.01	192→149 (7)	192→93 (27)

3.4.2.2 Optimization of derivatization reaction

The efficiency of derivatization reaction depends on the amounts of reagents. Accordingly, the quantity of PCF, PrOH, ACN and py have to be optimized. The best conditions to perform

derivatization were determined by the use of experimental design for investigating the different factors in the entire experimental domain and the possible synergetic effects between variables [178, 250]. The derivatizing reagents, ACN, PrOH, PCF and py, were optimized by using a central composite design (CCD). This design allowed the simultaneous estimation of the linear, quadratic and two-way interaction effects of the factors. The complete design provided a 2^4 factorial design, with six star points positionated at ±α from the center of the experimental domain. The total experiments number were 30 ((2^4 + (2 × 4) + 6)) where 4 is the factors number and 6 is the number of star points. The ranges of the variables to investigate were: piridine 100- 300 µL, propanol 100-300 µL, Acetonitrile 50- 250 µL and propyl chloroformate 50- 250 µL. The matrix experiments was reported in ***table 3.4.1***. A synthetic urine spiked at 10 mg/L was submitted to derivatization procedure according to the different quantity specified in table 3.4 and then the analytes derivatized were extracted with ethyl acetate. The subsequent analysis were performed in GC-MS acquiring signals in full scan mode.

Table 3.4.1: Design matrix in the CCD for optimization of derivatization reaction. (C): central point.

Exp	Acetonitrile (µl)	Pyridine (µl)	Propanol (µl)	Propyl chloroformate (µl)
1	100	150	150	100
12	200	150	250	200
16	200	250	250	200
6	100	250	150	200
29 (C)	150	200	200	150
4	100	150	250	200
30 (C)	150	200	200	150
2	100	150	150	200
24	150	200	200	250
23	150	200	200	50
11	200	150	250	100
20	150	300	200	150
7	100	250	250	100
18	250	200	200	150
3	100	150	250	100
19	150	100	200	150
10	200	150	150	200
17	50	200	200	150
13	200	250	150	100
26 (C)	150	200	200	150
14	200	250	150	200
21	150	200	100	150
22	150	200	300	150
25 (C)	150	200	200	150
28 (C)	150	200	200	150
9	200	150	150	100
8	100	250	250	200
5	100	250	150	100
27 (C)	150	200	200	150
15	200	250	250	100

When several responses corresponding to the studied analytes have to be taken into account it is required to look for a compromise among the responses. In order to convert a multi-response problem into a single-response one, the Derringer's desirability function was used. In the first istance, the response is converted in a particular desirability function (d_i) that varies from 0 to 1. Maximum or a

fully desired response has the desirability 1 whereas desirability 0 is for non-desirable situations or minimum. The single desirability scores for the predicted values and for each dependent variable are then combined into an overall desirability function D by computing the geometric means of different values:

$$D = \sqrt[n]{d_1^{p1} x d_2^{p2} x d_3^{p3} x \ldots . x d_n^{pn}}$$

where pn is the weight of the response, n is the number of responses and d_n is the individual desirability function of each response. Since the response of each analyte was considered equally important in the overall desirability function equal weights were given to the responses and in this case p1=p2=p3=1. This is a way to transform a multivariate problem into a univariate problem. The overall desirability function gives the optimized parameters with a higher response for all investigate analytes. The optimized working conditions for pyridine and propyl chloroformate were 238 µL and 223 µL, respectively. Acetonitrile and propanol gave the best results at the lower end of the experimental range investigated, as shown from the desiderability function obtained (***Figure 3.4.4***).

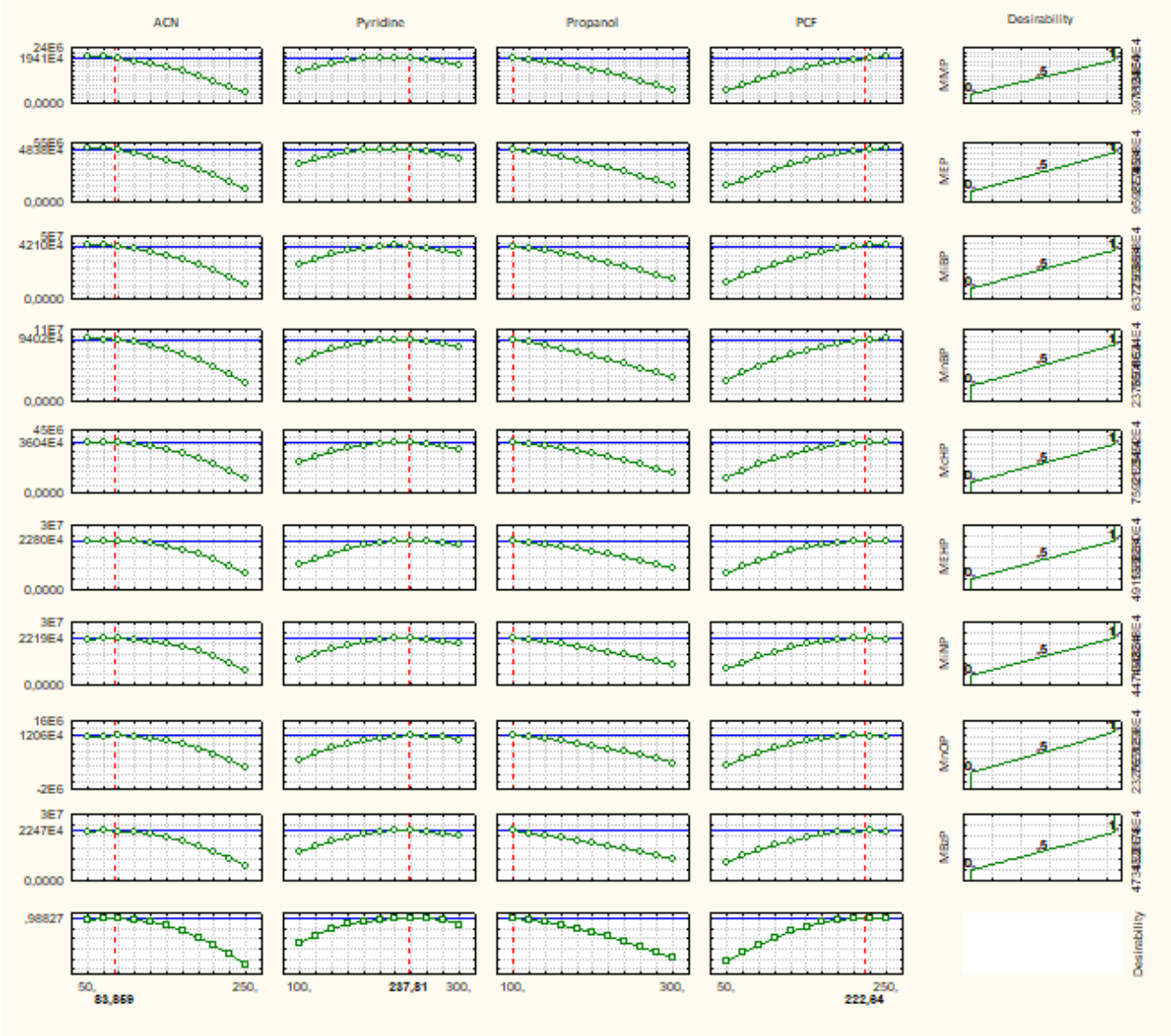

Figure 3.4.4: Desirability functions obtained by performing the CCD design for the optimization of derivatization reaction.

Therefore, a further optimization procedure was necessary for these two reagents. A full factorial design on three levels and two factors was carry out for evaluating the amount of propanol and acetonitrile. The experimental domain was 30-130 µL for PrOH and 0-100 µL for ACN whereas the optimized quantities of py and PCF were used. The matrix design of the experiments constituted from 9 experiments (3^2) is shown in the following table (***Table 3.4.2***).

Table 3.4.2: Design matrix for the optimization of ACN and PrOH. (C): central point.

Exp	Acetonitrile (µl)	Propanol (µl)
4 (C)	50	80
7	0	30
2	100	130
5	100	80
9	0	80
6	0	130
10 (C)	50	80
3	50	30
1	500	130
11 (C)	50	80
8	100	30
12 (C)	50	80
4	80	130

The best signal for these variables was obtained by considering the lowest quantities for both ACN and PrOH for all analytes. Further investigations were performed for the evaluation of PrOH volume in absence of ACN. In particular, three replicates of synyhetic urine sample spiked at 5 µg/mL were carried out for three different amounts of PrOH (10, 20 and 30 µL) and the results obtained are reported in ***figure 3.4.5***.

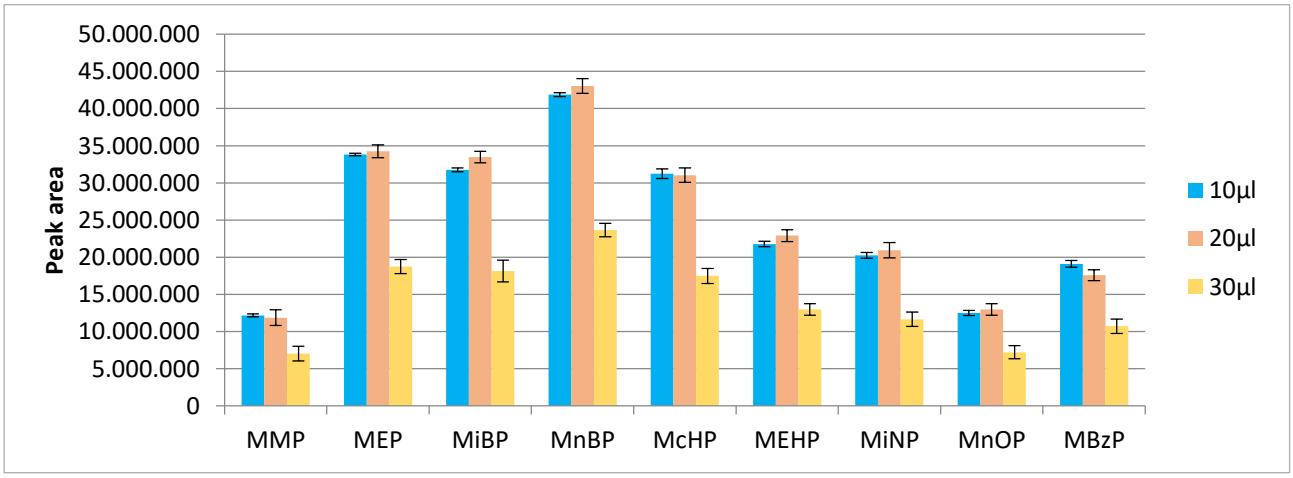

Figure 3.4.5: Signals obtained for each analyte at different volumes of PrOH and at concentration of 5 µg/mL.

The obtained results clearly showed that derivatization carried out with 10 µL and 20 µL leads to higher responses respect to 30 µL, However, since the differences between the signals obtained with 10 µL and 20 µL are negligible for all the analytes, less amount was chosen. In conclusion, the optimal working conditions for the derivatization reaction were: 238 µL of pyridine, 223 µL of propyl chloroformate and 10 µL of propanol in absence of acetonitrile.

3.4.2.3 Optimization of SPME variables

The crucial steps in SPME extraction are the selection of the SPME fiber and the evaluation of other factors that can influence the extraction process. For the first one, five different fibers namely polyacrylate 85 µm (PA), carboxen/polydimethylsiloxane 85 µm (Car/PDMS), divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm (DVB/Car/PDMS), polydimethylsiloxane/divinylbenzene 65 µm (PDMS/DVB), and polydimethylsiloxane 100 µm (PDMS) were tested. The samples used for the fiber screening were prepared as described in experimental section at 5 µg/mL and the analyses were acquired in triplicates, in SRM mode. The results obtained are shown in ***figure 3.4.6.***

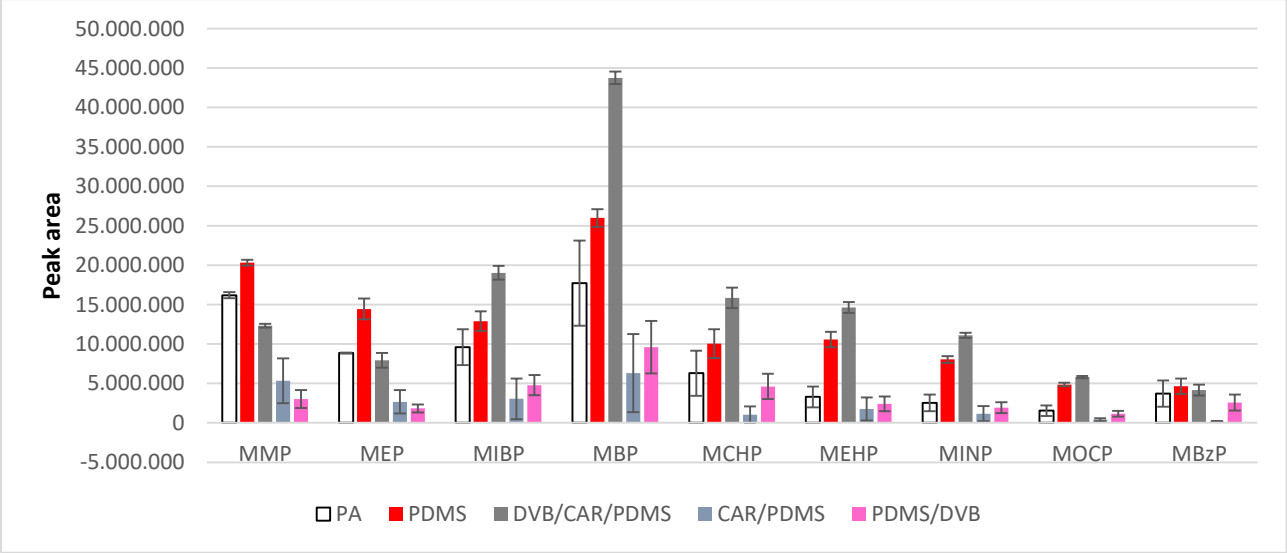

Figure 3.4.6: Peak areas obtained by performing analyses with five different fibers with each analyte at 5 µg/mL.

An overall evaluation of obtained peak areas showed the highest signals are obtained by using DVB/Car/PDMS fiber, except for MMP and MEP. Moreover, the sample blank after three analyses with the same fiber was evaluated and the chromatogram with DVB/Car/PDMS resulted cleaner for

all analytes than that obtained with PDMS fiber. Only for MBP a high signal is present performing analysis with both fibers due to the coelution of the derivatized MBP with di-n-butyl phthalate that is present in most of the laboratory equipment. In the light of these findings, the DVB/Car/PDMS fiber was used for the further investigations. The other variables that can influence the extraction efficiency such as extraction temperature, extraction time and percentage of sodium chloride were optimized with the multivariate approach of experimental design. In particular, a central composite design constituted from a 2^3 factorial design with six star points positioned at ±α from the center of experimental domain was carried out. The number of experiments to carry out is 20 experiments, (2^k + (2 × k) + n), with k=3 and n=6. The experimental domain used for the variables were the following: extraction time 15-45 min, extraction temperature 40-80 °C and percentage of NaCl 0-10%. In ***table 3.4.3*** the planning of experiments is reported:

Table 3.4.3: Design matrix in the CCD design for the optimization of SPME variables.

Exp	Extraction time (min)	Extraction temperature (°C)	% NaCl
11	30	40	5
1	21	48	2
5	39	48	2
2	21	48	8
19 (C)	30	60	5
9	15	60	5
6	39	48	8
13	30	60	0
15 (C)	30	60	5
17 (C)	30	60	5
16 (C)	30	60	5
18 (C)	30	60	5
14	30	60	10
7	39	72	2
4	21	72	8
8	39	72	8
3	21	72	2
20 (C)	30	60	5
12	30	80	5
10	45	80	5

The results were evaluated with the desiderability functions obtained from the CCD and shown in ***figure 3.4.7.***

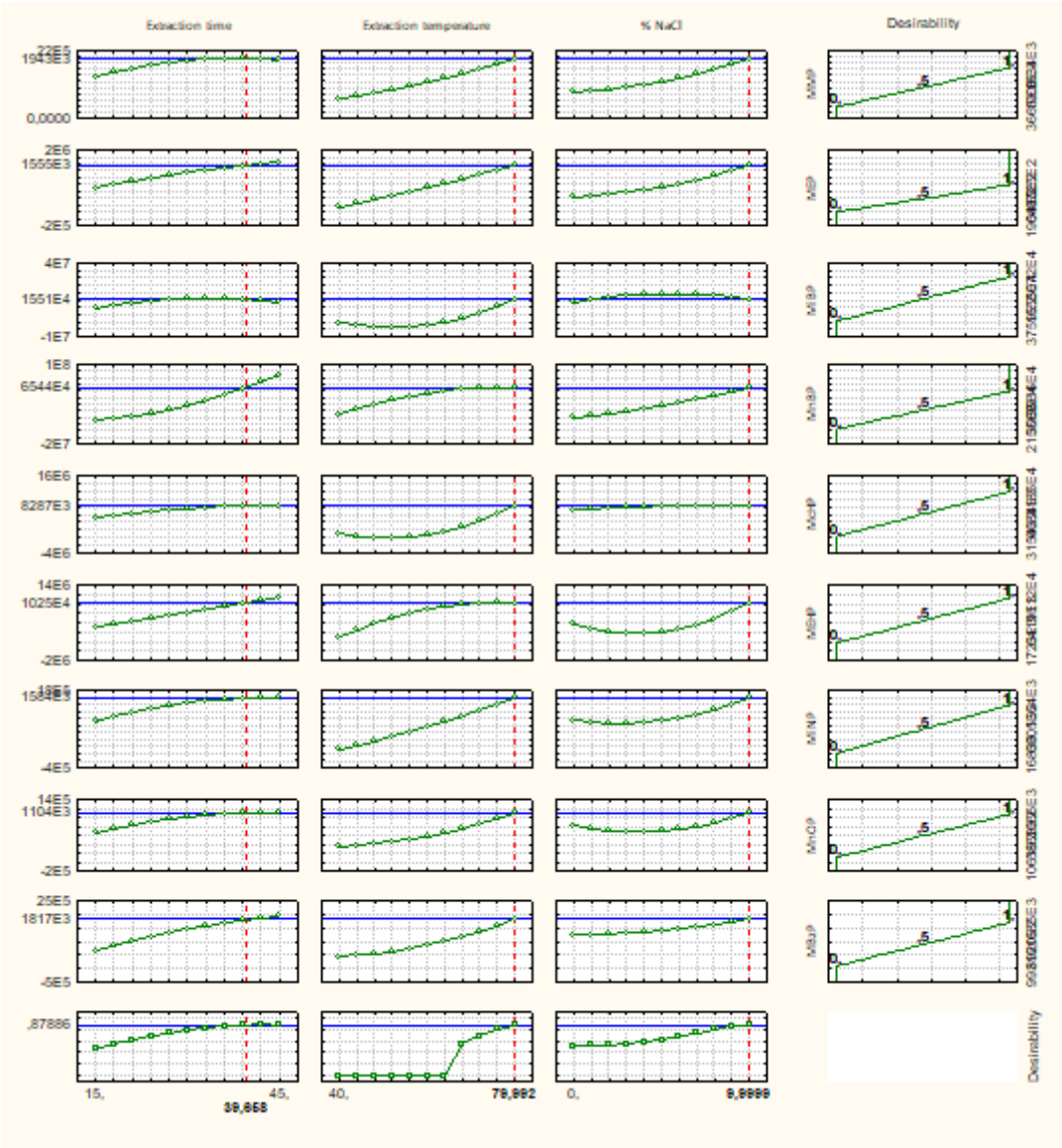

Figure 3.4.7: Desirability functions obtained by performing the CCD design for the optimization of SPME extraction parameters.

The optimal working SPME conditions were extraction time 40 min at an extraction temperature of 80 °C by adding a 10% of NaCl.

3.4.3 Conclusions and perspectives

In this work a SPME-GC-MS/MS method was developed for the determination of phthalates monoesters in urine. The derivatization reaction was carried out directly in urine with propyl chloroformate and propanol and then SPME was used in the same vial as microextraction technique to transfer analytes directly in the injector of gas chromatograph. The analyses were fully automated. Experimental design was applied to optimize the variables affecting derivatization reaction and SPME extraction. This novel method demonstrated that the combination of alkyl chloroformate and SPME extraction represents a convenient approach in the identification of the investigated analytes by an easy and fast method involving a minimal handling of sample. This work is still under construction; in particular, the next step will be the validation of the developed method through the evaluation of the lower limit of quantification, linearity, matrix effect and analytical performances.

3.5 References

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Chapter 4

Microextraction by packed sorbent and gas chromatography coupled with triple quadrupole mass spectrometry analysis in environmental applications

4.1 Introduction

In the last years the research field was very attracted by the presence of chemical contaminants in several environmental matrices and in biological fluids. These contaminants represent chemical substances continually introduced into the environment as anthropogenic pollutants due to urbanization and industrialization processes. The main problem of the presence of contaminants is represented by the adverse effects on environment and ecosystem and consequently on the human health. For this reason, the development of new methods for the identification of environmental contaminants is growing rapidly. Sample preparation is an important stage in the determination of components of interest from complex matrices and strongly influences the reliability and accuracy of analysis and data quality. The classical sample preparation techniques present several drawbacks because they involve many steps increasing the error sources and making the method too laborious. In the light of this, microextraction techniques represent a very interesting alternative in environmental and clinical fields. The novel MEPS extraction technique offers several advantages for the extraction of contaminants from environmental matrices and biomarkers from urine. The combination of MEPS extraction with a high efficiency separation system such as gas chromatography coupled with mass spectrometry triple quadrupole allows to achieve simple and high-throughput protocols.

In this part of the thesis two MEPS-PTV-GC-MS/MS methods were developed for analysis in environmental matrices and in urine. In particular, a novel method for the assay of organophosphate flame retardant in tap water, river water and wastewater was developed and optimized. Finally, the initial part of the development of a new method for the determination of phthalate monoesters in urine was presented.

Multivariate optimization of a microextraction by packed sorbent-programmed temperature vaporization-gas chromatography-tandem mass spectrometry method for organophosphate flame retardant analysis in enviromental aqueous matrices [252]

4.2 Introduction

Organophosphate esters (OPEs) represent a common class of flame retardants (OPFRs). They are semi-volatile, have good solubility in water, some of them are thermally stable whereas other OPEs have better elasticizing properties. For this reason, they can be used as flame retardants or plasticizers in several materials such as foams, paints, textiles and plastics [253]. OPFRs belong to the class of Emerging Pollutants and are easily released into the environment due to their extensive use [254]. A worrying property of some OPFRs is the ability to bioaccumulate such in the case of tricresylphosphate (TCP), tris(2-ethylhexyl) phosphate (TEHP), (2-ethylhexyl)-diphenyl phosphate (EHDPP) with several adverse effects on human health [255]. They are stable toward biodegradation, in particular the chlorinated OPFRs such as tris(1-chloro-2-propyl) phosphate (TCPP) [256]. Tris(2-chloroethyl) phosphate (TCEP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(1-chloro-2-propyl) phosphate (TCPP) are suspected carcinogenic [253, 257, 258] whereas tripropyl phosphate (TPP) and tris(1,3-dichloro-2-propyl) phosphate (TDCPP) have a potential endocrine disruption activity [259, 260]. Tri-n-butyl phoshate (TBP) and triphenyl phosphate (TPhP) can have neurotoxic effects and inhibit the activity of carboxyl esterase [253, 261].

Chromatographic techniques such as gas chromatography (GC) and liquid chromatography (LC) was extensively proposed for the analysis of OPFR. Many studies report their identification in water [253, 262, 263], soils [263, 264], air [253, 262], biota samples and sediments [263]. Liquid chromatography for the analysis of OPFR was used coupled to tandem mass spectrometry [265, 266] whereas initially GC was used in combination with mass spectrometry in electron ionization [267, 268] and nitrogen-phosphorous detector (NPD) [269, 270]. The possibility to interface GC with tandem mass spectrometry for OPFR analysis [271-274] allowed to perform simple operations with high separation efficiency and reliability improving selectivity and sensitivity of the proposed method [275-279]. Generally, the extraction methods for sample preparation in environmental analysis involve many steps such as extraction and purification before the instrumental analysis. OPFR extraction is normally performed by mean of liquid-liquid extraction (LLE) or solid phase extraction (SPE) [262]. Some of microextraction techniques were also used for OPFR quantification. For example, solid-phase microextraction (SPME) allowed to extract OPFR in several environmental matrices with

advantages in terms of time saving and eco-compatibility. In particular, SPME was used as sample preparation of OPFR in air [280], water [281, 282], wastewater [283], and soil [284, 285]. Microextraction by packed sorbent (MEPS) is a miniaturization of solid phase ectraction (SPE) with several advantages in terms of cost, environment protection and applications [286, 287]. MEPS extraction in analysis of flame retardants was used for the determination of brominated diphenyl ether in sewage sludge [288].

The purpose of the work herein presented was the development of a simple and fast method for the extraction of organophosphate flame retardants ***(figure 4.2)*** by microextraction by packed sorbent (MEPS) in environmental waters. In order to improve the sensitivity of method the programmed temperature vaporization (PTV) was chosen as gas chromatographic injection technique. The MEPS and PTV working conditions were optimized in univariate and multivariate approach with the use of experimental design. Tandem mass spectrometry was exploited so as to set up a high specific protocol capable of a reliable and sensitive analyte quantification. Finally, the developed method was applied to real samples of tap water, river water and simulated wastewater.

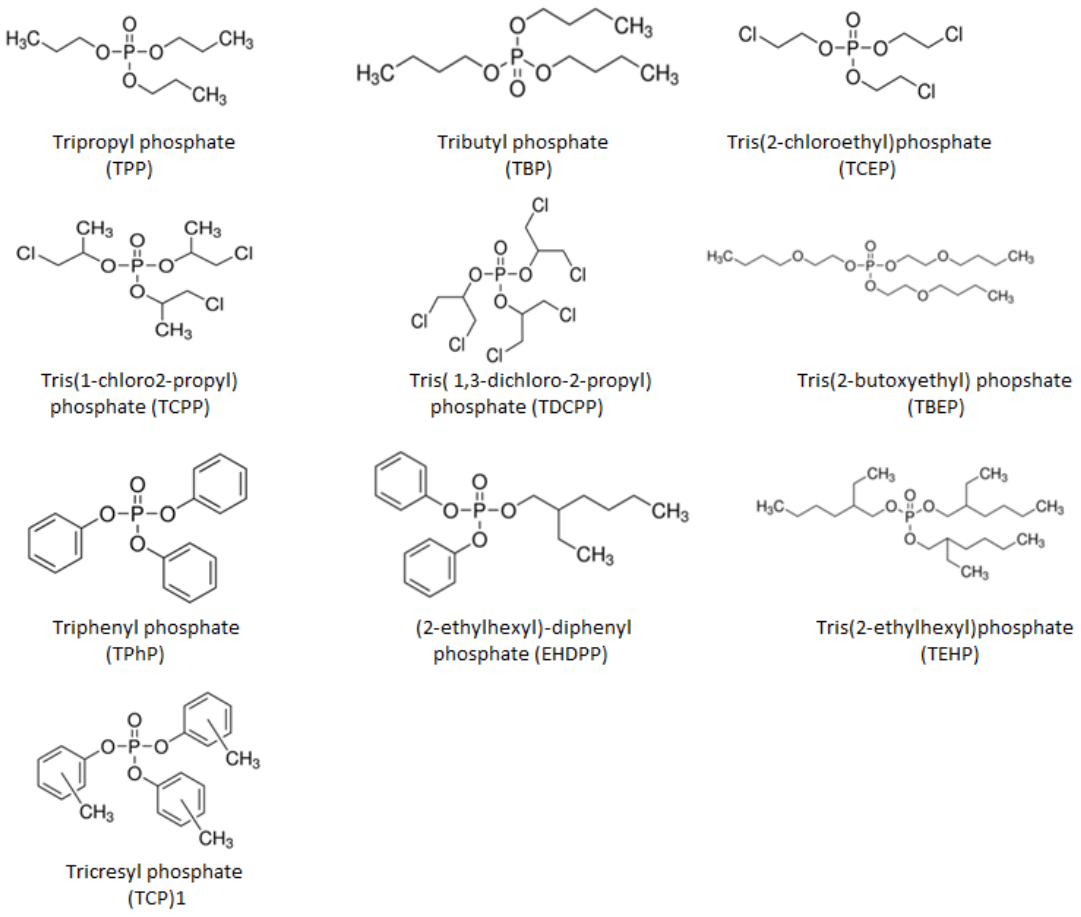

Figure 4.2: Chemical structures of the investigated analytes. 1: mixture of isomers.

4.2.1 Experimental section

4.2.1.1 Chemicals and materials

The studied analytes tripropyl phosphate (TPP), tributyl phosphate (TBP), tris(2-chloroethyl) phosphate (TCEP), tris(1-chloro-2-propyl) phosphate (TCPP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tris(2-butoxyethyl) phosphate (TBEP), triphenyl phosphate (TPhP), (2-ethylhexyl)-diphenyl phosphate (EHDPP), tris(2-ethylhexyl) phosphate (TEHP), tricresylphosphate (TCP) (technical mixture of isomers) were bought from Sigma–Aldrich (Milan, Italy). The deuterated internal standards, tris(2-chloroethyl) phosphate-d12 (TCEP-d12) and tributylphosphate-d27 (TBP-d27) were provided by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) whereas the triphenylphosphate-d15 (TPhP-d15) was bought from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile (ACN), dichloromethane (DCM), hexane (Hex) and methyl tert-butyl ether (MTBE) were obtained from Sigma-Aldrich (Milan, Italy). Methanol (MeOH) was obtained from VWR Chemicals (France), ethyl acetate (EtAc) and trichloromethane (TCM) were obtained from Panreac Quimica (Barcelona, Spain). The certified reference material "Wastewater" from RTC International (Laramie, WY, USA) was commercialized by Sigma-Aldrich. Micropore filters (0.45 µm pore size) were purchased from Millipore (Bedford, MA). MEPS cartridges (C2, C8, C18, Silica, DVB) and semiautomatic electronic syringe (eVol®) were provided by SGE Analytical Science (Melbourne, Australia). Aqueous solutions were prepared using ultrapure water obtained from a Millipore Milli-Q plus system (Bedford, MA). Oasis hydrophilic lipophilic balance (HLB) solid phase extraction cartridges (6 mL, 500 mg) were purchased from Waters (Milan, Italy).

4.2.1.2 Real samples and MEPS extraction

The real samples collected for this study were the following: five tap water samples from public water supply of Rende (Cosenza, Italia) after allowing the tap to flow for 5 min; three surface water samples of three different rivers (Campagnano, Crati and Busento) located in the city of Cosenza (Italy) and the wastewater sample simulated by spiking the certified material at LLOQ value for all analytes. The sampled waters were collected in glass amber bottles, previously washed with acetonitrile to avoid the presence of any possible contamination [289]. Wastewater sample was filtrated through 0.45 µm filters and all samples were stored under refrigerated conditions (4 °C) and then analyzed without any pre-treatment. MEPS extraction was carried out with a semiautomatic electronic syringe consisting of a 500 µL gas-tight syringe with a barrel contained 4 mg of a solid-phase silica-DVB material. The

MEPS optimized conditions provided for the conditioning of sorbent first with 500 µL (2×250 µL) of methanol and then with 500 µL (2×250 µL) of ultrapure water at a flow rate of 8 µL/s. The analytes were extracted by aspiring and then discarding into the waste the sample (4×500 µL) at a flow rate of 8 µL/s. Next, the cartridge was dried by pumping air through it (10×500 µL) at a flow rate of 25 µL/s. Elution was carried out with 60 µL of ACN (3×20 µL), pumped up through the sorbent and down into a vial with conical insert at a flow rate of 0.7 µL/s. After each extraction process, the sorbent was rinsed with 7 cycles of 250 µL of acetonitrile (4 µL/s) in order to avoid carryovers.

4.2.1.3 Instrumentation and data processing

The analyses were performed with a TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a triple quadrupole mass spectrometer (QqQ-MS) Quantum, and equipped with a programmable temperature vaporizer (PTV) injector and TriPlus autosampler. Chromatographic separation of the analytes was performed using a Restek Rxi-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness, 95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 80 °C for 4 min, then ramped at 20 °C/min to 140 °C and held for 2 min; then ramped again at 4°C/min to 180 °C; and finally ramped again at 20°C/min to 280 °C held at this temperature for 6 min. The total runtime was 30 min. Nine microliters of sample were injected at 50 µL/s using a 10 µL syringe in an empty Siltek deactivated baffled liner (120 mm × 2 mm i.d.). The initial temperature of PTV injector was set at 60 °C for 0.4 minutes during the sample injection and solvent evaporation steps and the split valve was opened at 50 mL/min. After the solvent venting step, the split valve was closed and the injector was heated to 350 °C at a rate of 14.5 °C/s, and maintained at this value for 2 min during the analyte transfer step from the liner to the capillary column. The carrier gas was helium at 1 mL/min of purity 99.999% and argon at a pressure of 1.0 mTorr was used as collision gas for tandem mass spectrometry acquisition. The transfer line and ionization source temperatures were set at 280 °C and 250 °C, respectively. As regard the mass spectrometer conditions the filament emission current was set at 25 µA. The scan width was set at 0.8 m/z for all segments and peak width of Q1 was fixed at 0.7 amu. The mass spectrometer was operated in electron ionization (EI) in full scan mode for identification (50–500 m/z as mass range) and in selected reaction monitoring (SRM) mode for quantification. The analytes were identified using the NIST 02 database (NIST/EPA/NIH Mass Spectral Library, version 2.0).

The data processing was performed with a Xcalibur software 2.0.0 as well as the instrument control. Experimental data were evaluated by use of Excel and the experimental design was conducted with Statistica 8.0 (StatSoft 2007 Edition, Tulsa, USA).

4.2.1.4 Limit of detection, lower limit of quantification and calibration procedure

Limit of detection (LOD) for all investigated analytes was calculated as the lowest concentration that gave a signal-to-noise ratio of three (S/N = 3) in accordance to the guideline of the International Conference for Harmonization [290]. The Food and Drug Administration (FDA) guidance were used to evaluate the lower limit of quantifications (LLOQ) and linearity [291]. LLOQs for each analyte, were evaluated by preparing ultrapure water samples spiked with the analytes at different concentrations, extracted as described above and analyzed in quintuplicates. LLOQs were determined as the lowest concentration that provides a signal at least five times the response compared to blank response and that can be quantified with suitable accuracy (80-120%) and precision (RSD<20%). Calibration curves consisted of a blank sample (ultrapure water without internal standards), a zero sample (ultrapure sample with internal standards) and seven non-zero samples. The seven non-zero samples consist of ultrapure water spiked with known amount of analytes covering a range between their quantification limits and 5 or 10 ng/mL. 0.5 ng/mL of internal standards TBP-d27 and TCEP-d12 were added to the calibration standards before MEPS extraction. Three replicates for each calibration level were performed.

4.2.1.5 Recovery, matrix effect and figures of merit

The Food and Drug Administration (FDA) guidance were considered for evaluating accuracy and recovery, within-run and between-run precision [291], whereas the method proposed by Matuszewski et al. was used to determine the matrix effect (ME) [292]. Three quality control samples (QC) were used to determine all these parameters. These samples were prepared at levels covering the range of calibration curves for each matrix, i.e. tap and river waters and wastewater. The low QC level was prepared at 1.5 × the LLOQ, the middle QC level near the center of the calibration range and finally, the high QC sample at 0.8 × the upper boundary of the standard curve [291]. The recovery was evaluated for each analyte as the ratio of the peak area obtained from the analysis in quintuplicate of the QC samples against that of a standard solution in acetonitrile with an equivalent concentration. The matrix effect was determined by splitting a sample for each matrix and a sample of ultrapure water into two aliquots. The first aliquot was spiked only with the internal standards at 0.5 ng/mL whereas the second aliquot was used to prepare samples with internal standards at 0.5 ng/mL and a concentration of analytes at the three QC levels. In this way the spiked matrix samples and spiked ultrapure water sample were obtained. Each sample was analyzed in quintuplicate and ME was calculated according the following formula:

$$ME = \frac{A_{sm} - A_{bm}}{A_{sw}} \times 100 \qquad Eq.\ 4.2$$

where A_{sm} is the analyte peak area in the spiked matrix sample, A_{bm} is the peak area in the blank matrix sample, and A_{sw} is the peak area in the spiked ultrapure water sample. Precision was expressed as percentage relative standard deviation (RSD%) whereas accuracy was calculated as percentage ratio between the concentration estimated from the calibration curve and the spiked concentration.

4.2.2 Results and discussion

4.2.2.1 GC-MS/MS analysis and optimization of MEPS cartridge and elution solvent

The use of tandem mass spectrometry allowed to perform the analyses in selected reaction monitoring improving the sensitivity and selectivity of the analytical protocol. For each analyte two SRM transitions were chosen, the transition yielding the best S/N ratio was selected for quantification and the second more sensitive was considered to confirm recognition and therefore to avoid an unambiguous identification of the analytes. The chosen SRM transitions and the other instrumental parameters are reported in ***table 4.2.***

Table 4.2: Instrumental parameters, SRM transitions and corresponding collision energies for all analytes.

Compound	**RT (min)**	**Scan time (s)**	**SRM transition, (m/z)**	
			Quantification	Identification
TPP	12.66	0.2	99→81 (13)	183→99 (9)
TBP	18.93	0.1	99→81 (13)	211→99 (11)
TCEP	20.96	0.07	205→143 (6)	249→125 (10)
TCPP	21.44	0.150	125→99 (10)	277→125 (10)
TDCPP	25.46	0.05	209→99 (8)	381→159 (11)
TBEP	25.78	0.05	153→125 (8)	199→101 (5)
TPhP	25.96	0.1	215→168 (13)	326→215 (18)
EHDPP	26.08	0.05	251→77 (27)	251→152 (22)
TEHP	26.19	0.05	99→81 (8)	211→99 (9)
TCP[a]	28.07 28.40 28.75	0.2	368→165 (27)	368→243 (22)
TBP-d27	18.49	0.1	103→83 (12)	167→103 (10)
TCEP-d12	20.81	0.07	261→131 (10)	213→148 (5)

[a] acquired as sum of isomers

The selected transitions were evaluated in both centroid and profile acquisition. The results obtained by the analysis in profile mode showed a significant increase of sensitivity for all investigated analytes and comparable performance in terms of specificity. For these reasons, the further investigations were carried out in profile mode.

The several variables influencing the efficiency of MEPS extraction were optimized combining univariate and multivariate approaches. The examined variables were solid sorbent material, elution solvent, elution volume, sample volume, number of sample draw cycles, sample draw, and elution flow rate. MEPS extractions were executed in extract-discard mode in which the aspired sample is not re-injected in the same vial but discarded into waste [293, 294]. Sorbent materials and elution solvents were taken into account as the first parameters to be optimized. Five commercially available MEPS cartridges, namely C2, C8, C18, Silica, and DVB, were tested for sorption of the investigated OPEs. For each cartridge, seven solvents, namely acetonitrile, methanol, ethyl acetate, methyl tert-butyl ether, hexane, dichloromethane, and trichloromethane, were evaluated. Ultrapure water samples

spiked with the investigate analytes at 200 ng/mL were extracted using the electronic eVol equipped with a 500-µL syringe. The other experimental conditions were fixed following previously reported MEPS studies [288, 295]. Firstly, the cartridge was washed two times, each with 250 µL of methanol, followed by a conditioning step with water (3×250 µL at 8µL/s). After that, 1000 µL of spiked ultrapure water were aspirated through the MEPS cartridge (5×200 µL at 2µL/s) and discarded into the waste at 7 µL/s. The cartridge was then dried in an empty vial flushing with 500 µL of air ten times. The analytes were eluted with 300 µL (3×100 µL) of solvent aspirated at 2µL/s and then ejected at 3 µL/s into a vial with conical insert. One microliter of this solution was injected into the GC-MS system and analyzed in full scan mode. The results obtained are reported in ***figure 4.2.1*** as responses normalized by taking higher extraction efficiency as 100%.

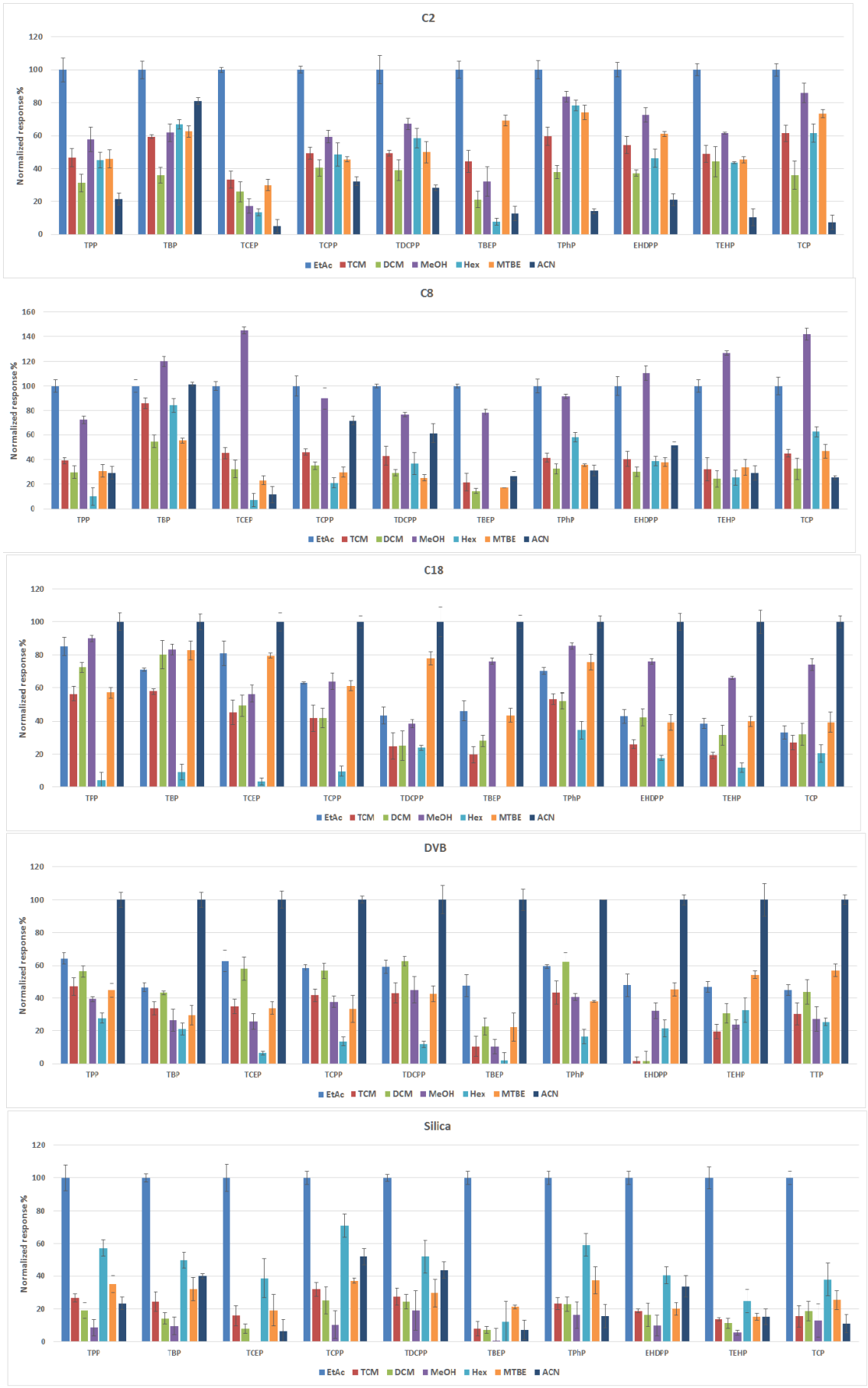

Figure 4.2.1: MEPS extraction with different cartridge sorbents and elution solvents.

The highest responses were observed for the most polar sorbents, i.e. C2 and Silica, when ethyl acetate was used as elution solvent, whereas for DVB and C18 the best results were achieved when the elution was carried out with acetonitrile. Finally, the higher response for C8 was obtained with methanol as elution solvent. In the following figure a summary of the results obtained is shown ***(figure 4.2.2)***. The responses were normalized by taking the signal of the best analyte responses for DVB/ACN as 100%.

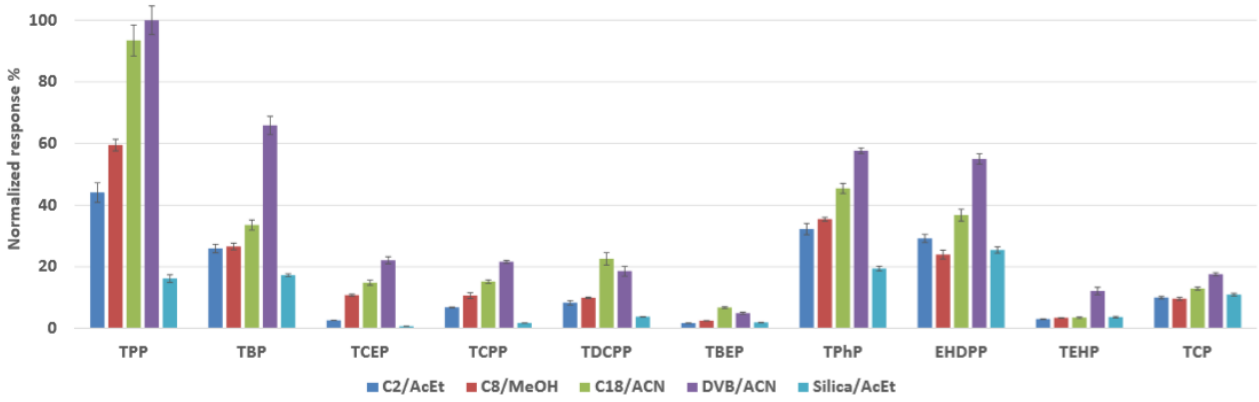

Figure 4.2.2: Comparison of the results for all the best sorbent/solvent pairs for each analyte at concentration of 200 ng/mL.

The best results were obtained with DVB/ACN pair for all analytes, except for TDCPP and TBEP for which C18/ACN showed the highest response.

4.2.2.2 Optimization of extraction and elution conditions

The parameters that can influence the MEPS extraction such as, solvent volume, sample volume, number of sample draw cycles, sample draw and elution solvent were optimized for both sorbent/solvent pairs C18/ACN and DVB/ACN with the use of experimental design. This approach allowed the simultaneous investigation of all factors and the possible synergic effects between variables [296-298]. Firstly, to evaluate the parameters with a significant effect on MEPS extraction a screening design was carried out. A full factorial design (2^5) was performed to estimate the first-order effects and all first order-interactions. In the following table ***(Table 4.2.1)*** a design matrix constituted of 32 randomly experiments are reported.

Table 4.2.1: Design matrix for the screening of MEPS working conditions for the sorbent/solvent pairs DVB/ACN and C18/ACN.

Exp	Sample volume (µl)	Sample draw cycles	Sample draw time (s)	Solvent volume (µl)	Elution time (s)
1	500	2	200	60	200
25	500	2	200	300	30
5	500	2	30	60	200
26	2000	2	200	300	30
29	500	2	30	300	30
10	2000	2	200	300	200
24	2000	10	30	60	30
20	2000	10	200	60	30
6	2000	2	30	60	200
15	500	10	30	300	200
18	2000	2	200	60	30
14	2000	2	30	300	200
11	500	10	200	300	200
7	500	10	30	60	200
13	500	2	30	300	200
30	2000	2	30	300	30
17	500	2	200	60	30
21	500	2	30	60	30
19	500	10	200	60	30
32	2000	10	30	300	30
22	2000	2	30	60	30
9	500	2	200	300	200
16	2000	10	30	300	200
3	500	10	200	60	200
31	500	10	30	300	30
4	2000	10	200	60	200
8	2000	10	30	60	200
28	2000	10	200	300	30
2	2000	2	200	60	200
27	500	10	200	300	30
23	500	10	30	60	30
12	2000	10	200	300	200

The experimental range for each variable was selected according to the literature data regarding OPFRs and the characteristics of eVol system: solvent volume 60-300 µL, sample volume 500-2000 µL, number of sample draw cycles 2-10, sample draw and elution time 30-200 seconds [288, 295]. Recovery percentage values were used as response in the experimental design and the effect of each variable were evaluated by ANOVA and Pareto charts. For the DVB/ACN pair, the Pareto charts show that all the variables have no statistically significant effect ($p < 0.05$) on the response of all the considered analytes, except for TEHP ***(Figure 4.2.3)***. Indeed, for this compound sample draw time is a significant variable though with a modest contribution.

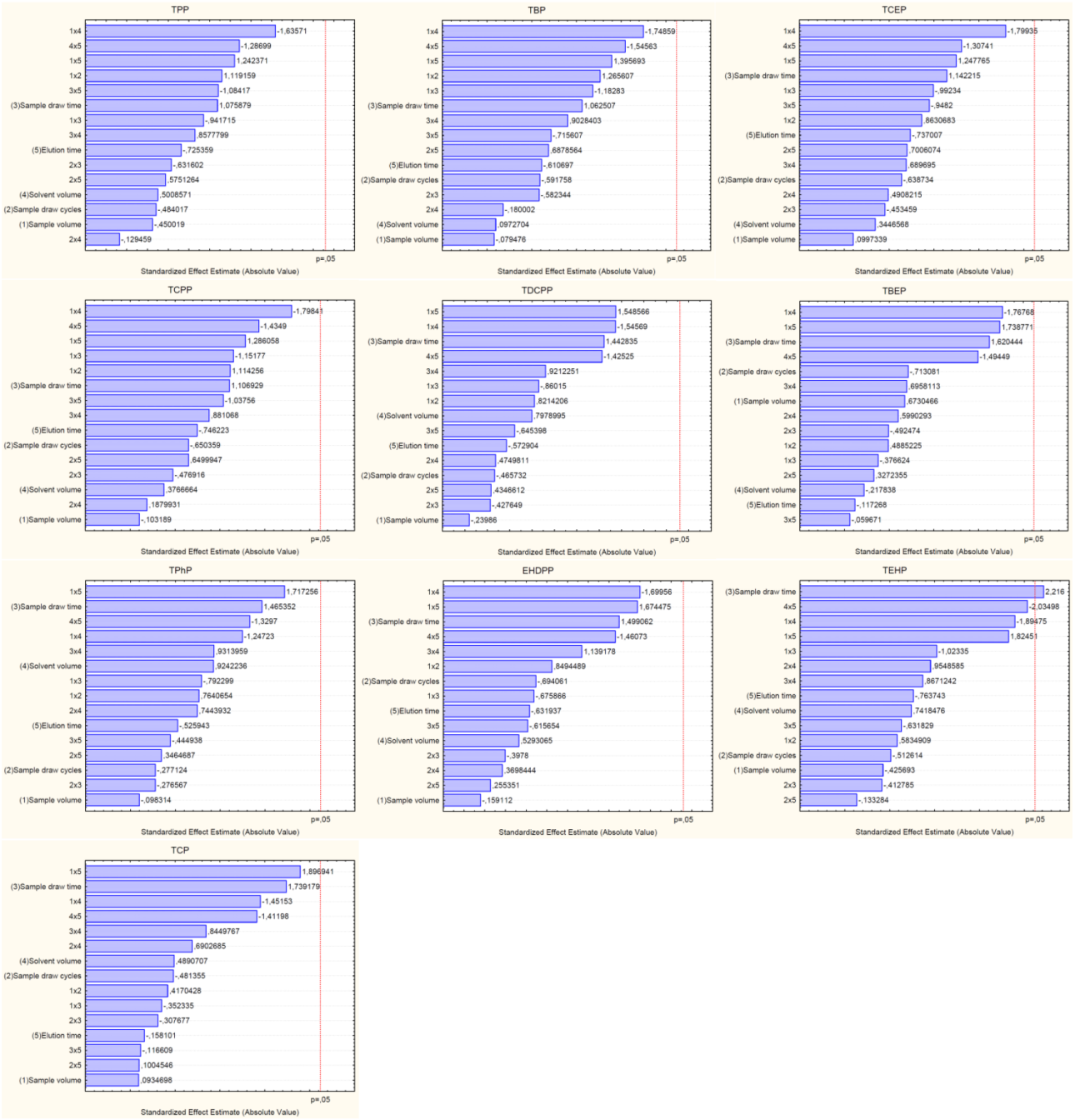

Figure 4.2.3: Pareto charts obtained from the screening of MEPS variables using DVB/ACN pair.

From these results the variables were set in accordance to analyst's convenience. In particular, to simplify the protocol four cycles of sample draw were selected whereas sample draw time and elution time were set at 60 seconds (speed 8 µL/s) and 30 seconds (speed 0.7 µL/s), respectively. Sample volume and solvent volume are the variables which influence significantly the sensitivity of the method. Therefore, the highest value for sample volume (2000 µL) and the lowest value for elution volume (60 µL, 3×20 µL) were chosen. The Pareto charts obtained for the C18/ACN pair showed that the interaction term between sample volume and elution time significantly affects the response of TBEP, EHDPP, TEHP and TTP ***(Figure 4.2.4).***

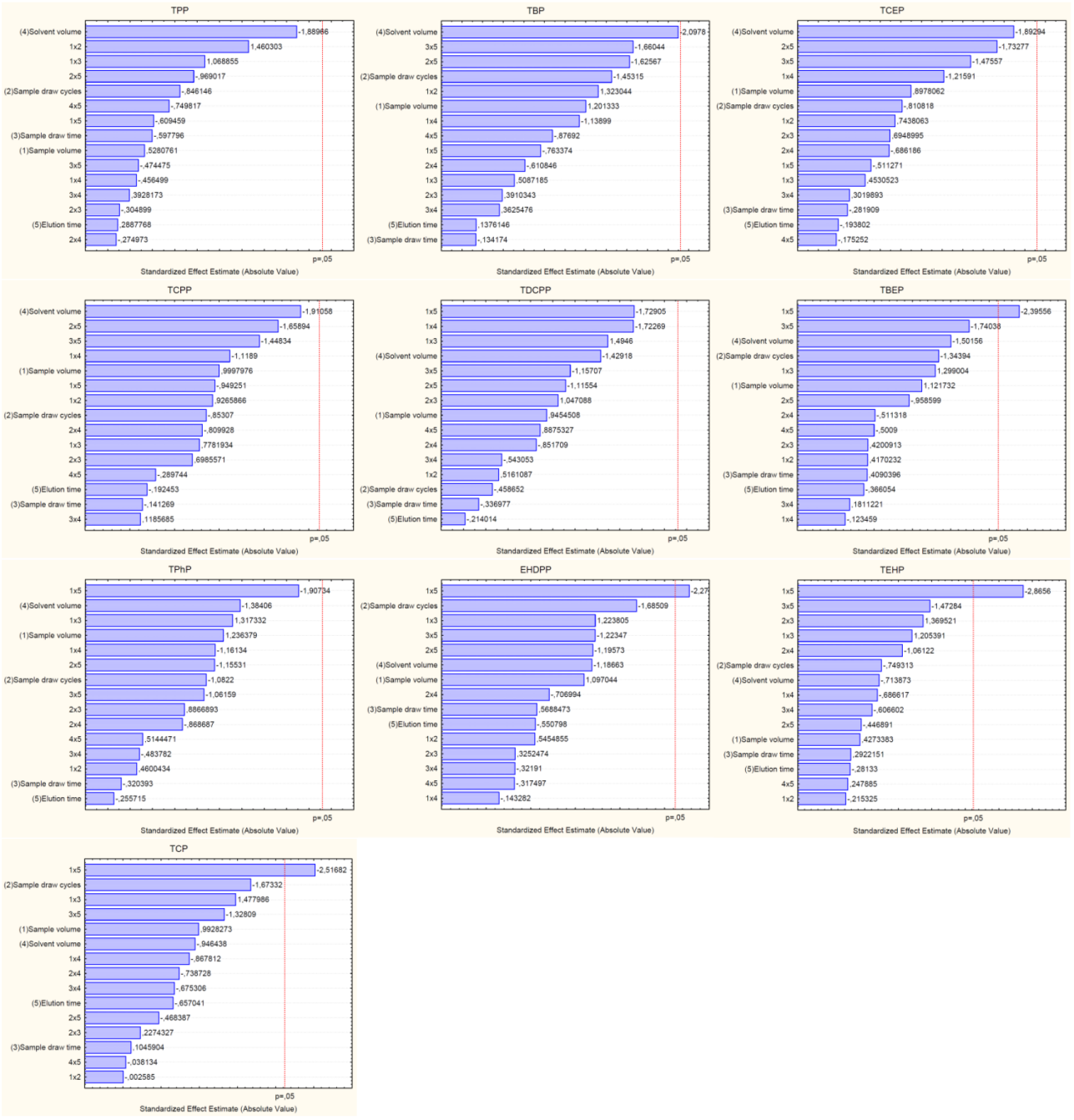

Figure 4.2.4: Pareto charts obtained from the screening of MEPS variables using C18/ACN pair.

To optimize these variables a three level full factorial design was used. This design allows to estimate the linear effects, the interactions between pairs of variables and the quadratic effects. The design matrix constituted of nine experiments (3^2) is reported in the following table (***Table 4.2.2***).

Table 4.2.2: Design matrix for the optimization of sample volume and elution time for the sorbent/solvent pair C18/ACN. (C): central point.

Exp	Sample volume (µl)	Elution time (s)
2	500	200
6	1250	200
8	2000	115
5(C)	1250	115
1	500	30
9	2000	200
10(C)	1250	115
7	2000	30
12(C)	1250	115
11(C)	1250	115
3	500	115
4	1250	30

The trends of analyte responses obtained from the full factorial design for the C18/ACN pair were evaluated with the response surfaces. These graphs show that higher recovery values are achieved close to the highest value of sample volume and the lowest value of elution time (***Figure 4.2.5***).

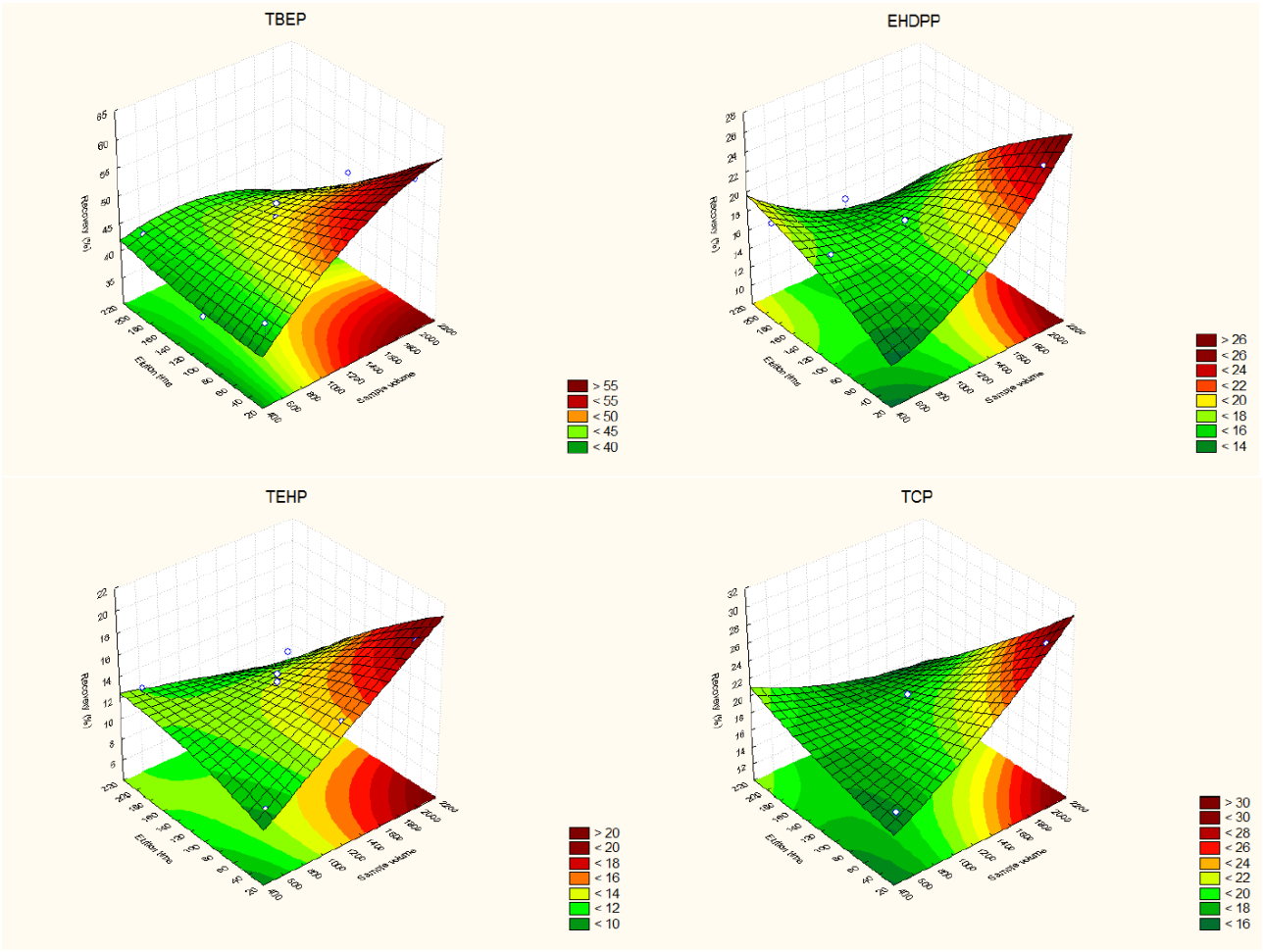

Figure 4.2.5: Surface responses obtained by the three level full factorial design for the optimization of sample volume and elution time.

The optimized conditions found for both DVB/ACN and C18/ACN couples were used to perform three extractions of ultrapure water samples spiked with all analytes at 200 ng/mL for each pair and then calculate the recovery values. The comparison between recoveries attained with C18/ACN and DVB/ACN pairs (figure ***4.2.6***) showed that the DVB sorbent allowed for better recovery values for all the investigated compounds, ensuring a comparable precision respect to C18 cartridge.

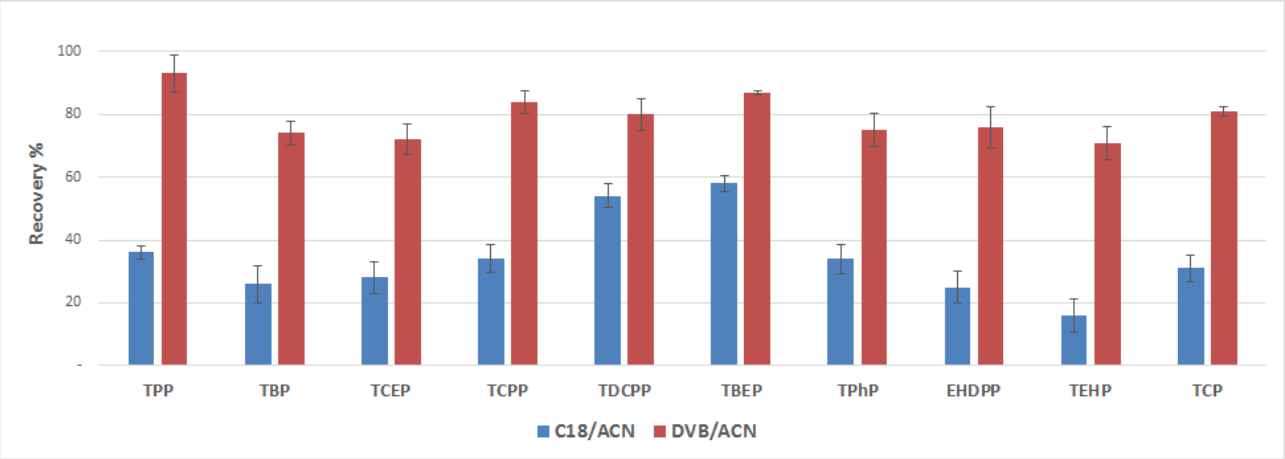

Figure 4.2.6: Comparison in terms of recovery between C18/ACN and DVB/ACN pairs under the optimized MEPS conditions.

4.2.2.3 Optimization of PTV injection

Programmed temperature vaporization (PTV) injection is a kind of sample introduction into gas chromatographic system so as to improve the sensitivity of an analytical protocol. There are many variables that can influence the performance of PTV injection, such as injection volume, initial temperature, injection speed, evaporation time, evaporation temperature, and solvent vent flow. Also in this case, they were optimized in multivariate way with the use of experimental design. The injection volume, in this work, was set at 9 µL which is the maximum injectable volume by the used autosampler. For the other variables, a 2^5 full factorial screening design was firstly performed to evaluate their influence on the response. The experiments were performed with a standard sample of the investigated OPFRs at 500 ng/mL in ACN acquired in SRM mode. The matrix design provided the planning of 32 experiments, reported in ***table 4.2.3.***

Table 4.2.3: Design matrix for the screening of PTV working conditions.

Exp	Injection speed (µl/s)	Initial temperature (°C)	Solvent vent flow (ml/min)	Evaporation temperature (°C)	Evaporation time (min)
21	10	45	200	60	2.0
3	10	60	50	60	0.2
18	50	45	50	60	2.0
15	10	60	200	100	0.2
31	10	60	200	100	2.0
25	10	45	50	100	2.0
13	10	45	200	100	0.2
4	50	60	50	60	0.2
10	50	45	50	100	0.2
1	10	45	50	60	0.2
8	50	60	200	60	0.2
26	50	45	50	100	2.0
7	10	60	200	60	0.2
17	10	45	50	60	2.0
22	50	45	200	60	2.0
12	50	60	50	100	0.2
2	50	45	50	60	0.2
28	50	60	50	100	2.0
24	50	60	200	60	2.0
5	10	45	200	60	0.2
19	10	60	50	60	2.0
27	10	60	50	100	2.0
6	50	45	200	60	0.2
16	50	60	200	100	0.2
20	50	60	50	60	2.0
14	50	45	200	100	0.2
30	50	45	200	100	2.0
29	10	45	200	100	2.0
9	10	45	50	100	0.2
11	10	60	50	100	0.2
32	50	60	200	100	2.0
23	10	60	200	60	2.0

Also in this case, the results were interpreted with the aid of the Pareto charts. The graphs show that the evaporation temperature is the most statistically significant variable ($p<0.05$) for all analytes except TPP and TBEP whereas evaporation time is significant for TCEP and TPhP (***figure 4.2.7***).

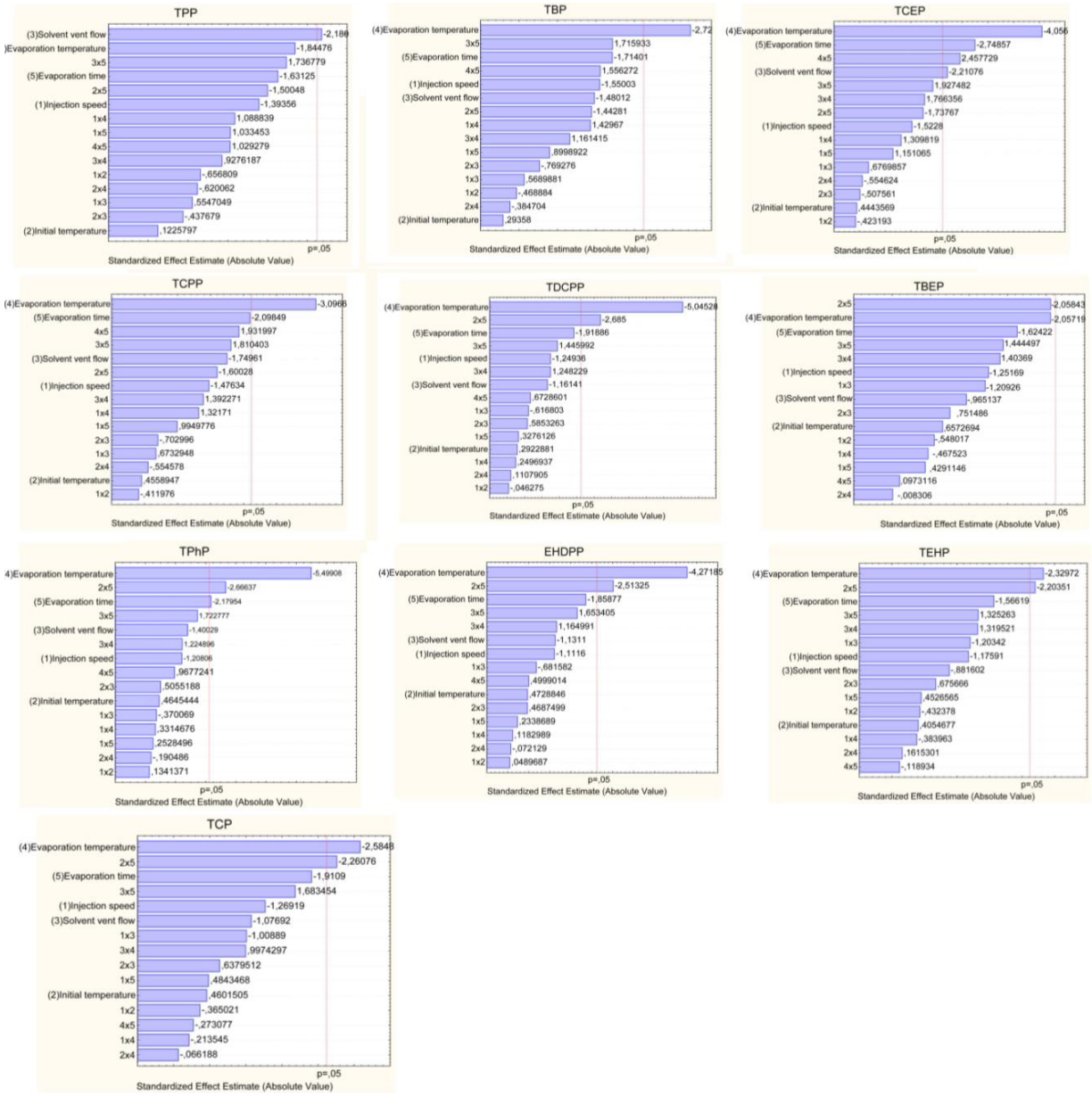

Figure 4.2.7: Pareto charts obtained from the screening of PTV variables.

Evaporation temperature influences the responses of almost all analytes whereas the two factor interaction term between initial temperature and evaporation time significantly affects the response of higher boiling analytes. Therefore, to consider the synergic effect of both variables and to find the optimal working conditions for initial temperature, evaporation time and evaporation temperature a

central composite design (CCD) was performed. A CCD consisting of a 2^3 factorial design with six star points positioned at ±α from the center was performed. The design matrix included the planning of 20 experiments (2^n + (n x k) + k), where n=3 and k=6 (***table 4.2.4***).

Table 4.2.4: Design matrix for the optimization of PTV working conditions.

Exp	Initial temperature (°C)	Evaporation time (min)	Evaporation temperature (°C)
18 (C)	52.5	1.10	80.0
14	52.5	1.10	100.0
4	48.0	1.64	91.9
12	52.5	2.00	80.0
9	45.0	1.10	80.0
17 (C)	52.5	1.10	80.0
19 (C)	52.5	1.10	80.0
1	48.0	0.57	68.1
3	48.0	1.64	68.1
16 (C)	52.5	1.10	80.0
20 (C)	52.5	1.10	80.0
13	52.5	1.10	60.0
7	57.0	1.64	68.1
10	60.0	1.10	80.0
15 (C)	52.5	1.10	80.0
8	57.0	1.64	91.9
6	57.0	0.57	91.9
5	57.0	0.57	68.1
2	48.0	0.57	91.9
11	52.5	0.20	80.0

The Derringer's desirability function was used to evaluate the optimal working conditions. From the evaluation of the responses of the three variables there was no quadratic effect on the response of the investigated analytes except for evaporation time on the response of TCEP (***Figure 4.2.8***).

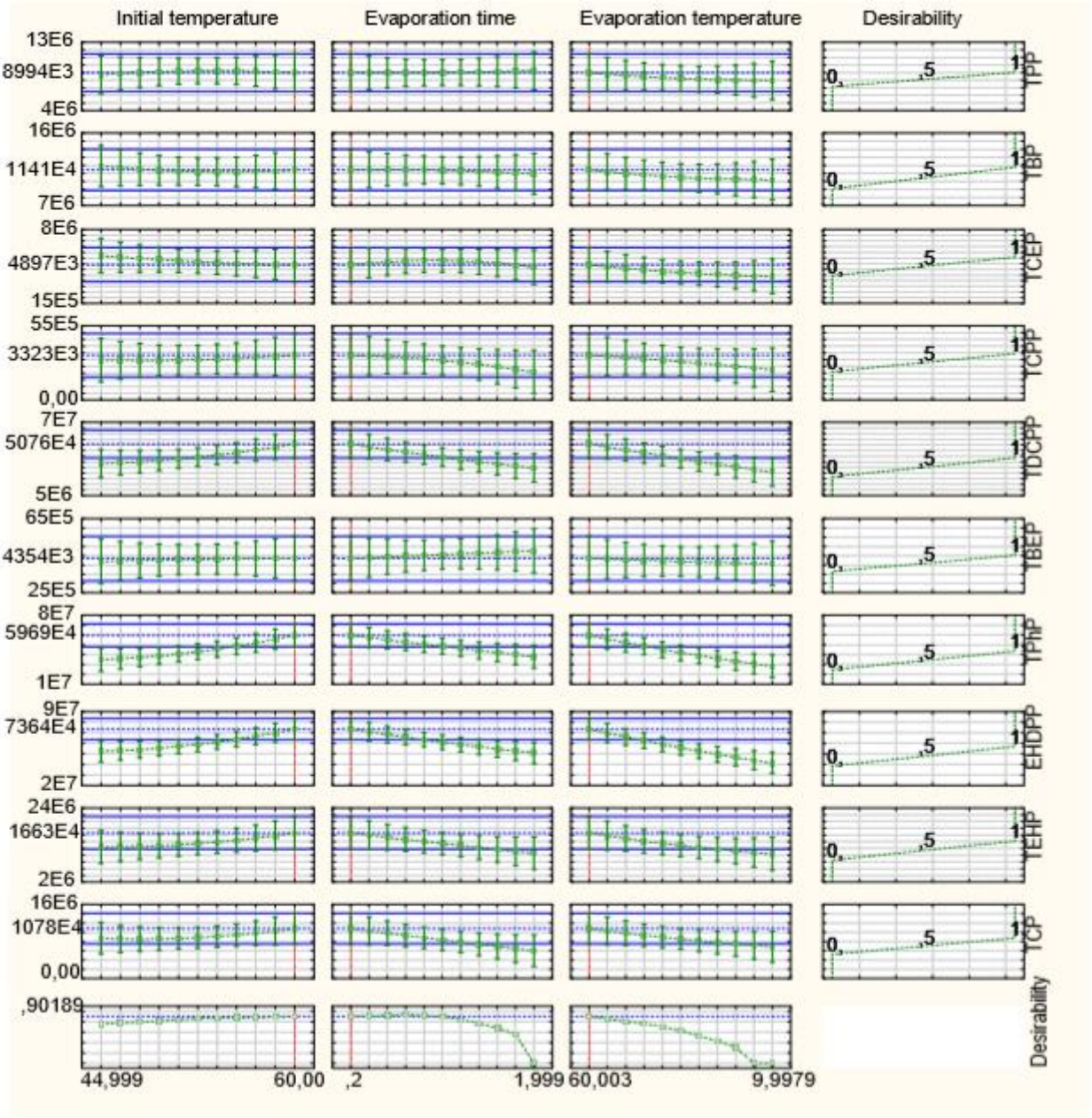

Figure 4.2.8: Desirability functions obtained by performing the CCD design for the optimization of PTV parameters.

The highest response intensities are achieved by setting the evaporation time and evaporation temperature at the lowest values (0.2 min and 60 °C, respectively) and initial temperature at the highest value (60 °C). The other two parameters, i.e. solvent vent flow and injection speed, were set according to analyst's convenience at 50 mL/min and 50 µL/s, respectively.

4.2.2.4 Clean-up of the MEPS cartridge and blank contamination

Carryover effect was observed mainly for TBP, TCEP and TCPP after the analysis of ultrapure water sample spiked with the analytes at 1 ng/mL. In according to other works [288, 293, 295] a clean-up step of the DVB cartridge was carried out after MEPS procedure. Since ACN was demonstrated to be the most suitable solvent for the elution of analytes, the same solvent was selected for the clean-up step. In particular three different amount of ACN were tested: 60 µL, 120 µL, and 250 µL, washing times 30 s and 60 s and washing cycles 1, 3, 5, and 7. The lower response in terms of peak area were achieved aspirating 250 µL of solvent in 60 seconds for 7 cycles. Under these conditions, no carryover was observed for all the analytes. The blank contamination occurs for all analytes except for TBEP and TCP. This contamination, already reported by other authors [289], can be attributed to several sources such as vial septa, leftover analytes in the gas chromatograph injector, elution solvents and ultrapure water. This latter represents the principal contamination source due to polymeric materials used in water purification. To minimize the presence of investigated analytes in blank samples, water was passed through a SPE cartridge previously soaked with ACN [289]. A cleaning of the glassware and MEPS syringe was also carried out with ACN in accordance with the procedure suggested by Liang et al. [289]. After the cleaning steps three blank samples in ultrapure water were extracted and analyzed as described in experimental section and the results showed no target analytes in the samples.

4.2.2.5 Limit of detection, lower limit of quantification and linearity

Limits of detection (LODs) were evaluate for each matrix by analysing samples with decreasing concentration of the analytes until a response equivalent to three times the background noise was observed. The LOD values achieved by the proposed method and reported in ***table 4.2.5*** are compatible with the levels of OPFRs detected in most of the real samples reported in literature [270, 299-305].

Table 4.2.5: LODs (ng/L) achieved by the proposed method in each considered matrix.

Compound	Tap water	River water	Wastewater
TPP	2.7	2.9	3.0
TBP	11	10	12
TCEP	12	12	12
TCPP	13	13	13
TDCPP	22	24	25
TBEP	87	95	101
TPhP	13	12	13
EHDPP	23	24	28
TEHP	26	28	28
TCP[a]	99	97	107

[a] acquired as sum of isomers

Lower limits of quantification (LLOQs) were determined for each analyte and the obtained values were chosen as the concentration of the lowest calibration standard point. The linearity was evaluated as described in experimental section by considering seven-point calibration curves built using spiked ultrapure water and internal standards. The calibration range ranged between the LLOQs for each analyte and 5 or 10 ng/mL with 0.5 ng/mL of each internal standard. TBP-d27, TCEP-d12, and TPhP-d15 were tested as internal standards to correct instrumental drift and matrix effect. The use of TPhPd15 was discharged because unsatisfactory results were obtained related to its instrumental response and chromatographic behavior.

Table 4.2.6: LLOQs for each analyte, linearity and internal standards used.

Compound	LLOQ (ng/mL)	Internal standard	Linear range (ng/mL)	Calibration curve	R^2
TPP	0.01	TBP-d27	0.01-10	y=2.485x+0.0028	0.9942
TBP	0.025	TBP-d27	0.025-10	y=2.251x+0.0033	0.9925
TCEP	0.025	TCEP-d12	0.025-10	y=1.218x+0.0014	0.9963
TCPP	0.025	TCEP-d12	0.025-5	y=4.679x+0.0034	0.9918
TDCPP	0.05	TCEP-d12	0.05-5	y=0.227x+0.0006	0.9971
TBEP	0.2	TCEP-d12	0.2-10	y=0.0069x+0.0001	0.9890
TPhP	0.025	TCEP-d12	0.025-10	y=1.013x+0.0034	0.9965
EHDPP	0.05	TCEP-d12	0.05-10	y=0.331x+0.0009	0.9985
TEHP	0.05	TCEP-d12	0.05-10	y=0.051x+0.0005	0.9973
TCP[a]	0.2	TCEP-d12	0.2-10	y=0.011x+0.0002	0.9908

[a] acquired as sum of isomers

Satisfactory results were obtained in terms of lower limit of quantification and linearity within the considered calibration ranges with correlation coefficient values ≥0.99 for all analytes except for TBEP (***table 4.2.6***). The LLOQs attained with the proposed method are comparable to the lowest calibration concentrations used by other microextraction protocols [270, 283].

3.2.2.5 Matrix effect, recovery and analytical performance

The evaluation of matrix effect is a crucial aspect of a newly introduced method. Indeed, the presence of the matrix may lead to an enhancement or a suppression of signal. ME values, calculated in according to the equation 4.2 described in experimental section, were high for the majority of the target compounds in all the considered matrices (only about 36% of overall data are between 70 and 130%). Therefore, in order to test the ability of internal standards to correct matrix effect ME was determined by considering the ratio between the analyte peak area and internal standard peak area. (the analyte/internal standard pairs considered are reported in the table 4.2.6). As shown in ***table 4.2.7***, the ME values ranging between 70 and 130% for the majority of the target analytes except for TPP at the lower QC demonstrated the noticeable capability of internal standard in balancing matrix effect.

Table 4.2.7: Matrix effect and recovery at each QC level. (RSD% calculated for five replicates).
[a] acquired as sum of isomers

Compound	Concentration (ng/mL)	Matrix effect (%)			Recovery (%)		
		Tap water	River water	Wastewater	Tap water	River water	Waste water
TPP	0.015	74(8)	72(10)	69(10)	88(10)	82(9)	72(12)
	0.5	84(11)	73(12)	72(14)	91(9)	87(8)	75(10)
	8	85(8)	88(6)	76(5)	94(5)	81(6)	68(8)
TBP	0.037	76(8)	117(7)	83(10)	66(12)	88(12)	87(8)
	0.5	118(9)	118(10)	81(8)	79(10)	82(7)	78(12)
	8	109(5)	90(7)	81(8)	67(9)	76(6)	62(10)
TCEP	0.037	94(9)	89(13)	82(12)	97(10)	92(15)	74(10)
	0.5	108(12)	111(6)	113(6)	84(9)	82(8)	78(8)
	8	97(8)	106(11)	94(12)	76(8)	68(9)	64(7)
TCPP	0.037	78(16)	75(13)	71(15)	97(13)	76(10)	73(12)
	0.5	115(9)	89(13)	83(10)	95(9)	79(7)	67(5)
	4	90(8)	105(7)	93(9)	82(9)	67(6)	61(9)
TDCPP	0.075	83(14)	80(18)	83(17)	92(12)	76(11)	71(13)
	0.5	93(9)	104(7)	108(10)	85(8)	78(10)	82(12)
	4	108(7)	113(7)	115(9)	82(9)	72(5)	75(8)
TBEP	0.3	90(14)	88(15)	82(12)	71(16)	67(13)	63(10)
	1	95(10)	94(9)	103(11)	78(12)	74(5)	63(6)
	8	104(6)	108(8)	109(7)	81(10)	77(10)	66(5)
TPhP	0.037	104(13)	90(19)	85(17)	71(15)	80(12)	68(14)
	0.5	108(8)	94(11)	89(8)	72(14)	77(5)	66(5)
	8	115(9)	77(8)	92(7)	79(10)	74(5)	67(6)
EHDPP	0.075	95(12)	113(17)	111(18)	65(13)	66(17)	69(10)
	0.5	115(13)	120(8)	114(10)	67(13)	68(10)	71(10)
	8	112(8)	106(8)	104(7)	72(10)	59(5)	62(6)
TEHP	0.075	86(12)	96(12)	85(15)	67(17)	61(11)	58(15)
	0.5	107(9)	92(7)	84(10)	63(12)	59(11)	61(11)
	8	108(10)	116(7)	108(11)	67(9)	64(8)	63(10)
TCP[a]	0.3	87(15)	86(11)	78(12)	74(12)	71(16)	69(14)
	1	91(5)	87(13)	85(8)	83(14)	81(10)	78(13)
	8	96(8)	94(10)	89(7)	73(5)	75(7)	67(7)
TBP-d27	0.5				81(10)	84(8)	79(12)
TCEP-d12	0.5				80(9)	81(9)	81(11)

Recovery for each investigated matrix was evaluated by analyzing the QC samples as described in the experimental section. The values obtained ranged from 58% to 97% (table 4.2.7). Lower recovery values were attained for more hydrophobic OPFRs as EHDPP and TEHP, probably due to adsorption onto the inner wall of glassware as reported in literature [289].

Intra-day and inter-day precision and accuracy were determined by analyzing the QC samples in quintuplicates once a day on six consecutive days. The results obtained, reported in ***table 4.2.8***, can be considered generally satisfactory except in few cases for TPhP, EHDPP and TCP at the lower concentration level.

Table 4.2.8: Accuracy and precision values obtained for each analyte.

Compound	**Concentration (ng/mL)**	**Intra-day accuracy [% theoretical value (RSD%)]**			**Inter-day accuracy [% theoretical value (RSD%)]**		
		Tap water	**River water**	**Wastewater**	**Tap water**	**River water**	**Wastewater**
TPP	0.015	102(6)	96(7)	95(12)	106(11)	97(13)	94(15)
	0.5	109(9)	92(11)	86(8)	110(14)	89(14)	90(13)
	8	81(4)	72(7)	85(9)	76(9)	70(8)	86(12)
TBP	0.037	121(9)	113(10)	109(12)	120(8)	118(11)	112(11)
	0.5	85(10)	94(8)	82(9)	84(8)	91(10)	86(8)
	8	89(5)	104(7)	92(8)	85(10)	106(6)	89(9)
TCEP	0.037	95(10)	109(9)	112(11)	95(12)	111(15)	113(18)
	0.5	107(11)	108(9)	103(12)	108(10)	109(9)	96(10)
	8	97(7)	96(12)	92(13)	93(11)	92(12)	90(14)
TCPP	0.037	114(12)	113(10)	109(15)	117(15)	113(10)	116(18)
	0.5	101(9)	111(12)	114(11)	103(12)	113(12)	118(16)
	4	82(6)	87(10)	85(9)	77(8)	86(10)	82(11)
TDCPP	0.075	93(15)	109(13)	118(9)	91(17)	114(13)	121(16)
	0.5	93(7)	115(12)	114(12)	94(13)	118(11)	115(12)
	4	108(6)	113(8)	103(6)	113(5)	114(8)	108(14)
TBEP	0.3	88(11)	87(12)	86(13)	85(13)	88(18)	82(15)
	1	108(7)	109(10)	94(11)	112(5)	107(10)	90(10)
	8	101(9)	107(5)	96(7)	96(9)	112(8)	92(9)
TPhP	0.037	117(12)	116(11)	121(8)	121(14)	123(13)	126(14)
	0.5	94(5)	85(9)	86(6)	92(8)	83(12)	88(11)
	8	85(5)	108(5)	98(8)	79(9)	104(8)	94(9)
EHDPP	0.075	84(13)	128(15)	117(14)	81(15)	131(17)	115(21)
	0.5	74(7)	113(5)	107(11)	71(9)	109(6)	106(12)
	8	111(7)	94(5)	85(5)	116(10)	95(6)	83(8)
TEHP	0.075	109(12)	75(16)	90(15)	116(11)	72(18)	87(15)
	0.5	110(9)	91(10)	106(8)	113(11)	90(14)	109(15)
	8	92(5)	108(11)	95(6)	87(10)	106(12)	96(11)
TCP[a]	0.3	93(12)	118(5)	125(13)	94(15)	124(10)	127(21)
	1	94(8)	89(11)	93(8)	90(10)	88(13)	89(16)
	8	105(10)	106(8)	102(7)	107(10)	108(9)	105(10)

The suitability of the cartridge washing procedure reported above was confirmed by the blank sample analysis obtained after the processing of ultrapure water sample at the higher concentration of the calibration range. Indeed, after the analysis in triplicate of ultrapure water sample spiked at 10 ng/mL, a no memory effect was observed for all the analytes.

4.2.2.6 Application to real samples

The developed method was applied to real water samples. In particular, the levels of OPFRs were determined in five tap waters and three river waters. They were extracted and analyzed under the optimized conditions, as described in experimental section. Concentrations below the LODs were found for all the target analytes in the examined samples, as expected due to the low pollution level and absence of industrial activities in the area where the waters were sampled. This result was in line with the literature that reports OPFR concentration below LOD in river not affected by urban and industrial pressures [306]. The method proposed was then applied for the analysis of OPEs in the considered real samples and wastewater material spiked at LLOQ valued for all analytes. The accuracy value obtained were satisfactory for all samples, as demonstrated by their values in a range 85-122% for tap water, 75-131% for river water and 76-132% for wastewater. The chromatogram of the wastewater sample spiked at the LLOQ concentrations is reported in ***figure 4.2.9***.

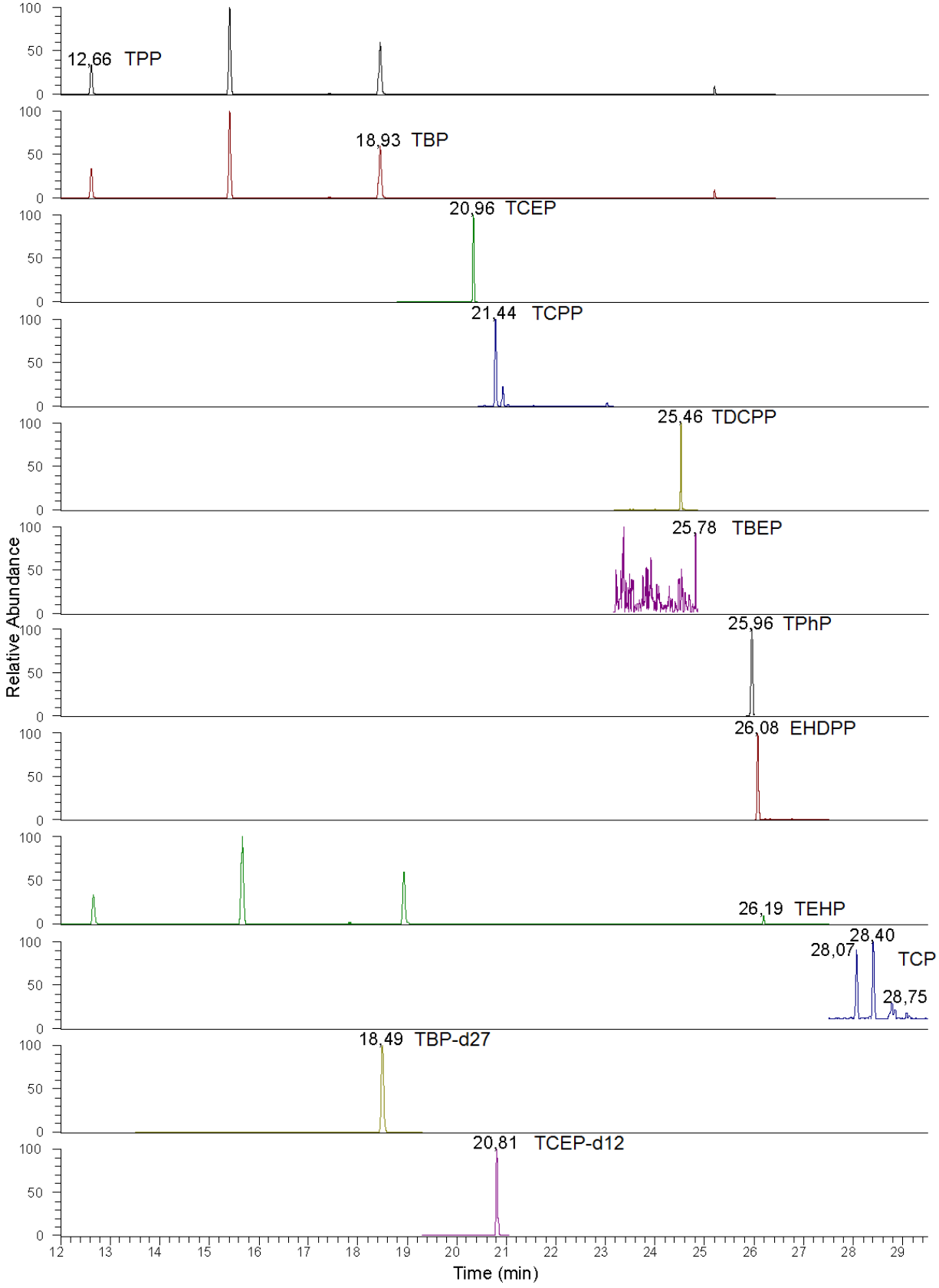

Figure 4.2.9: Chromatogram of wastewater sample spiked at LLOQ concentrations for each of the investigated OPFRs.

4.2.3 Conclusions

A fully optimized MEPS-PTV-GC-MS/MS protocol for the assay of OPFRs in environmental aqueous matrices is proposed. Programmed temperature vaporization was successfully implemented to further improve the method sensitivity by means an increased injectable amount of analytes. Univariate and multivariate approaches were applied so as to optimize the variables affecting MEPS extraction and sample vaporization in the PTV injector. In particular, the use of DoE permitted the establishment of the best working conditions for all the investigated analytes, taking in account the possible synergistic effects between the optimized parameters. The sample preparation protocol is fast and automated reducing significantly the preparation time required per sample and minimizing user-made mistakes. MEPS allowed the development of a user-friendly analytical protocol with many advantages in terms of cost and environment protection. Indeed, MEPS is simpler, faster and requires less sample volume and solvent consumption compared to SPE. The concentration step is eliminated, decreasing in this way sample preparation time (about 30 minutes) and avoiding any analyte losses because target compounds are eluted directly in a suitable solvent volume. The possibility to work in selected reaction monitoring acquisition mode allowed to identify and quantify the analytes in a sensitive and selective way. Finally, all the validation parameters, evaluated in accordance to the guidance issued by FDA and ICH, were satisfactory and comparable to the outcomes reported in literature for other microextraction method.

Development of a microextraction by packed sorbent-programmed temperature vaporization-Gas chromatography-triple quadrupole mass spectrometry method for phthalate monoesters assay in human urine

4.3 Introduction

The principal object in this work was the development of a novel method based on microextraction by packed sorbent-programmed temperature vaporization-gas chromatography-triple quadrupole mass spectrometry (MEPS-PTV-GC-QqQ-MS) analysis for the determination of nine monoester phthalates (***Figure 4.3***) in urine. The derivatization reaction was directly carried out in urine with propyl chloroformate in order to obtain a fast and simple protocol [307]. The extraction ability of five MEPS cartridges and seven elution solvents were evaluated in univariate mode, while the variables affecting the MEPS analysis, PTV system were optimized by the multivariate approach of "Experimental design" (DoE) [308].

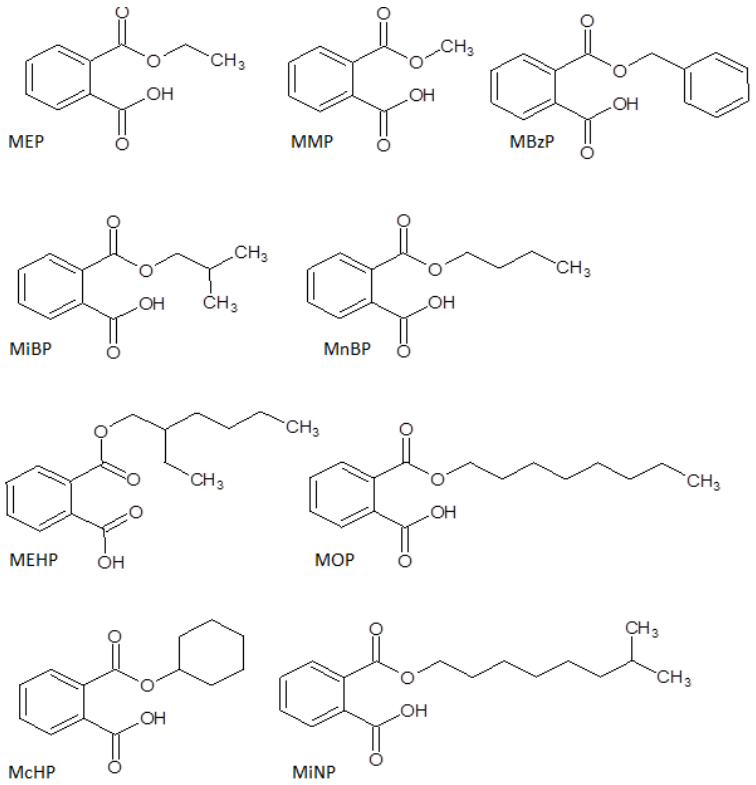

Figure 4.3: Chemical structures of the investigated analytes.

4.3.1 Experimental section

4.3.1.1 Chemicals and materials

Monomethyl phthalate (MMP), monoethyl phthalate (MEP), monobenzyl phthalate (MBzP), mono-isobutyl phthalate (MiBp), mono-*n*-butyl phthalate (MnBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-*n*-octyl phthalate (MnOP), monocyclohexyl phthalate (McHP), mono-isononyl phthalate (MiNP) were purchased from Chemical research 2000 (Rome, Italy). Bis(2-ethylhexyl) phthalate-3,4,5,6-d4 (DEHP-d4) used as internal standard was bought from Sigma-Aldrich (Milan, Italy). Pyridine, sodium chloride, propyl chloroformate and propanol were bought from Sigma Aldrich (Milan, Italy). β-glucuronidase from *E. coli* K12 was bought from Sigma-Aldrich (Milan, Italy). Synthetic urine (negative urine control) produced from Cerilliant was commercialized by Sigma-Aldrich (Milan, Italy). Acetonitrile (ACN), dichloromethane (DCM), hexane (Hex) and methyl tert-butyl ether (MTBE) were obtained from Sigma-Aldrich (Milan, Italy). Methanol (MeOH) was obtained from VWR Chemicals (France) whereas ethyl acetate (EtAc) and trichloromethane (TCM) were obtained from Panreac Quimica (Barcelona, Spain). MEPS cartridges (C2, C8, C18, Silica, DVB) and semiautomatic electronic syringe (eVol®) were provided by SGE Analytical Science (Melbourne, Australia). Ultrapure water was obtained from a Milli-Q plus system (Millipore, Bedford, MA).

4.3.1.2 MEPS extraction for the sorbent/solvent pairs screening

Firstly, the investigated analytes at concentration of 0.1 mg/L in synthetic urine were derivatized in according to the optimized protocol described in section 3.4. MEPS extraction was carried out with a semiautomatic electronic syringe (eVol®) consisting of a 500 µL gas-tight syringe with a barrel contained 4 mg of a solid-phase material, using different sorbents and elution solvents. The MEPS extraction provided for the conditioning of sorbent first with 500 µL (2×250 µL) of methanol followed by 500 µL (2×250 µL) of ultrapure water at a flow rate of 8 µL/s. After that, 1000 µL of spiked syntethic urine were aspirated through the MEPS cartridge (5×200 µL at 2µL/s) and discarded into the waste at 7 µL/s; the cartridge was then dried in an empty vial flushing with 500 µL of air ten times and finally, the analytes were eluted with 300 µL (3×100 µL) of solvent aspirated at 2µL/s and then ejected at 3 µL/s into a vial with conical insert. After the extraction DEHP-d4 used as internal standard was added to sample at 0.5 mg/L. Nine microliters of this solution was injected at 50 µL/s into the GC-MS system with a programmed vaporization temperature, in order to improve the

sensitivity of the method. Signals were acquired in profile and SRM mode by considering the SRM transitions optimized in section 3.4. The PTV parameters chosen for the sorbent/solvent screening were the following: solvent vent flow 50 ml/min, evaporation rate 14.5 °C/s, evaporation temperature 60 °C, evaporation time 2 min, transfer rate 14.5 °C/s, transfer temperature and transfer time 300 °C and 2 min, respectively.

4.3.1.3 Instrumentations and data processing

A TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a triple quadrupole mass spectrometer (QqQ) Quantum and a Trace GC Ultra equipped with programmable temperature vaporizer (PTV) injector and a Triplus autosampler was used to perform analysis. The capillary column was Restek Rxi-5MS capillary column (30 m×0.25 mm i.d., 0.25 µm film thickness, 95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 70 °C for 5 min, then ramped at 20 °C/min to 250 °C, then ramped at 10 °C/min to 320 °C and held at this temperature for 10 min. The carrier gas was helium (purity 99.999%) at 1 mL/min, whereas argon (purity 99.999%) at a pressure of 2.3 mTorr was used as collision gas. A Thermo PTV straight Liner 0.75×2.75×105 mm was used in the GC injector. The triple quadrupole was operated in electron ionization (EI) and was used in full scan mode to identify the derivatized analytes and then in selected reaction monitoring (***Table 4.3***). The emission current was set at 25 µA and the transfer line and ion source were set at 280 °C and 250 °C respectively. The scan width was set at 1.2 m/z whereas the peak width of Q1 was 0.7 amu for all segments. The analyte acquisitions were carried out in profile mode.

Xcalibur software was used as instrumentall control whereas Excel (Microsoft, USA) and Statistica 8.0 (StatSoft 2007 Edition, Tulsa USA) were used to perform and evaluate experimental data and experimental design matrix.

Table 4.3: SRM transitions and collision energy for each analyte.

Compound	**SRM transition, m/z (collision energy, V)**	
	Quantification	Identification
MMP	163→77 (21)	163→92 (23)
MEP	177→149 (12)	195→149 (11)
MiBP	149→65 (22)	191→149 (7)
MBP	149→65 (22)	191→149 (7)
McHP	149→65 (22)	209→149 (9)
MEHP	149→65 (22)	209→149 (7)
MiNP	149→65 (22)	191→149 (7)
MnOP	149→65 (22)	209→149 (11)
MBzP	192→149 (7)	192→93 (27)
DEHP-d4	171→153 (6)	283→153 (12)

4.3.2 Results and discussions

4.3.2.1 MEPS cartridge and elution solvent

The first step in the optimization of MEPS variables was the evaluation of sorbent material and elution solvent. In particular, five MEPS cartridges namely C2, C8, C18, Silica and DVB were tested for the extraction of phthalates monoesters previously derivatized with propyl chloroformate, propanol and pyridine in synthetic urine at concentration of 0.1 mg/L. For each sorbent seven solvents, namely acetonitrile, methanol, ethyl acetate, methyl tert-butyl ether, hexane, dichloromethane, and trichloromethane, were evaluated. The MEPS conditions used during the extraction are reported in the experimental section. The signals were evaluated by considering the ratio between analyte peak area and internal standard peak area. Internal standard was added as final internal standard to correct

instrumental drift. The results obtained are reported in ***figure 4.3.1*** where the responses were normalized considering higher extraction efficiency as 100%.

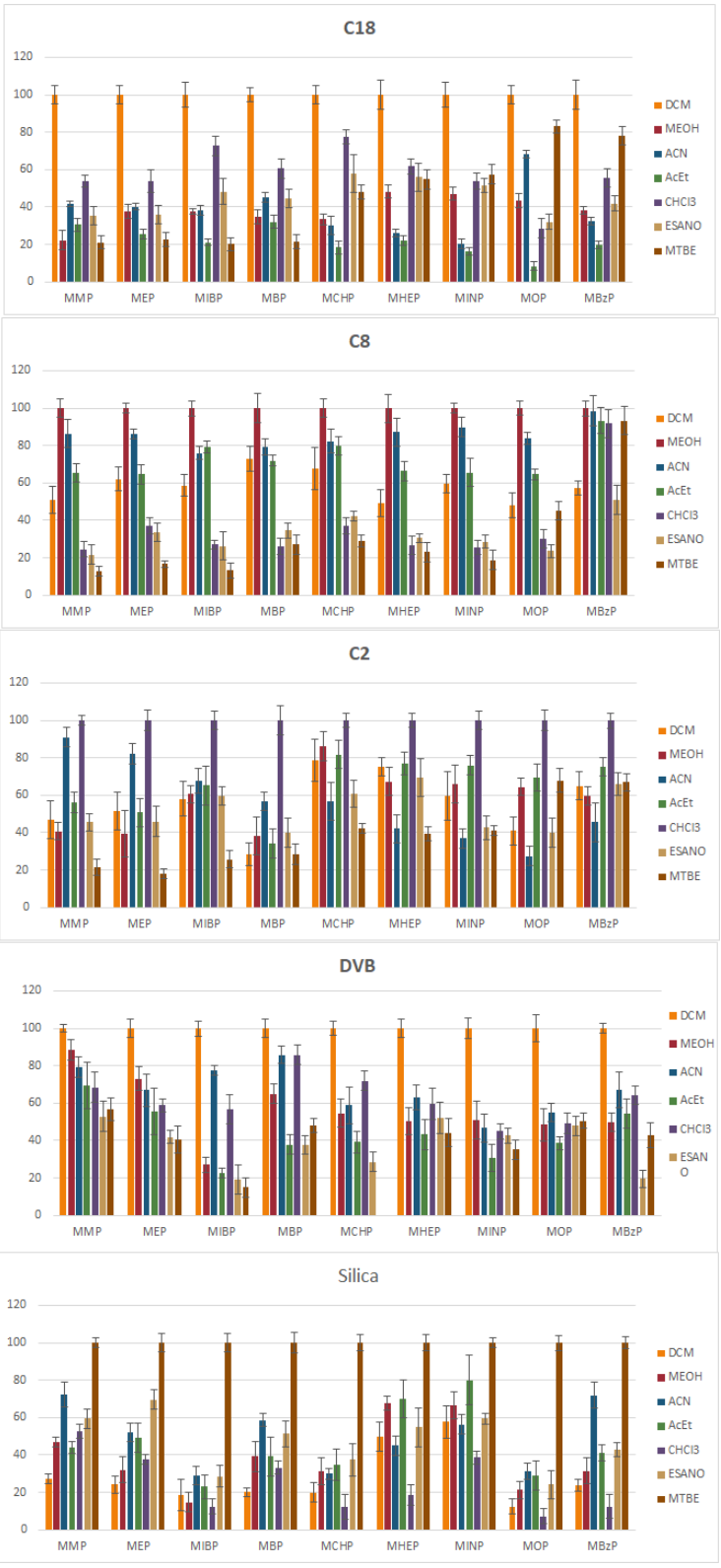

Figure 4.3.1: Normalized responses for MEPS extraction with different cartridge sorbents and elution solvents.

The highest responses for C18 and DVB were observed when dichloromethane was used as elution solvent, whereas for C8 the best results were obtained when the elution was carried out with acetonitrile and methanol. Finally, the best response for C2 was obtained with methanol, trichloromethane, acetonitrile and ethyl acetate as elution solvents whereas for Silica the highest signals were attained using methyl tert-butyl ether. A summary of the responses normalized by considering the signals obtained for all analytes with C18/DCM as 100% is shown in ***figure 4.3.2***.

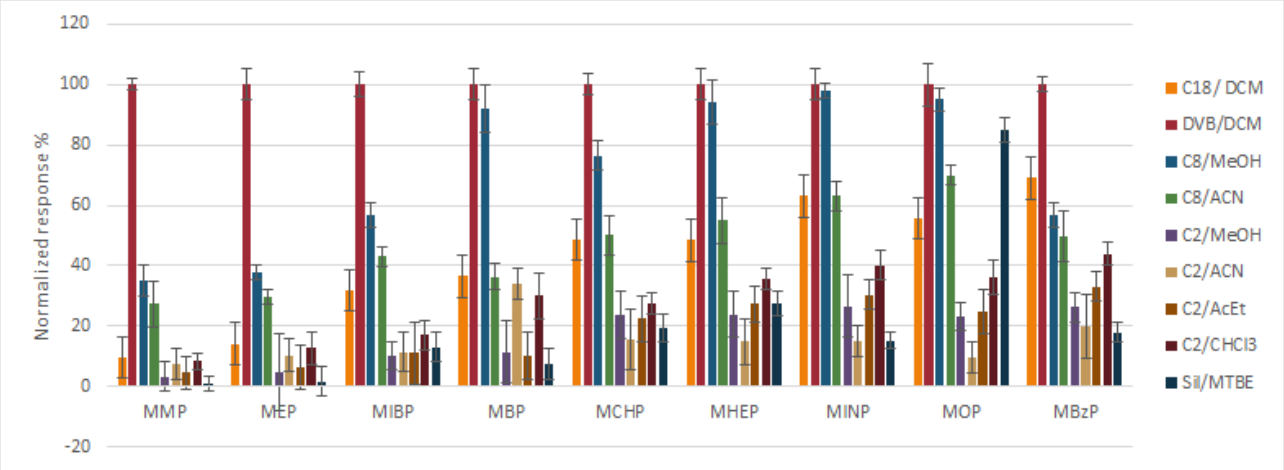

Figure 4.3.2: Comparison of the results between the best sorbent/solvent pairs for each analyte at concentration of 0.1 mg/L.

The comparison of data showed that the highest signals were achieved for all analytes with DVB/DCM and C8/MeOH pairs, therefore, these couples were used for the multivariate screening of MEPS and PTV variables.

4.3.2.2 Plackett-Burman design for the screening of MEPS and PTV variables

The effect of a high number of factors on a response can be evaluated by a screening design. This design provided less information compared to an optimization design but allows to select the significant factors. In this study, a screening design was applied to all variables of MEPS extraction and PTV process to take into account possible interaction between variables of the two procedures. The MEPS variables to evaluate were sorbent/solvent pair, elution volume, sample volume, number of sample draw cycles, sample draw and elution time whereas those for PTV process were liner type, initial temperature, injection speed, evaporation time, evaporation temperature and solvent vent flow.

A Plackett-Burman design was chosen on the 12 variables, providing for the planning of 16 experiments. In Plackett-Burman design the number of experiments is a multiple of four and the number of factors is one less the number of experiments. In our work 12 variables should be optimize but 12 experiments cannot be performed. Finally, a design with 16 experiments and 15 variables was used, setting 3 variables as dummy variables because have no effect on the response. The design matrix is reported in ***table 4.3.1***.

Table 4.3.1: Matrix of the Plackett-Burman design for the screening of MEPS and PTV variables.

Exp	Sorbent/solvent	Elution volume[a]	Sample volume[a]	Number of cycles	Elution time[b]	Sample draw time[b]	Liner	Initial temp[c]	Injection speed[d]	Evaporation time[e]	Evaporation temp[c]	Solent vent flow[f]	Dummy 1	Dummy 2	Dummy 3
5	DVB/DCM	60	2000	2	200	30	Baffled	45	50	0.2	80	50	A*	A	B
11	DVB/DCM	300	500	10	30	200	Straight	45	50	0.2	80	50	A	B	A
9	DVB/DCM	60	500	10	200	200	Straight	60	10	0.2	60	200	A	A	B
12	C8/MeOH	300	500	10	200	30	Baffled	45	50	0.2	60	200	B*	B	B
4	C8/MeOH	300	500	2	200	30	Straight	45	10	2	60	50	A	A	A
14	C8/MeOH	60	2000	10	30	200	Baffled	45	10	2	60	50	A	B	B
3	DVB/DCM	300	500	2	30	200	Baffled	45	10	2	80	200	B	A	B
1	DVB/DCM	60	500	2	200	200	Baffled	60	50	2	60	50	B	B	A
15	DVB/DCM	300	2000	10	30	30	Straight	60	50	2	60	50	B	A	B
7	DVB/DCM	300	2000	2	30	30	Baffled	60	10	0.2	60	200	A	B	A
2	C8/MeOH	60	500	2	30	30	Straight	60	50	2	80	200	A	B	B
10	C8/MeOH	60	500	10	30	30	Baffled	60	10	0.2	80	50	B	A	A
8	C8/MeOH	300	2000	2	200	200	Straight	60	10	0.2	80	50	B	B	B
6	C8/MeOH	60	2000	2	30	200	Straight	45	50	0.2	60	200	B	A	A
16	C8/MeOH	300	2000	10	200	200	Baffled	60	50	2	80	200	A	A	A
13	DVB/DCM	60	2000	10	200	30	Straight	45	10	2	80	200	B	B	A

[a]: µL; [b] s; [c] °C; [d] µL/s; [e]:min; [f:] ml/min; *A: high; *B: low

The results were evaluated with Pareto charts. In particular, the dummy variables are useful in this case because all variables that have a similar behaviour to dummy variables have no effect on the response (***Figure 4.3.3***)

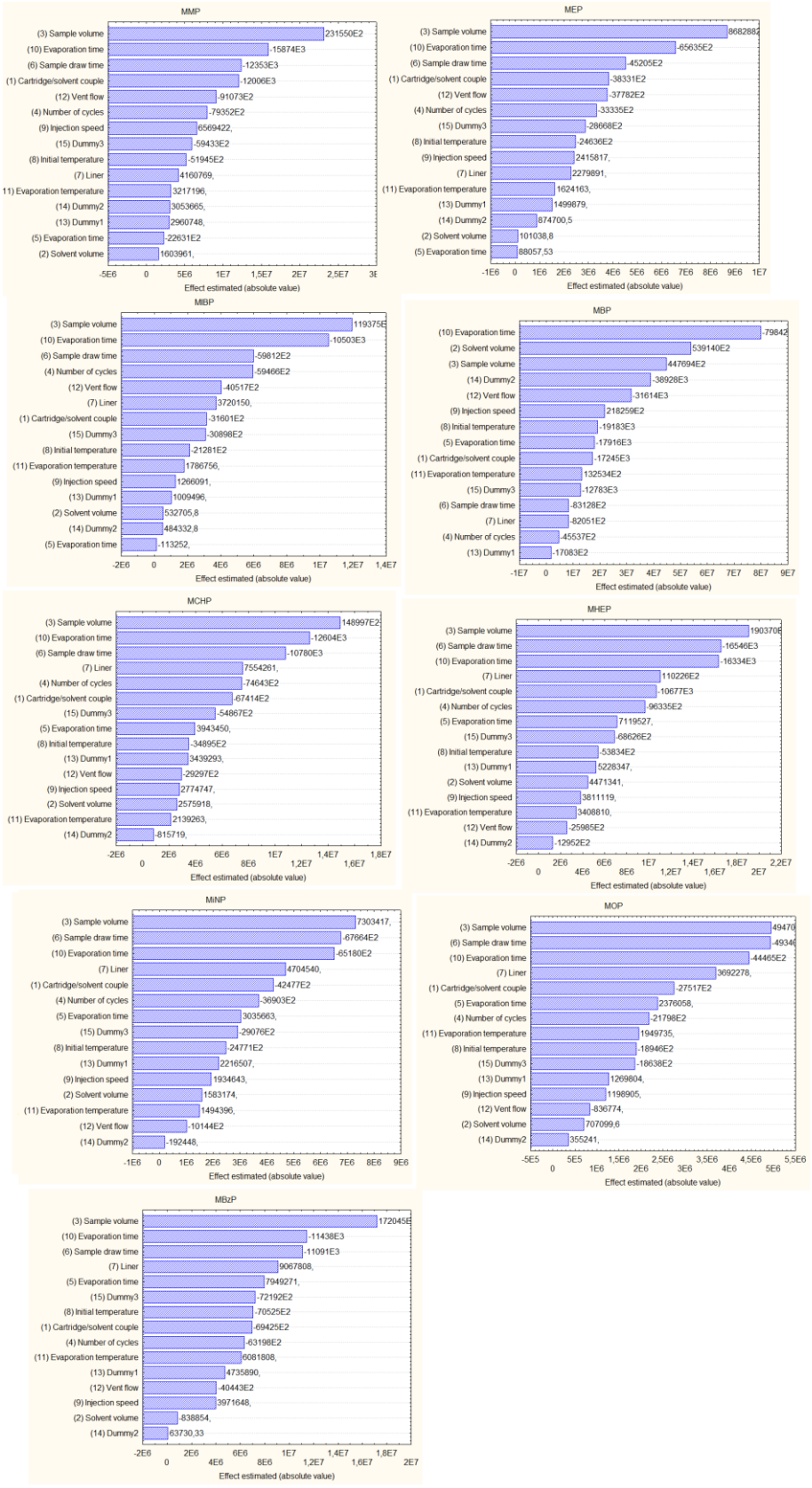

Figure 4.3.3: Pareto charts obtained by Plackett-Burman design for the screening of MEPS and PTV variables.

The variables having a significant effect for all analytes except for MBP are sample volume, evaporation time, sample draw time, number of sample draw cycles and tsorbent/solvent pair. For more volatile analytes, solvent vent flow has a significant effect, in particular with a negative sign that means an enhanced signal when the low value of range is considered. For less volatile analytes, liner represents a variable with a significant effect on the analysis. The different MBP behaviour can be attributed to the coelution of this analyte with dibuthyl phthalate.

4.3.3 Conclusions and perspectives

In this work, the initial part of the development of a MEPS-PTV-GC-MS/MS method for the analysis of phthalate monoesters in urine was presented. MEPS extraction was carried out in semi-automated way with the use of electronic pipette reducing the extraction time and using the minimal amount of organic solvent than other extraction techniques. The screening of sorbent and solvent was carried out in univariate mode. MEPS and PTV parameters were evaluated in multivariate mode with Plackett-Burman design. The use of tandem mass spectrometry allowed to perform an unambiguous identification of investigated analytes and improved the method sensitivity. The next steps for the development of the method provide for the optimization of parameters selected by screening design and the determination of limits of detection, lower limits of quantifications, linearity, matrix effect and analytical performance and, finally, the application to real samples.

SPME Arrow extraction for the evaluation of FAMEs in wastewater

4.4 Introduction

During my period as visiting Ph.D student at University Duisburg-Essen, Faculty of Chemistry, Instrumental Analytical Chemistry, I was part of the research group coordinated by Professor Torsten C. Schmidt. The object of my work was the extraction of fatty acid methyl esters (FAMEs) in wastewater by solid phase microextraction arrow (SPME arrow).

SPME Arrow is a new configuration of SPME fiber that allows to overcome some of its limitations [309, 310]. SPME Arrow combines the advantages of SPME fiber and Stir bar Sorptive extraction (SBSE) because it can be used in fully automated way with a PAL sampler and, at the same time, presents higher sorbent volume compared to classical SPME fiber (***Figure 4.4***). Moreover, the fragility of classical SPME fiber was improved by introducing a stainless steel rod.

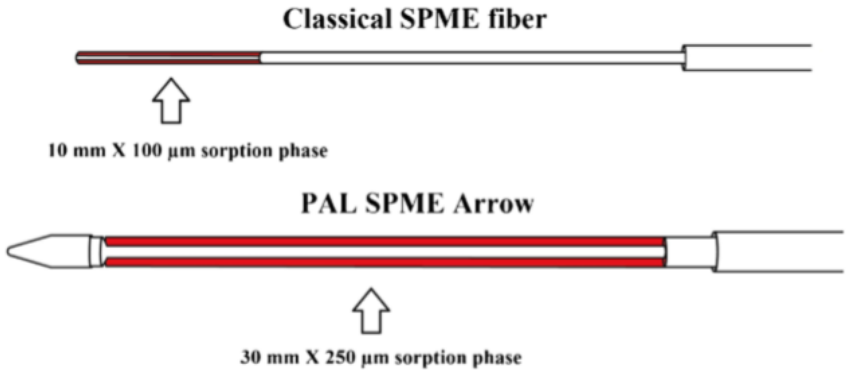

Figure 4.4: SPME fiber and PAL SPME Arrow.

As shown the SPME fiber presents a 100 µm × 10 mm of stationary phase whereas the SPME Arrow shows a 250 µm × 30 mm of sorbent phase [310]. SPME Arrow consists of a steel rod coated with an amount of stationary phase and is compatible with desorption in a standard GC liner due to its dimensions and sharp closed tip. The tip retains the sorption phase and allows to PAL SPME Arrow to enclose this sorption phase during transfer processes. To date, SPME Arrow was used to determine PAH in water and volatile compounds in wastewater and atmosphere [310-312].

Fatty acid methyl esters (FAMEs) can derive from several process such as that commonly used to produce commercial biodiesel from the conversion of vegetable oils or fats. Biodiesel presents several advantages over conventional diesel such as low toxicity and lower emission of particulate matter. However, the biodiesel process is energy consuming and produces high amount of wastewater that

requests an appropriate treatment. Wastewater can contain organic molecule with a low biodegradability coefficient and the removal of these components is the main concern in wastewater plants. The presence of lipids causes many problems for the treatment of fatty wastewater due to the production of long chain fatty acids that contribute to the generation of floating microbial flocks [313, 314]. Two methods were proposed to carry out the separation and quantification of FAMEs using GC-MS technique in combination with solid phase microextraction in direct immersion and headspace extraction [315, 316]. Non-aqueous reverse phase HPLC with different detection systems, such as ultraviolet spectroscopy, refractive index or evaporative light-scattering detector, to assay fatty acids methyl esters were used [317-319].

The object of the present work was to develop a method for the extraction of FAMEs from wastewater by SPME Arrow gas chromatography mass spectrometry analysis and establishing the extraction differences between SPME Arrow and SPME.

4.4.1 Chemicals and instrumentation

A standard containing 37 FAMEs from C4 to C24 in dichloromethane was bought from Sigma Aldrich (Germany). The PDMS SPME Arrow and SPME fiber were obtained from CTC Analytics AG (Zwingen, Switzerland). The ultrapure water used to prepare the samples was obtained from a PURELAB Ultra analytic water purification system (Lohmar, Germany). The analyses were performed with a GC-MS-QP2010 Ultra (Shimadzu) equipped with a PAL RTC autosampler. The chromatographic column used was a capillary column SLB-IL111 (200 m × 0.25 mm I.D., 0.20 µm 1,5-Di (2,3-dimethylimidazolium) pentane bis (trifluoromethylsulfonyl) imide). The best chromatographic behaviour was achieved using the following temperature ramp: 40°C for 3 min, then ramped at 5°C/min to 130°C and then ramped at 2.5°C/min to 200°C for 3 min. The injector was set at 270 °C with a split ratio 1:10. At first, mass spectra were acquired in full scan mode for identification of analytes, after in selected ion monitoring (SIM) to increase sensitivity.

4.4.2 Results and discussion

In order to select the best chromatographic conditions, several tests were performed with SPME fiber, also taking into account literature data. In particular, a water sample spiked with a mix of FAMEs at 0.5 mg/L was prepared and analyses were carried out by a PDMS (100µm) fiber with an extraction time of 30 min and desorption time of 3 min. A chromatogram obtained under the optimized conditions is shown in ***figure 4.4.1***.

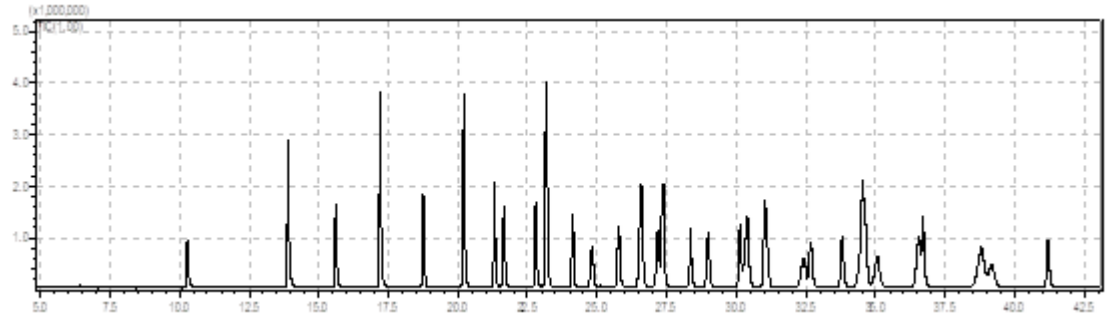

Figure 4.4.1: Chromatogram obtained by extracting FAMEs at 0.5 mg/L by SPME fiber (PDMS as stationary phase) under the optimized chromatographic conditions.

32 FAMEs were recognized and the most abundant ions were selected for the following acquisition in selected ion monitoring. Under the same extraction and chromatographic conditions, SPME Arrow with PDMS (250 µm and length 20 mm) as sorbent phase was tested. In this case, a loss of intensity was observed, as shown in ***figure 4.4.2***.

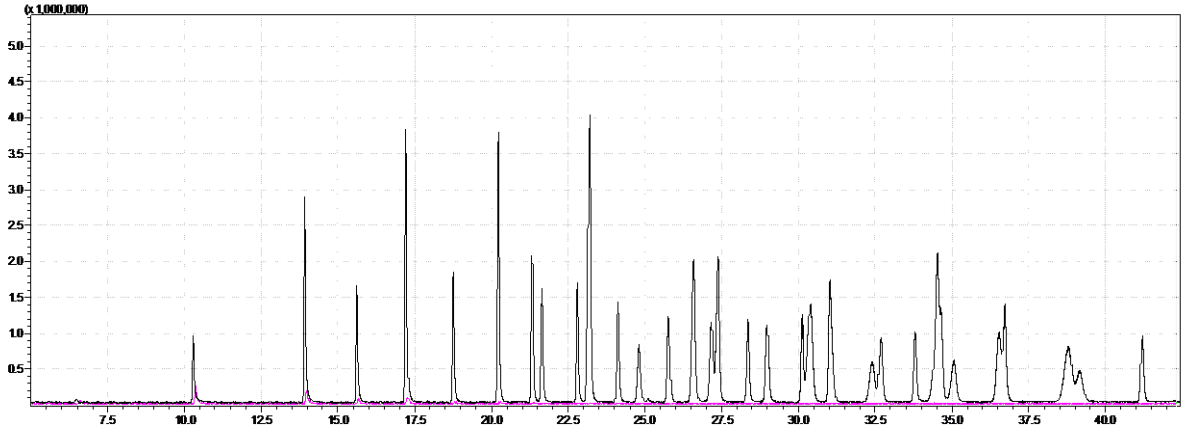

Figure 4.4.2: Chromatograms obtained with SPME fiber (Black) and SPME arrow (pink) in the same conditions.

Therefore, extraction ability of SPME Arrow was tested at two different extraction times: 30 min and 50 min. A considerable increase of signals was observed for all the FAMEs recognized (***Figure 4.4.3***) and, consequently, an extraction time of 50 min was selected for further investigations of SPME Arrow with PDMS coating.

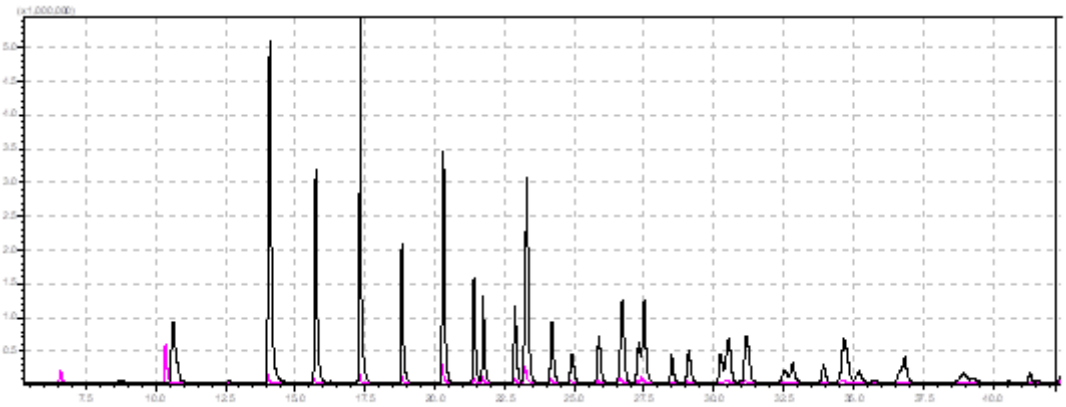

Figure 4.4.3: Chromatogram obtained using an extraction time of 30 min (pink) and 50 min (black).

4.4.2.1 Agitators comparison

Three different agitator modules were tested by performing analysis in triplicate for each agitator: IKA agitator that uses a magnetic stirrer, conventional agitator and heatex stirrer module. The results obtained are reported in ***figure 4.4.4.***

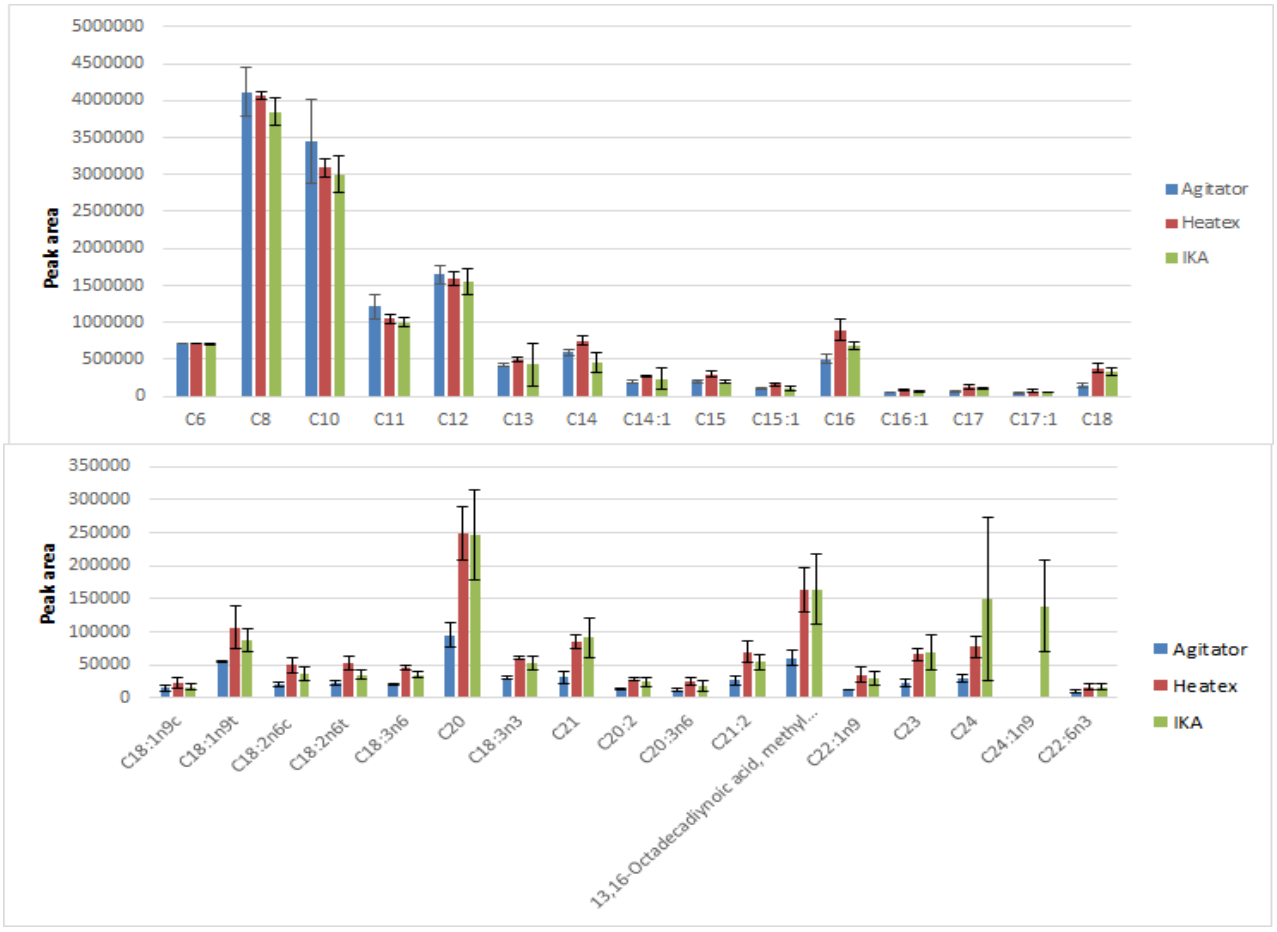

Figure 4.4.4: Peaks area for each analyte obtained using different agitators.

Different behaviors were observed depending on the length of the chain of analytes. Indeed, analytes with short chain gave higher responses when agitator and, in some cases, heatex were used. On the other hand, better responses for analytes with long chain were obtained when IKA or heatex were used. The RSD% values obtained for the agitator are between 0.3% and 28.3% whereas those obtained for heatex and IKA ranged from 0.4% to 36.4% and from 0.5% to 82.8%, respectively. Other experiments were carried out with agitator and heatex at different stirring rate. The maximum value of 750 rpm for agitator and 750 rpm and 1000 rpm for heatex were tested in triplicate under the same experimental conditions (***Figure 4.4.5***).

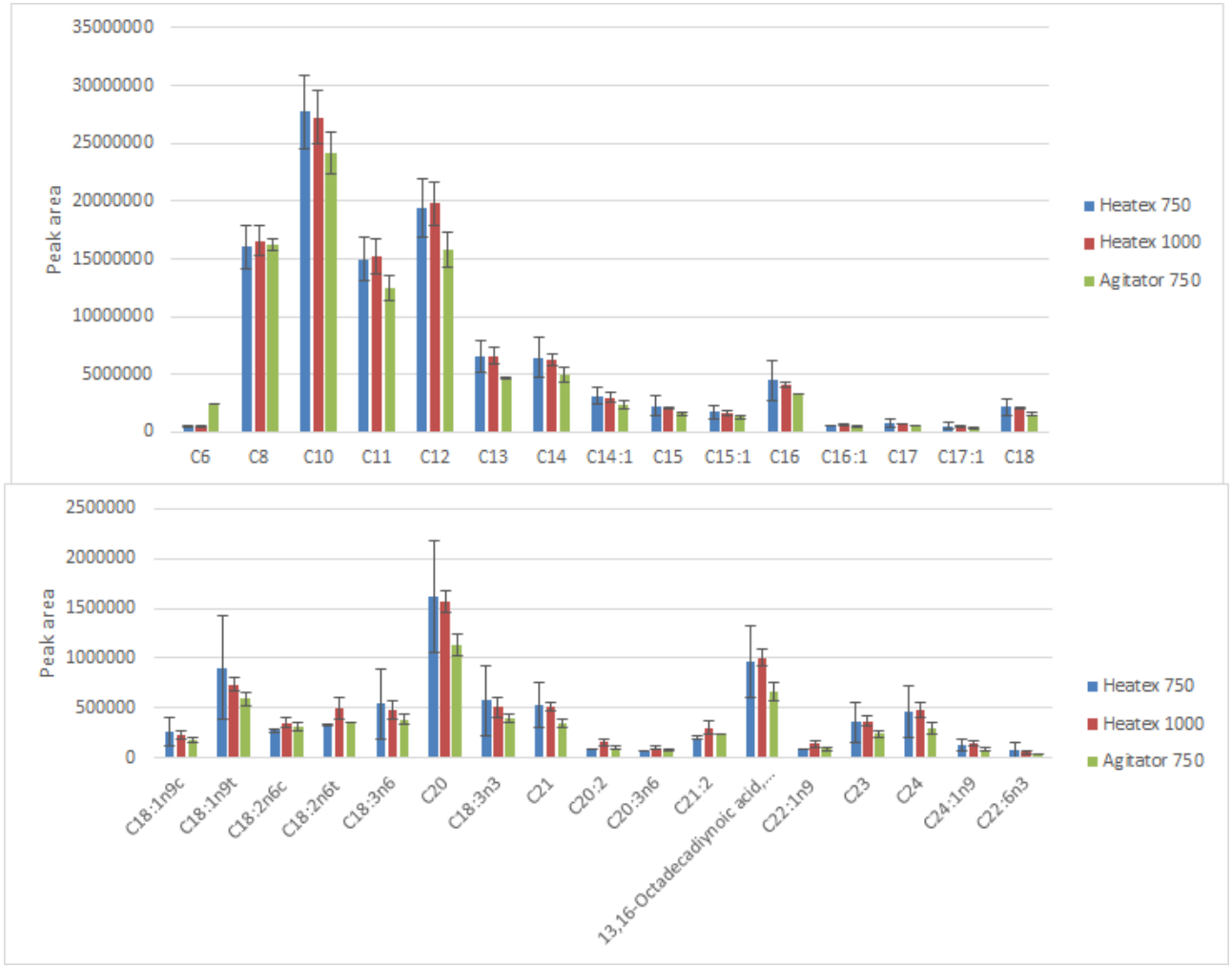

Figure 4.4.5: Results obtained for heatex and agitator tests at different stirring rates with each analyte at 0.5 mg/L.

Results very similar were obtained by using heatex with a speed of 750 and 1000 rpm for all analytes except for C6. Heatex with a speed of 1000 rpm was used for further investigations because had lower RSD % values.

4.4.2.2 Extraction time profile and carryover effect

The parameters that can influence the extraction with SPME Arrow are the same affecting SPME technique. At first, extraction time was evaluated analyzing a sample spiked with FAMEs at 0.5 mg/L at different extraction times (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 min). Results, reported in ***figure 4.4.6*** only for few representative analytes, demonstrated that there are not significant differences between the extraction time 80, 90 and 100 min. For this 80 min was selected as extraction time for further investigations.

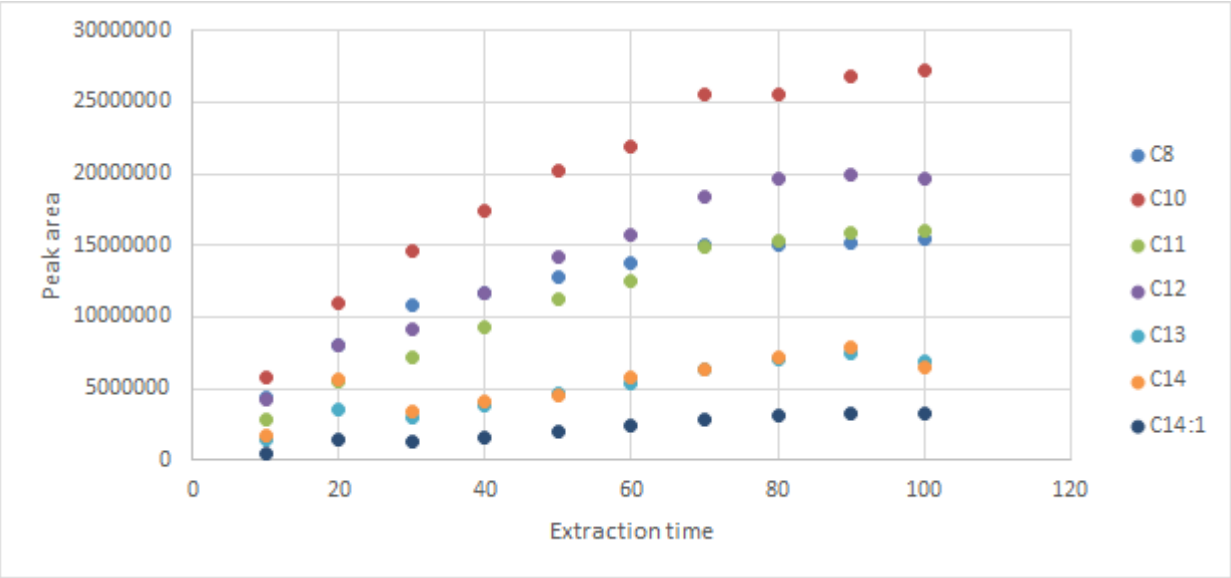

Figure 4.4.6: Extraction time profile obtained with SPME Arrow.

Finally, the carry over effect was evaluated testing different conditioning times after analysis: 15, 20 25 and 30 min (***Figure 4.4.7***). The results showed the increase of all the signals passing from 15 to 20 minutes of conditioning time. The lowest responses were obtained for 30 minutes, although the signals of analytes with short and medium chain are still present.

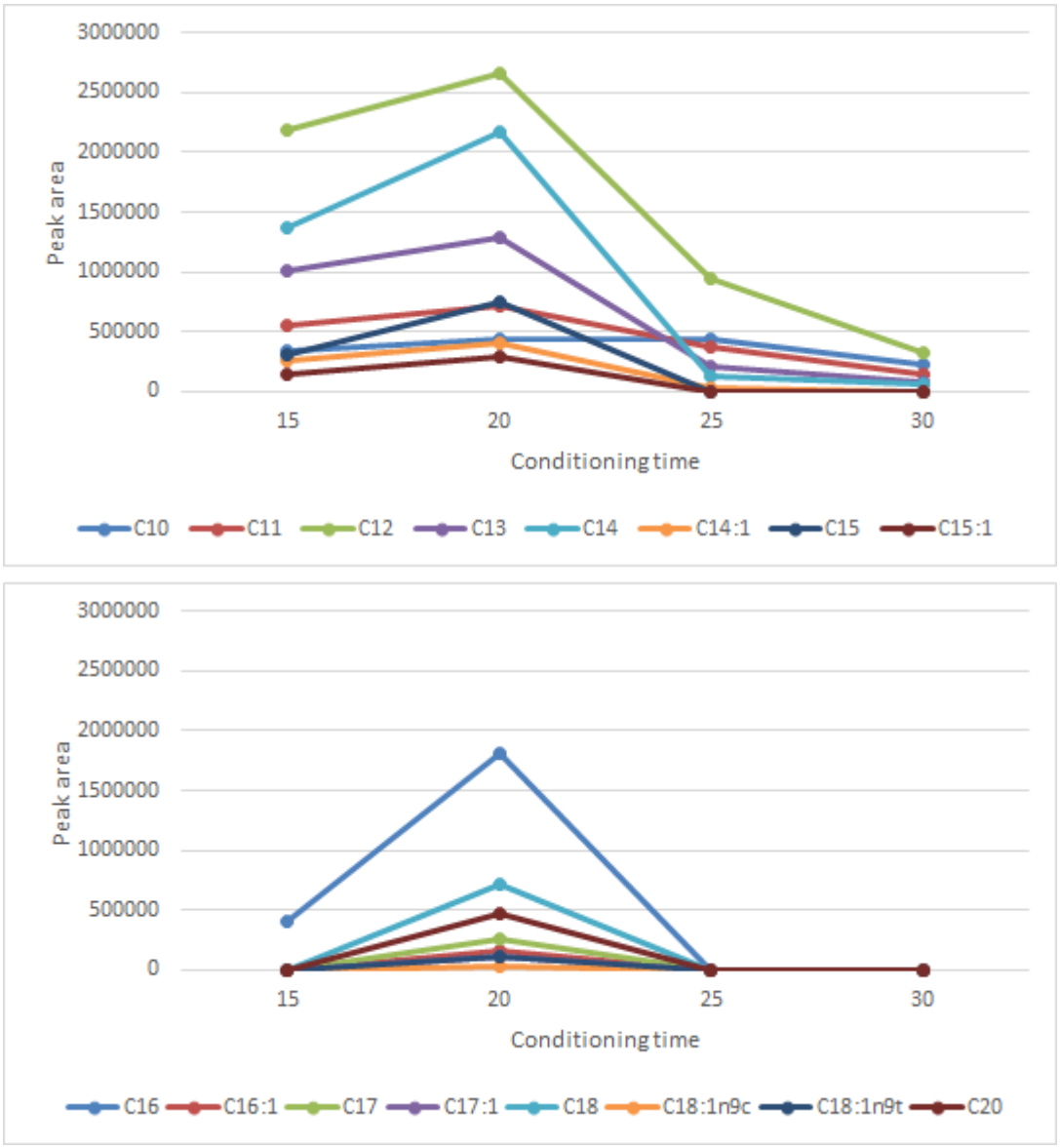

Figure 4.4.7: Results obtained at different conditioning times.

4.4.3 Conclusions and perspectives

The preliminary experiments described above demonstrated that the extraction of FAMEs can be performed with SPME Arrow. In this initial part of the work, analyses with SPME Arrow were conducted to evaluate different agitator modules, different extraction times and carry over effect. The next steps provide for the completion of the SPME Arrow experiments and SPME optimization in order to compare the extraction efficiencies.

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Appendix

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List of abbreviations

LLE	Liquid-liquid extraction
SPE	Solid phase extraction
METs	Microextraction techniques
SPME	Solid phase microextraction
MEPS	Microextraction by packed sorbent
BTEX	benzene, toluene, ethylbenzene, *o, m, p*-xylenes
PA	Polyacrylate
PDMS	Polydimethylsiloxane
PDMS-DVB	Polydimethylsiloxane- divinylbenzene
CAR-PDMS	Carboxen-PDMS
DVB/CAR-PDMS	Divinylbenzene/Carboxen-polydimethylsiloxane
PEG	Polyethylenglyco
PAH	Polyciclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
CNT	Carbon nanotube
MOF	Metal organic framework
MIP	Molecularly imprinted polymer
DI-SPME	Solid phase microextraction in direct immersion
HS-SPME	Solid phase microextraction in headspace
GC	Gas chromatography
GC-MS	Gas chromatography-mass specterometry
VOC	Volatile organic contaminant
NTD	Needle trap device
TFME	Thin-film microextraction
SIL	Silica
SCX	Sulfonic acid bonded silica
PS-DVB	Polystyrene-divinylbenzene
RAM	Restricted access material
OFAT	One factor at a time
CCD	Central composite Design
SPME-GC-MS/MS	Solid phase microextraction-gas chromatography-tandem mass spectrometry
LC	Liquid chromatography
MS	Mass spectrometry

SRM	Selected reaction monitoring
SIM	Selected ion monitoring
NAP	Naphthalene
ACY	Acenaphthylene
ACE	Acenaphthene
FLE	Fluorene
PHE	Phenantrene
ANT	Anthracene
FLT	Fluoranthene
PY	Pyrene
BaA	Benz[a]anthracene
CHR	Chrysene
BkF	Benzo[k]fluoranthene
BbF	Benzo[b]Fluoranthene
BaP	Benzo[a]pyrene
IP	Indeno[1,2,3-cd]pyrene
DahA	Dibenz[a,h]anthracene
BghiP	Benzo[ghi]perylene
EI	Electron ionization
PTV	Programmable temperature vaporizer
GC-ToF/MS	Gas chromatography-time of flight mass spectrometry
FDA	Food and drug Administration guidance
LLOQ	Lower limit of quantification
ME	Matrix effect
CV	Coefficient of variation
QC	Quality control
QCL	Quality control lifetime
S/N	Signal/noise ratio
HPLC	High-performance liquid chromatography
SPME-GC-QqQ-MS	Solid phase microextraction-gas chromatography triple quadrupole mass spectrometry
Put	Putrescine
Cad	Cadaverine
Spd	Spermidine
Spm	Spermine
N^1-AcSpm	N^1-Acetylspermine
N^8-AcSpd	N^8-Acetylspermidine

N^1-AcSpd	N^1-Acetylspermidine
1,6-DAH	1,6-diaminohexane
RSD	Relative standard deviation
MW	Molecular weight
DoE	Experimental design
MEP	Monoethyl phthalate
MMP	Monomethyl phthalate
MBzP	Monobenzyl phthalate
MiBP	Mono-isobutyl phthalate
MnBP	Mono-*n*-butyl phthalte
MEHP	Mono-(2-ethylhexyl) phthalate
MnOP	Mono-*n*-octyl phthalate
McHP	Monocyclohexyl phthalate
MiNP	Mono-isononyl phthalate
Py	Pyridine
PCF	Propyl chloroformate
PrOH	Propanol
ACN	Acetonitrile
MEPS-PTV-GC-MS/MS	Microextraction by packed sorbent-programmed temperature vaporization-gas chromatography-tandem mass spectrometry
TPP	Tripropyl phosphate
TBP	Tributyl phosphate
TCEP	Tris (2-chloroethyl) phosphate
TCPP	Tris (1-chloro2-propyl) phosphate
TDCPP	Tris (1,3-dichloro-2-propyl) phosphate
TBEP	Tris (2-butoxyethyl) phosphate
TPhP	Triphenyl Phosphate
EHDPP	(2-ethylhexyl)-diphenyl phosphate
TEHP	Tris (2-ethylhexyl) phosphate
TCP	Tricresylphosphate
DCM	Dichloromethane
Hex	Hexane
MTBE	Methyl tert-butyl ether
MeOH	Methanol
EtAc	Ethyl acetate
TCM	Trichloromethane

Attività svolte durante il Dottorato di Ricerca in Medicina Traslazionale XXXI ciclo:

Pubblicazioni Scientifiche:

Pubblicazioni riguardanti il progetto di Ricerca del Dottorato:

- Naccarato, **R. Elliani**, B. Cavaliere, G. Sindona, A. Tagarelli, Development of a fast and simple gas chromatographic protocol based on the combined use of alkyl chloroformate and solid phase microextraction for the assay of polyamines in human urine, *J. Chromatogr. A*, 1549 (2018) 1-13.
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- P. De Luca, I. Bernaudo, **R. Elliani,** A. Tagarelli, J. B. Nagy, A. Macario, Industrial Waste Treatment by ETS-10 Ion Exchanger Material, *Materials*, 11 (2018) 1-15.
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R. Elliani, A. Naccarato, A. Tagarelli, *Development of a fast and simple method for the assay of urinary phthalate monoesters by solid-phase microextraction-Gas Chromatography-Triple Quadrupole Mass Spectrometry. (O).* ISBN: 978-88-94952-04-9 O6SS.

R. Elliani, A. Naccarato, A. Tagarelli, *Development of a microextraction by Packed Sorbent-Programmed Temperature Vaporization-Gas Chromatography-Triple Quadrupole Mass Spectrometry method for phthalate monoesters assay in human urine. (P).* ISBN: 978-88-94952-04-9 P072.

VIII Convegno Nazionale sul particolato atmosferico, Matera (23-25 Maggio 2018):

A.Naccarato, A. Tassone, S. Moretti, **R. Elliani**, F. Sprovieri, N. Pirrone, A. Tagarelli, *Organophosphate ester determination in particulate matter: a greener microwave-assisted extraction approach coupled with solid-phase microextraction gas chromatography-tandem mass spectrometry. (O)*

XXVI Congresso Nazionale della Società Chimica Italiana, Paestum, Salerno (10-14 Settembre 2017):

E. Furia, **R. Elliani**, L. Malacaria, A. Tagarelli, *Coumarin-3-carboxylic acid as ligand: acid-base properties, interactions with* Fe^{3+} *and characterization of complexes in aqueous solution. (O)*

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R. Elliani, A. Naccarato, G. Sindona, A. Tagarelli, *Development and multivariate optimization of a MEPS-PTV-GC-MS/MS method for organophoshate flame retardants analysis in environmental aqueous matrices. (O)*

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R. Elliani, A. Naccarato, B. Cavaliere, G. Sindona, A. Tagarelli, *A rapid and simple method for the assay of polyamines in human urine by solid-phase microextraction-gas chromatography-triple quadrupole mass spectrometry. (P)*

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R. Elliani, A. Naccarato, G. Sindona, A. Tagarelli, *Determination of urinary metabolites from exposure to benzene and toluene by SPME- GC-MS/MS. (P) e Comunicazione orale su invito della Commissione Scientifica.*

L'autore che ha presentato è sottolineato. P: Poster; O: Presentazione orale.

Attività di tutoraggio

Tutor Didattico sull'insegnamento di Laboratorio di Chimica Analitica Qualitativa, CHIM/01, Corso di Laurea Triennale in Chimica, Dipartimento di Chimica e Tecnologie Chimiche, Università degli Studi della Calabria. (Marzo - Maggio 2018)**.**

Tutor Didattico sull'insegnamento di Laboratorio di Chimica Analitica Qualitativa, CHIM/01, Corso di Laurea Triennale in Chimica, Dipartimento di Chimica e Tecnologie Chimiche, Università degli Studi della Calabria. (Marzo - Maggio 2017).

Tutor Didattico sull'insegnamento di Chimica Analitica Strumentale e Laboratorio (Modulo di Laboratorio di Chimica Analitica Quantitativa), CHIM/01, Corso di Laurea Triennale in Chimica, Dipartimento di Chimica e Tecnologie Chimiche, Università degli Studi della Calabria. (Marzo - Maggio 2016).

Seminari

10 Dicembre 2015: *Atomistic view of human diseases.* Dott. ssa Alessandra Magistrato. SISSA Trieste. Presso il Dipartimento di Chimica e Tecnologie Chimiche (Università della Calabria, Rende, Italia)

1 Marzo 2016: *Effective models for complex materials.* Prof. Michele Pavone (Università Federico II, Napoli, Italia). Presso il Dipartimento di Chimica e Tecnologie Chimiche (Università della Calabria, Rende, Italia).

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6 - 9 Settembre 2016: *Salute e Sicurezza sui Luoghi di Lavoro.* Ing. Pompeo Runco (Università della Calabria, Rende, Italia).

28 Marzo 2017: *Intuvo Roadshow*. Presso Università della Calabria, Rende, Italia.

18 Aprile 2017: *The chemical composition and biological activity of essential oils.* Dr. Zeynep Firat (Anadolu University, Eskisehir, Turkey). Presso il Dipartimento di Chimica e Tecnologie Chimiche (Università della Calabria, Rende, Italia).

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9 Maggio 2018: *European health systems. Reforming patients rights by applying the European legislative framework in the field/ sistemi sanitari europei.* Prof. Cristina Luiza Erimia (Università ovidius, Constants, Romania); Prof. Carmelo Nobile (Università della Calabria, Rende, Italia).

Corsi

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Partecipazione a Congressi e Scuole

Dal 16 al 20 Settembre 2018: *XXVII Congresso della Divisione di Chimica Analitica*, Bologna, Italia.

> **R. Elliani**, A. Naccarato, A. Tagarelli, *Development of a fast and simple method for the assay of urinary phthalate monoesters by solid-phase microextraction-Gas Chromatography-Triple Quadrupole Mass Spectrometry. (Presentazione orale).*

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Dal 9 al 16 Aprile 2016: NATO SPS ASI 984915, Molecular Technologies for the Detection of Chemical and Biological Agents.

R. Elliani, A. Naccarato, G. Sindona, A. Tagarelli, *Determination of urinary metabolites from exposure to benzene and toluene by SPME- GC-MS/MS. (Poster e presentazione orale).*

Periodo all'estero

Settembre 2017 - Febbraio 2018: 6 mesi presso il gruppo di Ricerca del Prof. Torsten. C. Schmidt, Chimica Analitica Strumentale, Università di Essen, Germania.

Ringraziamenti

Sono giunta alla fine del mio percorso di Dottorato durante il quale ho avuto modo di conoscere persone meravigliose. La prima persona che vorrei ringraziare è il Prof. Antonio Tagarelli, mio supervisore durante i 3 anni, al quale devo tanto per tutti gli insegnamenti in campo professionale. Grazie Prof. perché con la sua brillante preparazione è riuscito a trasmettermi diverse nozioni e ragionamenti utili ai fini di una buona ricerca. Grazie per tutti quegli insegnamenti utili che poi si riflettono anche nella vita non professionale, è davvero una gran bella persona. Grazie alla Dott. ssa Emilia Furia perché ogni attimo di pausa trascorso con lei si traduce in un attimo di divertimento e felicità… come faremmo se non ci fossi tu!! Al mio collega e amico di pausa pranzo Dott. Attilio Naccarato che con il suo atteggiamento da duro è riuscito comunque ad incoraggiarmi in alcuni momenti bui… "hai finito con sta tesi"???!!! Alla mia amica Dott.ssa Lucia Bartella con la quale durante questi anni ci siamo legate e abbiamo imparato a conoscerci. Spero che questo legame possa solo rafforzarsi… è bello avere un'amica come te e grazie per tutti i consigli utili che mi dai durante le nostre chiacchierate. A tutta la mia famiglia vorrei dire un grosso Grazie…. A mio marito che mi ha supportato e soprattutto sopportato in questi anni e maggiormente in questi ultimi mesi. Grazie per essermi stato sempre vicino anche a 2000 km di distanza, sei Speciale. Ai miei genitori con i quali condivido ogni momento della mia vita, grazie Mamy e Babbo per avermi sempre detto parole confortanti in qualsiasi momento. Ad Ale e Tomas, mia sorellona e mio cognatone, per tutte le volte che li ho messi da parte in questo ultimo mese grazie perché senza di voi nulla avrebbe senso siete fantastici!!!!!

Thank you to Professor Schmidt for giving the opportunity to spend 6 months of my Ph.D student in his research group. I'm tankful for the availability and welcome, this time was very professionally stimulating and at the same time I met many new German friends.