UNIVERSITÀ DELLA CALABRIA

Facoltà di Scienze Matematiche Fisiche e Naturali

Dipartimento di Chimica

Scuola di Dottorato Bernardino Telesio - Scuola di Scienza e Tecnica

OMPI - Organic Materials of Pharmacological Interest

XXV CYCLE (CHIM/01)

PhD Thesis

Development and Optimization by Experimental Design of Solid Phase Microextraction Gas Chromatography Triple Quadrupole Mass Spectrometry Methods in Aqueous Matrices

Supervisor
Dott. Antonio TAGARELLI

Coordinator
Prof. Bartolo GABRIELE

Director
Prof. Roberto BARTOLINO

Candidate
Dott. Marcello MONTELEONE

Anno Accademico
2011/2012

To my Wife Anna

TABLE OF CONTENTS

Abstract

Il presente lavoro di tesi relativo all'attività di ricerca svolta durante il triennio di dottorato ha riguardato la messa a punto di metodi analitici per la determinazione di analiti in due distinte aree di interesse. La prima di ambito clinico ed ha riguardato la quantificazione in urina di metaboliti riconosciuti come marker in diagnostica clinica. In particolare ci si occupati della sarcosina come biomarker del tumore alla prostata, e di tre acidi: acido omovanillico (HVA), acido vanilmandelico (VMA) ed acido 5-idrossindoloacetico (5-HIAA) come marker urinari del neuroblastoma. Il secondo ambito di lavoro ha riguardato la quantificazione di inquinanti in matrici acquose, vale a dire carbammati ed acidi perfluoroalchilici. In particolare gli analiti, previa derivatizzazione con alchilcloroformiati (eccetto i carbammati), sono stati estratti dalle matrici acquose (acqua e urina) tramite la tecnica della microestrazione in fase solida (SPME) e successivamente analizzati mediante un gascromatografo con analizzatore di massa a triplo quadrupolo (GC-QqQ-MS). Le variabili significative della microestrazione in fase solida in ciascun metodo sono state ottimizzate tramite l'approccio multivariato dell' " Experimental Design". L'utilizzo della tecnica SPME ha consentito di poter estrarre gli analiti direttamente dal campione da analizzare minimizzando i tempi di preparazione dello stesso e riducendo l'uso di solventi organici, ottenendo metodi poco costosi e basso impatto ambientale. L'utilizzo dello spettrometro di massa triplo quadrupolo, ha consentito di raggiungere livelli di sensibilità molto elevati e, nel contempo, di identificare gli analiti con maggiore sicurezza. In tutti i metodi sviluppati, sono stati ottenuti ottimi risultati in termini di linearità accuratezza e precisione. Anche i valori dei limiti di rilevabilità (LOD) e dei limiti di quantificazione (LOQ) ottenuti in ciascun metodo possono essere considerati soddisfacenti.

Introduction

The development of new analytical methods is a very active field of research. Analytical methods involve various processes such as sampling, sample preparation, separation, detection and analysis of data. The most of analysis time is spent during the step of sampling and sample preparation such as extraction, concentration, fractionation, and isolation of analytes. Each additional step in analytical procedures, however, increases the possibility of loss of analyte, contamination of the sample and analytical error. Therefore, it is convenient to minimize the number of steps used in the preparation of the sample, but without lower the quality of the analysis. Often sample preparation involves various problems such as complicated operations, which require a lot of time, large amounts of sample and organic solvents organic.The latter ones cause environmental pollution, health hazards of the laboratory staff, as well as extra costs related to the disposal of these solvents.The possibility of improving each step of the analysis allows to obtaining protocols with as high performance reliability and sensitivity without increasing costs and working time. The purpose of this thesis is to develop analytical methods in which the analytes are extracted from aqueous matrices by SPME and analyzed using a gas chromatograph triple quadrupole (GC-QqQ-MS). The evaluation and optimization of the parameters affecting the methods were performed by the multivariate approach of the "experimental design". The application of the methods developed involves two specific areas of interest: clinical diagnostic and monitoring of pollutants.

CHAPTER 1

SOLID PHASE MICROEXTRACTION (SPME)

Introduction

The sampling is a crucial step in sample preparation. The objective of the sample preparation step is to isolate the components of interest from a sample matrix. Sample preparation involves extraction procedures and can also include ‘clean-up’ procedures for very complex ‘dirty’ samples. This step must also bring the analytes to a suitable concentration level for detection; therefore, sample preparation methods typically include enrichment.

Solid phase microextraction (SPME) was first introduced in 1990 by Pawliszyn and coworkers (1), and is a sampling technique wherein sample extraction and pre-concentration could be achieved in a single step.

The most widely used technique of sampling with solid phase microextraction consists of exposing a small amount of extracting phase (fiber coating) to the sample for a predetermined amount of time. The principle of SPME is based on the interactions of analytes between the sample matrix and the fiber coating via absorption or adsorption (depending on the nature of the coatings). The transport of analytes from the sample matrix to the fiber coating occurs immediately after contact between the two phases.

Commercial SPME devices consist of 2 major parts: a thin piece of fiber and a fiber holder. The fiber part is comprised of a tensioning spring, sealing septum, septum-piercing needle, fiber attachment needle, and fused-silica fiber, as shown in Figure 1

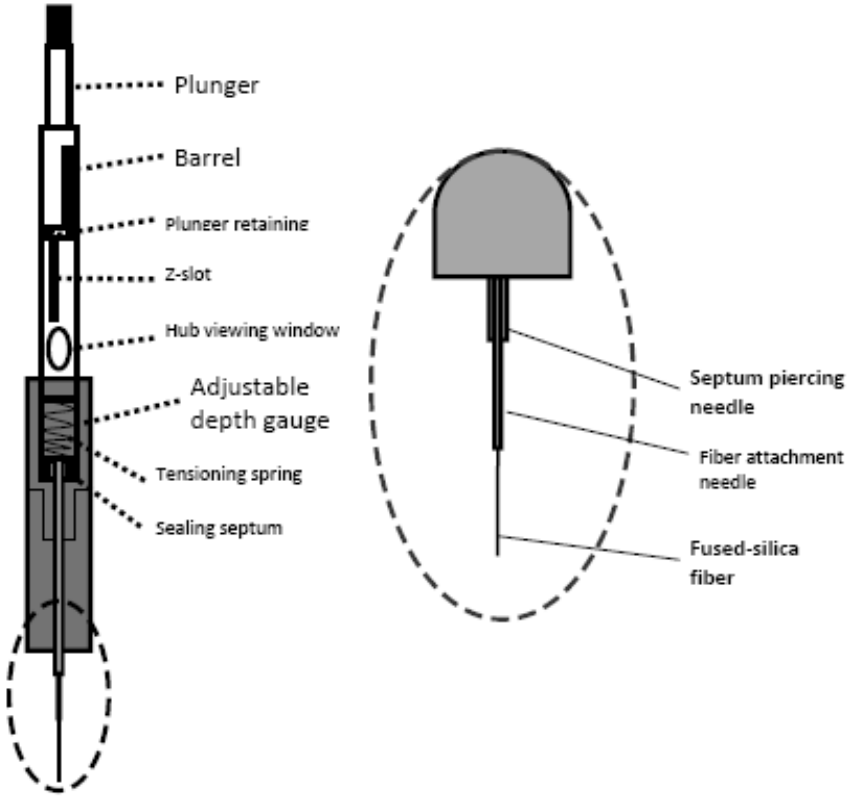


Figure 1. *Commercial SPME Device Made by Supelco (2)*

The fused-silica fiber, coated with a thin film of polymeric extraction phase, is mounted on the stainless-steel fiber attachment needle. It is protected by the septum-piercing needle. The septum-piercing needle is used for protecting the fiber during storage and carrying, and during the sampling procedure, the rigid stainless-steel material plays a dual rule by piercing the septum as well as protecting the fiber.

The fiber holder consists of a plunger, a barrel, a plunger retaining screw, a Z-lot, a hub viewing window, and an adjustable depth gauge. When sampling, the fiber is assembled into the holder, and the insertion depth (the length of fiber exposed during sampling or injection) is adjusted appropriately (2).

SPME can be performed manually or by an autosampler. The fiber configuration of solid-phase microextraction (SPME) is best suited for

automation with gas chromatography (GC), due to its similarity to the traditional GC syringe for liquid injection. In principle, any autosampler that is able to perform syringe injection can be modified to be capable of automated SPME-GC.

There are two typical SPME applications, sampling gases (headspace, HS) or sampling solutions (direct immersion, DI) In either case the SPME needle is inserted into the appropriate position the needle protecting the fiber is retracted and the fiber is exposed to the environment. The polymer coating acts like a sponge, concentrating the analytes by the absorption process After sampling, the fiber is retracted into the metal needle, and the next step is the transfer of the analyte from the fiber into the chromatography instrument. Gas chromatography (GC) is one of the preferred used techniques. In this case, thermal desorption of the analyte takes place in the hot GC injector. The main advantages of SPME extraction compared to solvent extraction are the reduction in solvent use, the combination of sampling and extraction into one step and the ability to examine smaller sample sizes.

1.1 Basic Theory of Solid-Phase Microextraction

Typically, SPME is considered to be complete when the analyte concentration has reached distribution equilibrium between the sample matrix and the fiber coating. Equilibrium extraction is the most frequently used quantification method for SPME. Equilibrium conditions in a two-phase system including extraction phase and aqueous matrix can be described according to the law of mass conservation (Equation 1.1)

$$C^0_s V_s = C^\infty_f V_f + C^0_s V_s \quad \text{Equation 1.1}$$

Where C^0_s is the initial concentration of a given analyte in the sample, V_s is the sample volume, V_f is the fiber coating volume, and C^∞_f and C^0_s, are the

equilibrium concentrations of the analyte in the fiber and the sample matrix, respectively. The fiber/sample matrix distribution coefficient K_{fs} is defined as:

$K_{fs} = C^{\infty}_f / C^{0}_s$ *Equation 1.2*

Equations (1.1) and (1.2) can be combined and rearranged into :

$$C^{\infty}_f = C_0 \frac{K_{fs} V_s}{K_{fs} V_f + V_s}$$ *Equation 1.3*

Finally, the mass of the analyte absorbed by the fiber $n = C^{\infty}_f V_f$ can be described as

$$n = C_0 \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s}$$ *Equation 1.4*

Equation 1.4 indicates that the amount of analyte extracted onto the fiber coating is directly proportional to the analyte concentration in the sample. This is the analytical basis for quantification using SPME.

When the the volume of the sample is very large compared to the volume of the coating ($V_s >> K_{fs} V_f$) the eqauation 1.4 becomes :

$n = C_0 K_{fs} V_f$ *Equation 1.5*

In Equation 1.5, the amount of extracted analyte is independent of the volume of the sample. Therefore, there is no need to collect a defined amount of sample prior to analysis. Thus, the fiber can be exposed directly to the ambient air, water, production stream, etc., and the amount of extracted analyte will correspond directly to its concentration in the matrix. This greatly accelerates the analytical process, while errors associated with the loss of

analyte through decomposition or absorption onto sampling-container walls is prevented. When sample volume is very small, and the distribution coefficient of the analyte between the fiber coating and the sample matrix is very large ($V_s << K_{fs} V_f$) as occurs when sampling of semivolatile organic compounds (semi-VOCs) in small volumes of a sample matrix, the equation 1.5 becomes:

$n = C_s^0 V_s$ *Equation 1.6*

The equation 1.6 shows that all of the analytes in the sample matrix are extracted on to the fiber coating. Therefore, the analyte concentration in the sample can be easily calculated with the amount of analyte extracted by the fiber coating and the volume of the sample (3,4).

1.2 Extraction Modes

There are three basic modes of performing SPME-based on the position of the extraction fiber. They are: direct extraction (DI), headspace extraction (HS) and membrane protection extraction.

In the DI-SPME mode, the fiber is inserted into the sample medium and the analytes are transported directly to the extraction phase. For aqueous matrices, more efficient agitation techniques, such as fast sample flow, rapid fiber or vial movement, stirring or sonication are required. These actions are undertaken to reduce the effect caused by the "depletion zone" which occurs close to the fiber as a result of fluid shielding and slow diffusion of analytes in the liquid media. In the headspace sampling mode, the analyte is transported through a layer of gas before reaching the coating. This protects the fiber coating from damage by high molecular weight substances and other non-volatile concomitants present in the liquid sample matrix,. The amount of analyte extracted at equilibrium using DI or HS sampling are identical as long as the sample and gaseous headspace volumes are the same. This is a result of

the equilibrium concentration being independent of the fiber location in the sample/headspace system. If the above condition is not satisfied, a significant sensitivity difference between the direct and headspace technique exists only for very volatile analytes.The extraction technique should be selected relative to the nature of the sample matrix. In general, DI-SPME is more sensitive than HS-SPME for analytes predominantly present in a liquid. However, HS-SPME exhibits lower background than DI-SPME, and is suitable for the extraction of more-volatile analytes in most gaseous, liquid and solid samples. In-tube SPME using an open tubular capillary column as the SPME device was developed to couple directing with HPLC or LC-MS With the in-tube SPME technique, organic compounds in aqueous samples are directly extracted from the sample into the internally coated stationary phase of a capillary column, and then desorbed by introducing a moving stream of mobile phase or static desorption solvent when the analytes are more strongly absorbed onto the capillary coating. The capillaries selected have coatings similar to those of commercially available SPME fibers.

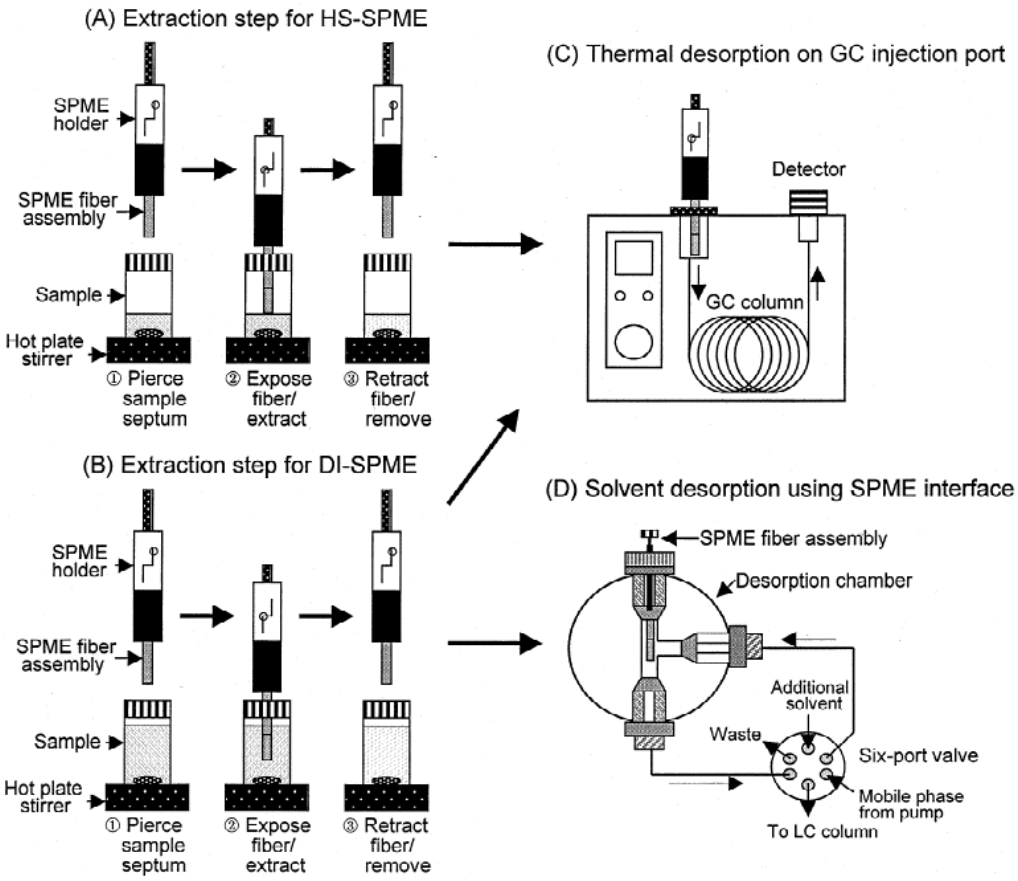


Figure 2 : *Extraction Process by HS-SPME and DI-SPME, and Desorption Systems for GC and HPLC Analyses (2)*

1.3 Fiber Types

Several types of coating fibers are currently available for the extraction of analytes. The fiber is coated with a thin polymeric film, which concentrates the organic analytes during absorption or adsorption from the sample matrix. The SPME coatings can be classified primarily into four categories: by the type of coating, by the coating thickness, by polarity and by whether the coating is an absorbent or an adsorbent. In the table 1 is shown a list of commercially available fibers.

Table 1 . *Types of Commercially Available SPME Fibre Coatings*

Type of Coating	Extraction Mechanism	Polarity
7µm PDMS	Absorbent	Non polar
30µm PDMS	Absorbent	Non polar
100µm PDMS	Absorbent	Non polar
85 µm PA	Absorbent	Polar
60µm PEG (Carbowax)	Absorbent	Polar
15µm Carbopack Z-PDMS	Absorbent	Bipolar
65µm PDMS-DVB	Absorbent	Bipolar
55µm/30µm DVB/Carboxen-PDMS	Absorbent	Bipolar
85µm Carboxen-PDMS	Absorbent	Bipolar

CW - Carbowax
DVB - Divinylbenzene
PA - Polyacrylate
PDMS - Polydimethylsiloxane

The chemical structures of polymers commonly used as SPME coatings are shown in figure 3.

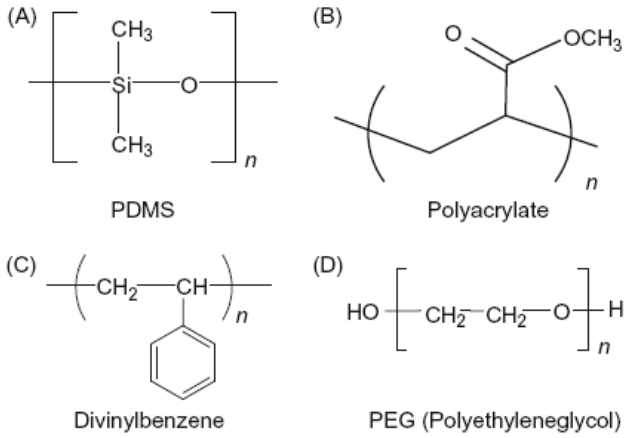


Figure 3 . *Chemical structures of common polymers used as SPME coatings.*

The polarity of the coating is determined by type of phase applied. Stationary phases are immobilized by non-bonding, partial cross-linking or high cross-linking. Non-bonded phases are stable with some water-miscible organic

solvents (up to 20% organic content). Bonded phases are compatible with the majority of organic solvents except for some non-polar solvents (hexane, dichloromethane). Partially cross-linked phases are stable in most water-miscible solvents. Highly cross-linked phases are equivalent to partially cross-linked phases, except that some bonding to the core has occurred. (5) Both PDMS and PA phases extract samples via the absorption of analytes, which dissolve and diffuse into the coating material. The remaining types (Carbowax–DVB, PDMS–Carboxen and PDMS–DVB) are mixed coatings and extract via adsorption of analytes staying on the surface (as a monolayer) of the fiber (6).The choise of appropriate fibre coating depending on the analyte polarity and volatility. The type of fibre used affects the selectivity of extraction: in general, polar fibers are used for polar analytes and non-polar fibers for non-polar analytes as with conventional GC stationary phase (5).

1.4 Optimization of Extraction

Several factors, in addition a fiber types influence the SPME efficiency and these are evaluated during method development. The primary parameters influencing analyte absorption into the stationary phase are extraction time and temperature, ionic strength, pH, sample volume and agitation. For SPME-GC, the analyte desorption is a function of time and temperature.

1.4.1 Extraction Time and Temperature

In the fiber SPME method, the amount of analyte extracted onto the fiber depends not only on the polarity and thickness of the stationary phase, but also on the extraction time and the concentration of analyte in the sample. An optimal approach to SPME analysis is to allow the analyte to reach equilibrium between the sample and the fiber coating. The equilibration time is defined as the time after which the amount of analyte extracted remains

constant and corresponds, within the limits of experimental error, to the amount extracted after an infinite time. Determination of the amount extracted at equilibrium allows calculation of the distribution constants. Extraction time is mainly determined by the agitation rate and the partition coefficient of of the analyte between the fiber coating and sample matrix. Although SPME has a maximum sensitivity at the equilibrium point, full equilibration is not necessary for accurate and precise analysis by SPME because of the linear relation ship between the amount of analyte adsorbed by the SPME fiber and its initial concentration in the sample matrix in non-equilibrium conditions (7). Extraction temperature is very important, especially for the extraction of semivolatile compounds.Temperature has a great influence on the vapor pressure of the analytes.Extraction temperature is closely related to equilibrium time because an increase of temperature results in an increase of Henry‟s Law constant and of the diffusion coefficient between the headspace and sample. This will lead to a decrease of the equilibrium time and will accelerate the analytical process considerably release of analytes from the sample matrix. An increase in extraction temperature causes an increase in extraction rate, and simultaneously a decrease in the distribution constant.

1.4.2 Ionic Strength

Typically, analyte solubility decreases as ionic strength increases. A decrease in analyte solubility improves sensitivity by promoting analyte partitioning into the stationary phase. This “salting out” effect is compound-specific. The addition of salts is preferred for HS-SPME because the fiber coatings are prone to damage during agitation by DI-SPME. The effects of salt addition to enhance the extracted amount of an analyte by SPME have been studied in detail (8-10). Salting with the addition of sodium chloride is well known to improve extraction of organics from aqueous solution. Although salt addition

usually increases the amount extracted, the opposite behavior is also observed (11, 12). A high salt concentration in the sample matrix facilitates salt deposition on the fiber which decreases extraction efficiency over time by DI-SPME (13, 14). In general, the effects of salt addition increase with the polarity of the compound.

1.4.3 pH

Matrix pH can be adjusted to optimize the SPME of acidic and basic analytes. Extraction efficiency for acidic compounds increases as pH decreases. At low pH, the acid-base equilibria of acidic compounds are shifted towards the neutral form and analyte partitioning into the stationary phase is enhanced. Conversely, basic compounds shift towards the ionized from as pH decreases and extraction efficiency decreases. Generally, extraction is more effective if the compounds are kept undissociated, which is similar to the LLE and SPE procedures. In DI-SPME, contact of the fiber with high and low pH solution would increase damage to the coating.

1.4.4 Agitation

The analyte equilibrium between the sample matrix and the stationary phase depends on the rate of mass transfer of the analytes in the aqueous phase. So, agitation is required to facilitate mass transport between the bulk of the aqueous sample and the fiber. Although the equilibration time progressively decreases with increasing agitation rate, faster agitation tends to be uncontrollable and the rotational speed might cause a change in the equilibration time and poor measurement precision. Several agitation methods can be used in SPME, depending on the type of application: magnetic stirring, intrusive stirring, needle vibration, moving vial (vortex stirring), flow-through

stirring, sonication and orbital shaking. Table 2 summarizes the properties of several agitation methods which have been tested with SPME (15).

Table 2: *Agitation methods in SPME*

Method	Advantages	Disvantages
Static (no agitation)	Simple ,performs well for gsaseous phase	Limited to volatile analytes and HS-SPME
Magnetic stirring	Common equipement, good performance	Requires stirring bar in the vial
Intrusive stirring	Very good performance	Difficult to seal the sample
Vortex/moving vial	Good performance, no need for stirring bar in the vial	Stress on needle and fiber
Fiber moviment	Good performance, no need for a stirring bar in the vial	Stress on needle and fiber, limited to small volume
Flow through	Good agitation at rapid flows	Potential for cross contamination, requie constant flows
Sonication	Very short extraction times	Noisy, heats up the sample

1.4.5 Sample Volume

The sensitivity achieved with SPME methodology is dependent solely on the number of moles of analyte extracted from the sample. When sample volume is large, the amount of analyte extracted is an insignificant portion of the total amount of analyte in the system. Therefore, analyte concentration in the sample remains constant during extraction, resulting in optimum sensitivity and better precision because the variation in sample volume does not affect the amount of analyte extracted. When sample volume is small, a substantial depletion of sample concentration occurs during extraction, resulting in loss of sensitivity and precision. In HS-SPME, the volume and sample/gas contact area affects the kinetics of the process, since the analytes need to be

transported through the interface and the headspace, in order to reach the fiber. The smaller the gas phase is with respect to the sample, the more rapid is the transport of analytes from the sample matrix to the fiber coating.

1.4.6 Desorption Time and Temperature

Efficient thermal desorption of an analyte in a GC injection port is dependent on the analyte volatility, the thickness of the fiber coating, injection depth, injector temperature and exposure time.

For a regular liquid sample injection in a split/splitless injector, the insert has to have a large volume (3-5 mm i.d.) because of the solvent expansion Since little or no solvent is present in the case of SPME, a narrow bore (0.75 mm i.d.) unpacked injection liner is required to ensure a high liner gas flow, to reduce desorption time and prevent peak broadening. Injections are carried out in the splitless mode to ensure complete transfer of analyte to increase sensitivity (4) .Generally, the optimal desorption temperature is approximately equal to the boiling point of the least volatile analyte. To prevent peak broadening, the initial GC column temperature should be kept low, or even cooled. Thus, pre-concentration of analytes at the head of the column is achieved.The desorption time depends on the injector temperature and the linear flow rate around the fiber. For non-polar, volatile compounds, desorption is virtually complete in a few seconds, but the desorption should be continued for another one or two minutes to ascertain that no carryover occurs when a blank is inserted after a sample.

1.5 Application of SPME

The SPME technique has been widely used for analysis of different compound in several areas of interest. Many methods have been developed in the recent years in environmental, food and biological analysis. SPME is generally applied to analysis of

organic compound and can be applied for the extraction of various analytes from several types of matrices. SPME is often used for the analysis of environmental pollutants in air, water, soil and sediment samples, in biomedical analysis and food quality and safety control.

1.5.1 Environmental applications

SPME had been widely used for analysis of environmental pollutants in air, water, soil, and sediment samples, hundreds of papers addressing environmental analysis by SPME were published in recent years. The majority of applications have been developed for aqueous matrices in particular for analysis of BTEX, PAH (16-22), pesticides and herbicides (23-25). Derivatization techniques have been used for analysis of organometallic compounds (26-33), phenols (34) , aromatic amines (35,36) and other compounds (37,38) in aqueous samples. Aqueous sampling by SPME can be performed by direct immersion (DI), the headspace (HS) method. SPME is also used for the determination of trace contaminants in air (39-42). The analytes are extracted by the SPME fiber either by direct exposure or by use of the headspace method. Most applications involve the use of a commercial SPME fiber. Generally, the sensitivity of the HS-SPME procedure can be improved by manipulation of the matrix (e.g. addition of acetone–water (70: 30)) (43). Most SPME air sampling is performed on-site or in the laboratory by collecting the air sample in a bag or solid phase extraction (SPE) device; the analytes are extracted by the SPME fiber either by direct exposure or in the headspace (HS).

Sampling of soil and sediments by SPME is normally performed by the HS or DI methods. Different procedures have been described for quantitative analysis of solid samples such as soils sediments and sludges. HS-SPME is used for analysis of alkylbenzenes, aromatic amines,(44) chlorophenols,(45) phthalates,(46,47) PAHs and hexachlorobenzenes in soils, (48-50)

organometallics in sediments(51) and alkylphenol ethoxylate surfactants in sludges.(52) The analysis by DI-SPME is performed by immersion of the fibre in solid solution (50–52) or in an aqueous extract of the solid.(48, 49).

1.5.2 Food Application

Food analysis is important for the evaluation of nutritional value, for quality control of fresh and processed products and the monitoring of food additives and other toxic contaminants. There are many methods in the literature using the SPME for the analysis of various components and contaminants in food samples. Aroma and flavor are one of the most important quality criteria of fresh and processed foods, and both qualitative and quantitative information is desired for characterizing aroma producing compounds (2). In general, flavour is sensitive to compositional alterations. In the case of food (fruit, wine, etc.) flavours the volatile aromatic compounds are produced through metabolic pathways during ripening, harvest, post-harvest and storage, and their production depends on many factors related to the species, variety and type of technological treatment.(53,54). Foodstuffs are prone to deterioration by light, heat, oxidation and contamination from the container during storage. Extensive research is being focused on the early detection of microorganisms responsible for food contamination and spoilage, and thus, preventing the outbreak of food-borne calamities. SPME has been used for the extraction of volatiles due to the action of spoilage microorganisms in potatoes and cured ham (55), wheat grain (56, 57), milk (58), and pathogenic organisms (59-61). SPME can serve as a key tool in investigating the presence or absence of desired favor compounds in a particular food product and hence be of immense help in quality control. Some investigations on the use of SPME to extract volatiles from food additives and flavorings regard honeys (62) spices (63) virgin olive oil (64) cane and beet sugars (65) rapeseed oil (66).

HS-SPME is one of the most popular extraction techniques for the characterization of different alcoholic drinks based on their volatile composition (67-71) or to extract specific trace components from the HS (72,73). One of the most important and well-studied areas of the food analysis is the analysis pesticides, herbicides, fungicides and other agrochemical products in foods. Various pesticide and fungicide residues in vegetables and fruits have been analyzed by SPME coupled with GC/MS (74-77).

1.5.3 Biomedical Application

Biomedical analyses of drugs, metabolites, poisons, environmental and occupational pollutants, disease biomarkers and endogenous substances in body fluids and tissues are important in the development of new drugs, therapeutic monitoring, forensic toxicology, patient diagnosis, and biomonitoring of human exposure to hazardous chemicals. In these analyses, sample preparation is essential for isolation of desired components from complex biological matrices and greatly influences their reliable and accurate determination. Solid-phase microextraction is an effective sample preparation technique that has enabled miniaturization, automation and high-throughput performance. The use of SPME has reduced assay times, as well as the costs of solvents and disposal (78). Urine is a relatively simple biological fluid to collect and is frequently used for drug screening, forensic purposes, monitoring workplace exposure to chemicals and other investigations as it contains the target analytes together with diagnostic metabolites (5). A variety of drugs (amphetamines, amphetamines, antihistamines, tricyclic antidepressants, (79) corticosteroides (80), organometallics,(81-83) inorganic mercury,(84-85) pesticides and industrial chemicals (86-87) can be measured in urine by SPME techniques. Various SPME methods, also has been developed in blood analysis. A range of compounds can be extracted and analysed such as industrial solvent residues (88), insecticides,(89) pesticides,

(90) amphetamines, anaesthetics, diazepines (91) and different drug metabolites. Amphetamine and related compounds are one of the ‘favourite’ target components in SPME blood analysis, similar to urine analysis. Endogenous substances such as neurotransmitters, hormones and various bioactive compounds are useful as diagnostic or prognostic biomarkers in disorders or healthcare, and are often analyzed during population screening, disease diagnosis, and biomonitoring. For this purpose most methods have been used to analyze blood (92-95), urine (96-99) saliva (100-102), skin (103,104) and breathe (105-107). Hair analysis is frequently used for the long-term monitoring of drug and alcohol users.HS-SPME as the advantage of producing a high purity of the extract with no interferences. It is a convenient one-step method for the measurement of many lipophilic basic drugs such as nicotine, amphetamine and related compounds, local anaesthetics, phencyclidine, ketamine, methadone, tricyclic antidepressants and phenothiazines (108-113). In summary, SPME has proved to be a very useful complement to the analytical equipment in a biomedical laboratory and should always be taken into consideration when a method for an analyte has to be updated or introduced.

References

1. Arthur C.L., Pawliszyn J. *Anal. Chem*. 62 (**1990**) 2145.
2. Kataoka H, Lord H.L. Pawliszyn J. *J. Chromatogr. A* 880 (**2000**) 35 –62
3. Zhang Z., & Pawliszyn, J. *Anal. Chem* 65 (**1993**) 1843–1852.
4. Pawliszyn J., Pawliszyn B., Pawliszyn M., *Solid Phase Microextraction SPME* 1 / Vol . 2, N. 4 *The Chemical Educator*
5. Vas G., Vekey K., *J. Mass Spectrom.* 39 (**2004**) 233–254
6. Chen J., Pawliszyn J., *Anal. Chem.* 67 (**1995**) 2530
7. Ai J., *Anal. Chem*. 69 (**1997**) 1230.
8. Zambonin, C.G., Cilenti, A. , Palmisano F., *J. Chromatogr. A.* 967 (**2002**) 255-260.
9. Zuin,VG., Lopes, A.L., Yariwake, J.H., Augusto F. *J. Chromatogr. A* 1056 (**2004**) 21-26.
10. Beltran J., Peruga, A., Pitarch, E., Lopez, F.J., Hernandez, F. *Anal. Bioanal. Chem*. 376 (**2003**). 502-511
11. Magdic, S. and Boyd-Boland, A. *J. Chromatogr. A* 736 (**1996**) 219-228.
12. Scheyer A. Morville, S. *Anal. Bioanal. Chem*. 384 (**2006**) 475-487.
13. Jinno K. , Muramatsu, T. *J. Chromatogr. A.* 754 (**1996**) 137-144.
14. Berrada, H., Font, G., Molto, J.C. *J. Chromatogr. A* 890 (**2000**) 303-312.
15. Pawliszyn J., *Handbook of Solid Phase Microextraction Elsevier* **2012**
16. Eisert R, Pawliszyn J. *Anal. Chem.* 69 (**1997**) 3140–3147
17. Gou Y., Eisert R., Pawliszyn J. *J Chromatogr. A* 873 (**2000**) 137–147

18. Gou Y, Pawliszyn J. *Anal. Chem.* 72 (**2000**) 2774–2779
19. Gou Y., Tragas C., Lord H., Pawliszyn J. *J Microcol. Sep.* 12 (**2000**)125–134
20. Hirayama Y., Ohmichi M., Tatsumoto H. *J. Health. Sci.* 51(**2005**) 526–532
21. Takino M., Daishima S., Nakahara T. *Analyst* 126 (**2001**) 602–608
22. Kataoka H, Mitani K, Takino M *Method Biotechnol* 19 (**2006**)365–382
23. Penalver A., Pocurull E., Borull F., Marce R.M. *J. Chromatogr. A* 839 (**1999**) 253.
24. Natangelo M., Tavazzi S., Fanelli R., Benfenati E. *J. Chromatogr. A* 859 (**1999**) 193.
25. Eisert R., Levsen K. *J. Am. Soc. Mass Spectrom.* 6 (**1995)** 1119
26. Le Gac M., Lespes G., Potin-Gautier M. *J Chromatogr A* 999 (**2003**)123–134
27. Arambarri I., Garcia R., Millan E. *Chemosphere* 51 (**2003**) 643–649
28. Devos C., Vliegen M., Willaert B., David F., Moens L., Sandra P. *J. Chromatogr. A* 1079 (**2005**) 408–414
29. Chou C.C., Lee M.R. *J. Chromatogr. A* 1064 (**2005**) 1–8
30. Centineo G., Blanco Gonzalez E., Sanz-Medel A. *J. Chromatogr. A* 1034 (**2004**) 191–197
31. Parkinson D., Bruheim I., Christ I., Pawliszyn J. *J. Chromatogr. A* 1025 (**2004**) 77–84
32. Mishra S., Tripathi R.M., Bhalke S., Shukla V.K., Puranik V.D. *Anal. Chim. Acta* 551 (**2005**) 192–198
33. Abranko L., Yang L., Sturgeon R.E., Fodor P., Mester Z. *J. Anal. Atom. Spectrom.* 19 (**2004**) 1098–1103

34. Urbanczyk A., Staniewski J. *Chem. Anal.* (Warsaw) 50 (**2005**) 749–759
35. Herraez-Hernandez R., Chafer-Pericas C., Campins-Falco P. *Anal. Chim. Acta* 513 (**2004**) 425–433
36. Zimmermann T, Ensinger WJ, Schmidt T.C. *Anal. Chem.* 76 (**2004**)1028–1038
37. Salgado-Petinal C., Alzaga R, Garcia-Jares C., Llompart M., Bayona J.M. *Anal. Chem*. 77 (**2005**) 6012–6018
38. Tsai S.W., Chang C.M. *J. Chromatogr. A* 1015 (**2003**)143–150
39. Svendsen MR, Glastrup *J. Atmos. Environ*. (**2002**) 36 3909.
40. De Angelis F., 21st *Informal Meeting on Mass Spectrometry*, May 11–15 (**2003**), Antwerp, *Book of Abstracts.* 69.
41. Hook G.L., Kim G.L., Hall T., Smith .PA. *Trends Anal. Chem.* 21 (**2002**) 534.
42. Tuduri L., Desauziers V., Fanlo J.L. *J. Chromatogr. A* 963 (**2002)** 49.
43. Sarrion M.N., Santos F.J., Galceran M.T. *J. Chromatogr. A* 819 (**1998**) 197.
44. Müller L., Fattore E., Benfenati E. *J. Chromatogr. A* 791 (**1997**) 221.
45. Ribeiro A., Neves M.H., Almeida M.F., Alves A., Santos L. *J. Chromatogr. A* 975 (**2002**) 267.
46. Luks-Betlej K., Popp P., Janoszka B., Paschke H. *J. Chromatogr. A* 938 (**2001**) 93.
47. Penalver A., Pocurull E., Borrull F., Marce R.M. *J. Chromatogr. A* 872 (**2000**) 191.
48. Pino V., Ayala J.H., Afonso A.M., Gonzalez V. *Anal. Chim. Acta* 477 (**2003**) 81.

49. Popp P., Kalbitz K., Oppermann G. *J. Chromatogr. A* 687 (**1994)** 133.

50. Boyd-Boland A.A., Magdic S., Pawliszyn J. *Analyst* 121 (**1996**) 929.

51. Tutschku S., Mothes S., Wennrich R., Fresenius J. *Anal. Chem*. 354 (**1996**)587.

52. BoydBoland A.A., Pawliszyn J. *Anal. Chem*. 68 (**1996**) 1521.

53. Vas G., Lorincz G. *Acta Aliment*. 28 (**1999)** 95.

54. Vas G., Koteleky K., Farkas M., Dobo A., Vekey K. *Am. J. Enol. Vitic.* 49 (**1998**) 100.

55. Jones, P.R.H., Ewen, R.J., Ratcliffe, N.M. *J. Food Comp. Anal.* 11 (**1998**) 274–279.

56. Jelen H.H., Majcher M., Zawirska-Wojtasiak R., Wiewiorowska M., Wasowicz E. *J. Agric. Food Chem*. 51 (**2003**) 7079–7085.

57. Turner N.W., Subrahmanyam, S., Piletsky S.A. *Anal. Chim. Acta*, 632 (**2009**) 168–180.

58. Marsili R.T. *J. Agric. Food Chem*. 47 (**1999**) 648–654.

59. Senecal A.G., Magnone J., Yeomans W., Powers, E.M., (**2002**). *Proceedings of the SPIE, Edited* by Jensen J. L. and Burggraf L. W., 4575, 121–131

60. Syhre M., Chambers S.T. *Tuberculosis* 88 (**2008**) 317–323.

61. Schilling, M.W., Yoon Y., Tokarskyy O., Pham A.J., Williams, R.C., Marshall D.L.. Meat Science, 85 (**2009**) 705–710.

62. Perez R. A., Brunete C.S., Calvo, R.M., Tadeo J.L. *J. Agric. Food Chem*. 50 (**2002**) 2633–2637.

63. Maroto M.C.D., Coello M.S.P., Cabezudo, M.D. *Chromatographia* 55(11/12) (**2002**) 723–728.

64. Vichi S., Castellote A. I., Pizzale L., Conte L.S., Buxaderas S., Tamames, E.L. *J.. Chrom.* 983 (**2003**) 19 33.
65. Batista, R.B., Grimm, C.C., Godshall, M.A. *J. Chrom. Sci,* 40 (**2002**)127–132.
66. Jelen H.H., Mildner-Szkudlarz S., Jasinska I., Wasowicz E. J. Am.Oil Chem. Soc. 84(6) (**2007**) 509–517.
67. Vas G., Koteleky K., Farkas M., Dobo A., Vekey K., *Am. J. Enol. Vitic.* 49 (**1998**) 100.
68. Jelen H.H., Wlazly K., Wasowicz E., Kaminski E. *J. Agric. Food Chem*. 1998; 46: 1469.
69. Pino J., Marti M.P., Mestres M., Perez J., Busto O., Guasch J. *J. Chromatogr. A* 954 (**2002**) 51.
70. Vas G.. *Supelco Rep*. 16(4) (**1997**) 7.
71. Ebeler S.E. *Food Rev. Int*. 17 (**2001**) 45.
72. Evans T.J., Butzke C.E., Ebeler S.E. *J. Chromatogr. A*; 786 (**1997**) 293.
73. Luan T., Li G., Zhang Z. Anal. Chim. Acta 424 (**2000**) 19.
74. Navalon A., Prieto A., Araujo L., Vilchez J.L. *J. Chromatogr. A,* 975 (**2002**) 355.
75. Ahmed E.F. *Trends Anal. Chem*. 20 (**2001**) 649.
76. Wu J., Tragas C., Lord H., Pawliszyn J. *J. Chromatogr. A* 976 (**2002**)357.
77. Hu R.W., Hennion B., Urruty L., Montury M. *Food Addit. Contam.* 16 (**1999**) 111.
78. Kataoka H., Saito K., *J. Pharm. Biomed. Anal.* 54 (**2011**) 926–950
79. Imaizumi M., Saito Y., Hayashida M., Takeichi T., Wada H., Jinno K. *J. Pharm. Biomed. Anal.* 30 (**2003**) 1801.

80. Volmer D.A, Hui J.P.M. *Rapid Commun. Mass Spectrom.*; 11(**1997)** 1926.
81. Wooten J.V., Ashley D.L., Calafat A.M. J. *Chromatogr. B*; 772 (**2002**) 147.
82. Mester Z., Pawliszyn J. *J. Chromatogr. A* 873 (**2000**) 129.
83. Rodil R., Carro A.M., Lorenzo R.A., Abuin M., Cela R. *J. Chromatogr. A* 963 (**2002**) 313.
84. Dunemann L., Hajimiragha H., Begerow J. Fresenius' *J. Anal. Chem.*; 363 (**1999**) 466.
85. Guidotti M., Vitali M. J. *High Resolut. Chromatogr.* 21 (**1998**) 665.
86. Fustinoni S., Giampiccolo R., Pulvirenti S., Buratti M.,Colombi A. *J. Chromatogr. B* 723 (**1999)** 105.
87. Bergamaschi E., Brustolin A., De Palma G.,Manini P.,Mozzoni P., Andreoli R., Cavazzini S., Mutti A. *Toxicol. Lett.* 108 (**1999**) 241.
88. Liu J.T., Hara K., Kashimura S., Hamanaka T., Tomojiri S., Tanaka K. *J. Chromatogr. A*; 731 (**1999**) 217.
89. Namera A., Yashiki M., Nagasawa N., Iwasaki Y., Kojima T. *Forensic Sci. Int.*; 88 (**1997)** 125.
90. Kusakabe T., Saito T., Takeichi S. *J. Chromatogr. B* 761 (**2001**) 93.
91. Mullett W.M., Levsen K., Lubda D., Pawliszyn J. *J. Chromatogr. A* 963 (**2002**) 325.
92. Musteata F.M., Musteata M.L., Pawliszyn J., *Clin. Chem*. 52 (**2006**) 708–715.
93. Es-haghi A., Zhang X., Musteata F.M., Bagheri H., Pawliszyn J., *Analyst* 132 (**2007**) 672–678.

94. Zhang X., Es-haghi A., Musteata F.M., Ouyang G., Pawliszyn J., *Anal. Chem.* 79 (**2007**) 4507–4513.
95. Wen Y., Fan Y., Zhang M., Feng Y.Q., *Anal. Bioanal. Chem.* 382 (**2005**) 204–210.
96. Oliveira A.F., Maia P.P., Paiva. M.J., Siqueira, M.E., *J. Anal. Toxicol.* 33 (**2009**) 223–228.
97. Poli D., Manini P., Andreoli R., Franchini I., Mutti A., *J. Chromatogr. B* 820 (**2005**) 95–102.
98. Salgado-Petinal C., Lamas J.P., Garcia-Jares C., Llompart M., Cela R., *Anal. Bioanal. Chem*. 382 (**2005**) 1351–1359.
99. Saito K., Yagi K., Ishizaki A., Kataoka H., *J. Pharm. Biomed. Anal.* 52 (**2010**) 727–733
100. Djozan D., Baheri T., *J. Chromatogr. Sci*. 48 (**2010**) 224–228.
101. Cha D., Cheng D., Liu M., Zeng Z., Hu X., Guan W., *J. Chromatogr. A* 1216 (**2009**) 1450– 1457.
102. Kataoka H., Inoue R., Yagi K., Saito K., *J. Pharm. Biomed. Anal.* 49 (**2009**) 108–114.
103. Zhang Z.-M., Cai J.-J., Ruan G.-H. Li G.-K, *J. Chromatogr. B* 822 (**2005**) 244–252.
104. Schulz K., Schlenz K., Malt S., Metasch R., Römhild W., Dressler J., Lachenmeier D.W., *J. Chromatogr. A* 1211 (**2008**) 113–119.
105. Fuchs P., Loeseken C., Schubert J.K, Miekisch W., *Int. J. Cancer* 126 (**2010**) 2663–2670.
106. Ligor M., Ligor T., Bajtarevic A., Ager C., Pienz M., Klieber M., Denz H.,.Fiegl M, Hilbe W., Weiss W., Lukas P., Jamnig H., Hackl M., Buszewski B., Miekisch W., Schubert J., Amann A., *Clin. Chem. Lab. Med.* 47 (**2009**) 550–560.

107. Buszewski B., Ulanowska A., Ligor T., Denderz N., Amann A., *Biomed. Chromatogr.* 23 (**2009**) 551–556.
108. Sporkert F., Pragst F., *Forensic Sci. Int.* 107 (**2000**) 129.
109. Gentili S, Torresi A, Marsili R, Chiarotti M, Macchia T. *J. Chromatogr. B* 780 (**2002**) 183.
110. Liu J.T., Hara K., Kashimura S., Kashiwagi M., Kageura M., *J. Chromatogr. B* 758 (**2001**) 95.
111. Sporkert F., Pragst F., *J. Chromatogr. B* 746 (**2000**) 255.
112. Pragst F, Spiegel K, Sporkert F, Bohnenkamp M., *Forensic Sci. Int.* 107 (**2000**) 201.
113. Hartwig S., Auwarter V., Pragst F., *Forensic Sci. Int.* 131 (**2003**) 90.

CHAPTER 2

DERIVATIZATION IN GAS-CHROMATOGRAPHY

Introduction

Derivatization in gas chromatography (GC) is necessary when the compounds to be analyzed are not quite volatile. For example conversion of analytes into volatile derivatives allows to separate and analyze by GC amino acids, sugars , ecc. The presence of different polar groups in molecules is one the principal problem in GC. Groups with active hydrogen such as -SH, -OH, -NH and -COOH capable to form hydrogen bonds are responsible both for the low volatility of the compounds and for other phenomena that make direct GC either difficult or impossible, e.g., strong adsorption on the support of the stationary phase and asymmetry of peaks eluted from it, and thermal and chemical instability of the compounds, which cause losses of the sample compounds in the chromatographic system, i.e., their non-quantitative elution or the elution of decomposition products (1). Since GC is used to separate volatile organic compounds, modification of the functional group of a molecule by derivatization enables the analysis of compounds that otherwise can not be readily monitored by GC. With the derivatization important aspects of GC as suitability, efficiency and detectability can be improved. Suitability is the form of compounds that is amenable to the analytical technique, for GC, it is a requirement that the compound to be analyzed should be volatile to gas chromatographic analysis conditions.

Efficiency is the ability of the compound of interest to produce good peak resolution and symmetry for easy identification and practicability in GC analysis. Interactions between the compounds themselves or between the

compounds and the GC column may reduce the separation efficiency of many compounds s and mixtures. Derivatization of analyte molecules can reduce these interactions that interfere with analysis. Detectability is the outcome signal that emanates from the interaction between the analyte and the GC detector. Increasing the amounts of materials will impact the range at which they can be detected in Gas chromatography. This can be achieved either by increasing the bulk of the compound or by introducing onto the analyte compound, atoms or functional groups that interact strongly with the detector and hence improve signal identification. Detectability is the outcome signal that emanates from the interaction between the analyte and the GC detector. Increasing the amounts of materials will impact the range at which they can be detected in Gas chromatography. This can be achieved either by increasing the bulk of the compound or by introducing onto the analyte compound, atoms or functional groups that interact strongly with the detector and hence improve signal identification (2). Derivatizations in GC sample preparation are micro-scale synthetic reactions that commonly replace active hydrogens in functional groups with less polar and thermally stable groups. Chemical reactions that are frequently employed to achieve this include silylation, alkylation, acylation and various condensation reactions. Several functional groups may be affected by a single reaction, such as silylation occurring on carboxyl, hydroxyl, and amine functionalities. Alkylation can form ethers, esters and enol ethers from hydroxyls, carboxyls and carbonyls respectively. The type of derivatizing reagent utilized and the selection of reaction conditions determine the reactivity toward a specific functional group. The main requirement for a successful derivatization reaction are: a single derivative should be formed for each compound; the derivatization reaction should be simple and rapid, and should occur under mild condition; the derivative should be formed with a high and reproducible yield and should be

stable in the reaction medium; in quantitative analyses, the calibration curve should be linear (3).

Derivatization reactions used in gas chromatography can be classified in three general reaction types; Alkylation of which the general process is esterification, Silylation and Acylation.

2.1 Alkylation

Alkyl derivatives are formed by the replacement of active hydrogen with an alkyl or aryl group to form esters and ethers. The main functional group subjected to alkylation reactions are carboxylic acid, phenols, alcohols and thiols. Ammines, amides and sulfonylamides may be also alkylated, but are considered better targets for silylation or acylation reactions. The most common reagents in alkylation reactions are low molecular weight alkyl halides (e.g. iodomethane, iodoethane, iodopropane etc.), followed by benzyl- and substituited benzyl bromides (e.g. pentafluorobenzyl bromide, PFBBr). Most of the alkylation reactions using these reagents take place in the presence of a catalyst such a dry potassium carbonate, sodium hydride, silver oxide etc. alkyl derivatives show good chromatographic performance an due to the possible selection of relatively small alkyl groups can derivatize sterically hindered groups.(4-7). Alkylation of carboxylic acids can also be achieved by esterification with alcohols. Methanol or ethanol containing an acidic catalyst, such as hydro chloric acid, sulfuric acid or boron trichloride, have been used to form methyl or ethyl esters (8-9). Diazoalkanes have been used to alkylate moderately acidic functional groups, such as carboxylic and sulfonic acids, phenols and enols. Lewis acids, such as boron trifluoride etherate, have been used as catalysts to promote the alkylation of less reactive hydrogens, such as aliphatic alcohols, but their use is not recommended for extremely acid-labile compounds.

The range of possible reaction is extensive; the possibility of side reactions during alkylation of new compounds with diazoalkanes should be interpreted with care. Diazomethane is the diazoalkane most frequently used (10-12). Diazomethane (N_2CH_2) is the quickest and cleanest method available for the preparation of analytical quantities of methyl esters. The reaction of diazomethane with a carboxylic acid is quantitative and essentially instantaneous in ether solutions. In the presence of methanol as catalyst, diazomethane reacts rapidly with fatty acids, forming methyl esters. The derivatization reaction of carboxylic acids to methyl esters with diazomethane can be represented as follows:

$RCOOH + {}^{-}CH_2N^{+}N \rightarrow RCOOCH_3 + N_2$

Pentafluorobenzyl bromide ($C_7H_2F_5Br$) and also Pentafluorobenzyl-hydroxylamine hydrochloride can be used to esterify phenols, thiols, and carboxylic acids. The general scheme of the reaction for $C_7H_2F_5Br$ is as follows:

$R`OR + C_7H_2F_5Br \rightarrow R`OC_7H_2F_5 + RBr$

When using benzyl bromide as a derivatizing, it reacts with the acid part of an alkyl acid to form an ester, and therefore increase the volatility of the analyte of interest. Benzyl bromide is used for the determination of long chain perfluorinated acids (13)

The following equation shows the chemical reactions of acetylation of perfluorooctanoic acid to the respective ester.

$CF_3(CF_2)_6COOH + C_7H_2F_5Br \rightarrow C_{15}H7F_{15}O_2 + HBr$

N,N-Dimethylformamide dialkyl acetals react with carboxylic acids, phenols and thiols to form the corresponding alkyl derivatives. The reagents are sensitive to moisture and the reaction must be performed under dry conditions. Dialkylacetals have a wider applicability for the derivatization of a number of functional groups containing reactive hydrogens. Because the principal reaction product is dialkylacetals (DMF), the isolation of the derivative is not required and the reaction mixture can be injected directly into the gas chromatograph (14)

The reaction between N, N-dimethylformamide dimethylacetal and Carboxylic acid is as follows:

$$CH_3CH_3NCHORORR + R`COOH \rightarrow R`COOR + ROH + CH_3CH_3NCHO$$

Although carboxylic acids, phenols, and thiols react quickly with DMF, to give the corresponding alkyl derivatives, hydroxyl groups are not readily methylated.

Acidic compound can be derivatized in the anionic form, such as ionized carboxylic acids and sulfonamides (15-17) using extractive alkylation. The acidic substance is extracted as an ion pair with a quaternary ammonium hydroxide into an appropriate immiscible organic solvent. The alkylation reaction involving nucleophilic displacement with an alkyl halide occurs in the organic phase. Alkylation of acidic compounds can be carried out by thermal decomposition of quaternary alkylammonium salts of the acid in the heated injector port of the gas chromatograph. Tetramethylammonium droxide, trimethylanilinium hydroxide or phenyl tri methylammonium hydroxide are usually used to prepare methyl derivatives (18-21). The injector temperature should be set to 250–300°C.

2.2 Silylation

Silylation is probably the most widely used derivatization technique for GC–MS analyses (22-24). Silylation is the introduction of a silyl group into a molecule, usually in substitution for active hydrogen such as dimethylsilyl [$SiH(CH_3)_2$], t-butyldimethylsilyl [$Si(CH_3)_2C(CH_3)_3$] and chloromethyldimethylsilyl [$SiCH_2Cl(CH_3)_2$]. Replacement of active hydrogen by a silyl group reduces the polarity of the compound and reduces hydrogen bonding. The silylated derivatives are more volatile and more stable and thus yielding narrow and symmetrical peaks (25). The silylation reaction is driven by a good leaving group, which means a leaving group with a low basicity, ability to stabilize a negative charge in the transitional state, and little or no back bonding between the leaving group and silicon atom (22). The mechanism involves the replacement of the active hydrogens (in -OH, -COOH, -NH, -NH2, and –SH groups) with a trimethylsilyl group. Silylation then occurs through nucleophilic attack (SN_2), where the better the leaving group, the better the siliylation. The general reaction of silylation is shown figure 4.

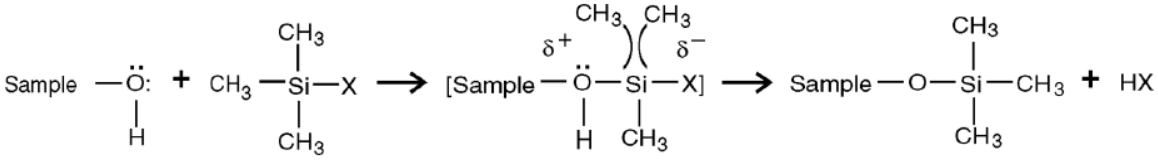


Figure 4. *General scheme reaction of silylation*

Nearly all protic functional groups present in organic compounds can be converted to silyl ethers or esters. The ability of various functional groups to form silyl derivatives is as follows: alcohols > phenols > carboxylic acids > amines > amides.

The most common silylation procedure is trimethylsilylation. Higher alkyl homologous or halogen containing analogous have been used to increase

hydrolytic stability of the derivative, to improve detectability with some particular detectors, to improve resolution or to obtain mass spectra of higher diagnostic value (24,26).

The advantage of these derivatives is evident with compounds that have different functional groups in the molecule: all groups are converted into the derivative in a one-step reaction. However, it should be noted that silyl derivatives have not always been successful and are not such ideal derivatives as was originally expected (1)

Many methods for the preparation of TMS derivatives have been developed. Commercial reagents prepared for immediate use are available. Mixtures of reagents with solvents are supplied for methods elaborated for individual substrates.

Individual types of reagents can be classified into four groups

(i) trimethylchlorodisilane, pure or with an acceptor of the acid or with a catalyst;

(ii) hexamethylsilazane (HMDS), mostly with addition of trimethylchlorosilane (TMCS) as a catalyst;

(iii) silylamines, such as trimethylsilyldiethylamine and trimethylsilylimidazole;

(iv) silylamides and others; N,O-bis(trimethylsilyl)acetamide (BSA), N,Obis(trimethylsily1)-trifluoroacetamide (BSTFA), and N-methyl-trimethylsilyltrifluoroacetamide, (MSTFA) are often used.(1)

HMDS is a mild reagent. It is used with the addition of TMCS, for the silylation of hydroxyl groups (27). Stronger reagents such as BSA (28) and BSTFA (29) are used for the silylation of less reactive groups (-NH2, -NH-) and of sterically hindered groups. N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) is the most volatile of the trimethylsilyl acetamides. It is most useful for the analysis of volatile trace materials where the derivatives may be

near the reagent or by-product peak. The addition of a catalyst has been used to increase the silylating power of these reagents to derivatize sterically hindered functions or to enhance reaction rates. Trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSIm), trimethyliodosilane (TMSI), or potassium acetate have been used as catalysts (30). BSTFA with 1% TMCS as a catalyst has been widely used to analyze drugs of abuse and their metabolites (31-34). Pyridine or another solvent with a large solvation capacity (acetonitrile, dimethylformamide) are mostly used as solvents in the silylation reactions. Pyridine provides on some phases a broad tailing peak and can overlap lower components. In silylation derivatisation, anhydrous conditions are essential because the derivatives are decomposed by traces of water (1).

2.3 Acylation

Acylation is another widely used derivatization method in GC–MS. It consists of the introduction of an acyl group in a molecule holding reactive atom of hydrogen. Compounds that contain active hydrogens (e.g., -OH, -SH and -NH) can be converted into esters, thioesters and amides, respectively, through acylation (35). Acylation reactions can be performed using three main types of reagents: acyl halides, acid anhydrides or reactive acyl derivatives such as acylated imidazoles. The general reaction of acylation is shown in figure 5:

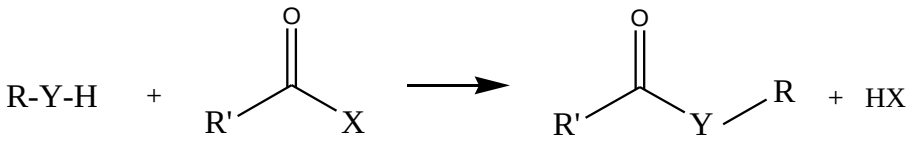


Figura 5 *General scheme reaction of acylation*

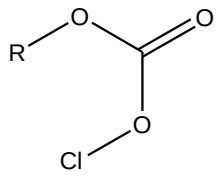
Acyl halides are highly reactive and are very useful in case of compounds that are difficult to acetylate. When acyl halides are used, a basic acceptor for halides acid produced in the reaction is required. The elimination of the excess acylating reagent is preferable prior to GC analysis to prevent deterioration of the column. The use of acetic anhydride (AA), generally in the presence of pyridine as acidic acceptor may be preferred because the excess reagent is easier to remove. The strongly acidic conditions of the reaction medium when used acyl halides are and anhydrides can lead to undesirable side reactions (dehydration, enolization, etc.). Acylation of acid sensitive compound can be performed using reagents that have a high acylation reactivity, such as acylimidazoles, and in which the by-product of the reaction is a basic leaving group. Haloalkylacyl derivatives are the most common acyl derivatives.

Perfluoroacyl derivatives such as trifluoroacetyl (TFA), pentafluoropropionyl (PFP) and heptafluorobutyryl (HFB), are the most widely used in practice (3). The preparation of perfluoroacyl derivatives can be performed by re action with the appropriate acid anhydrides sometimes in the presence of a basic catalyst (36-40) or by reaction with perfluoroacylimidazoles. Alcohol and primary and secondary amines can be acetylated by imidazole reagents. These reagents are very sensitive to moisture and undergo hydrolysis, whereby when the derivatization products are stable enough, the excess reagent can be removed, by using a wash with an aqueous solution. N-methyl bis(trifluoroacetamide (BSTFA) or bis(trifluoroacetamide) (BSTFA) can be used for trifluoroacetylation, also under mild condition, of amine, hydroxyl and thiol groups (41) . These reagents are highly volatile and do not interfere in the GC analysis, and the reaction mixture can be directly analyzed with no adverse effects on GC column. Phenolalkylamines, hydroxy amines and

amino acids can be selectively derivatized as N-TFA-O-TMS (42-49) using MSTFA as trimethylsilylating reagent followed by MBTFA as trifluoroacylating agent. Also the extractive acylation was performed using different reagents. TFA derivatives of primary and secondary amines can be obtained using a mixture of ether and MBTFA at alkaline pH (50). Amines and phenols can be also derivatizated by extractive acylation using acetic anhydride or pentafluorobenzoyl chloride (51)

2.4 Derivatization with Alkyl Chloroformates

Alkyl chloroformates (figure 6) are compound known as rapid esterification agents. The chloroformates commonly used are those with simple alkyls, i.e. methyl, ethyl propyl or isobutyl.



R: methyl, ethyl propyl or isobutyl.

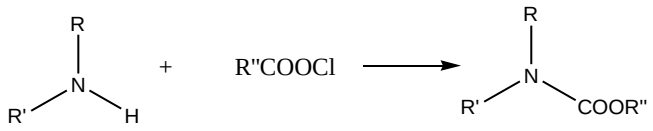
Figure 6. *Alkyl chloroformate*

The alkyl chloroformate has been used for several years for trating ammino groups in GC, and later proved to be excellent for treating. Esterification of carboxylic acids, catalyzed by pyridine in the analytical microscale gives excellent results, in contrast to the poor results obtained with with chloroformate-mediated ester formation in organic chemistry.In addition of the catalizer an alcohol should also be present in the medium. Most derivatization reactions use apolar reagents contrary to that, chloroformates have been widely used for derivatization in aqueous media (52).

Optimizing the reaction conditions for various classes of carboxylic acids is possible to obtain a unique rapid derivatization of amino acids in aqueous medium. This allows the treatment of several analytes as acidic metabolites, or the simultaneous analysis of amino and fatty acids or of amines and their acidic catabolytes directly in the aqueous matrix.

2.4.1 Derivatization of Amino Groups

Chloroformates have been widely used to convert amines into carbamates in buffered aqueous media.

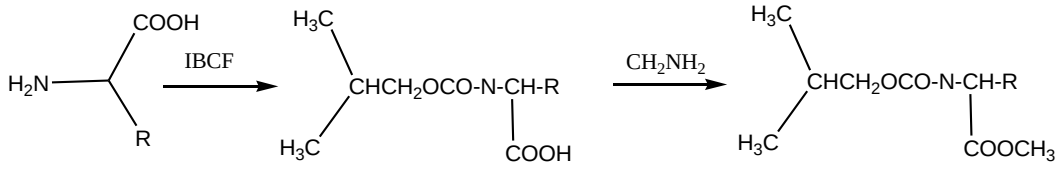


R'': methyl, propyl , ethyl or isobutyl

Methylchloroformate (MCF) and ethylchloroformate (ECF) have been used in the early 80s for derivatize biogenic amines (53-55).

Many derivatization of amines have been performed using isobuthylchloroformate (IBCF) because it represent the best compromise between a derivative of higher mass and one of greater stability. Urinary aliphatic amines (56) and 57 amines in aqueous samples (52) can be derivatized by extractive alkylation with IBCF in organic solvent and the analytes in basified water. Alkyl chloroformates can derivatize both the phenolic and the amino groups. In the latter' case the yeald of derivatization is pH- dependent. Some authors (57) reported maximal yields in the pH range 7–8.5, others introduced a pH shift from 7.5 to 9 or more to provide for phenolic hydroxyl and to improve reaction of the amine functions (58,59), or treated the amines at pH 12 (60). Another important application of chloroformate is the derivatization of amino acids. The first applications were

carried out by Makita et al. (61-63) and concerned the treatment of amino acids with IBCF in aqueous carbonate. This procedure, based in two steps, consist in the alkylation of the amino group and most of the side-chain groups with IBCF in the carbonate (10 min at 20°C) and subsequent methylation of the carboxyl groups with diazomethane in ethereal extract from the preceding.



An important improvement in the methodology in the treatment of amino acids is the one–step derivatization. (64, 65) The modification of condition reaction using reagent and alcohols with different alkyls has led to considerable improvements of the procedure. Alcohol plays an important role in the derivatization reaction the proposed mechanism is based on the formation of an intermediate mixed carboxylic—carbonic acid anhydride followed by the exchange with an alcohol. The authors examined various combinations of reagents and alcohols in mutual inter-reaction and concluded that the mixed anhydride underwent an exchange reaction with the alcohol present (pathway A) leading to the principal product: A small amount of minor product with the same alkyl group as that of the reagent was also found. In view of this, either the decarboxylation mechanism (route B) or the alcoholysis by the action of the alcohol, liberated from the reagent (route C), was proposed.

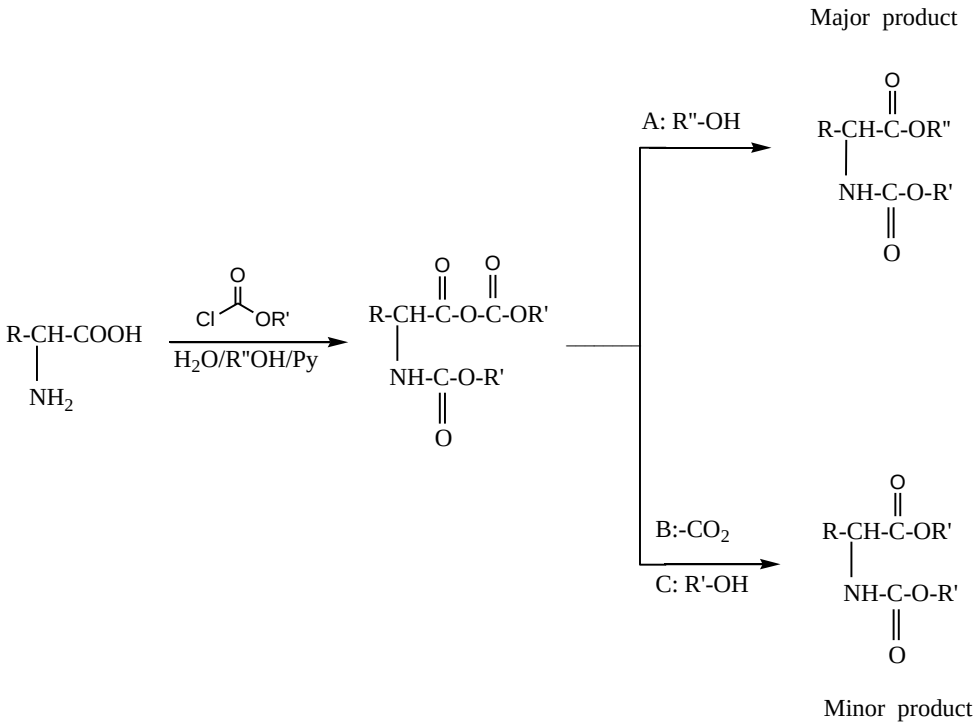


Figure 7 *Reaction of alkyl chloroformates with amino acids (Different pathways of reaction)*

It is therefore evident that to have a single product of derivatization is necessary to use the alkyl chloroformate in presence of the corresponding alcohol (66). Alkyl chloroformates were used for the analysis of amino acids in various matrices: serum (67), plasma, (68) urine (69) and biological fluids (70).

2.4.1 Derivatization of Carboxylic acids

As previously mentioned above, the carboxylic acids can be converted in esters by alkyl chloroformate in presence of pyridine. Fatty acids at different length of alkyl chain can be converted to alkyl esters virtually instantaneously (71).The optimum composition of the reaction i.e. acetonitrile–pyridine–

alcohol in a ratio of 22:2:1, is identical for short and long-chain fatty acids (70, 71). Also the aliphatic keto acids can be esterified directly without oximation of the keto group, as with silylation procedure (72). Various keto acids can be derivatized with good results (73). The yield of the derivatization is strongly influenced by the composition of the action medium, in which acetonitrile is the prevalent solvent and the water content, if any, should not exceed 25%, and beyond this limit is observed, a decline of the reaction yield. Polycarboxylic fatty acids (di, tri, etc.) are not directly esterified with acidified alcohols. Dicarboxylic acid from C2 to C12 can be converted to esters with MCF and ECF with the yield of derivatization that is independent of the composition of the medium (73). The yeald of derivatizzation of oxalic, succinic and glutaric acids when acetonitrile prevailed in the reaction medium is negligible or low, especially with ECF. These acids prefer to form alternative products even under conditions of ester formation. To promote the formation of esters, alcohol should replace acetonitrile in the reaction medium partially or fully and the reagent should be added successively.

Another important class of compounds is that of the aromatic acids. Compounds such as phenolic acids (benzoic, phenilacetic phenylpropenoic, phenylglycolic, phenyl- propionic and their variously substituited form) are partially present in body fluids, in beverage, etc. These compounds were easily converted into their methyl and ethyl esters, with much better results than the esterification of aromatic acids chloroformate-induced in organic chemistry (74). Addition of up to 30–40% of water to the acetonitrile–alcohol medium did not seem to influence the reaction yields much. Only the benzoic acid and some of its substituents, manifest a different behavior. This class of compounds, with a carboxylic group directly attached to the benzene ring, has behaviour different from others. When these react with chloroformates, different products of reaction are observed: the alkyl ester, the free acid and

the alkoxycarbonyl ester (the mixed anhydrides). The changing of the medium composition doesn't affect the yield of reaction. The mixed anhydrides if treated with aqueous bicarbonate decompose nearly entirely to the salts and possible to favor the reaction toward the formation of alkyl esters (52). The optimization of reaction medium is crucial for have good yields of derivatization.

In a study for the analysis of phenolic acids as chloroformate derivatives in aqueous media (75), the composition of reaction medium (acetonitrile, water, alcohol and pyridine) has been modified and optimized. The seven phenolic acids to considered (caffeic, ferulic, gallic, p-coumaric, protocatechuic, syringic and vanillic) has been derivatized into corresponding methyl/ethyl esters by methyl and ethyl chloroformate using two different composition of reaction medium for methyl and ethyl derivatives.

The path of the reaction can occur in two ways as shown in reaction scheme for the vanillic acid, and is dependent on the composition of the reaction medium. Mixed anhydrides are formed preferably, when occurs the straight coupling of the reagent to the carboxylic group excluding alcohol from the medium. Conversely, the alkyl esters can be formed by both by rapid decarboxylation of the mixed anhydrides in aqueous bicarbonate solution, or better by an exchange reaction with alcohol added to the reaction medium. Concerning the hydroxyl groups, they are simultaneously converted into ethers alkoxycarbonyl. The ratio of acetonitrile, water, methanol/ethanol and pyridine has been varied in order to get the best composition of the reaction medium.

The optimal compositions of the reaction medium obtained from these experiments are: CH_3CN/H_2O/MeOH/Pyr in ratio 14:10:2:3 in the derivatization with methyl chloroformate, and CH_3CN/H_2O/EtOH/Pyr in ratio 2:4:2:2 in the derivatization with ethyl chloroformate.

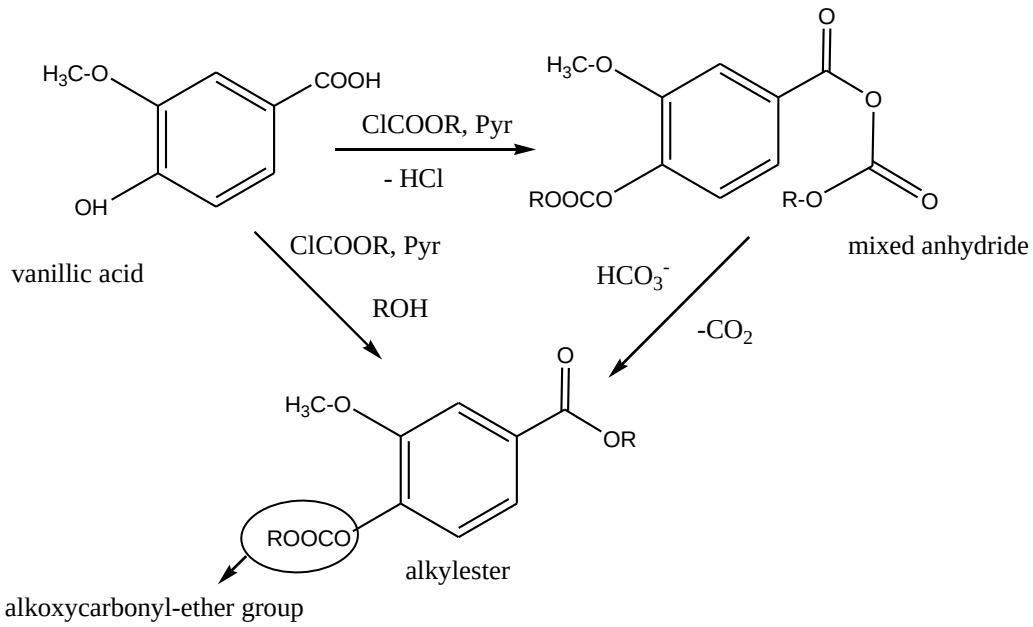


Figure 8. *Reaction scheme of the phenolic acids derivatization treated with chloroformates (Possible formation of mixed anhydride and alkylester via different pathways).*

Hydroxycarboxylic acids are another important class of acids that can be derivatized with alkyl chloroformates. When phenolic hydroxyl and alcoholic groups not adjacent to the carboxyl on an aliphatic are treated with chloroformates, the former group is always esterified smoothly, the latter remained untouched. Problems are observed in the derivatization of 2-hydroxycarboxylic acids (HAs), with MCF and ECF, due to an excessive one involving both the activated functional groups. Several metabolites present in body fluids belong to the category of the Has, and the possibility to have reproducible derivatization yields with these analytes is particularly important. The main reaction product of Has with chloroformates is the expected O-alkoxycarbonyl alkylesters (R'OCOO-CHR-COOR'). Besides the main product a number of side-products are observed, whose abundance appeared to be largely dependent on the reaction conditions chosen. The amount of alcohol in the reaction medium influences strongly the alkylation

yield of the α-positioned OH-group. Particularly, it was observed that the alcohol prevents an effective esterification of the hydroxyl group and from this point of view, its portion in the medium should be held as low as possible. The presence of water to 10% for treatment with MCF and up to 20% for that with ECF proved to be acceptable without noticeable changes in the yield (76). The content of pyridine to 10% or more increases the formation of main product. Exclusion of alcohol, lowering the pyridine amount below that of the reagent and a partial replacement of acetonitrile by acetone resulted in formation of the mixed anhydrides. A different behavior was been observed with lactic and butyric acids. It is identified the formation of product as inter-ester oligomers (dimers, trimers) with a mostly derivatized and partly underivatized terminal OH-group, plus the lactide dimmer. The process of inter-ester formation might be considered as a 'self-alcoholysis' caused by mutual interaction of an activated carboxyl of one molecule with an activated 2-OH-group of another molecule. With hydroxy acids having the alcohol group not adjacent to the carboxyl, i.e. with 3-OH or 4-OH butyric acids, the formation of inter-esters was not observed. Complete elimination of the side-products did not appear to be possible under the chosen reagents and reaction conditions. However, a solution was found to improve the results substantially. It was based on altering the sequence of base and reagent addition (77). With such a 'reversed mode' (base after reagent) addition, the yield of the main product was higher by 30% with MCF (Fig. 20) and by about 20% with ECF.Upon admixing 10 to 25% of water to the acetonitrile with 1% of methanol or 5% of ethanol for the MCF and ECF treatment, the lactic acid converted to the main product by more than 90%.To conclude, HAs, namely the lactic one, should be treated with MCF and ECF via the reversed mode in acetonitrile with 1 to 5% of alcohol. The presence of up to 10% of admixed water is tolerable.

References

1. Drozd J., Novák J. P., 19 *J. Chromatogr. Library* (**1981)**
2. Orata F. *Advanced Gas Chromatography – Progress in Agricultural, Biomedical and Industrial Applications*. (**2012)** InTech
3. Segura J., Rosa Ventura R., Jurado C., *J. Chromatogr. B,* 713 (**1998**) 61–90
4. Segura J, Rosa Ventura R, Jurado C., *J. Chromatogr. B* 687 (**1996**) 127.
5. Davis B., *Anal.Chem.* 49 (**1977**) 832
6. Galceran, M.T., Moyano E., Poza J.M., *J. Chromatogr. A,* 710 (**1995**) 139.
7. Sullivan W.R., Fox K.E., *J. Chromatogr.* 425 (**1988**) 396.
8. Segura J., Mestres M., Aubets J., de la Torre R., Ugena B., Cam´ı J., *Biomed. Environ. Mass Spectrom.* 16 (**1988**) 361.
9. Pfleger K., Maurer H.H. , Weber A., *Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and Their metabolites* VCH, Weinheim, 2nd ed., **1992.**
10. de Baere S.M,. Lambert W.E, van Bocxlaer J.F., de Leenheer A.P., *J. Anal. Toxicol.* 20 (**1996**) 159.
11. Szirmai M., Beck O., Stephansson N., Halldin M.M., *J. Anal. Toxicol*. 20 (**1996**) 573.
12. Orata F., Quinete N., Wilken R.D., *Bulletin of Environmental Contamination and Toxicology* 83 (**2009**) 630-635.
13. Ribeiro B., Guedes de Pinho P., Andrade B.P., Baptista P., Valentão P., *Micro chemical Journal* 93 (**2009**) 29–35.
14. Lisi A.M., Trout G.J., Kazlauskas R., *J. Chromatogr*. 563 (**1991**) 257.
15. Vessman N., Karlsson K.E., Gyllenhaal O., *J. Pharm. Biomed. Anal.* 4 (**1986**) 825.

16. Lisi A.M., Kazlauskas R., Trout G.J., *J. Chromatogr.* 617 (**1993**) 265.
17. Hagedorn H.W., Schulz R., *J. Anal. Toxicol.* 16 (**1992**) 194.
18. Barroso M.B., Meiring H.D., de Jong A., Alonso R.M., Jime´nez R.M., *J. Chromatogr. B* 690 (**1997**) 105.
19. Lillsunde P., Michelson L., Forsstrom T., Korte T., Schultz E., Ariniemi K., Portman M., Sihvonen M.L., Seppala T., *Forensic Sci. Int.* 77 (**1996**) 191.
20. Kintz P., Tracqui A., Jamey C., Mangin P., *J. Anal. Toxicol.* 20 (**1996**) 197.
21. Knapp D.R., *Handbook of Analytical Derivatization reactions, Wiley-Intercience*, New York, **1979**.
22. Pierce A.E., *Silylation of Organic Compounds*, Pierce Chemical Company, Chicago, IL, **1968**.
23. Evershed R.P., Blau K., Halket J.M. (Editors), *Handbook of Derivatives for Chromatography*, Wiley, Chichester, 2nd ed., (**1993)**, 51.
24. Kataoka, H., *J. Chromatogr. Library.* 70, (**2005**), 364-4
25. van Look G., *Silylating Agents*, Fluka Chemie AG, Buchs, Switzerland, **1995**.
26. Sweeley C.C., Bentley R., Makita M., Wells W.W., *J. Amer. Chem. Soc.* 85 (**1963**) 2497.
27. Wilk S., Gitlow S.E., Franklin M.J., Carr H.E., *Clin. Chim. Acra.* 10 (**1964**) 193.
28. Cummins L.M., Fourier M.J., *Anal. Lett.* 2 (**1969**) 403.
29. Opfermann G., Schaenzer W., Schaenzer W., Geyer H., Gotzmann A., Mareck-Engelke U., *Recent Advances in Doping Analysis* (4), Sport and Buch Strauss, Köln, **1997**, 247.

30. Wang W.L., Darwin W.D., Cone E.J., *J. Chromatogr. B* 660 (**1994**) 279.
31. Fritch D., Groce Y., Rieders F., *J. Anal. Toxicol*. 16 (**1992**) 112.
32. Black D.A., Clark G.D., Haver V.M., Garbin J.A.,. Saxon J.A, *J. Anal. Toxicol* 18 (**1994**) 185.
33. Nelson C.C., Foltz R.L., *Anal. Chem.* 64 (**1992**) 1578.
34. Zenkevich I.G. *Acids: Derivatization for GC Analysis Encyclopedia of Chromatography*, Third Edition **2009**,
35. Daeseleire E.A.I., De Guesquie`re A., van Peteghem C.H., *J. Chromatogr. Sci.* 30 (**1992**) 409.
36. Chen B.H., Taylor E.H., Pappas A.A., *J. Anal. Toxicol.* 14 (**1990**) 12.
37. Grinstead G.F., *J. Anal. Toxicol.* 15 (**1991**) 293.
38. Kuhlman J.J., Magluilo J., Cone E., Levine B., *J. Anal. Toxicol.* 20 (**1996**) 229.
39. Dallakian P., Budzikiewicz H., Brzezinka H., *J. Anal. Toxicol.* 20 (**1996**) 255.
40. Donike M., *J. Chromatogr.* 78 (**1973**) 273.
41. Solans M., Carnicero R., de la Torre J., Segura, *J. Anal. Toxicol*.19 (**1995**) 104.
42. Leloux M.S., de Jong E.G., Maes R.A.A., *J. Chromatogr.* 488 (**1989**) 357.
43. Donike M., *J. Chromatogr.* 103 (**1975**) 91.
44. Donike M., *J. Chromatogr.* 115 (**1975**) 591.
45. Leloux M.S., Maes R.A.A., *Biomed. Environ. Mass Spectrom.* 19 (**1990**) 137.
46. Lho D.S., Hong J.K., Paek H.K., Lee J.A., Park J., *J. Anal. Toxicol.* 14 (**1990**) 77.
47. Hemmersbach P., de la Torre R., *J. Chromatogr. B* 687 (**1996**) 221.

48. Kraft M., Bellotti P., Benzi G., Ljungqvist A. (Editors), *Official Proceedings: IInd I.A.A.F. World Symposium on Doping in Sport, International Athletic Foundation,* **1990** 93.
49. Coutts R.T., Torok-Both G.A., Tam Y.K., Chu L.V., Pascuto F.M., *Biomed. Environ. Mass Spectrom.*14(4) (**1987**) 173-82
50. Husék P., *J. Chromatogr. B* 717 (**1998**) 57 –91
51. Gyllenhaal O., Johansson L., Vessman J., *J. Chromatogr.* 190 (**1980**) 347.
52. DeJong A.P.J.M., Cramers C.A., *J. Chromatogr.* 276 (**1983**) 267.
53. Yamamoto S., Kakuno K., Okahara S., Kataoka H., Makita M., *J. Chromatogr.* 194 (**1980**) 399.
54. Lundh T., Akesson B., *J. Chromatogr.* 617 (**1993**) 191.
55. Kim K.R., Paik M.J., Kim J.H., Dong S.W., Jeong D.H., *J. Pharm. Biomed. Anal.* 15 (**1997**) 1309.
56. Gyllenhaal O., Johansson L., Vessman J., *J. Chromatogr.* 190 (**1980**) 347.
57. Kim K.R., Paik M.J., Kim J.H., Dong S.W., Jeong D.H., *J. Pharm. Biomed. Anal.* 15 (**1997**) 1309.
58. Lundh T., Akesson B., *J. Chromatogr.* 617 (**1993**) 191.
59. Makita M., Yamamoto S., Kono M., Sakai K., Shiraishi M., *Chem. Ind.* 19 (**1975**) 355.
60. Makita M., Yamamoto S., Kono M., *J. Chromatogr.* 120 (**1976**) 129.
61. Makita M., Yamamoto S., Kiyama S., *J. Chromatogr.* 237 (**1982**) 279.
62. Husék P., FEBS Lett. 280 (**1991**) 354.
63. Husék P., *J. Chromatogr.* 552 (**1991**) 289.
64. Wang J., Huang Z.H., Gage D., Watson J.T., *J. Chromatogr.* 663 (**1994**) 71.

65. Tao X. , Liu Y. , Wang Y. , Qiu Y. , Lin J. , Zhao A. , Su M. , Jia W *Anal. Bioanal. Chem.* 391 (2008) 2881–2889)
66. Husék P., *Journal of Chromatography B*, 669 (**1995**) 352-357
67. Qiu Y., Sua M., Liuc Y., Chena M., Gua J., Zhang J.,. Jiaa W., *Anal.Chim. Acta* 58 (**2007**) 277–283
68. Kaspar1 H., Dettmer K., , Gronwald W., Oefner W., *J. Chromatogr. B* 870 (**2008**) 222–232.
69. Gao X., Pujos-Guillot E., Martin Jean-F., Galan P., Juste C., Jia W. , Sebedio J.L., *Anal. Biochem* 393 (**2009**) 163–175
70. Husèk P., Rijks J.A., Leclercq P.A., Cramers C.A., *J. High Resol. Chromatogr.* 13 (**1990)** 633
71. Husèk P., *J. Chromatogr*. 615 (**1993**) 334.
72. Niwa T., J. *Chromatogr*. 379 (**1986**) 313.
73. Husèk P., *LC–GC Int*. 5(9) (**1992**) 43
74. Husèk P., *Chromatographia* 34 (**1992**) 621.
75. Citová I., Sladkovsk´y R., Solich P., *Anal. Chim. Acta* 573–574 (**2006**) 231–241.
76. Husèk P.,*J. Chromatogr.* 547 (**1991**) 307.
77. Husèk P., *J. Chromatogr*. 630 (**1993**) 429.

CHAPTER 3

DESIGN OF EXPERIMENTS

Introduction

Design of experiments is a series of tests in which purposeful changes are made to the input variables of a system or process and the effects on response variables are measured. Experimental design is an effective tool for maximizing the amount of information gained from a study while minimizing the amount of data to be collected. Factorial experimental designs investigate the effects of many different factors by varying them simultaneously instead of changing only one factor at a time. Factorial designs allow estimation of the sensitivity to each factor and also to the combined effect of two or more factors. Experimental design methods have been successfully applied to several fields of research. In research, development, and production, often half of the available experimental resources are spent on solving optimization problems. With the rapidly increasing costs of experiments, it is essential that these questions are answered with as few experiments as possible. Design of Experiments, DOE, is used for this purpose to ensure that the selected experiments are maximally informative. Design of experiments, also called experimental design, is a structured and organized way of conducting and analyzing controlled tests to evaluate the factors that are affecting a response variable. The design of experiments specifies the particular setting levels of the combinations of factors at which the individual runs in the experiment are to be conducted. This multivariable testing method varies the factors simultaneously. Because the factors are varied independently of each other, a causal predictive model can be determined. Data obtained from observational studies or other data not collected in accordance with a design of experiments

approach can only establish correlation, not causality. There are also problems with the traditional experimental method of changing one factor at a time, i.e., its inefficiency and its inability to determine effects that are caused by several factors acting in combination. (1-2)

Application of Design of Experiment (DOE)

- Development of new products and processes
- Enhancement of existing products and processes
- Optimization of quality and performance of a product
- Optimization of an existing manufacturing procedure
- Screening of important factors
- Minimization of production costs and pollution
- Robustness testing of products and processes

Sectors where DOE is used

- Chemical industry
- Polymer industry
- Car manufacturing industry
- Pharmaceutical industry
- Food and dairy industry
- Pulp and paper industry
- Steel and mining industry
- Plastics and paints industry
- Telecom industry

3.1 Fundamental Principles

The fundamental principles in design of experiments are solutions to the problems in experimentation posed by the two types of nuisance factors and serve to improve the efficiency of experiments. Those fundamental principles are:

- Randomization
- Replication
- Blocking
- Orthogonality
- Factorial experimentation

The purpose of randomization is to prevent systematic and personal biases from being introduced into the experiment by the experimenter. A random of subjects or experimental material to treatments prior to the start of the experiment ensures that observation that are favoured or adversely affected by unknown sources of variation are observation " selected in the luck of the draw " and not systematically selected. Lack of a random assignment of experimental material or subjects leaves the experimental procedure open to experimenter bias. Replication is the repetition of the treatments under investigation to different experimental units. Replication is the essential for obtaining a valid estimate experimental error and to some extent increasing the precision of estimating the pair wise differences among the treatments effects. Replication increases the sample size and is a method for increasing the precision of the experiment. Replication increases the signal-to-noise ratio when the noise originates from uncontrollable nuisance variables. A replicate is a complete repetition of the same experimental conditions, beginning with the initial setup. Blocking is the simplest technique to take care of the

variability in response because of the variability in the experimental material. To block an experiment is to divide, or partition, the observation into groups called blocks in such a way that the observation in each block are collected under relatively similar experimental conditions. If blocking is done well, the comparisons of two or more treatments are made more precisely than similar comparisons from an unblocked design. Blocking is a restriction of complete randomization, since both procedures are always applied to each batch. Blocking increases precision since the batch-to-batch variability is removed from the "experimental error." Orthogonality in an experiment results in the factor effects being uncorrelated and therefore more easily interpreted. The factors in an orthogonal experiment design are varied independently of each other. The main results of data collected using this design can often be summarized by taking differences of averages and can be shown graphically by using simple plots of suitably chosen sets of averages. In these days of powerful computers and software, orthogonality is no longer a necessity, but it is still a desirable property because of the ease of explaining results. Factorial experimentation is a method in which the effects due to each factor and to combinations of factors are estimated. Factorial designs are geometrically constructed and vary all the factors simultaneously and orthogonally. Factorial designs collect data at the vertices of a cube in p-dimensions (p is the number of factors being studied). If data are collected from all of the vertices, the design is a full factorial, requiring 2^p runs. Since the total number of combinations increases exponentially with the number of factors studied, fractions of the full factorial design can be constructed. As the number of factors increases, the fractions become smaller and smaller (1/2, 1/4, 1/8, 1/16, …). Fractional factorial designs collect data from a specific subset of all possible vertices and require 2^{p-q} runs, with 2^{-q} being the fractional size of the design. If there are only three factors in the experiment,

the geometry of the experimental design for a full factorial experiment requires eight runs, and one-half fractional factorial experiment (an inscribed tetrahedron) requires four runs (Fig. 9). Factorial designs, including fractional factorials, have increased precision over other types of designs because they have built-in internal replication. Factor effects are essentially the difference between the average of all runs at the two levels for a factor, such as “high” and “low.” Replicates of the same points are not needed in a factorial design, which seems like a violation of the replication principle in design of experiments. However, half of all the data points are taken at the high level and the other half are taken at the low level of each factor, resulting in a very large number of replicates. Replication is also provided by the factors included in the design that turn out to have non significant effects. Because each factors varied with respect to all of the factors, information on all factors is collected by each run. In fact, every data point is used in the analysis many times as well as in the estimation of every effect and interaction. Additional efficiency of the two-level factorial design comes from the fact that it spans the factor space, that is, puts half of the design points at each end of the range, which is the most powerful way of determining whether a factor has a significant effect. (1, 3)

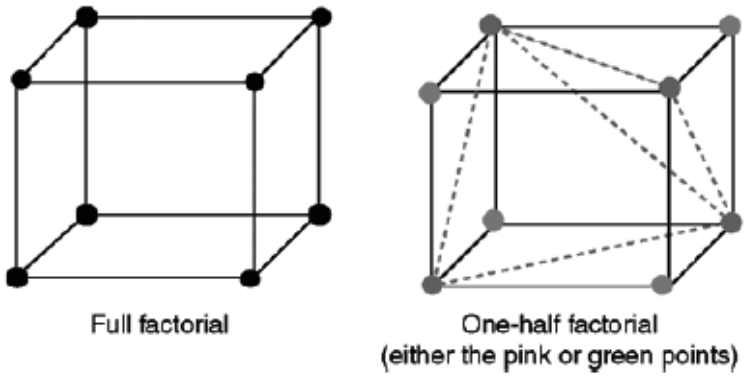


Figure 9. *Full factorial and one-half factorial in three dimensions.*

3.2 General Approach in DOE

Usually, an experimenter does not jump directly into an optimization problem, but the planning of DOE is performed in three different steps: screening, optimization, and robustness testing. Initial screening experimental designs are used in order to locate the most fruitful part of the experimental region in question. Screening is used at the beginning of the experimental procedure. The objective is (i) to explore many factors in order to reveal whether they have an influence on the responses, and to identify their appropriate ranges. With a screening design, the experimenter is able to extract a yes or no answer with regard to the influence of a particular factor. Information is also gained about how to modify the settings of the important factors, to possibly further enhance the result. Screening designs need few experiments in relation to the number of factors. Optimization is used after screening. The objective is to predict the response values for all possible combinations of factors within the experimental region, and to identify an optimal experimental point. However, when several responses are treated at the same time, it is usually difficult to identify a single experimental point at which the goals for all responses are fulfilled, and therefore the final result often reflects a compromise between partially conflicting goals. With an optimization design the experimenter is able to extract detailed information regarding how the factors combine to influence the responses. Optimization designs require many experiments in relation to the number of investigated factors.The robustness testing, and are applied as the last test just before the release of a method. When performing a robustness test of a method the objective is to ascertain that the method is robust to small fluctuations in the factor levels, and, if non-robustness is detected, to understand how to alter the bounds of the factors so that robustness may still be claimed.With a

robustness testing design, it is possible to determine the sensitivity of the responses to small changes in the factors. Where such minor changes in the factor levels have little effect on the response values, the analytical system is determined to be robust.

3.3 General Guidelines for Conducting DOE

DOE is not only a collection of statistical techniques that enable an engineer to conduct better experiments and analyze data efficiently. The general guidelines for planning efficient experiments will be here reported.

1. Clarify and State Objective. The objective of the experiment should be clearly stated. It is helpful to prepare a list of specific problems that are to be addressed by the experiment.

2. Choose Responses. Responses are the experimental outcomes. An experiment may have multiple responses based on the stated objectives. The responses that have been chosen should be measurable.

3. Choose Factors and Levels. A factor is a variable that is going to be studied through the experiment in order to understand its effect on the responses. Once a factor has been selected, the value range of the factor that will be used in the experiment should be determined. Two or more values within the range need to be used. These values are referred to as *levels* or *settings*. Practical constraints of treatments must be considered, especially when safety is involved. A cause-and-effect diagram or a fishbone diagram can be utilized to help identify factors and determine factor levels.

4. Choose Experimental design. According to the objective of the experiments, the analysts will need to select the number of factors, the number of level of factors, and an appropriate design type. For example, if the objective is to identify important factors from many potential factors, a

screening design should be used. If the objective is to optimize the response, designs used to establish the factor-response function should be planned. In selecting design types, the available number of test samples should also be considered.

5. Perform the Experiment. A design matrix should be used as a guide for the experiment. This matrix describes the experiment in terms of the actual values of factors and the test sequence of factor combinations. For a hard-to-set factor, its value should be set first. Within each of this factor's settings, the combinations of other factors should be tested.

6. Analyze the Data. Statistical methods such as regression analysis and ANOVA (Analysis of Variance) are the tools for data analysis. Engineering knowledge should be integrated into the analysis process. Statistical methods cannot prove that a factor has a particular effect. They only provide guidelines for making decisions. Statistical techniques together with good engineering knowledge and common sense will usually lead to sound conclusions. Without common sense, pure statistical models may be misleading.

7. Draw Conclusions and Make Recommendations. Once the data have been analyzed, practical conclusions and recommendations should be made. Graphical methods are often useful, particularly in presenting the results to others. Confirmation testing must be performed to validate the conclusion and recommendations.

The above seven steps are the general guidelines for performing an experiment. A successful experiment requires knowledge of the factors, the ranges of these factors and the appropriate number of levels to use. Generally, this information is not perfectly known before the experiment. Therefore, it is suggested to perform experiments iteratively and sequentially. It is usually a

major mistake to design a single, large, comprehensive experiment at the start of a study.

3.4 Types of Statistical Design

The DOE concept may be viewed as a framework for experimental planning. Following a few basic designs of this framework here reported, which are used to deal with the three major experimental objectives, and point out their common features and differences. Figure 10 provides a summary of the types of designs.

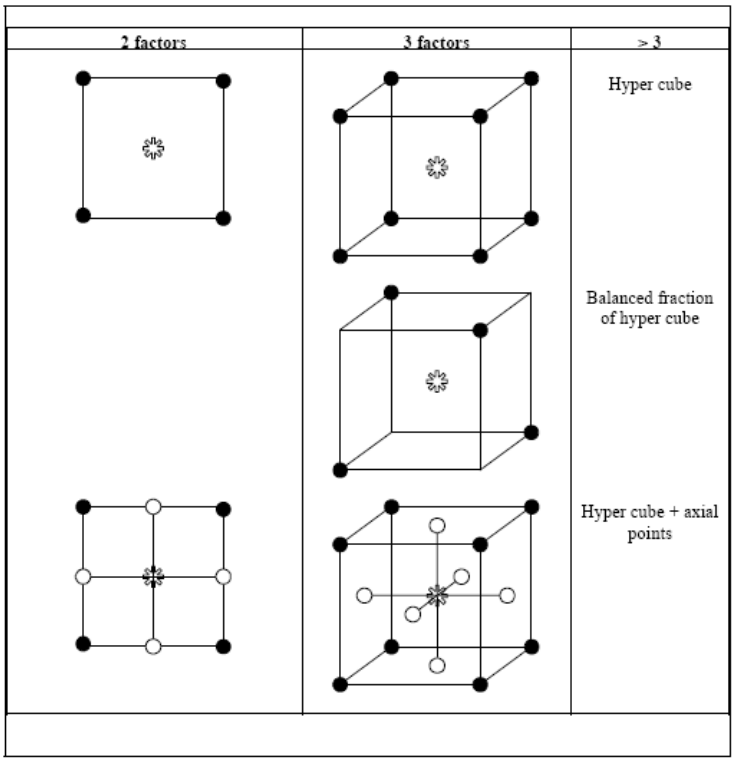


Figure 10 . *Examples of full factorial, fractional factorial, and composite designs used in DOE.*

The first row of Figure 10 shows complete, or full, factorial designs for the investigation of two and three factors. These are screening designs, and are called *full* because all possible corners are investigated. The snowflake in the interior part depicts replicated center-point experiments carried out to investigate the experimental error. Usually, between 3 -5 replicates are made. The second row in the figure also shows a screening design, but one in which only a fraction of all possible corners have to be carried out. It belongs to the *fractional factorial* design family, and this family is extensively deployed in screening. Fractional factorial designs are also used a lot for robustness testing. The last row of figure 10 displays designs originating from the *composite* design family, which are used for optimization. These are called composite designs because they consist of the building blocks, corner (factorial) experiments, replicated center-point experiments, and axial experiments, the latter of which are denoted with open circles (2).

3.4.1 Factorial Design

Factorial designs are widely used to investigate main effects and interactions. In a factorial design the influences of all experimental variables, factors, and interaction effects on the response or responses are investigated (4). There are L^k combinations of L levels of k factors in full factorial design.

If the combinations of *k* factors are investigated at two levels, a factorial design will consist of 2^k experiments (4). In full factorial designs (Fig. 11) every experiment is performed, while for fractional factorial designs a specific subset is performed that allows calculation of certain coefficients of the model The levels of the factors are given by – (minus) for low level and + (plus) for high level. A zero-level is also included, a centre, in which all variables are set at their mid value. Coded values in experimental design are used to simplify the comparison of the importance of the variables. The

physical scale for each variable is different, and to make the interpretation of significance for the different effects easier, all the variables are placed on a common scale (5). Three or four centre experiments should always be included in factorial designs, for the following reasons:

- The risk of missing non-linear relationships in the middle of the intervals is minimized, and
- Repetition allows for determination of confidence intervals.

A factorial design with three variables is illustrated in figure 11.If the value of the response for the centre point differs much from the mean value of the other experiments, it will be necessary to include quadratic terms in the model (4)

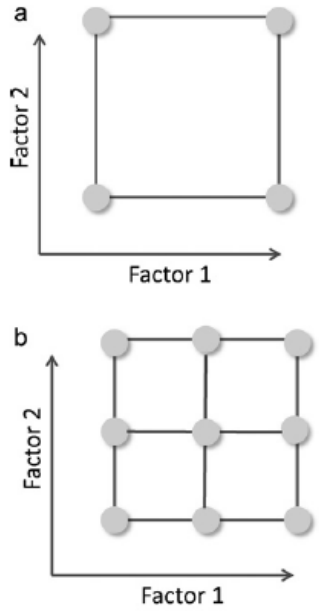


Figure 11 . *Full-factorial designs. (a) Two-factor, two-level design and (b) two-factor three-level design. Each point represents the factor values for one experiment*

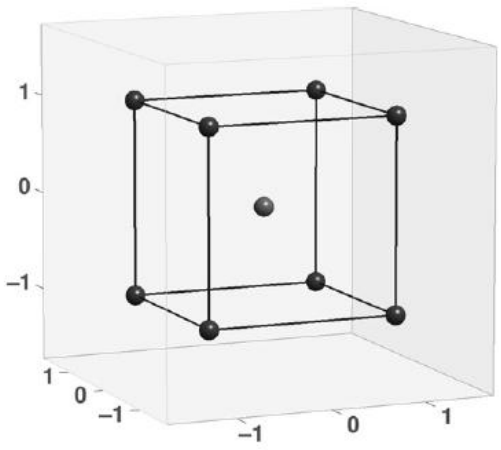


Figure 12 . *Full factorial designs with three variables*

What + and - should correspond to for each variable is defined from what is assumed to be a reasonable variation to investigate. In this way the size of the experimental domain has been settled. For two and three variables the experimental domain and design can be illustrated in a simple way. For two variables the experiments will describe the corners in a quadrate Fig. 13. While in a design with three variables they are the corners in a cube Fig. 14The sign for the interaction effect between variable 1 and variable 2 is defined as the sign for the product of variable 1 and variable 2 (Table 3) . The signs are obtained according to normal multiplication rules. By using these rules it is possible to construct sign columns for all the interactions in factorial designs.

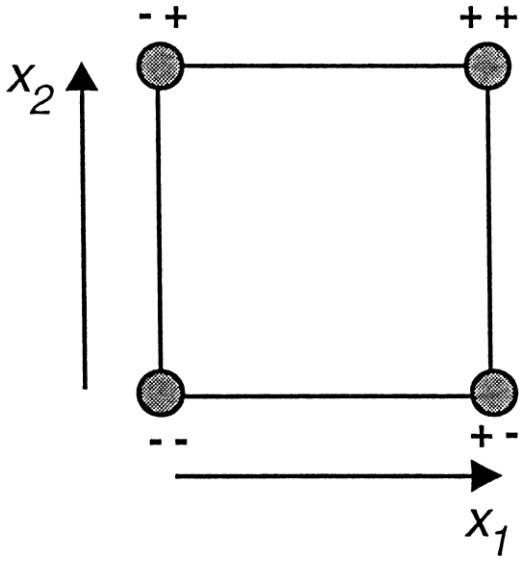


Figure 13 . *The experiments in a design with two variables*

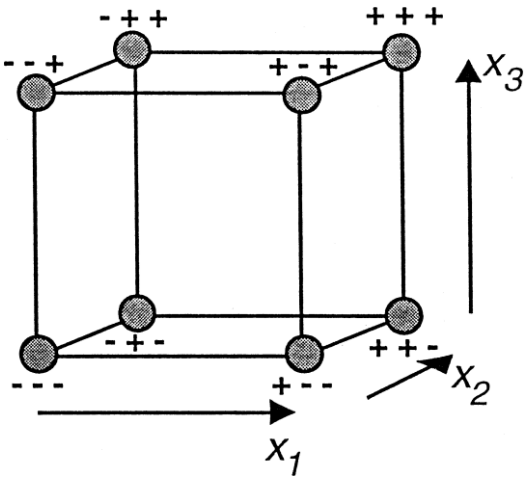


Figure 14 . *The experiments in a design with three variables*

Table 3 . *Sign of interaction effects* $X_1 X_2$

Sign of interaction effect $X_1 X_2$		
X_1	X_2	$X_1 X_2$
-	-	+
+	-	-
-	+	-
+	+	+

3.4.2 Fractional Factorial Design

An alternative to the full factorial design is the *fractional factorial design*, in which only a fraction of the experiments covered by the full design are performed. Fractional factorial designs are primarily used for screening purposes, where one focuses on identification of the more important variables and interactions among a larger set. To investigate the effects of *k* variables in a full factorial design, 2*k* experiments are needed. Then, the main effects as well as all interaction effects can be estimated. To investigate seven experimental variables, 128 experiments will be needed; for 10 variables, 1024 experiments have to be performed; with 15 variables, 32,768 experiments will be necessary. It is obvious that the limit for the number of experiments it is possible to perform will easily be exceeded, when the number of variables increases. In most cases, it is not necessary to investigate the interactions between all of the variables included from the beginning .In the first screening it is recommended to evaluate the result and estimate the main effects according to a linear model. After this evaluation the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having

to increase the number of experiments to the extreme. The fractions (1/2, 1/4, 1/8, 1/16 . . . $1/2^{p}$) of a factorial design with 2^{k-p} experiments are defined, where *k* is the number of variables and *p* the size of the fraction. The size of the fraction will influence the possible number of effects to estimate and, of course, the number of experiments needed. The construction of fractional factorial designs is performed considering the important principles. The design matrix in fractional factorial designs is defined using the model matrix from a factorial design. This means that columns in the model matrix **X** for a full factorial design are used to define the settings for the 'extra' variables in a series of experiments performed according to a fractional factorial design. The number of variables that can be included is limited by the number of columns in **X.** On the following tables, numbers is used to indicate the variable columns in the matrix. ***I*** is used to indicate the column used for calculating the mean value the constant term in the mode (4).

Example: A factorial design with two variables is shown in table 4 The columns a, b and ab define the settings for three variables, x_1, x_2 and x_3, in four experiments. The column ab is the product of a·b. See table 5 for the obtained design matrix.

Table 4 . *Model matrix of factorial design*

I	**a**	**b**	**ab**
1	-1	-1	1
1	1	-1	-1
1	-1	1	-1
1	1	1	1

Table 5 . *Design matrix factorial design*

Exp n°	X_1	X_2	X_3
1	-1	-1	1
2	1	-1	-1
3	-1	1	-1
4	1	1	1

Table 6 . *Experimental plan factorial design*

Exp n°	X_1	X_2	X_3
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1

This is a half fraction of a factorial design with three variables and it is found that the selected experiments correspond to experiment 5, 2, 3 and 8 in the factorial design table 6. In figure 15, it is illustrated how the experiments for a half fraction are distributed in the experimental domain spanned by three variables. It is also shown that the experiments have the form of a tetrahedron. This is the largest possible volume spanned by four corners in three dimensions. This shows another important property of the fractional factorial designs, that the experiments cover as much as possible of the experimental domain. The whole experimental domain cannot be covered by a limited

number of experiments, but a fractional factorial design 'selects' those experiments that cover a maximal volume of the domain in a limited number of experiments.

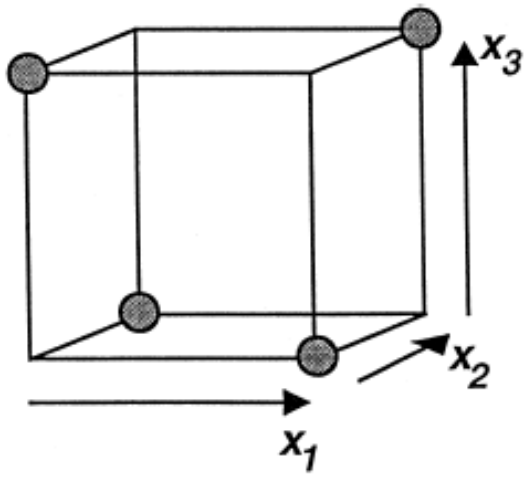


Figure 15 . *Distribution of the experiment in a 2^{3-1} fractional factorial design*

3.4.3 Central Composite Designs

Two-level designs can only lead to linear models of responses and so cannot give information about maxima or any non-linear relationships. However a drawback of full factorial designs at levels greater than two is the great number of experiments that must be done. Designs that allow greater numbers of levels without performing experiments at every combination of factor levels cover the factor space near the centre with more points than at the periphery. One such design is the *central composite design (CCD)*, so named

because it combines two-level full or fractional factorial designs with additional axial or star points and at least one point at the center of the experimental region being investigated (figure 16) (6). The star and factorial points can lie equidistant from the centre or the star points can lie within the space of the factorial design (inscribed design) or they can lie on the faces of the factorial design points (faced). Central composite designs require $L^k + Lk + n_c$ where n_c are the number of replicate centre points chosen.

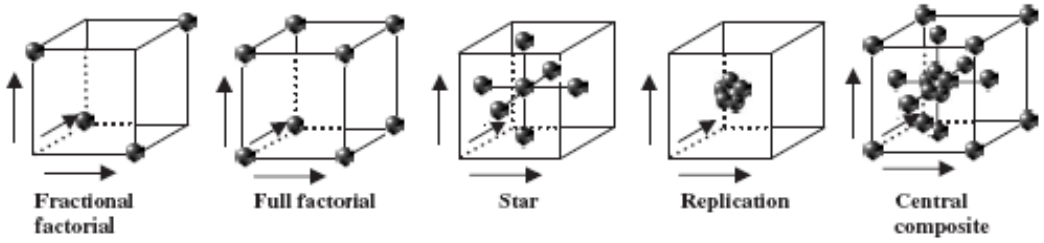


Figure 16 *Central Composite Designs (CCD)*

In general, a CCD for k factors, coded as (Xi, . . ., X_k), consists of three parts:

1. A factorial (or cubic) design, containing a total of n_{fact} points with coordinates $Xi = -1$ or $Xi = +1$, for i=1, . ..,k;

2. An axial (or star) part, formed by $n_{ax} = 2k$ points with all their coordinates null except for one that is set equal to a certain value α (or −α);

3. A total of n_c runs performed at the center point, where, of course, Xi = . . . X_k =0.

To build a central composite design, we need to specify each of these three parts (7). We have to decide how many cubic points to use and where they

will be, what will be the value of α, and how many replicate runs should be conducted at the center point.

3.5 Response Surface Methodology (RSM)

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design. After acquiring data related to each experimental point of a chosen design, it is necessary to fit a mathematical equation to describe the behavior of the response according to the levels of values studied. The mathematical model found after fitting the function to the data can sometimes not satisfactorily describe the experimental domain studied. The more reliable way to evaluate the quality of the model fitted is by the application of analysis of variance (ANOVA). The central idea of ANOVA is to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses (8). From this comparison, it is possible to evaluate the significance of the regression used to foresee responses considering the sources of experimental variance. In ANOVA, the evaluation of data set variation is made by studying its dispersion. The surfaces generated by linear models can be used to indicate the direction in which the original design must be displaced in order to attain the optimal conditions. However, if the experimental region cannot be displaced due to physical or instrumental reasons, the research must find the best operational condition inside the studied experimental condition by visual inspection. For quadratic models, the critical point can be characterized as maximum, minimum, or saddle. The visualization of the predicted model equation can be obtained by the surface response plot. This graphical representation is an n-dimensional surface in the (n + 1)-dimensional space.

Usually, a two-dimensional representation of a three-dimensional plot can be drawn. Thus, if there are three or more variables, the plot visualization is possible only if one or more variables are set to a constant value. Fig 3.9 illustrates some profile for the quadratic response surface plot in the optimization of two variables. In fig 17 (a and b) represents surfaces where the maximum point is located inside the experimental region. Surface shown in figure 17 (c), shows that the maximum point is outside the experimental region and that it is necessary to displace. The surface shown in figure 17 (d), presents a minimum point, and that shown in figure17 (e) presents a saddle point as the critical point (8).

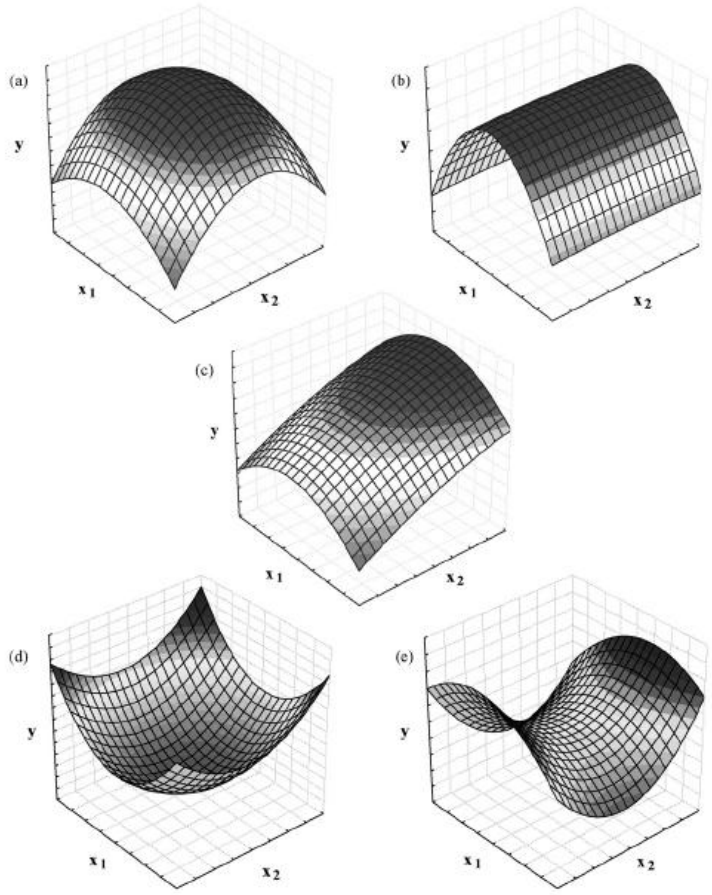


Figure 17 . *Some profiles of surface response generated from a quadratic model in the optimization of two variables. (a) maximum, (b) plateau, (c) maximum outside the experimental region, (d) minimum, and (e) saddle surfaces.*

References

1. Telford J.K., Hopkins J., *APL Technical Digest*, Vol.27, *N. 3* (**2007**) *Design of Experiments - Principles and Applications* UMETRICS AB,
2. Gupta V.K., Parsad R., *Fundamentals of experimental design*. I.A. S. R. I. Library.
3. Lundstedt T., Seifert E., Abramo L., Thelin B., Nyström A., Pettersen J., Bergman R., *Chemometrics and Intelligent Laboratory Systems* 42 (**1998**) 3–40
4. Skartland L.K., Mjøs S.A., Grung B., J. Chromatogr. A 1218 *(***2011***)* 6823– 6831
5. Hibbe D.B., *J.Chromatogr.B* Chromatogr. B (**2012**), doi:10.1016/j.jchromb.2012.01.020
6. Ferreira S.L.C.,, Bruns R.E., Galvão E., da Silva P., dos Santos W.N. L., Quintella C.M., David J.M., Bittencourt de Andrade J., Breitkreitz M.C., Fontes Jardim I.C.S., Barros Neto B., *J. Chromatogr. A* 1158 (**2007**) 2–14
7. Vieira S., Hoffman R., *Estatıstica Experimental*, Atlas, São Paulo, **1989**.
8. Bezerra M.A., Santelli R.E., Oliveira E.P., L.S. Villar , Escaleira L.A., *Talanta* 76 (**2008**) 965–977

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Gas chromatography Triple Quadrupole (GC-QqQ-MS) (1-2)

In gas chromatography (GC) the sample, which may be a gas or liquid, is injected into a stream of an inert gaseous mobile phase (often called the carrier gas). The sample is carried through capillary column, where the sample's components were on the basis of ability to distribute themselves between the mobile and stationary phases. A schematic diagram of a typical gas chromatograph is shown in figure 18.

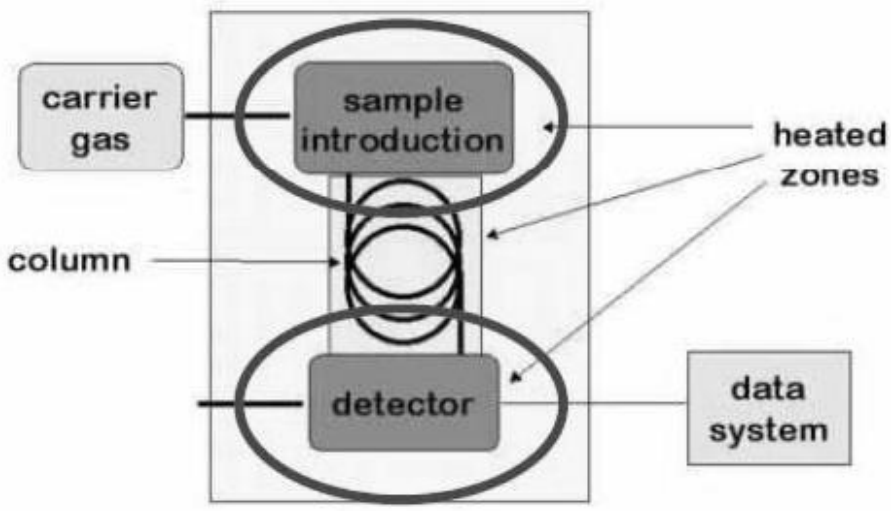


Fig 18. *Scheme of a typical gas chromatograph*

4.1.1 Detectors for Gas Chromatography

The final part of a gas chromatograph is the detector. The ideal detector has several desirable features, including low detection limits, a linear response over a wide range of solute concentrations (which makes quantitative work easier), responsiveness to all solutes or selectivity for a specific class of solutes, and insensitivity to changes in flow rate or temperature. There are different types of detectors used in GC, in this section we will of those using mass spectrometry in particular of the triple quadrupole mass spectrometer.

4.1.2 Mass Spectrometer

In GC–MS effluent from the column is introduced directly into the mass spectrometer's ionization chamber, in a manner that eliminates the majority of the carrier gas. In the ionization chamber all molecules (remaining carrier gas, solvent, and solutes) are ionized, and the ions are separated by their mass-tocharge ratio. Because each solute undergoes a characteristic fragmentation into smaller ions, its mass spectrum of ion intensity, as a function of mass-to-charge ratio provides qualitative information, that can be used to identify the solute. As a GC detector, the total ion current for all ions, reaching the detector is usually used, to obtain the chromatogram. Selectivity can be achieved by monitoring only specific mass-to-charge ratios, a process called selective ion monitoring. A mass spectrometer provides excellent detection limits, typically 25 fg to 100 pg, with a linear range, spanning five orders of magnitude.

4.1.3 Mass Analyzer: Triple Quadrupole

A mass analyzer measures gas phase molecules with respect to their mass-to charge ratio (m/z), where the charge is produced by the addition or loss of a proton(s), cation(s), anions(s) or electron(s) (3). The addition of charge allows

the molecules to be affected by electric fields thus allowing its mass measurement. There are a number of types of mass analyzers available and choice depends on the information required from the ionised analytes. Quadrupole mass analyzers have been important in mass analysis for many decades because they are relatively inexpensive, rugged, and have been implemented in a wide variety of instrumental configurations including triple quadrupole instruments which can do MS/MS experiments. A quadrupole mass analyzer consists of four parallel rods that have fixed DC and alternating RF potentials applied to them (see figure 19). Ions produced in the source are focussed and passed along the middle of the quadrupoles. Motion of these ions will depend on the electric fields so that only ions of a particular mass to charge ratio (m/z) will have a stable trajectory and thus pass through to the detector (4). Varying the RF brings ions of different m/z into focus on the detector and thus builds up a mass spectrum. Quadrupoles can also be placed in tandem to enable them to perform fragmentation studies - the most common set-up is the triple quadrupole (QqQ) (5) mass spectrometer which enables basic ion fragmentation studies (tandem mass spectrometry MS/MS) to be performed. These instruments are particularly sensitive in selected ion monitoring modes and hence ideal for trace analysis and pharmacokinetic applications. In a triple quadrupole, the sample enters the ion source and is usually fragmented by either an electron impact or chemical ionisation process.

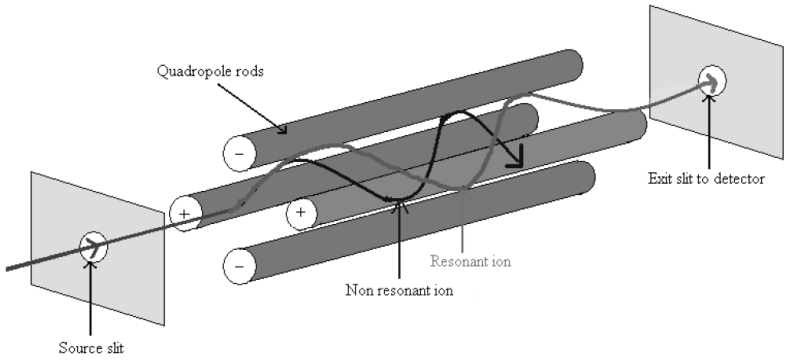


Figure 19. *Adapted schematic of a Quadrupole Mass Analyzer*

The first quadrupole acts as a filter for the ion of interest; the various charged ions are separated in the usual way and then pass into the second quadrupole section sometimes called the collision cell. This one acts as a collision chamber for the MS/MS to form the product ions of interest (6).

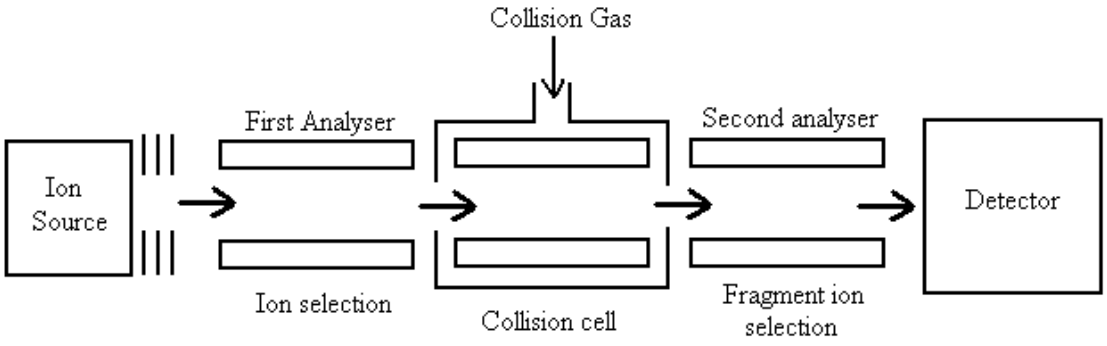


Figure 20. *Schematic of the Triple Quadrupole Mass Spectrometer*

These product ions are then passed into the third quadrupole which functions as a second analyser to detect the aforementioned ions for quantitation. The second analyser segregates the product ions into their individual masses, which are detected by the sensor, producing the mass spectrum (originally from ions of one mass only). In this way, the exclusive mass spectrum of a particular molecular or product ion can be obtained from the myriad of ions

that may be produced from the sample in the first analyser. A diagram of a triple Quadrupole Mass Spectrometer is shown in figure 20. QqQ-MS is an extremely powerful analytical system that can handle exceedingly complex mixtures and very involved molecular structures. The system has more than adequate resolving power and is valuable for structure. A triple quadrupole-mass spectrometer permits to carry out experiments in multiple reaction monitoring (MRM). In this acquisition mode, the analytical method sensitivity is improved by a significant reduction of background and, at the same time, a more reliable identification of analytes can be achieved because a specific precursor-product transition is monitored for the compound of interest.

4.2 Development of Analytical Method for Clinical Diagnostics

This section describes the development of two analytical methods, in particular the assay of the sarcosine in the humane urine as a marker of prostate cancer progression, and the quantification of neuroendocrine tumor markers (Homovanillic acid (HVA), vanylmandelic acid (VMA), and 5-hydroxyindoleacetic acid (5-HIAA)) in human urine.

4.3 Sarcosine as a marker in prostate cancer progression: a rapid and simple method for its quantification in human urine by solid-phase microextraction–gas chromatography–triple quadrupole mass spectrometry (7)

Introduction

Tumor marker comprise a wide spectrum of biomacromolecules found in the blood, urine, or body tissues that can be synthesized in excess concentration by a wide variety of neoplastic cells. The markers could be endogenous

products of highly active metabolic malignant cells or the products of newly switched on genes, which remained unexpressed in early life or newly acquired antigens at cellular and sub-cellular levels. The appearance of tumor marker and their concentration are related to the genesis and growth of malignant tumors in patients. An ideal tumor marker should be highly sensitive, specific, and reliable with high prognostic value, organ specificity and it should correlate with tumor stages. However, none of the tumor markers reported to date has all these characteristics. Inspite of these limitations, many tumor markers have shown excellent clinical relevance in monitoring efficacy of different modes of therapies during entire course of illness in cancer patients. Additionally, determination of markers also helps in early detection of cancer recurrence and in prognostication (8). In particular, the diagnosis and treatment of prostate cancer was significantly affected by the detection of prostate-specific antigen (PSA) in the blood test. The PSA test has become an indispensable marker also for follow-up of patients, particularly after radical prostatectomy. Nevertheless the PSA test has significant limitations, such as its lack of specificity, failure to detect a significant number of PSA-negative tumors, and its high levels in both cancerous and healthy tissues, resulting in significant numbers of false-positive cases. The possibility of having highly specific markers is a useful instrument to obtain early diagnosis and reliable. Recently, the metabolic approach has been applied by Sreekumar et al (9) to identify prognostic biomarkers that improve the clinical management of prostate cancer and this was supported by y Jamaspishvili et al. (10). In this study, we analyzed the metabolic profile of 1126 molecules present in 262 biological samples derived from plasma, urine and prostatic tissue derived from healthy or prostate cancer. The results showed that the concentration of sarcosine (N-methylglycine) in urine increased greatly during prostate cancer progression

to metastasis. From these findings suggest that sarcosine is a potentially important metabolic marker for prostate cancer noninvasive diagnosis and could be used successfully for a screening test. Many methods have been developed to determine amino acids in biological fluids which use capillary electrophoresis (11–14), high-performance liquid chromatography and ion-exchange chromatography with UV detection (15–18), and mass-spectrometric detection (19–24) .GC-MS is an analytical approach that will still be widely used in many laboratories. Analysis of amino acids by GC requires your conversion in more volatile derivatives. For this purpose, different procedures are used, which are based on the use of silylating agents such as BSTFA (25–28) and N-methyl-N-(tert butyldimethylsilyl)trifluoroacetamide (29–31) or derivatization by pentafluoropropyl anhydride/2- propanol (32, 33), trifluoroacetic anhydride/2-propanol (34), and pentafluorobenzyl bromide (35–37). All these procedures requires a tedious sample preparation procedure and can be carried out only at high temperature (above 80°C) and, above all, in organic solvents. Alkyl chloroformate can be used for performing simultaneous derivatization of amino acid directly in aqueous phase (38).Usually, the N(O,S)-alkoxycarbonyl alkyl esters were extracted using organic solvents prior to GC analysis. In a few works, alkyl chloroformate derivatives were extracted by solidphase microextraction (SPME) (39–41).

4.3.1 Derivatization of Sarcosine

The derivatization of sarcosine was performed by ethyl chloroformate in presence of ethanol and pyridine mixture (3:1 v/v) in according to method used by Vonderheide et al. (39). In the derivatization reaction shown in figure 21 it happens the esterification of carboxylic group catalized by pyridine and contemporarily acylation of amino group by ethyl chloroformate.

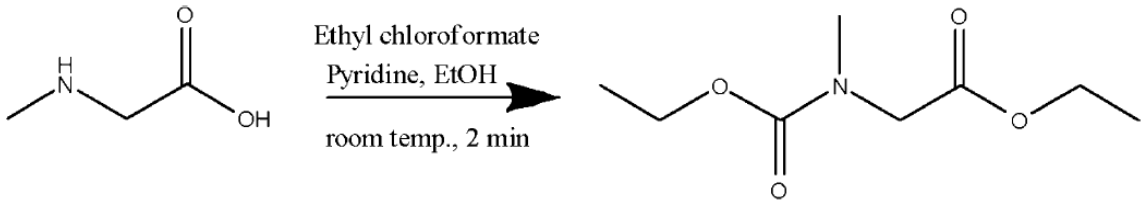


Figure 21 . *Reaction between sarcosine and the derivatizing agent*

4.3.2 Optimization of SPME Conditions

Preliminary test of SPME was performed by immersion of a fiber in a urine sample spiked at 5 μg/ml. Several experiments with different extraction times was performed for each fiber considered, in order to evaluate the extraction efficiency. The best results were obtained by utilizing the DVB/CAR/PDMS fiber but regrettably, a progressive deterioration of the coating was noted and a complete breakup of the coating was observed after ten analyse. The fiber with coating of PDMS and polyacrylate not undergo appreciable degradation in the work of Myung et al. (41) where an about threefold dilution of the sample was performed with deionized water. In presence of organic solvent in solution the lifetime of the fiber can be increased performing the analysis SPME in headspace mode. Therefore, the extraction efficiencies of five fibers (85 μm CAR/PDMS, 85 μm PA, 50/30 μm DVB/CAR/ PDMS, 65 μm PDMS/DVB, and 100 μm PDMS) were evaluated by sampling the analyte in headspace mode without sample dilution and in immersion mode after derivatization and addition of 6 ml of water to 1.5 ml of urine. All the experiments was performed under the same conditions (synthetic urine spiked at 5 μg/ml, room temperature, extraction time 20 min). The highest peak areas were obtained by performing analysis with PA, DVB/CAR/PDMS, and

PDMS/DVB fibers in immersion mode with dilution of the sample and no fiber damage was observed (figure 22).

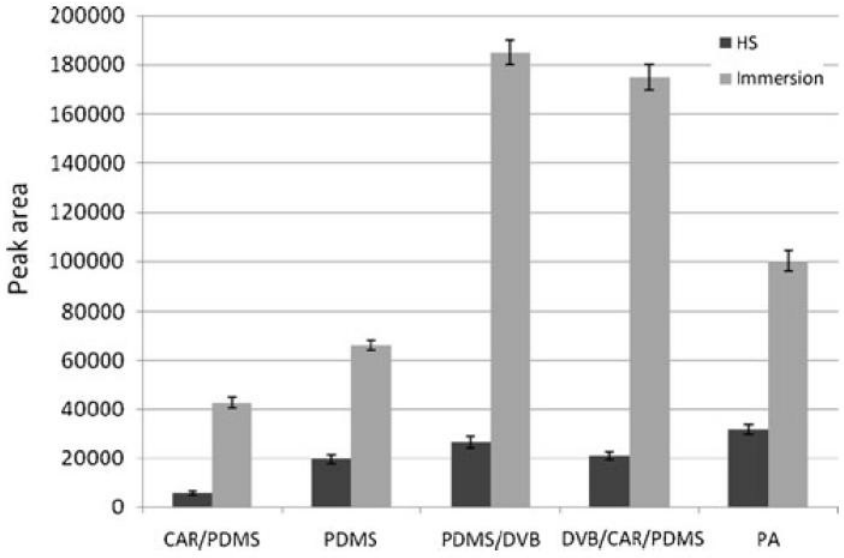


Figure 22. *Peak areas obtained by performing analyses in headspace (HS) and immersion modes (n=3) with five different fibers. CAR Carboxen, PDMS polydimethylsiloxane, DVB divinylbenzene, PA polyacrylate*

These fibers were selected for optimization of the parameters of SPME by experimental design. The experimental design was performed carrying out experiments with samples of synthetic urine spiked a 1 µg/ml and considering as the variables.The extraction time, the desorption temperature, and the concentration of sodium chloride added. A central composite design consisting of a 23 factorial design with six star points positioned at±α from the center of the experimental domain was performed for each of the fibers. In total, the experimental design matrix had 20 runs [2^3+(2×3)+6]. The ranges of the parameters evaluated in experimental design have been defined considering the results of preliminary tests and are : extraction time 2–20 min, concentration of NaCl ranging from 0 to 10%, and a desorption temperature in the range 220–270°C for PDMS/DVB fiber, 230-270°C for DVB/

CAR/PDMS fiber, and 240–300°C for PA fiber. Table 7, shows the design matrix. To find the optimal values for the three evaluate variables, all response surfaces were drawn (figure 23).The highest response was achieved using DVB/CAR/PDMS fiber at 270°C desorption temperature, 10% NaCl, and 20-min extraction time. The Pareto chart shows the influence that each factor investigated has on the response as well as the possible cross-effect among these factors. The Pareto chart obtained for DVB/CAR/PDMS fiber (figure 24) shows the effects of the variables studied and the possible cross-effect among these factors have on the response. In these charts, 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 crossing this line had a significant effect on the response. As can be seen in figure 24, the percentage of salt is the variable that affects more the response both for linear and for quadratic coefficients. Furthermore the extraction time was found to be significant and, also in this case, the effect was positive. While the desorption temperature has no significant effect on the signal of sarcosine.

Table 7 . *Design matrix in the central composite design for determination of sarcosine by solid-phase microextraction for each fiber*

Exp	NaCl (%)	Extraction Time (min)	Desorption Temperature (°C)		
			PDMS/DVB	DVB/CAR/PDMS	PA
18 (C)	5	11	245	250	270
6	8	16.4	230	238	252
9	5	2	245	250	270
16 (C)	5	11	245	250	270
19 (C)	5	11	245	250	270
13	0	11	245	250	270
3	2	5.6	260	262	288
4	8	5.6	260	262	288
15 (C)	5	11	245	250	270
20 (C)	5	11	245	250	270
11	5	11	220	230	240
14	10	11	245	250	270
8	8	16.4	260	262	288
1	2	5.6	230	238	252
12	5	11	270	250	300
5	2	16.4	230	250	252
10	5	20	245	262	270
17(C)	5	11	245	238	272
7	2	16.4	260	262	288
2	8	5.6	230	238	252

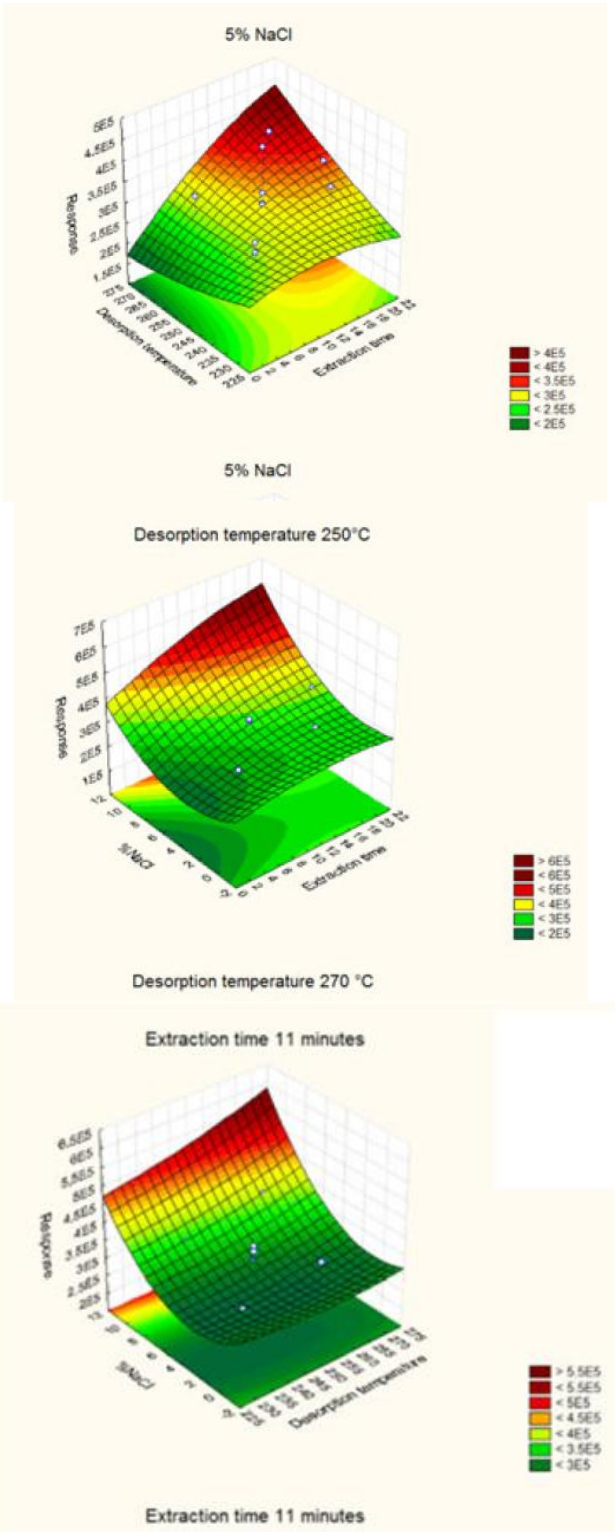


Figure 23 *Response surfaces estimated from the central composite design: peak area of derivatized sarcosine for extraction time versus desorption temperature, extraction time versus concentration of NaCl, and desorption temperature versus concentration of NaCl. DVB/CAR/PDMS fiber.*

,

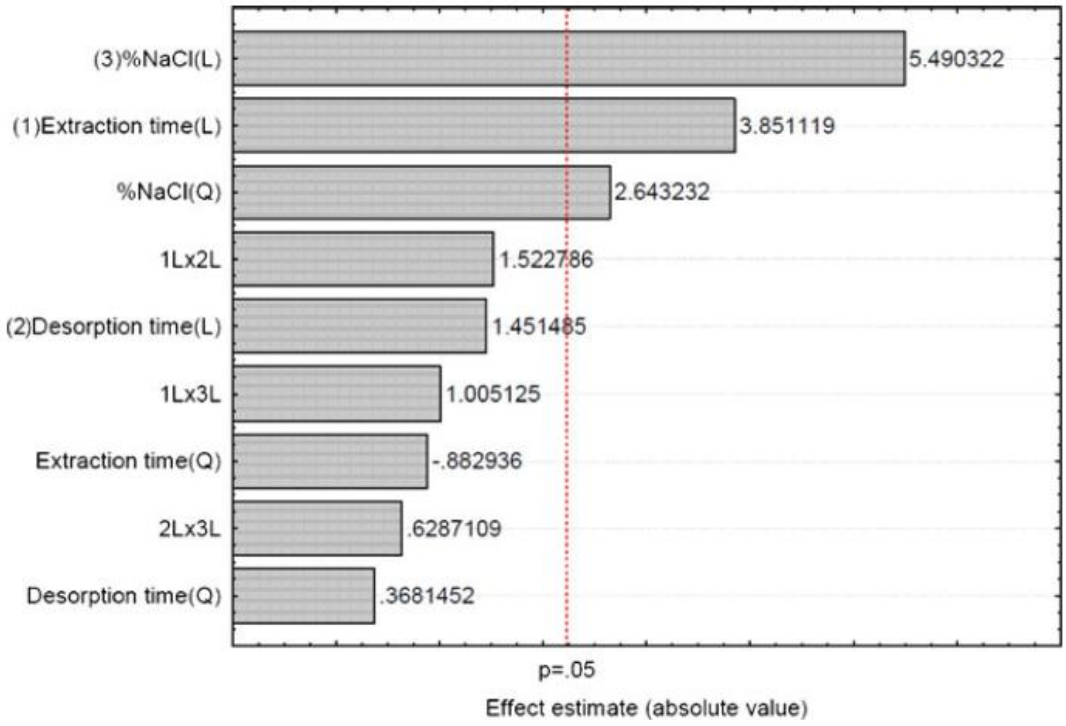


Figure 24. *Pareto chart obtained from the central composite design for DVB/CAR/PDMS fiber*

4.3.3 GC-QqQ-MS/MS Analysis

L-alanine is an amino acid normally present in urine real. This compound being an isomer of sarcosine represents an interfering compound in the quantification of sarcosine in real urine samples. As can be seen in fig the full-scan mass spectra of the two isobaric compounds are similar, and the MS/MS spectra by selecting m/z 116 and m/z 189 as precursor ions for both isomers show the same characteristic fragments (figure 25). Several GC oven temperature programs were tested to obtain an acceptable resolution for the sarcosine/alanine pair. The best result taking account also of the analysis time was achieved by using the temperature program described in "Experimental section".

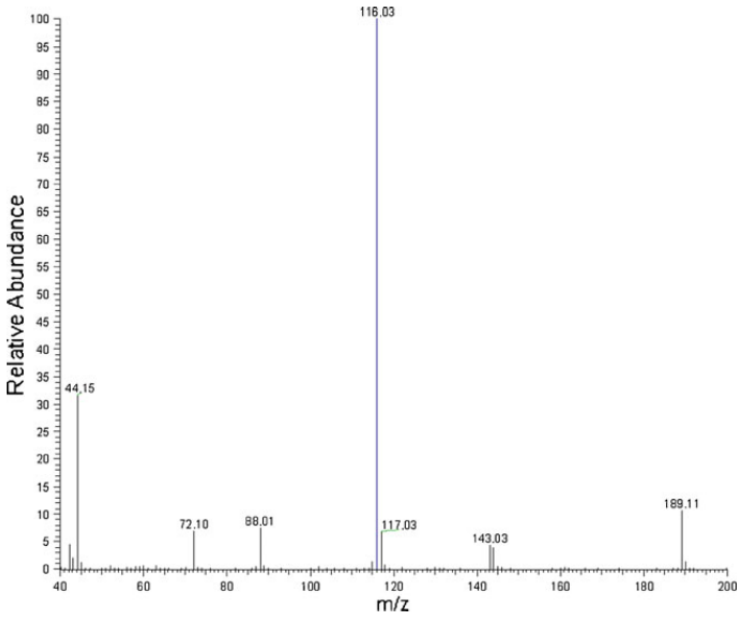


Figure 25 . *Electron ionization (EI) full-scan spectrum of derivatizated sarcosine*

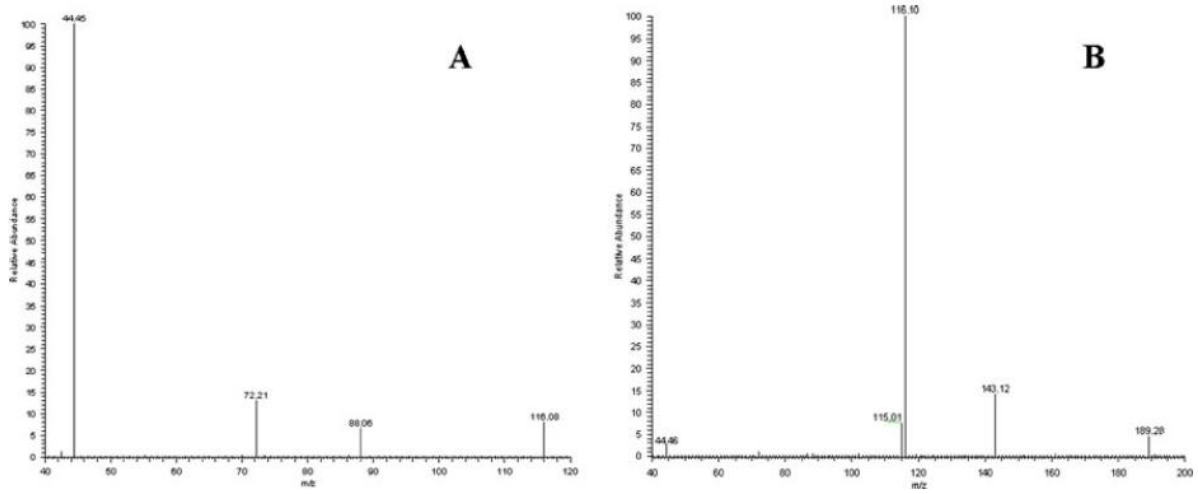


Figure 26. *EI tandem mass spectrometry (MS/MS) spectra of sarcosine and L-alanine by selecting m/z 116 (a) and m/z 189 (b) as precursor ions*

Initially the ion 116 m/z was selected as parent ion in MS/MS approach to achieve more sensitivity (ion with higher abundance) and more specificity (ion with higher m/z ratio). The transition m/z 116→m/z 44 was chosen and

the collision energy and scan time were optimized. Unfortunately, in this operating condition, the high concentration of L-alanine in urine in healthy individuals does not allow to obtain a good separation between L-alanine and sarcosine. Considering the ion m / z 189 in the full-scan mass spectra of alanine and sarcosine is evident that its value of ion current for alanine is significantly lower than the corresponding ion current of sarcosine. The reaction paths taken by sarcosine and alanine in the gas phase are identical (figure 27).

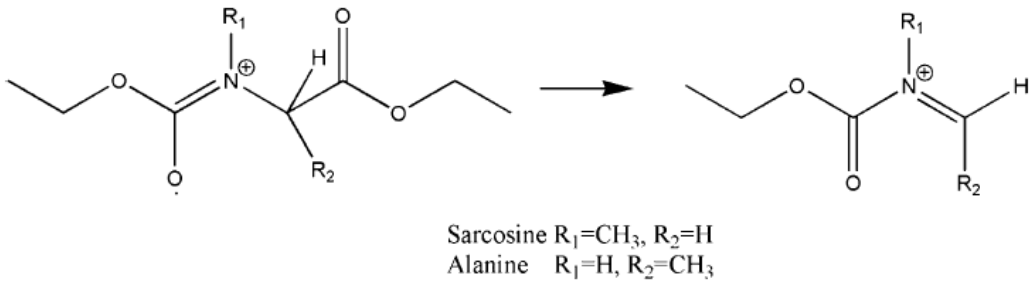


Figure 27. *Reaction path taken by sarcosine and alanine in the gas phase*

In the reconstructed chromatogram obtained by GC-MS, an equimolar amount of sarcosine and alanine showed a ratio of 21.4 for the ion current of m/z 189. A similar ratio was obtained from the SRM scan (transition m/z 189→m/z 116). Taking into account these considerations, the transition m/z 189→m/z 116 was considered for the assay of sarcosine in urine sample in SRM mode. As can be seen in the figure 28 a satisfactory separation was obtained because the response factor of alanine is smaller than that of sarcosine

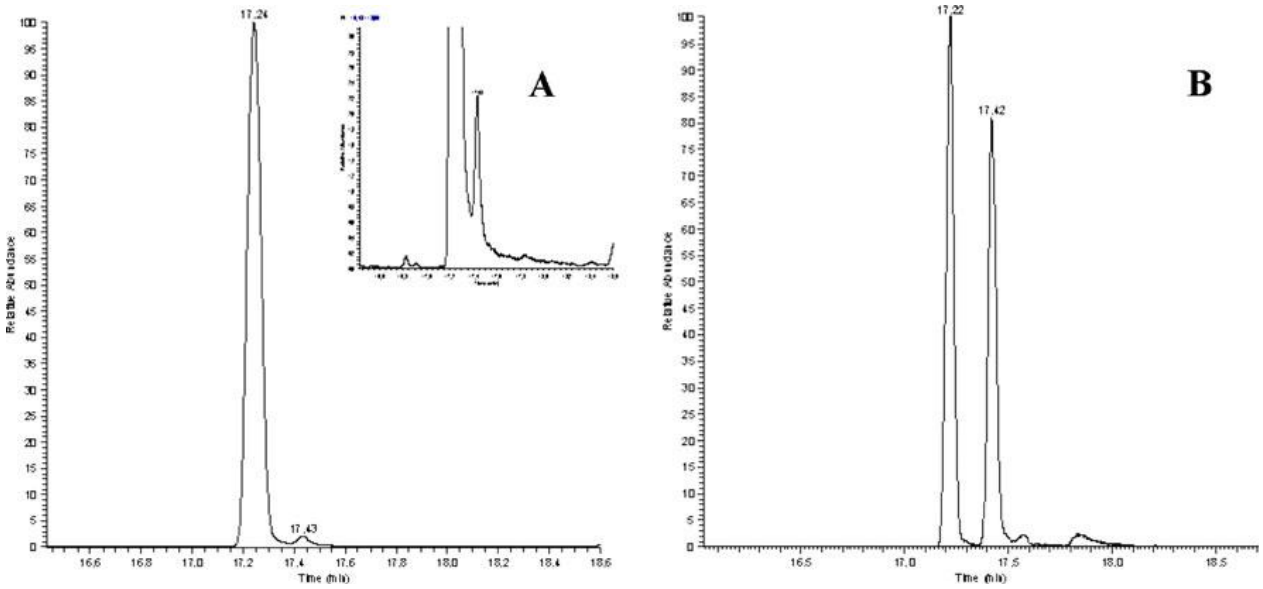


Figure 28 . *Solid-pase microextraction–gas chromatography–MS/MS (selected reaction monitoring) chromatogram of a real urine sample selecting the a transition m/z 116→m/z 44 (inset y-axis zoomed chromatogram) and b the transition m/z 189→m/z 116. Alanine and sarcosine were detected at 17.22 and 17.42 min, respectively*

4.3.4 Analytical Performances

The linearity of the calibration curve was evaluated in the concentration range of 50–1,000 ng/ml and using as internal standard the sarcosine-d_3 at 400 ng/ml. The response function (y=1.095x+0.007) was found to be linear, with a correlation coefficient (R2) of 0.9999 in the range considered. Accuracy and precision were evaluated at three concentrations (70, 250, and 800 ng/ml) in synthetic urine by analyzing a spiked sample five times. The accuracy values (111.4%, 103.6%, and 99.0% for the three concentrations, respectively) and the relative standard deviations (0.74%, 0.59%, and 0.13%) obtained can be considered satisfactory (table 8).

Table 8. *Summary of mean accuracies (%) and relative standard deviations (RSD; n=5)*

Spiked values (ng/ml)	Found Value (ng/ml)	RSD (%)	Accuracy (%)
70	78.8 ±0.6	0.74	111.4
3250	256±1.5	0.59	103.6
800	792±1.0	0.13	99.0

Very satisfactory results were obtained also for the values of limit of detection (LOD) and limit of quantitation (LOQ): LOD=0.10 ng/ml, LOQ=0.16 ng/ml.

4.3.5 Application to Real Samples

The method developed was applied to the analysis of ten urine samples from healthy individuals .The mean value of the concentration of sarcosine was 211 ng/ml and the minimum and maximum values were 73.4 and 470 ng/ml respectively. The data obtained are compatible with the range of urinary sarcosine concentrations observed in urine samples collected by the INTERMAP Study and analyzed by GC-MS.(42)

4.3.6 Conclusion

In this work, a rapid and easy method for the quantification of sarcosine in urine samples was developed for noninvasive diagnosis and prognostic evaluation of prostate cancer. The combined use of chloroformate as a derivatizing agent and SPME as an extraction technique allows one to minimize the presence of organic solvents in the final protocol. The capability of QqQ-MS to minimize matrix interference and improve the signal-to-noise ratio was confirmed. Moreover, an appropriate choice of the SRM transition (m/z 189→m/z 116) together with a suitable gas chromatograph oven

temperature program permitted a very satisfactory separation between L-alanine and sarcosine. Finally, the excellent performances in terms of linearity, accuracy, precision, and LOD and LOQ values make the method developed suitable to be adopted for quantification of sarcosine in urine samples as a potentially important metabolic marker for prostate cancer diagnosis and for a screening test.

4.4 A reliable and Simple Method for the Assay of Neuroendocrine Tumor Markers in Human Urine by Solid-Phase Microextraction Gas Chromatography-Triple Quadrupole Mass Spectrometry (43)

Introdution

Vanilmandelic (4-hydroxy-3-methoxymandelic) acid (VMA), homovanillic (4-hydroxy-3-methoxy- phenylacetic) acid (HVA) and 5-hydroxyindoleaceticacid (5-HIAA) are the main acidic catabolites of norepinephrine, dopamine and serotonin, respectively and are excreted in urine. The concentrations of catecholamines, serotonin, and their precursor and metabolites are essential for diagnosis of neurological disorder. HVA and VMA are biological markers of neuroblastoma, the most solid tumor in children. (44-47). the determination of the urinary concentra tions of 5-HIAA is fundamental for the diagnosis of the carcinoid syndrome, a serotonin secreting tumor (48). VMA, HVA and 5-HIAA levels are usually measured by high performance liquid chromatography methods with electrochemical detection (49-53), fluorometric detection (54,55) and mass spectrometric acquisition (56,57). However, there are few works present in literature using gas chromatography coupled to flame ionization detection (GC-FID) (58), mass spectrometry (59) and biosensors (60). Gas chromatography coupled

with mass spectrometry is still widely used in many laboratories and is able to offer high analytical performance while keeping costs low instrumental. Certain classes of compounds, such as carboxylic acids, in order to be analyzed by gas chromatography require derivatization into compounds more volatile and less polar. The carboxylic acids are mainly derivatized by silylation, using reagents such as N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (61). These methods require lengthy procedures involving extraction step with an organic solvent, evaporation under nitrogen, high temperatures and long reaction times (59). Alkyl chloroformate can be used for obtaining an reliable and simple esterification of acidic moiety in acqueous phase (62). This method, which is compatible with the use of SPME (63, 64), to date has not yet was used for the derivatization of carboxylic acids in urine. The main purpose of the present work was to develop a simple and rapid method for the analysis of VMA, HVA and 5-HIAA in urine samples by SPME-GC-QqQ-MS/MS after a derivatization step with ethyl chloroformate.

4.4.1 Optimization of Solid Phase Microextraction Variables

Derivatization reaction was performed using an variation of the method developed by Solich et al. (65) in which methyl and ethyl chloroformate were used to derivatize the phenolic acids in aqueous medium consisted of acetonitrile, water, methanol/ethanol and pyridine.The efficiency of extraction for each fiber depends on the type of alkyl chloroformate used in the derivatization.Three alkyl chloroformate were used that in particular: methyl-ethyl and propyl chloroformate as shown in the reaction scheme (figure 29). The ratio of the compounds used in the derivatization reaction (acetonitrile, sample, alcohol and pyridine) were 14:10:2:3 in the case of

methylchloroformate/methanol and 2:4:2:2 for the remaining mixtures.The extraction efficiency of five fibers (carboxen/polydimethylsiloxane 85 µm (CAR/PDMS), polyacrylate 85 µm (PA), divinylbenzene/ carboxen/polydimethylsiloxane 50/30 µm (DVB/CAR/PDMS), polydimethylsiloxane/divinylbenzene 65 µm (PDMS/DVB) and polydimethylsiloxane 100 µm (PDMS)) was evaluated by univariate method extracting the derivatized analytes in immersion mode under the same condition (synthetic urine spiked at 5 mg L-1, room temperature, extraction time 20 min).

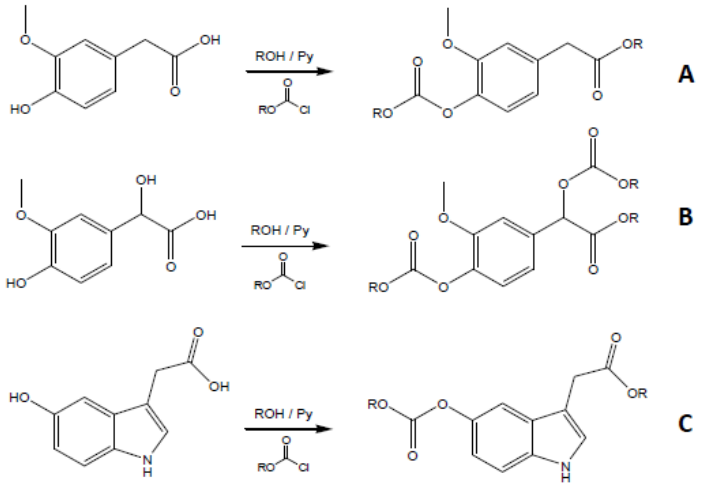


Figure 29. *Reaction between HVA (A), VMA (B), 5-HIAA (C) and the derivatizing agents*

The evaluation of the efficiency of extraction for each fiber, was made by considering the area of the chromatographic peak for each analyte. As can be seen in the figure 30, the most abundant signals were obtained by derivatizing with ethyl chloroformate and extracting the analytes with the PA fiber. Moreover, in these conditions similar values of the signals for the three analytes were achieved (figure 30 B, PA fiber).

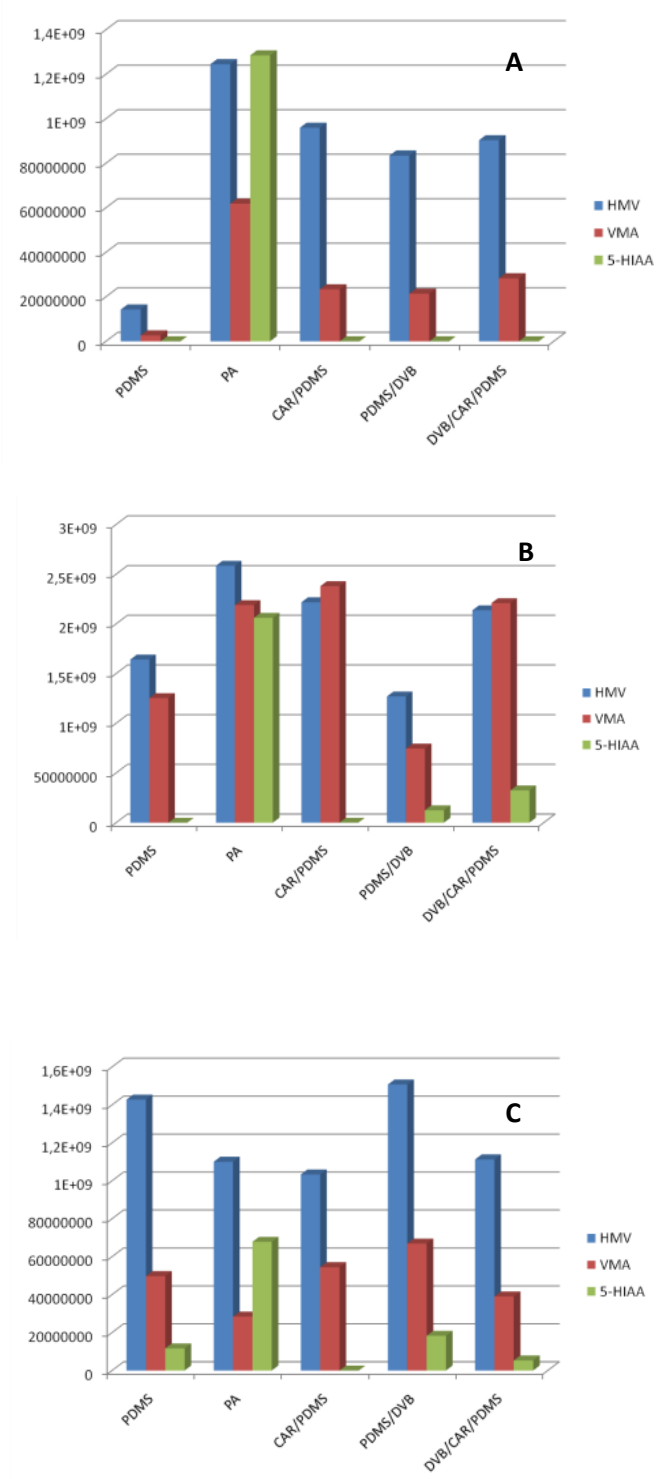


Figure 30. *Peak areas for the three analytes obtained by performing analyses in immersion mode with five different fibers and three derivatizing mixtures (A: methyl chloroformate/methanol; B: ethyl chloroformate/ethanol; C: propyl chloroformate/propanol*

In the light of these results, PA fiber was selected for applying experimental design to obtain the best experimental conditions of SPME analysis. The variables considered in the experimental design were extraction temperature, extraction time and concentration (%) of sodium chloride. Other variables such as pH, the desorption time and desorption temperature were not taken into account because the pH does not significantly affect the SPME extraction when non-ionic compounds are involved and desorption time and desorption temperature usually give an important contribution to the response only if dealing with thermally labile compounds (66). A Central Composite Design (CCD) consisting of a full factorial design 23 with six star points located at ±α from the center of the experimental domain and six central points was performed for PA fiber using ethyl chloroformate as derivatizating reagent. The choice of the ranges for the variables evaluated in the experimental design, was made on the basis of the results of preliminary tests, and was the following: NaCl 0-10 %; extraction time 5-30 min; extraction temperature 40-60 °C. A total of 20(i.e. 23+(2×3)+6) randomly-performed experiments (table 9) were performed using a synthetic urine sample spiked at 5 mg /l of analytes. In the Pareto chart (figure 31) shows the effects of the variables studied and the possible cross-effect among these factors have on the response. As you can see the effect of the variables examined for the HMV and VMA was very similar (figure 31A and B). NaCl percentage and extraction time were found to be significant and of positive sign. The third most important coefficient is the quadratic term of percentage of NaCl but this value is statistically significant only for the HVA. Regarding the response of 5-HIAA , the most important coefficients were found to be the quadratic term of % NaCl and linear term of extraction time. In particular the negative sign of the quadratic term of the percentage of salt indicates the presence of a

maximum in the trend of signal as function of % NaCl. Finally, the extraction temperature is not found to be influential on the response for all three analytes.The response surface shown in figure 32, help to assess the trend of the most important variables for the three analytes.

Table 9. *Design matrix in the central composite design (CCD) for optimization of SPME parameters*

Exp	NaCl (%)	Extraction time (min)	Extraction Temperature (°C)
14	5	17.5	60.0
17 (C)	5	17.5	50.0
9	0	17.5	50.0
5	8	10.1	44.1
8	8	24.9	55.9
16 (C)	5	17.5	50.0
13	5	17.5	40.0
15 (C)	5	17.5	50.0
11	5	5.1	50.0
2	2	10.1	55.9
4	2	24.9	55.9
19 (C)	5	17.5	50.0
7	8	24.9	44.1
6	8	10.1	55.9
1	2	10.1	44.1
3	2	24.9	44.1
12	5	29.9	50.0
10	10.0	17.5	50.0
18 (C)	5.0	17.5	50.0
20 (C)	5.0	17.5	50.0

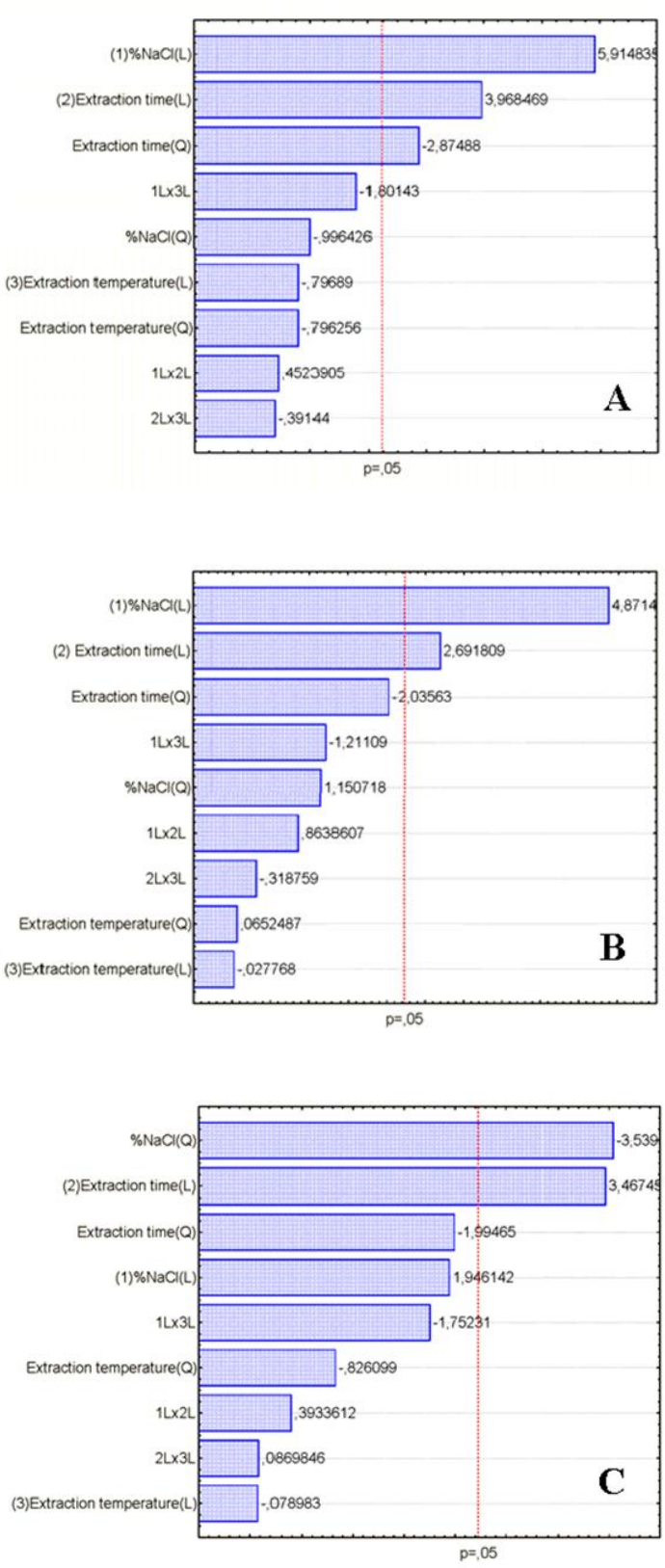


Figure 31. *Pareto charts obtained from the central composite design for derivatized HVA (A), derivatized VMA (B) and derivatized 5-HIAA (C)*

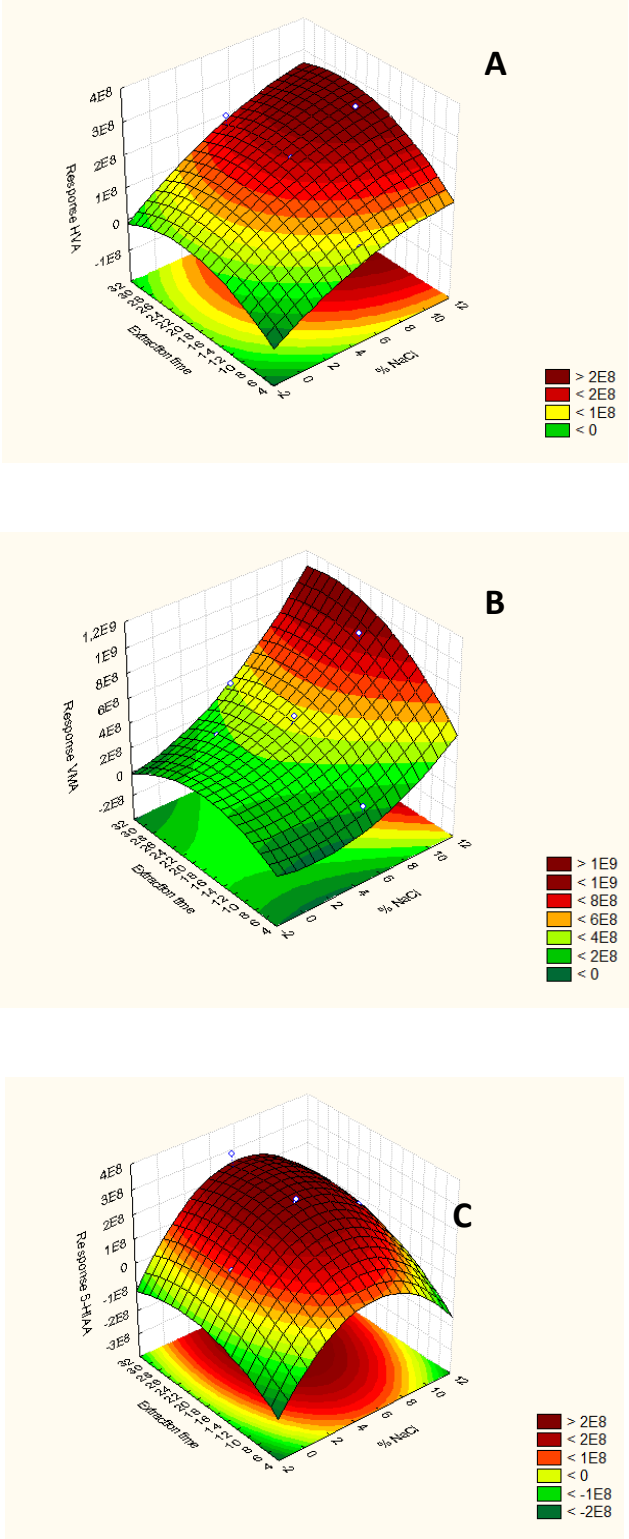


Figure 32.*Response surfaces estimated from the central composite design for extraction time versus percentage of NaCl: peak area of derivatized HVA (A), derivatized VMA (B) and derivatized 5-HIAA (C)*

The single desirability scores for the predicted values for each dependent variable are then combined into overall D by computing their geometric mean of different di values:

$$D = \sqrt[n]{d_1{}^{p_1} \times d_2{}^{p_2} \times d_3{}^{p_3} \times \ldots \times d_n{}^{p_n}}$$

Where pi is the weight of the response, n is the number of responses and di is the individual desirability function of each response (67).Since each response was considered equally important in the overall desirability function, equal weights were given to the responses, i.e. p1 = p2 = p3 = 1. The desirability function applied to the results obtained from the experimental design has provided the following values: 9.5% NaCl, extraction time 25.8 min, extraction temperature 40 °C. Whereas the extraction temperature does not have a significant effect on all three responses, we decide to carry out analysis at room temperature to make the method more simple and rapid

4.4.2 GC-QqQ-MS/MS Analysis

As previously mentioned, the tandem mass spectrometry is a technique, which is in degrees to provide high performance in terms of sensitivity and specificity, allowing discriminating any interfering substances in complex matrices. The determination and optimization of the parameters of tandem mass spectrometry takes place through the choice of the precursor ion, the ion product and optimization of the energy of collision. As precursor ions to be tested, were selected more abundant ions present in the mass spectrum of each analyte, which was acquired in full scan (figure 33-35)

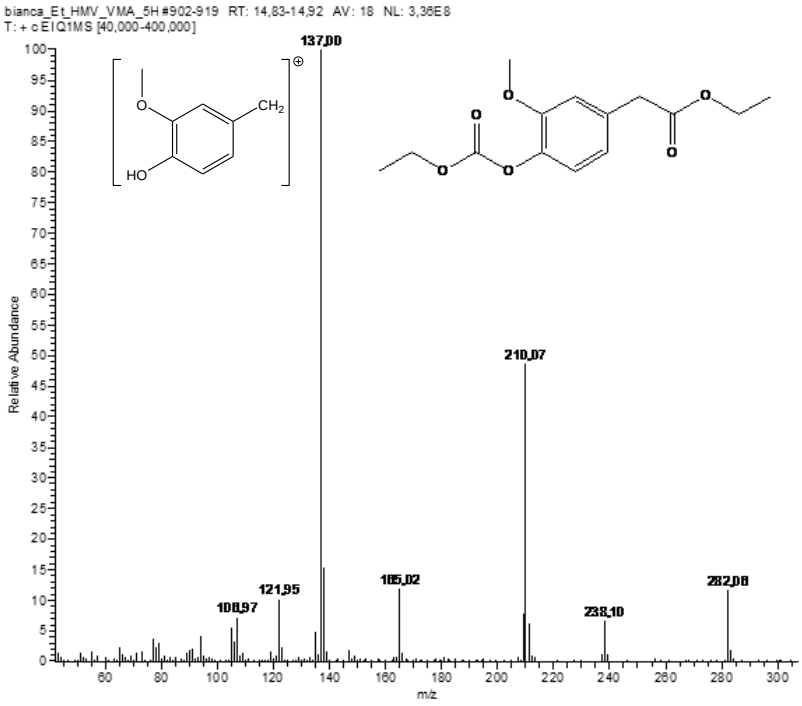


Figure 33. *Mass spectra of ethyl derivatives of HMV*

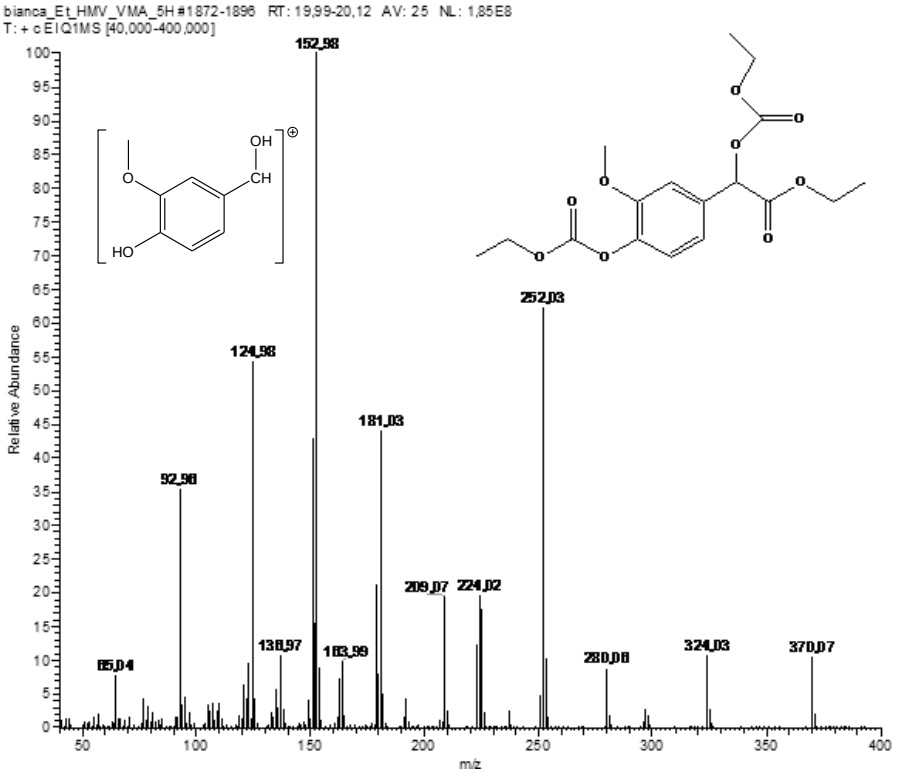


Figure 34. *Mass spectra of ethyl derivatives of VMA*

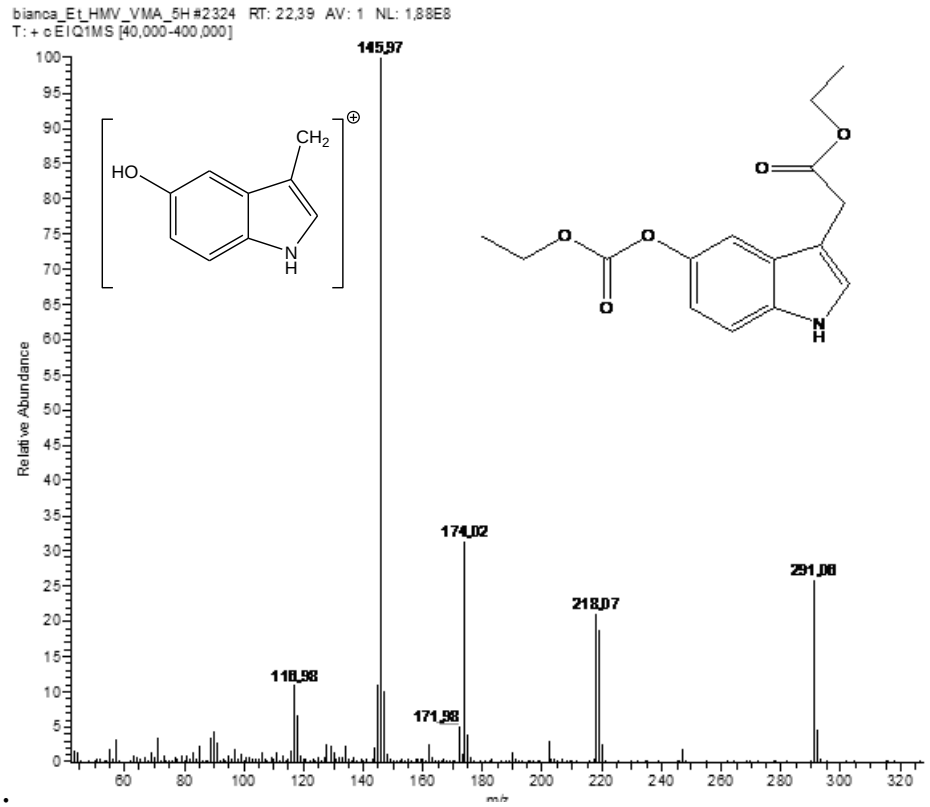


Figure 35. *Mass spectra of ethyl derivatives of 5-HIAA*

Therefore, three precursor ions were tested for VMA (m/z 370, m/z 252, m/z 153) and 5-HIAA (m/z 291, m/z 174, m/z 146) whereas two ions were selected for HVA (m/z 210, m/z 137). The MRM transition that gave the best results in terms of sensitivity is been used for quantification and the second best transition is chosen for the identification of` analyte (table 10).

Table 10. *Retention time (RT) and electron ionization tandem mass spectrometry (EI-MS/MS) parameters (Collision energies (eV) are indicated in parenthesis)*

Compound	Retention time (RT)	MRM transition, m/z (collision energy, V)	
		Quantification	Identification
HVA	14.60	137→122 (13)	137→94 (19)
VMA	19.67	252→151 (16)	153→93 (11)
5-HIAA	21.97	174→146 (16)	146→91 (19)
HVA-d5 (IS)	14.55	142→127 (12)	-
VMA-d3 (IS)	19.64	255→154 (16)	-
5-HIAA-d5 (IS)	21.91	179→151 (16)	-

4.4.3Analytical Performances

The linearity of the developed method was evaluated in a concentration range between 0.5 mg/l and 100 mg/l with 10 mg/l of each of the deuterated internal standard (HVA-d5, VMA-d3, 5-HIAA-d5). Very good values of linearity were achieved in the range examined, with correlation coefficient values > 0.99 for all the analytes (table 11).

Table 11. *Linearity and correlation coefficient*

Compound	Curve	R^2
HVA	y= 0.0938 x - 0.0012	0.9998
VMA	y= 0.0865 x + 0.0084	0.9989
5-HIAA	y= 0.0661 x - 0.0013	0.9998

Table12. *Summary of calibration parameters, mean accuracies, relative standard deviations (RSD (n = 5), %, in parentheses) and limits of detection (LODs) and limits of quantitation (LOQs) in multiple reaction monitoring (MRM) acquisition*

1 mg/l	10 mg/l	80 mg/l	LOD (mg/l)	LOQ (mg/l)
100.8 (8.9)	97.6 (1.9)	99.2 (1.8)	1.30	2.70
91.3 (8.2)	102.0 (1.2)	102.0 (0.5)	0.046	0.063
106.6 (6.3)	98.5 (5.9)	102.3 (2.9)	24.3	49.6

Accuracy and precision were evaluated at three concentrations (1, 10, and 80 mg /l) in synthetic urine by analyzing five samples. Satisfactory values were obtained for both parameters since accuracy ranged between 91.3 and 106.6 % whereas RSD values were in the range between 0.5 and 8.9 %. The limit of detection (LOD) and the limit of quantitation (LOQ) values obtained the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry can be considered satisfactory even though a fiftyfold dilution of the sample was performed. Indeed, these values are far below lower limit of the range of normal values in 24 h-human urine (2.0, 1.0 and 1.0 mg/l for HVA, VMA and 5-HIAA, respectively).

4.4.4 Application to Real Samples

The developed method has been applied to the analysis of ten urine samples (five males and five females) from healthy individuals. Each sample was analyzed in triplicate and a typical chromatogram was shown in Figure 36. The mean values of the concentration of HMA, VMA and 5-HIAA were 5.16, 5.41 and 5.51 mg/l, respectively, with minimum and maximum values of 0.45 and 11.1 mg/l for HVA, 0.38 and 13.2 for VMA, 0.52 and 10.5 mg/l for 5-HIAA. The concentrations obtained are compatible with the range of normal

values in 24 h-human urine (2.0-10.0 mg/l for HVA, 1.0-11.0 mg /l for VMA and 1.0-9.0 mg /l for 5-HIAA (58).

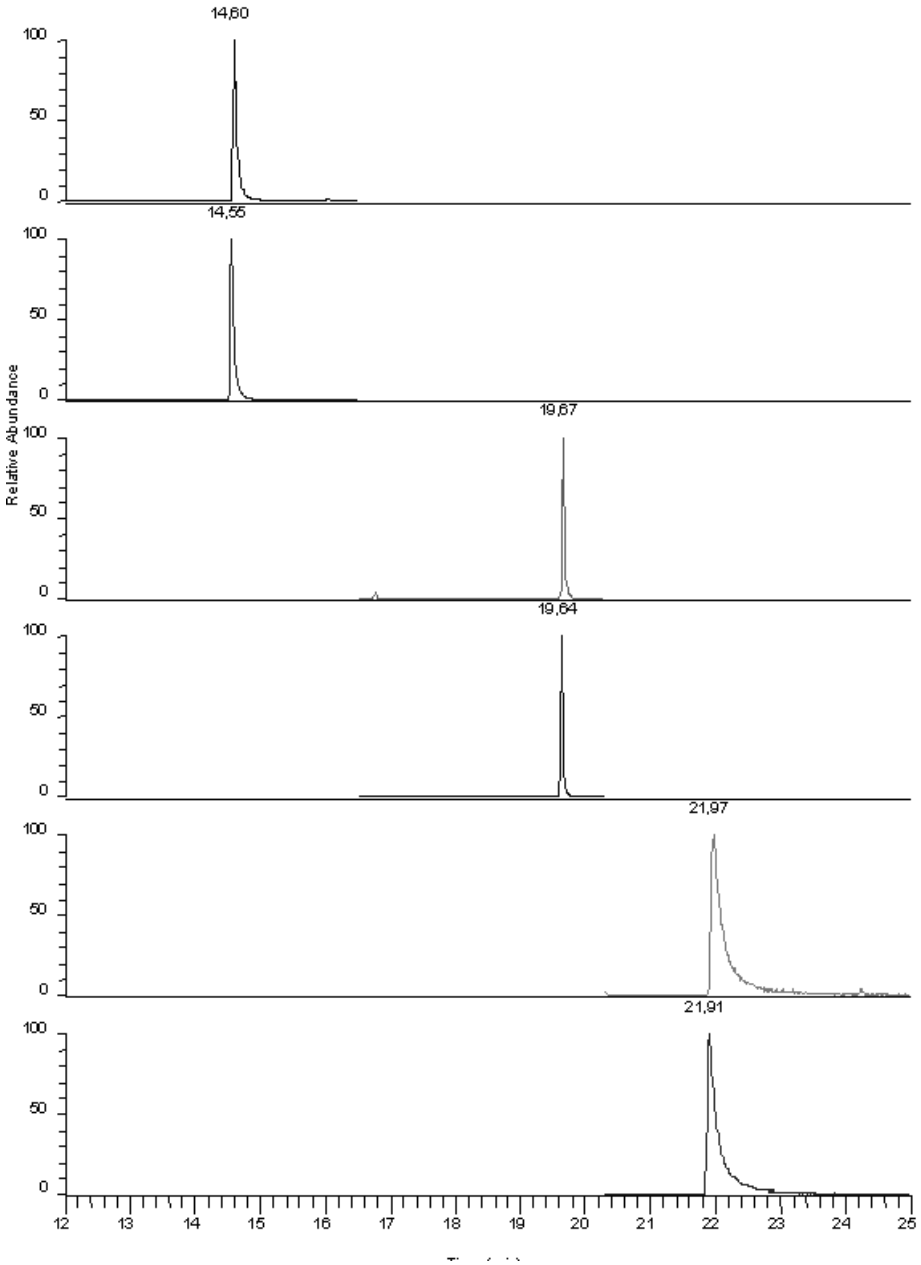


Figure 36. *SPME-GC-MS/MS (multiple reaction monitoring) chromatogram of a real urine sample from an healthy individual (r.t. 14.60 HVA; 14.55 HVA-d5; 19.67 VMA; 19.64 VMA-d3; 21.97 5-HIAA; 21.91 5-HIAA-d5)*

4.4.5 Conclusions

A reliable and rapid method for the determination of HVA, VMA and 5-HIAA, as markers of neuroendocrine tumors, was developed by SPME-GC-QqQ-MS/MS analysis following a preliminary derivatization using ethylchloroformate/ethanol mixture. The combined use of alkylchloroformate and SPME has permitted of analyzing these metabolites by an easy and automated method involving a minimal handling of sample and no consumption of toxic and not environmentally friendly organic solvents. Therefore, the possibility of identifying analytes without ambiguity and very satisfactory performances in terms of linearity, accuracy, precision, and sensitivity make the method developed suitable to be adopted for the quantification of these acidic biomarkers in urine samples as important diagnostic test of neuroendocrine tumors.

4.5 Development of Analytical Method for Analysis of Pollutants in Aqueous Matrices.

This section will describe two methods developed, for the analysis of pollutants in aqueous matrices.In particular, we have developed two protocols of analysis: the first one concerned the quantification of carbamates pesticides, whereas in the second method the determination of perfluoroalkyl acids, by SMPE - GC-QqQ-MS was considered.

4.6 A Solid-Phase Microextraction-Gas Chromatographic Approach Combined with Triple Quadrupole Mass Spectrometry for the Assay of Carbamate Pesticides in Water Samples (66)

Introduction

Carbamate pesticides, a class of highly effective commercial pesticides, are used worldwide against insects, fungi and weeds. They are increasingly used instead of organochlorine and organophosphorous pesticides due to their lower environmental persistence. However, since they are acetylcholinesterase inhibitors, carbamate pesticides are suspected carcinogens and mutagens (68). Aquatic systems and further becomes a potential hazard to human environment. Carbamate pesticides have been on the priority blacklist released by the US Environmental Protection Agency (EPA). Moreover, the European Union Directive (98/83/EC) established that the maximum allowed concentration of individual pesticides is 0.1 µg/l and total pesticides is 0.5µg/l in drinking water. The determination in trace concentration represents an important tool to guarantee the consumers' health and confidence. For their determination in different matrices exist numerous methodologies involving the use of high-performance liquid chromatography (HPLC) with different detector (69–91), gas chromatography (92–102), micellar electrokinetic chromatography (MEKC) (103,104) and biosensor (105). Carbamates of the compounds are thermally unstable, for which undergo decomposition in the injector when they are analyzed by gas chromatography. This aspect is a problem in GC analysis, and precautions have been taken in order to be able to obtain good results in terms of sensitivity and reproducibility. An approach provides for the use of derivatization to block the NH group. Various reactions have been applied by

using several electrophilic compounds as derivatization reagents (106-109). However, the methodology of derivatization does not allow the assay of the carbamates at low concentration. However, the direct analysis is possible if you can minimize the degradation of carbamates. Minimum degradation and was obtained using a temperature program with a gradual gradient, a longer syringe needle, and a column with less modified stationary phase (100). It was demonstrated that solvent the thermolability of carbamates because the high activity of solvent induced by elevated temperatures can modify the degradation process (101,110). The use of SPME is a viable alternative that allows you to control the thermal degradation of carbamates obtaining reliable and reproducible results (93). The aim of this work was to value the applicability of SPME technique coupled to GC-QqQ-MS in developing a new protocol for the assay of carbamates in real water samples that meets the requirements of sensitivity established by European and international legislations.

4.6.1 Optimization SPME and Gas Chromatographic Parameters

In this study were considered the following carbamates: Carbaryl, aldicarb, carbofuran, methiocarb, propoxur and pirimicarb, while as internal standards for their quantification were tested: 4-Bromo-3,5-dimethylphenyln-methylcarbamate (BDMC), carbaryl-d7 and 2,3,5-Trimethacarb. Propoxur, carbofuran, carbaryl and methiocarb gave rise to their phenolic forms together with the parent compound signals whereas a single chromatographic peak was detected for pirimicarb indicating that no decomposition of this pesticide occurs. For aldicarb only the degradation product was detected.

This work started on the basis of preliminary results obtained in the same laboratory research in which this activity took place. In the preliminary phase of work critical variables of SPME and gas –chromatographic parameters

were evaluated using the multivariate method of the Experimental Design (111).In this part of the study the performances of five SPME fibers was evaluated in univariate mode. While, the SPME variables extraction temperature, extraction time, percentage of sodium chloride, pH and desorption temperature has been studied by experimental design. In particular, an fractional factorial design was first performed in order to determine which factors have a significant influence on the experimental response. The variables with greater statistical significance were found to be: concentration of NaCl, pH and extraction time. Later, these three variables were optimized using a central composite design, obtaining the following working conditions: 10% NaCl, 30 min of extraction time and pH 7.

4.6.2 GC-QqQ-MS/MS Analysis

For the optimization parameters tandem mass spectrometry were first acquired of mass spectra in full scan both of the analytes that of the internal standards (figure 37-44). From these, for each analyte was chosen the precursor ion to be subjected to collision-induced dissociation (CID) with argon, by applying collision energies 5-25 V., so as to obtain the ion product spectra. For both analytes and internal standard base peaks were selected as precursor ions. The only exception was made for propoxur whose base peak (m/z 110) was considered not much specific and therefore ion at m/z 151 was chosen as parent ion. The transition yielding the highest response for each pesticide was chosen for quantification and the second more sensitive transition was selected for unequivocal identification of carbamates (table 13).

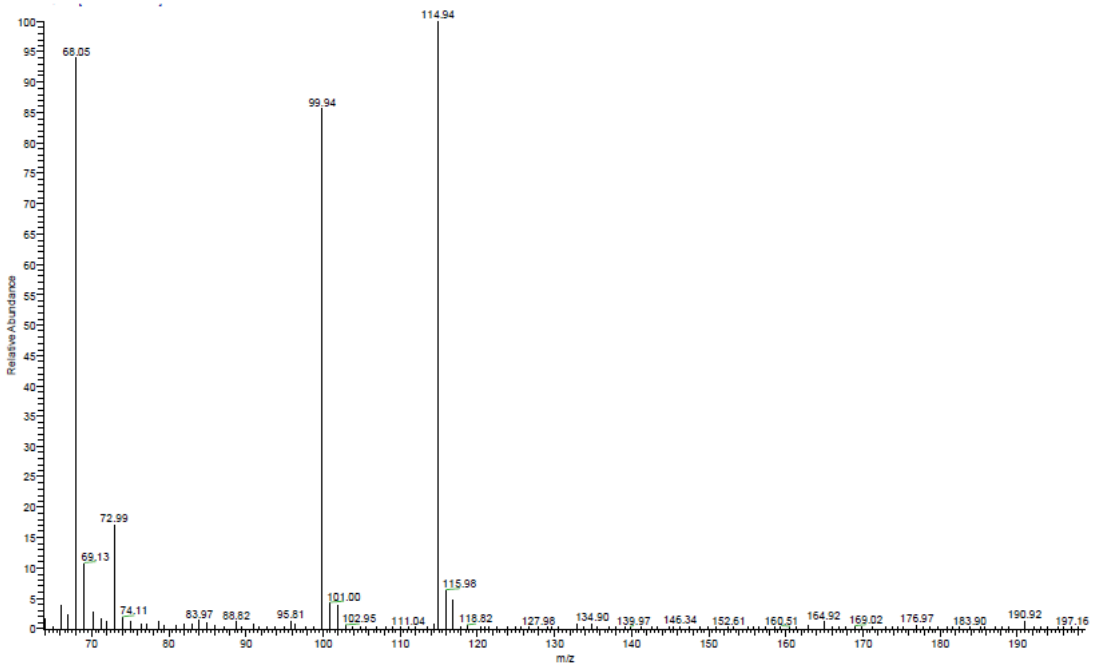


Figure 37. *Mass spectra EI full scan of aldicarb nitrile*

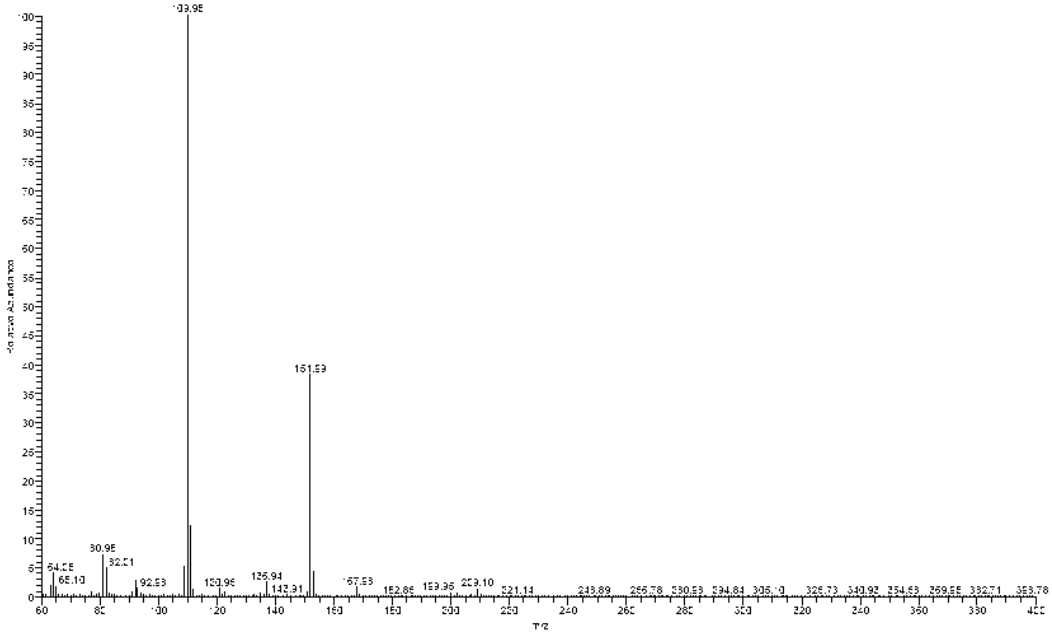


Figure 38. *Mass spectra EI full scan of propoxur*

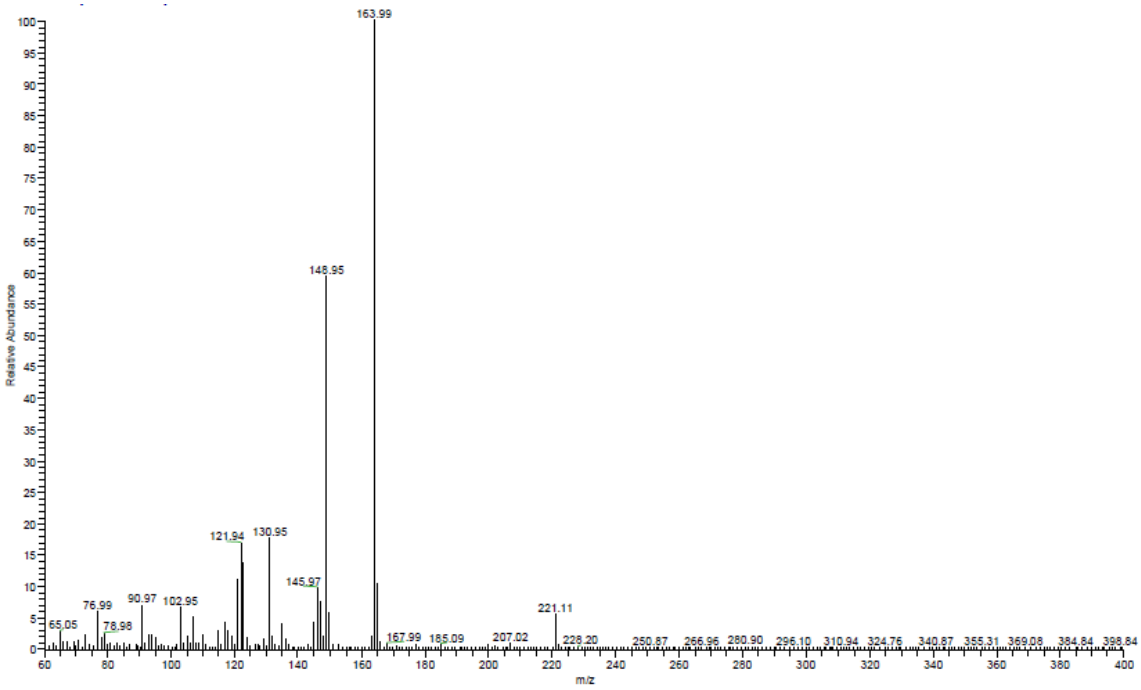


Figure 39. *Mass spectra EI full scan of Carbofuran*

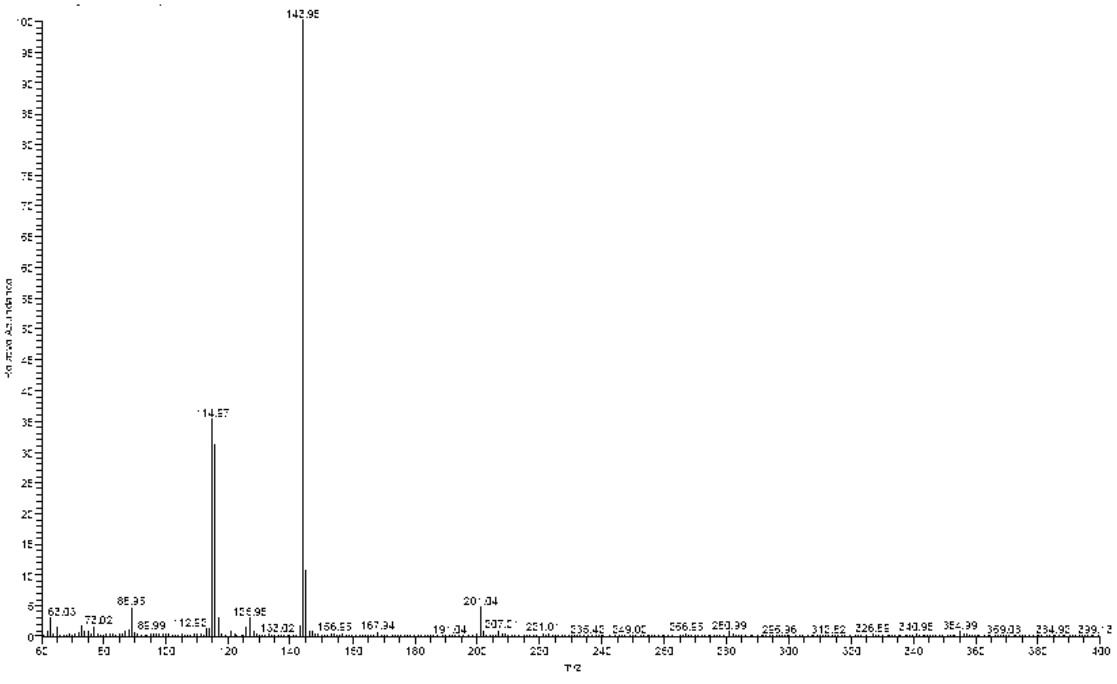


Figure 40. *Mass spectra EI full scan of carbaryl*

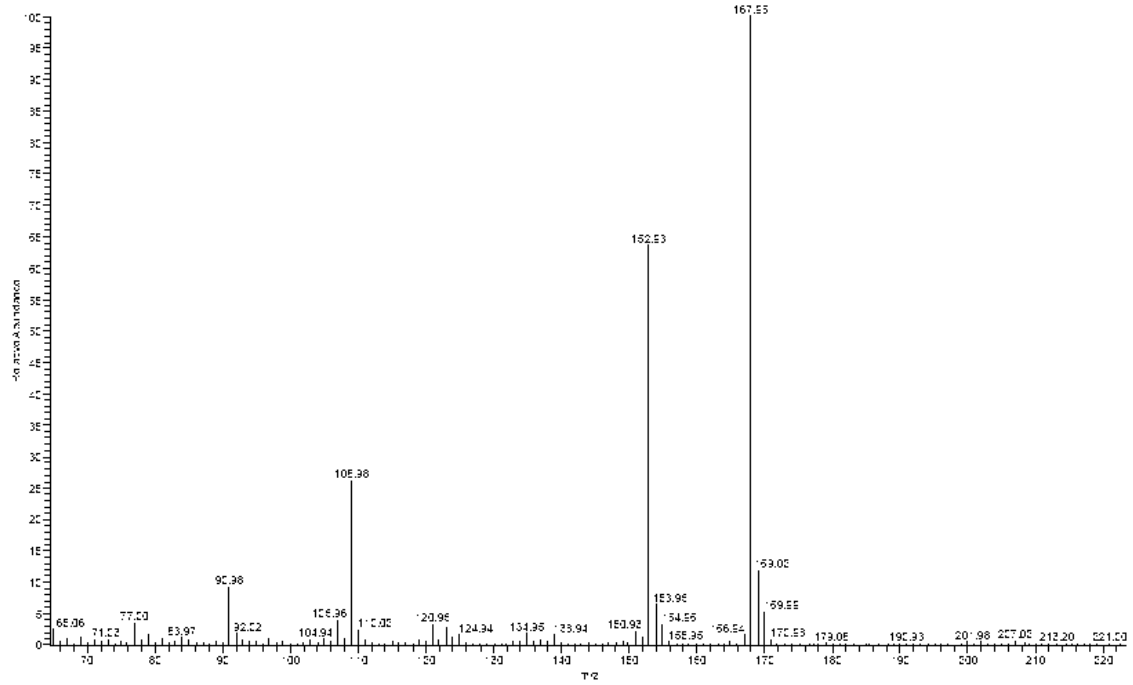


Figure 41. *Mass spectra EI full scan of Methiocarb*

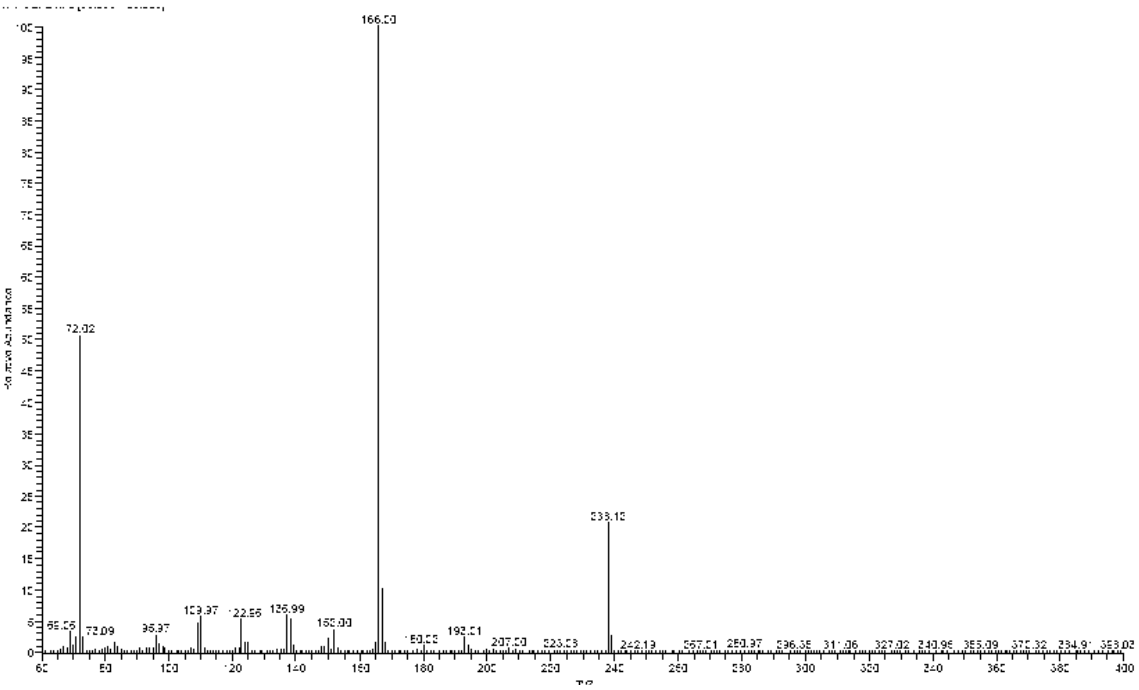


Figure 42. *Mass spectra EI full scan of pirimicarb*

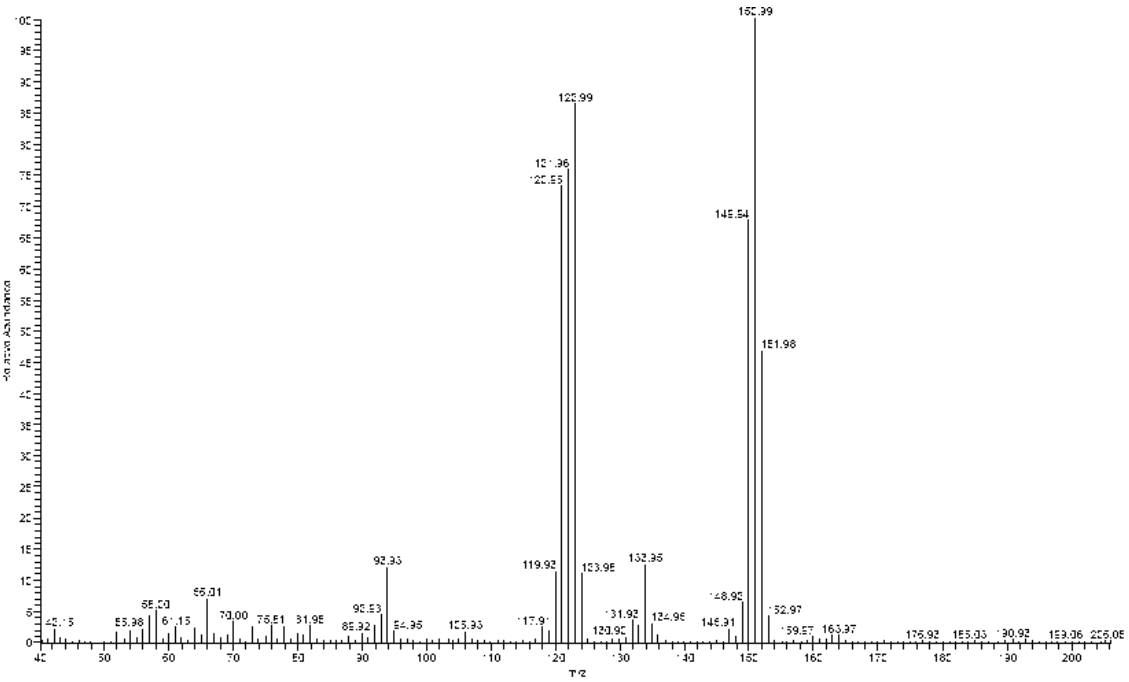


Figure 43. *Mass spectra EI full scan of Carbaryl d7*

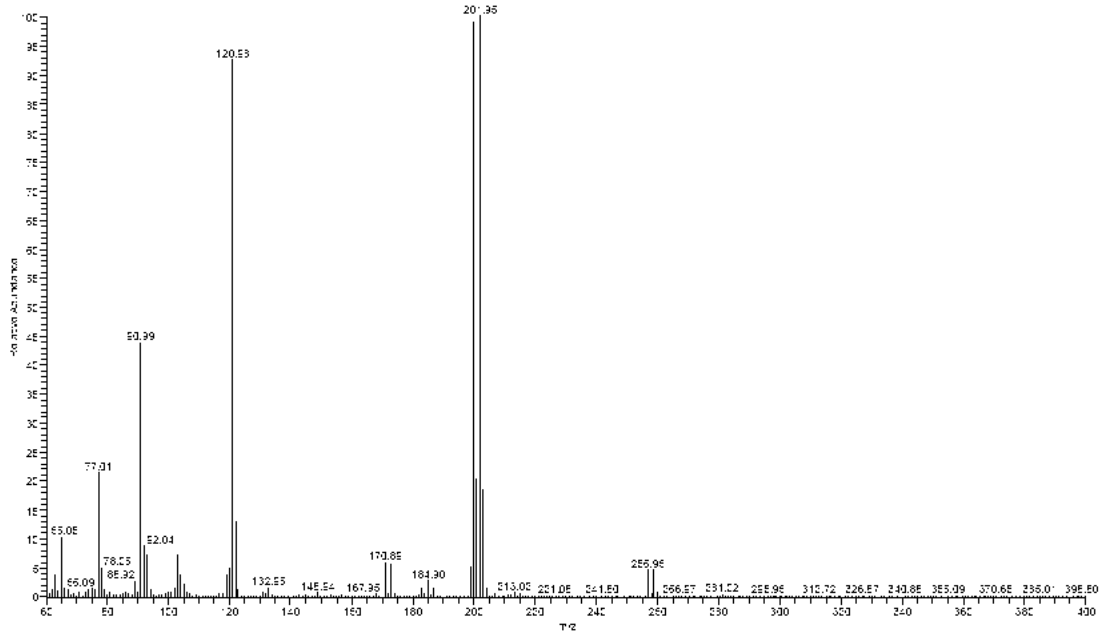


Figure 44. *Mass spectra EI full scan of BDMC*

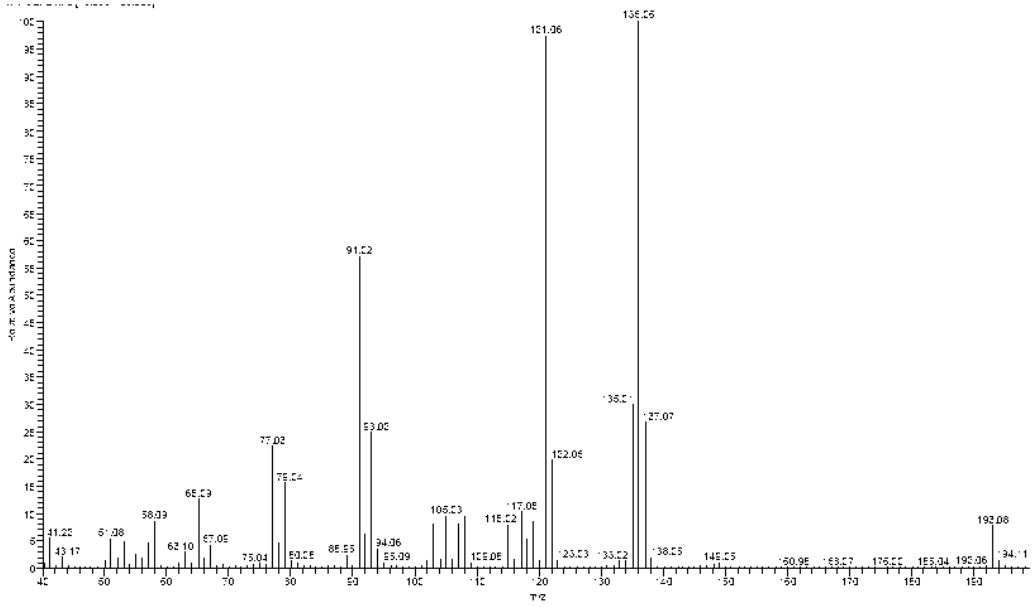


Figure 45. *Mass spectra EI full scan of Trimethacarb*

Table 13. *Retention time (RT) and electron ionization tandem mass spectrometry (EI-MS/MS) parameters (collision energies (eV) are indicated in parenthesis).*

Compound	Retention time (RT)	SRM transition, m/z (collision energy, V)	
		Quantification	Identification
Propoxur	13.94	152→ 110 (7)	110→ 64 (18)
Carbofuran	14.39	164→ 149 (9)	164→ 131 (9)
Pirimicarb	14.63	166→ 96 (14)	166→ 71 (14)
Carbaryl	14.98	144 115 (14)	115→ 89 (18)
methiocarb	15.07	168 →153 (9)	168 →109 (9)
2,3,5-Trimetacarb (IS)	14.11	136→ 121 (7)	
BDMC (IS)	14.81	200→ 121 (11)	
Carbaryl-d7 (IS)	14.96	151→121 (23)	

4.6.3 Analytical Performances

For compounds such as carbamates that undergo thermal degradation of the choice of internal standard is an important step in the method validation. In fact, the use of an internal standard with a thermal behavior similar to those of

analytes should involve a better reproducibility and robustness of the entire analytical method. The three internal standards (2,3,5-trimethacarb, BDMC and carbaryl-d7) were used for the determination of the analytical parameters of each analyte, in order to choose the one that gave the best performance. The linearity of the method was evaluated over the concentration range between 0.05 and 10µg/l with 0.5µg/l of each of the selected internal standard. BDMC has shown a significant memory effect due to an insufficient desorption of analyte from fiber and therefore it was immediately discarded. Accuracy and precision have been evaluated at three concentration levels (0.08, 5 and 3 µg/ l) by analyzing a spiked sample five times. As can be seen in the tables 14 and 15, an appropriate choice of internal standards allows to achieve satisfactory linearities in the tested calibration ranges for all carbamates. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry. The LOD and LOQ values achieved are very good since they range from 0.04 to 1.7 ng/l (table16).

Table 14. *Calibration parameters, internal standard used*

Compound	Calibration curve	R^2	Internal standard
Propoxur	y = 0.1676x + 0.0001	0.9962	Trimethacarb
Carbofuran	y = 0.1009x + 0.0002	0.9956	Trimethacarb
Pirimicarb	y = 0.2209x + 0.0033	0.9891	Trimethacarb
Carbaryl	y = 0.5885x + 0.0224	0.9992	Carbaryl- d7
Methiocarb	y = 2.4532x + 0.0084	0.9986	Carbaryl-d7

Table 15. *Mean accuracies, relative standard deviations*

(RSD (n = 5), %, in parentheses)

Compound	0.08 µg/ l	0.5 µg/ l	3 µg/ l
Propoxur	86.2 (9.0)	87.6 (4.1)	104.6 (5.5)
Carbofuran	86.2 (9.0)	93.6 (4.2	102.9 (7.6)
Pirimicarb	70.8 (1.0	90.4 (3.5)	79.9 (7.2)
Carbaryl	96.2 (2.2)	115.7 (3.8)	125.0 (5.5)
Methiocarb	100.8 (3.6)	80.5 (4.9)	107.7 (3.9)

Table 16. *limits of detection (LODs) and limits of quantitation (LOQs)*

Compound	LOD (ng /l)	LOQ (ng /l)
Propoxur	0.45	0.72
Carbofuran	1.5	2.0
Pirimicarb	0.7	0. 9
Carbaryl	0.39	0.64
Methiocarb	0.04	0.64

4.6.4 Application to Real Samples

The method was tested by carrying out the analysis of water samples real (mineral and tap water) for the determination of carbamates under examination. However, none of the five carbamates were detected in either mineral or tap water samples. Therefore, these samples were spiked at 0.1 µg /l of each pesticide and analyzed five times by the present method. A chromatogram of the five pesticides spiked into tap water sample was shown

in figure 46. Satisfactory results were obtained in terms of recovery and RSD (table 17), which allow to affirm that the matrix effect is negligible.

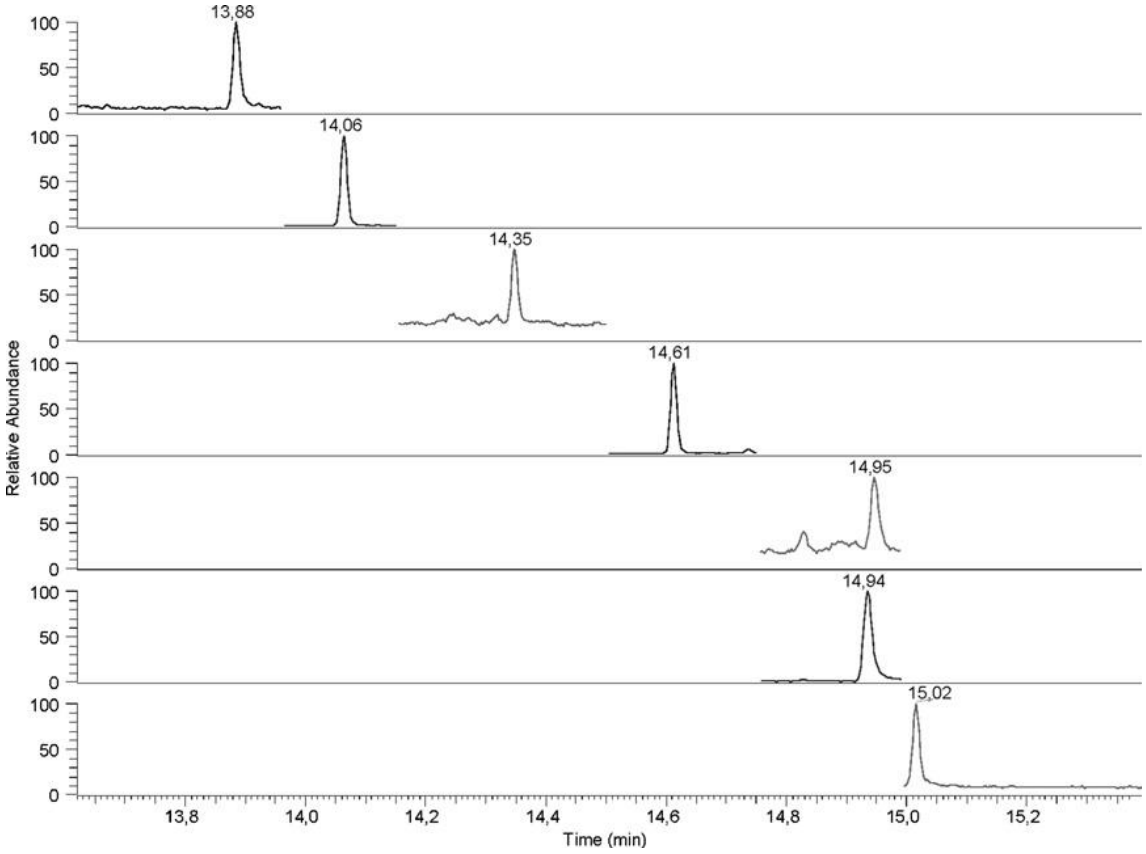


Figure 47. *Typical chromatogram of the extracted analytes from a tap water sample after spiking with 0.1 µg/ l. each of the five carbamates (13.88 propoxur; 14.06 2,3,5- trimetacarb (IS); 14.35 carbofuran; 14.61 pirimicarb; 14.95 carbaryl; 14.94 carbaryl-d7 (IS); 15.02 methiocarb)*

Table 17. *Mean recoveries and relative standard deviations (RSD (n = 5)) for analysis of a tapwater sample spiked at 0.1 µg/ l.*

Compound	Recovery (%)	RSD (%)
Propoxur	84.1	14.6
Carbofuran	92.0	11.1
Pirimicarb	79.4	11.0
Carbaryl	104.2	7.3
Methiocarb	107.6	5.4

4.6.5 Conclusions

This work has permitted the development and validation of a method for the quantification of carbamates in water matrix. For the first time a GC- QqQ-MS system has been used in this field of research that can reach very high levels of sensibility and, at the same time, identify the analytes with greater security . SPME approach athe proper choice of two internal standards (2,3,5-Trimethacarb and Carbaryl d7) has allowed us to obtain good linearity, accuracy and precision. The method also allows to reach high levels of sensibility, expressed as LOD and LOQ, because they represent the lowest values referring to the current literature on carbamates, regardless of the analytical method used.

4.7 A Rapid and Sensitive Assay of Perfluorocarboxylic Acids in Aqueous Matrices by Headspace Solid Phase Microextraction–Gas Chromatography–Triple Quadrupole Mass Spectrometry (112)

Introduction

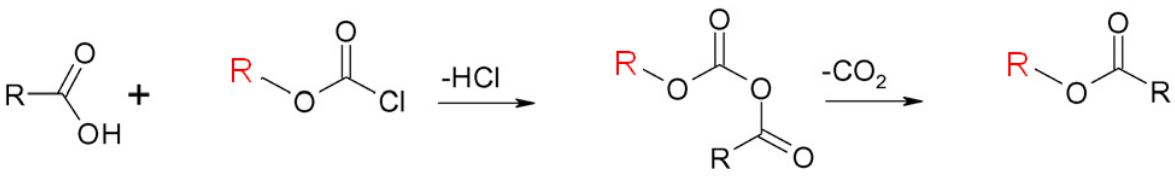
Perfluorocarboxylic acids (PFCAs), belong to the class of ubiquitous pollutants because they have been discovered in air, water, food and soil.(113-116). They are the compounds anthropogenic chemicals, with unique physical, chemical and biological properties, closely related to their high-energy carbon – fluorine bonds (485 kJ/ mol) (115). The latter property makes them resistant to chemical and thermal stress, hydrolysis, photolysis, biodegradation and to metabolic processes in living organisms (117,118). These compounds have been widely used for more than half a century in commercial and industrial applications such as surfactants, lubrificants,

polishes, paper and textile coatings, PTFE precursors, food packaging and fire-retarding foams among others (119-123). The widespread use of PFCAs, along with their high stability and bioaccumulation, has increased considerably over the years their presence in all parts of the environment. So, the most common acid, perfluorooctanoic acid (PFOA), appears to be widespread in the environment and in food. The human intake of PFCAs occurs through the contaminated food including drinking water (124-126). Toxicity of the PFCAs has been concerned by Upham and his coworkers since 1998 (127). According to their studies, the chain length of PFCAs affected an important mechanism of normal cell behavior and gap junction intercellular communication. PFC_8A and nonadecafluorodecanoic acid ($PFC_{10}A$) are peroxisome proliferators and hepatocarcinogens, they are known to promote liver cancer in rats even though DNA are not damaged, the mechanism usually associated with cancer development (127–129). Furthermore, higher concentrations of PFC_8Ain blood might be linked to changes in thyroid hormone levels and associated with thyroid disease in adult (130). PFCAs have been detected in various environmental waters such as river water (131–134), lake water (133–135), seawater (136,137), ground water (138), drinking water (139–141) and wastewater (142–146). The Science Advisory Board to the USEPA has recommended PFOA be classified as a “likely human carcinogen” (147). The technique mainly used for the determination of PFCAs is liquid chromatography–mass spectrometry and tandem mass spectrometry (LC–MS and LC–MS/MS) (117,131-133,135,137,138,148). With these methods achieve high levels of sensitivity and specificity, but require the use of solvents as mobile phase and often extraction procedures such as liquid–liquid and solid-phase extraction. Few are instead, methods that use gas chromatography for the determination of PFCAs in aqueous matrices (115,149-152).The high polarity of PFCs makes necessary the

derivatization step prior to GC analysis. Good results were obtained by derivatizing these compounds by isobutyl chloroformate (153) and ion-pair SPME extraction and following derivatization in GC injector (151). The main purpose of this work was to develop and validate a simple and rapid method for the analysis of PFCAs (Perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA) and perfluorododecanoic acid (PFDoA)) in aqueous samples by SPME–GC–MS/MS after a derivatization step.

4.7.1 Derivatization of PFCAs

Before being analyzed by the GC the PFCs were derivatized with alkyl chloroformates in accordance with the method developed by Čabala et al. (154).



R: $(CF_2)_n–F$ ***R: Alkyl***

Figure 48. *Scheme derivatization reaction of PFCAs with alkyl chloroformate*

The derivatization reaction is carried out by treating with alkyl chloroformate, the reaction mixture composed of the sample of PFCA, phosphate buffer at pH 2.5, pyridine and the corresponding alcohol of the alkyl chloroformate. The general mechanism of the reaction of derivatization is shown in figure 48.

4.7.2 Optimization of SPME Variables

In the first experiments were evaluated five fibers((carboxen/polydimethylsiloxane 85 µm (CAR/PDMS), polyacrylate 85 µm (PA), divinylbenzene/ carboxen/polydimethylsiloxane 50/30 µm (DVB/CAR/PDMS), polydimethylsiloxane/divinylbenzene 65 µm (PDMS/DVB) and polydimethylsiloxane 100 µm (PDMS)) to extract the analytes derivatized with three different alkyl chloroformates (ethyl, propyl and isobutyl), by means of direct immersion and headspace, under the same condition (water spiked at 10 mg/l, room temperature, extraction time 20 min). The most intense signals were obtained derivatizing all analytes with propyl chloroformate and extracting in headspace mode by means of the fibers : DVB/CAR/PDMS and CAR/PDMS. Accordingly, these two fibers were selected for carrying out two experimental designs to obtain the best experimental conditions for each fiber in headspace mode. Variables that were taken into account are: extraction temperature, percentage of sodium chloride and volume of sample, while the extraction time was subsequently studied by univariate method. A central composite design (CCD) was performed for each of the selected fibers. In total, the experimental design matrix had 20 runs (2^3 + (2 × 3) + 6), six of them in the central point (table 18). The range of variables considered were chosen considering the results of preliminary tests and are: extraction temperature in the range 25–60 ◦C, concentration of NaCl ranging from 0 to 20% and sample volume in the range

2–6 ml. All the design experiments were carried out using an aqueous solution of PFCAs at 10 mg /l. The two fibers tested gave very similar results, but CAR/PDMS fiber produced more selective chromatograms and then this fiber was chosen for further development of method. To obtain the best values of the variables studied all response surfaces were drawn for the PFCAs analyzed except for PFHxA which was not detected in all experiments of the design matrix (figure 49). As you can see in fact the higher response was obtained with the lower percentage of NaCl, the lower extraction temperature and the higher sample volume for all the analytes. This result is very surprising, because generally the increase in extraction temperature and the presence of salts promotes the extraction HS-SPME. In this case it is likely that the increase in temperature and the presence of salt involves an increase of interfering species in gaseous phase which compete with the adsorption of analytes on the fiber. Summing the values of variables optimized by experimental design for the CAR/PDMS fiber are: room temperature, absence of NaCl and with a sample volume of 6 ml. The extraction time were optimized, extraction time was studied by univariate method carrying out HS-SPME analysis under previously optimized conditions at three different times: 5, 10 and 20 min. The most intense signals were obtained for all analytes with an extraction time of 10 minutes (figure 48).The observed trend indicating that signals are quadratically dependent of this variable.

Table 18. *Design matrix in the central composite design (CCD) for determination of PFCAs by HS-SPME. (C), central point*

Exp	Extraction Temperature (°C)	Sample volume (ml)	NaCl (%)
18 (C)	42,5	4,0	10,0
6	52,9	2,8	15,9
9	25,0	4,0	10,0
16 (C)	42,5	4,0	10,0
19 (C)	42,5	4,0	10,0
13	42,5	4,0	0,0
3	32,1	5,2	4,1
4	32,1	5,2	15,9
15 (C)	42,5	4,0	10,0
20 (C)	42,5	4,0	10,0
11	42,5	2,0	10,0
14	42,5	4,0	20,0
8	52,9	5,2	15,9
1	32,1	2,8	4,1
12	42,5	6,0	10,0
5	52,9	2,8	4,1
10	60,0	4,0	10,0
17 (C)	42,5	4,0	10,0
7	52,9	5,2	4,1
2	32,1	2,8	15,9

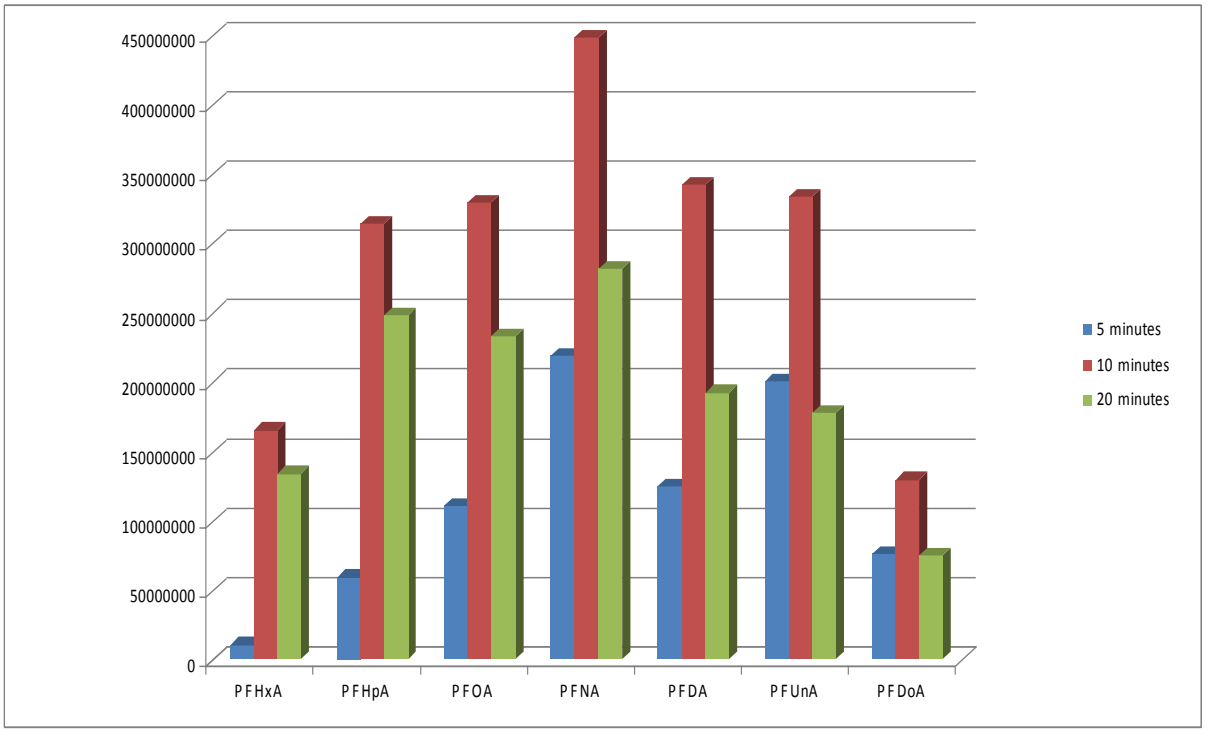


Figure 48. *PFCAs areas Vs extraction time*

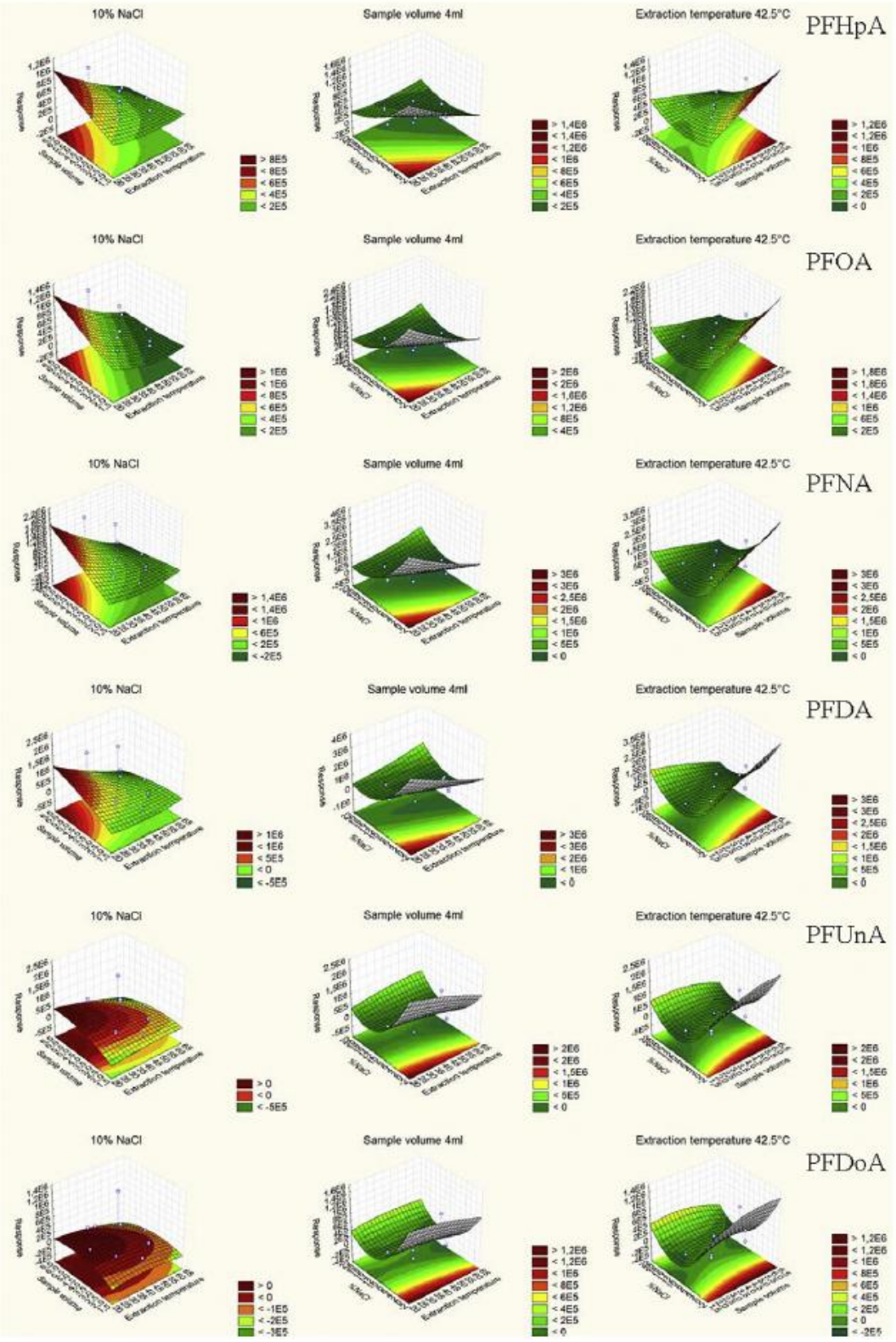


Figure 49. *Response surfaces estimated from the central composite design: peak area of derivatized PFCs for sample volume versus extraction temperature, % NaCl versus extraction temperature, and % NaCl versus sample volume.*

4.7.3 GC–QqQ–MS/MS Analysis

The mass spectrums acquired in EI full scan of the PFCAs as propyl esters , all showed the same pattern of fragmentation with a base peak m/z 43 and different fragment ions with varying carbon–fluorine proportion ($[CF3]^+$ = m/z 69, $[C3F5]^+$ = m/z 131, $[C3F7]^+$ = m/z 169, $[C7F15]^+$ = m/z 369). EI mass spectrum of PFOA-Pr, is reported in figure 50

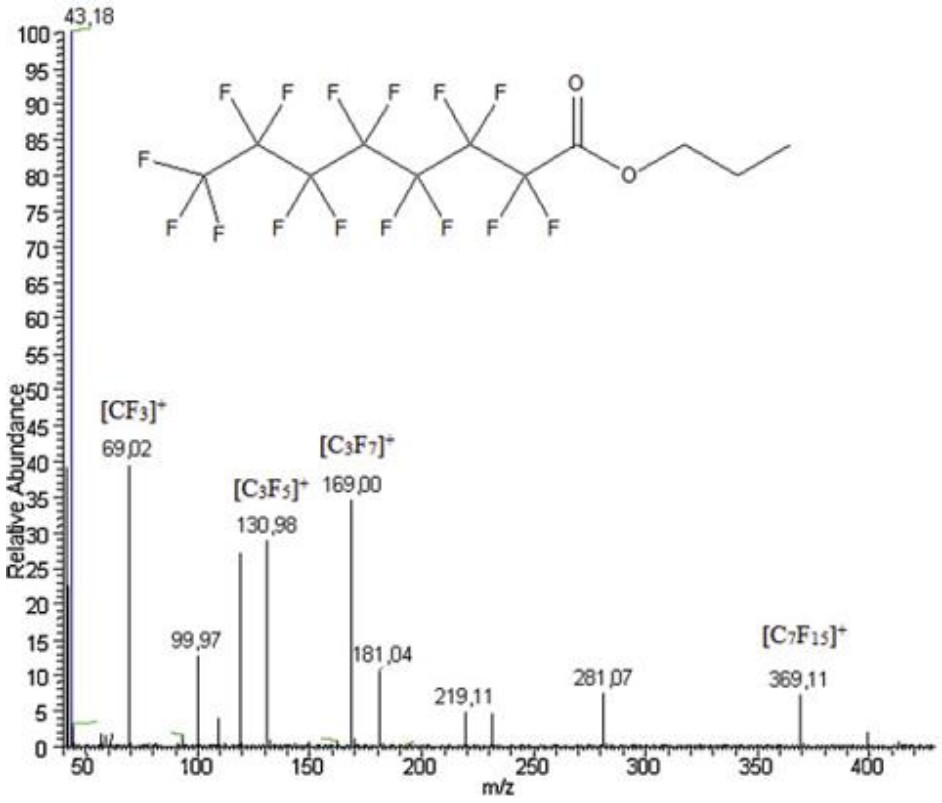


Figure 50. *EI mass spectra of PFOA-Pr*

In order to select the precursor ions for the optimization of the parameters of tandem mass spectrometry, the more abundant ion (m/z 43 and m/z 69) are not usable because of their low mass value, whereas ion at m/z 169 cannot be selected as parent ion, because the corresponding ion for the internal standard ($PF^{13}C4OA$-Pr) does not contain the four ^{13}C atoms and then it has the same m/z value of PFOA-Pr. For which it was necessary to obtain mass spectra with ions at higher values of m/z ratio. For this reason have been acquired

mass spectra of all analytes in negative chemical ionization (NCI) mode using ammonia as reagent gas, as demonstrated by Bayona and Alzaga (151). Also in NCI mode, all PFCAs-Pr spectra follow the same fragmentation pattern (figure 51) .The ions obtained in these conditions are suitable for quantitative determination by tandem mass spectrometry

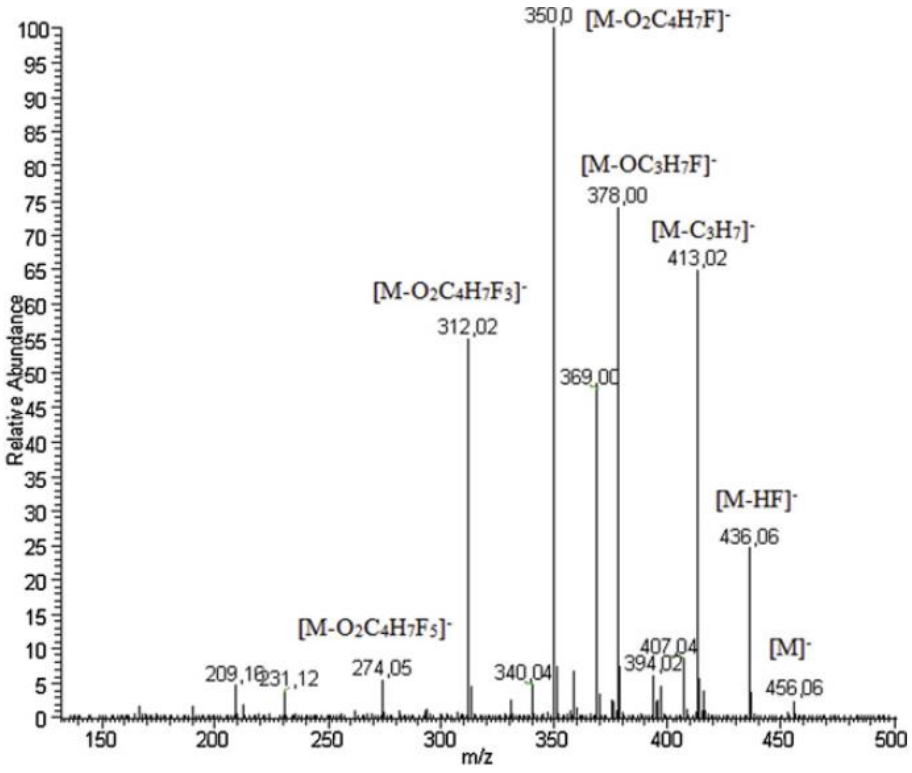


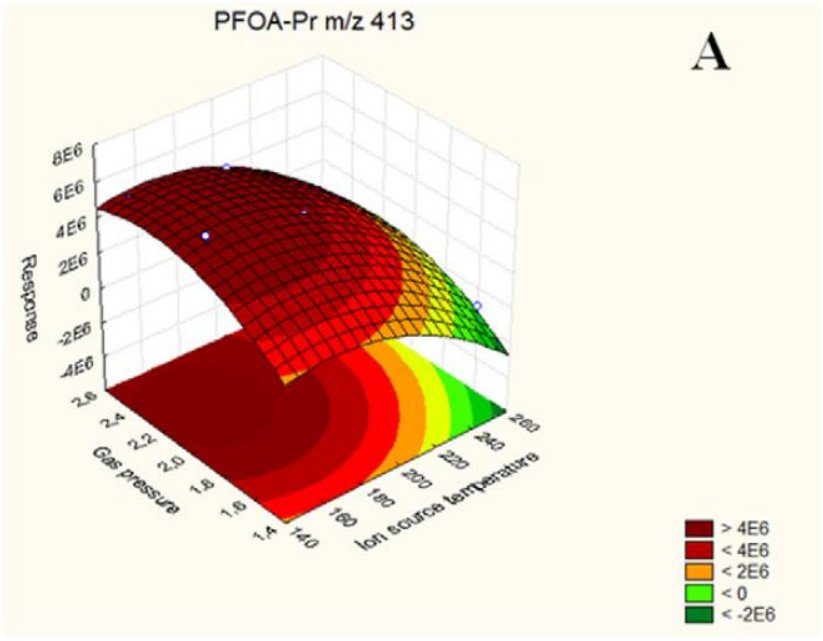
Figure 51. *NCI mass spectra of PFOA-Pr. Ammonia was used as reagent gas*

Four appropriate precursor ions were chosen from the NCI full scan spectra of PFCAs. The corresponding product ion spectra were acquired by collision-induced dissociation (CID) with argon applying collision energies from 5 to 20 V. The results obtained with the optimized SRM transitions for the quantitative and qualitative determination of PFCAs under examination are summarized in the table 19. As it has been demonstrated by Bayona and Alzaga (151), the distribution of ion currents in mass spectra was significantly influenced by ion source temperature and reagent gas pressure at ion source and, therefore, these variables were optimized. A full factorial design with

three levels consisting of 3^2 experiments was performed in the range 150–250 ◦C for ion source temperature and ranging from 1.5 to 2.5 mTorr for reagent gas pressure. The response surfaces obtained by selecting the parent ion of MRM transition for each PFC showed that two trends can be distinguished. The first one regards PFHxA, PFHpA, PFOA and PFNA (for example, response of PFOA-Pr was shown in figure 52 (**A)**) and the second one is common for PFDA, PFUnA and PFDoA (response of PFUnA was shown in figure (**B**)). Is evident that there is a correspondence between the optimal conditions of the two groups, and then it is required to look for a compromise among the responses. For this purpose has been used the Derringer's desirability function, that allows of convert a multi-response problem into a single-response one (67). From this mathematical processing were obtained the following values: ion source temperature 167 ˚C and gas pressure 2.3 mTorr.

Table 19. *Negative chemical ionization tandem mass spectrometry (NCI-MS/MS) parameters (collision energies (eV) are indicated in parenthesis*

Compound	**SRM transition, m/z (collision energy, V)**	
	Quantification	**Identification**
PFHxA	313→ 269 (7)	278 → 209
PFHpA	363→ 319 (6)	328 → 209
PFOA	413→ 369 (8)	350 → 231
$PF^{13}C_4OA$ (IS)	417 → 372 (7)	
PFNA	463 → 419 (7)	419 → 219
PFDA	450 → 331 (9)	412 → 243
PFUnA	462 → 243 (17)	550 → 331
PFDoA	512→ 243 (18)	578 → 209



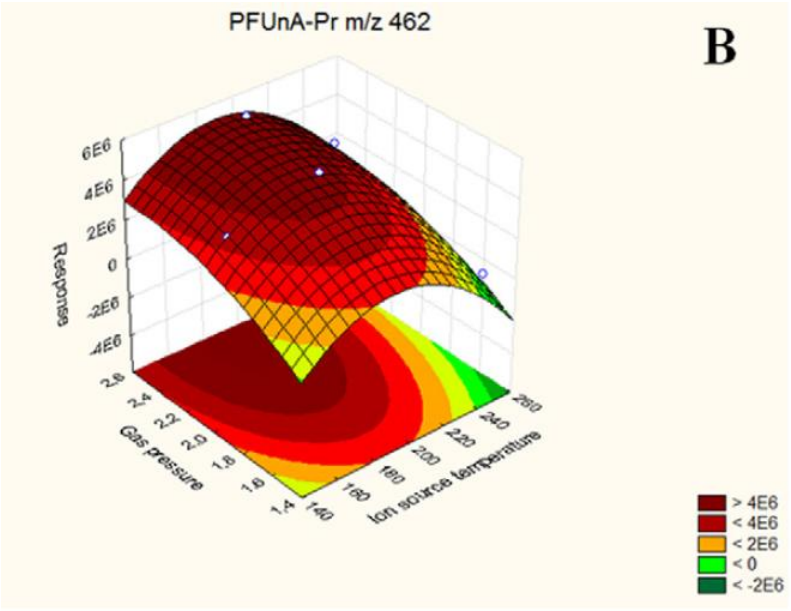


Figure 52. *Response surfaces in NCI mode estimated from the full factorial design for (A) PFOA-Pr (m/z 413) and for (B) PFUnA-Pr (m/z 462).*

4.7.4 Analytical Performances

The linearity test was performed in the concentration range between 0.5 µg/ l and 50 µg/l with 5 µg/l of $PF^{13}C_4OA$ as internal standard. The results

obtained can be considered very satisfactory, indeed the value of correlation coefficient for all analytes is > 0.99 (table 20). Values of accuracy and precision were calculated at three concentration levels, (0.8, 8 and 30 µg/ l) by analyzing a spiked sample three times. As can be seen in the table 21, accuracy values were in the range between 84.4% and 116.8% whereas the relative standard deviations obtained were acceptable because the RSD values were between 0.4% and 14.5%. The LOD and LOQ values achieved by the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry are very good since they range from 0.08 ng/l to 6.6 ng /l. It is important to highlight that the developed method allows to quantify PFCs at concentration levels of sub to low nanogram per liter.

Table 20. *Linearity and correlation coefficient*

Compound	**Curve**	**R^2**
PFHxA	y = 0.2273x + 0.0011	0.9927
PFHpA	y = 0.5289x + 0.1066	0.9986
PFOA	y = 0.8121x + 0.0809	0.9999
PFNA	y = 1.2810x + 0.0379	0.9996
PFDA	y = 1.1363x + 0.1507	0.9941
PFUnA	y = 0.6726x + 0.0184	0.9939
PFDoA	y = 0.2275x + 0.0251	0.9916

Table 21.*Mean accuracies, relative standard deviations (RSD (n = 3), %, in parentheses) and limits of detection (LODs) and limits of quantitation (LOQs) in multiple reaction monitoring (MRM) acquisition.*

Compound	Accuracy and Precision			LOD ng/l	LOQ ng/l
	0.8 µg /l	8 µg /l	30 µg /l		
PFHxA	84.4 (12.1)	110.7 (14.5)	114.9 (11.4)	0.40	0.86
PFHpA	98.9 (9.5)	94.7 (10.8)	103.4 (7.0)	0.17	0.37
PFOA	95.7 (6.9)	103.3 (0.8)	105.4 (0.4	0.11	0.24
PFNA	109.2 (10.4)	98.0 (2.2)	106.2 (6.4)	0.08	0.17
PFDA	86.7 (11.7)	109.9 (8.4)	106.5 (8.2)	1.2	2.5
PFUnA	110.0 (13.1)	104.6 (9.2)	107.4 (8.3)	2.2	4.8
PFDoA	116.8 (12.8)	112.2 (14.1)	110.7 (11.6)	6.6	14.3

4.7.5 Application to Real Samples

The developed method was applied to the analysis of six samples of river water collected at corresponding rivers located in Calabria in January 2012. In particular, Campagnano (in the city of Cosenza), Mavigliano (Montalto Uffugo), Amusa (Caulonia), Allaro (Caulonia), Crati (Cosenza) and Busento (Cosenza) were considered. PFCAs concentrations for all the examined samples were found below LOD. This result is not surprising, because all the considered rivers are located in low environmental impact areas. To check the presence of interferences, a river water sample (Mavigliano) was spiked with the standards of the target analytes at the LOQ values. Three replicate experiments were made and the results were shown in the table 22. The good results obtained, shown in the table exclude the presence of interferents and confirm the efficiency of the method developed. The typical chromatogram of the extracted analytes from the river water sample after spiking with a

concentration corresponding to LOQ value each of the seven PFCAs is showm in figure 53.

Table 22. *Mean recoveries and relative standard deviations (RSD (n = 3)) for analysis of a river water sample spiked at LOQ values.*

Compound	**LOQ values**	
	Recovery (%)	**RSD (%)**
PFHxA	81.5	16.2
PFHpA	79.2	11.8
PFOA	87.3	7.7
PFNA	106.1	13.1
PFDA	109.2	15.8
PFUnA	116.2	15.3
PFDoA	123.7	22.5

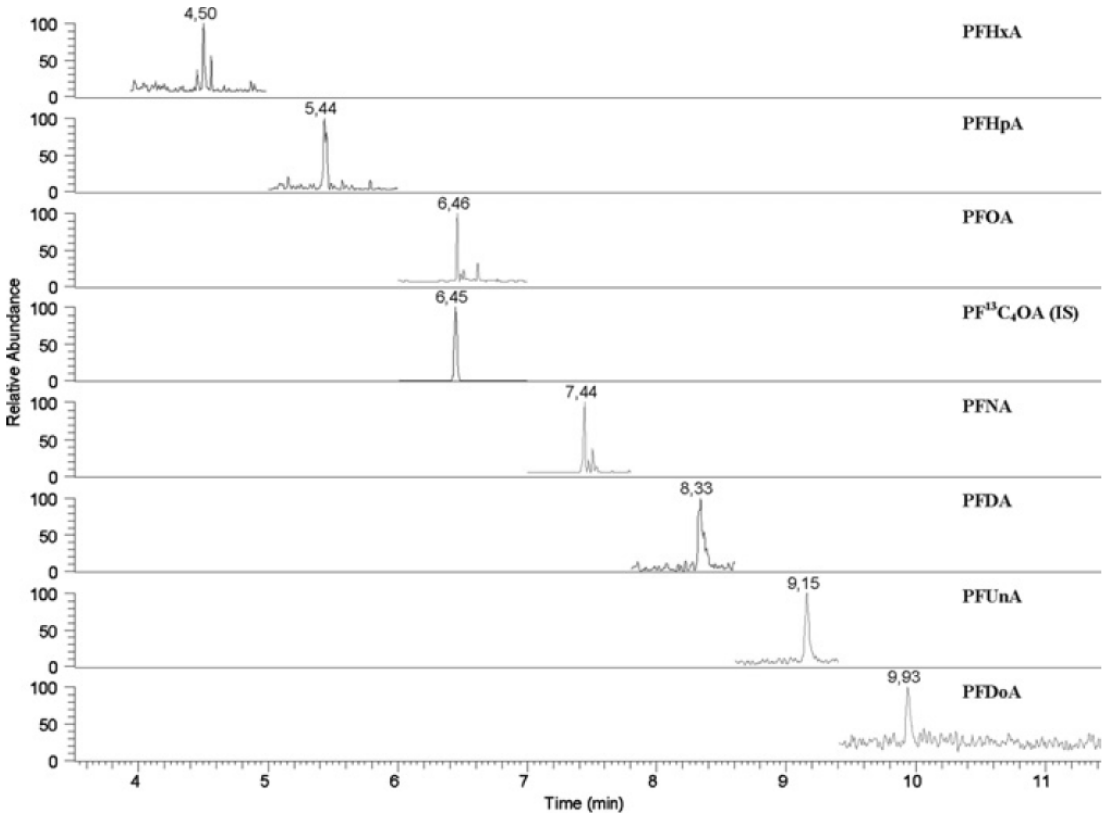


Figure 53. *Typical chromatogram of the extracted analytes from the river water sample after spiking with a concentration corresponding to LOQ value each of the seven PFCAs.*

4.7.6 Conclusions

In the present work, a method for the determination of PFCAs in aqueous sample was developed by HS-SPME–GC–NCI-MS/MS analysis following a preliminary derivatization using propylchloroformate/ propanol mixture. The use of both chloroformate as derivatizing reagent and SPME as extraction technique permits to minimize the amount of organic solvents in final protocol. Moreover, the ability of QqQ–MS in multiple reaction monitoring acquisition allows one to obtain more clear reconstructed chromatograms with well-defined peaks which are more easily integrated. An overall evaluation in terms of linearity, accuracy, precision and sensitivity shows that the proposed method represents a suitable tool for the monitoring of these analytes in environmental and drinking waters. In particular, the obtained LODs and

LOQs for PFHxA, PFHpA, PFOA and PFNA are the lowest values reported in literature. Finally, in this method two important advantages can be highlighted (i) a very rapid and automated procedure allowing higher-throughput screening; (ii) the known specificity of the NCI-MS/MS acquisition mode through the selection of appropriate parent–daughter ion couples improving the ability in analyte identification.

References

1. Harvey DePauw D., University, *Modern Analytical Chemistry*, (**2000)**, McGraw-Hill Higher Education
2. Jurgen H., Gross, *Mass Spectrometry* **2004**. Springer,
3. Westma Brinkmalm A. and Brinkmalm, G., *A Mass Spectrometer's Building Blocks. Mass Spectrometry.* (**2008**) John Wiley & Sons, Inc, 15-87
4. Chen, G., Zhang, L. and Pramanik, B.N.,. LC/MS: *Theory, Instrumentation, and Applications to Small Molecules. HPLC for Pharmaceutical Scientists.* (**2006**) John Wiley & Sons, Inc, 281-346.
5. Yost, R.A., Enke, C.G., *J. Am. Chem. Soc.,* 100(7) (**1978**). 2274-2275.
6. Hoffmann, E., Stroobant, V., *Mass spectrometry: principles and applications.* -(**2007)** J. Wiley
7. Cavaliere B., Macchione B., Monteleone M., Naccarato A., Sindona G., Tagarelli A., *Anal. Bioanal. Chem.* 400 (**2011**) 2903–2912
8. Malati T., *Ind. J. Clin. Biochem.* 22 (2) (**2007)** 17-31
9. Sreekumar A., Poisson L.M., Rajendiran T.M.,Khan A.P., Cao Q.,Yu J., et al *Nature* 457 (**2009**) 910–914
10. Jamaspishvili T., Kral M., Khomeriki I., Student V., Kolar Bouchal Z., *Prostate Cancer Prostatic Dis.* 13 (**2010**) 12–19
11. Smith J.T., *Electrophoresis,* 20 (**1999**)3078–3083
12. Oguri S., Yokoi K., Motohase Y., *J. Chromatogr. A* 787 (**1997**) 253– 260
13. Kolch W., Neusüß C., Pelzing M., Mischak H. *Mass Spectrom Rev.* 24 (**2005**) 959–977

14. Williams B.J., Cameron C.J., Workman R., Broeckling C.D., Sumner L.W., Smith J.T., *Electrophoresis*, 28 (**2007**)1371–1379
15. Le Boucher J., Charret C., Coudray-Lucas C., Giboudeau J., Cynober L., *Clin. Chem.* 43 (**1997**)1421–1428
16. Feste A.S., *J. Chromatogr. B Biomed Appl.* 574 (**1992**) 23–34
17. Biggs H.G., Gentilcore L.J., *Clin. Chem.* 30 (**1984**) 851–855
18. Gatti R., Gioia M.G., *Biomed. Chromatogr.* 22 (**2008**) 207–213
19. Qu J., Wang Y., Luo G., Wu Z., Yang C., Anal. Chem. 74 (**2002**) 2034–2040
20. Piraud M., Vianey-Saban C., Petritis K., Elfakir C., Steghens J.P., Morla A., Bouchu D., *Rapid Commun. Mass Spectrom.* 17 (**2003**)1297–1311
21. Rashed M.S., Bucknall M.P., Little D., Awad A, Jacob M., Alamoudi M., Alwattar M., Ozand P.T., Clin. Chem. 43 (**1997**) 1129–1141
22. Casetta B., Tagliacozzi D., Shushan B., Federici G., *Clin. Chem. Lab. Med.* 38 (**2000**) 391–401
23. Chace D.H., Hillman S.L., Millington D.S., Kahler S.G., Roe C.R., Naylor E.W., *Clin. Chem.* 41(**1995**) 62–68
24. Matsumoto I., Kuhara T., *Mass Spectrom. Rev.* 15 (**1996**) 43–57
25. Gehrke C.W., Nakamoto H., Zumwalt R.W., *J. Chromatogr. A* 45 (**1969**) 24–51
26. Duncan M.W., Poljak A., *Anal. Chem.* 70 (**1998**) 890–896
27. Woo K.L., Lee D.S., *J. Chromatogr. B Biomed. Appl.* 665 (**1995**)15–25
28. Shen X., Deng C., Wang B., Dong L., *Anal. Bioanal. Chem.* 384 (**2006**) 931–938

29. Simek P., Heydova A., Jegorov A., *J. High Resolut Chromatogr.* 17 (**1994**)145–152
30. Chaves das Neves H.J., Vasconcelos A.M.P., *J. Chromatogr.* 392 (**1987**) 249–258
31. Starke I., Kleinpeter E., Kamm B., *Anal. Bioanal. Chem.* 371(**2001**) 380–384
32. Pätzold R., Brückner H., *Amino Acids* 31 (**2006**) 63–72
33. Davis B.A., Durden D.A., (1987) *Biomed. Environ. Mass Spectrom.* 14 197–206
34. Zumwalt RW, Roach D, Gehrke CW J Chromatogr 53 (**1970**) 171–194
35. Fiamegos Y.C., Stalikas C.D., *J. Chromatogr. A* 1110 (**2006**) 66–72
36. Blount B.C., Duncan M.W., *Anal. Biochem.* 244 (**1997**) 270–276
37. Guidetti P., Schwarcz R., *Mol. Brain. Res.* 118 (**2003**)132–139
38. Hušek P., *J. Chromatogr. B* 717 (**1998**) 57–9
39. Vonderheide A.P., Montes-Bayon M., Caruso J.A., *Analyst* 127 (**2002**) 49–53
40. Deng C., Li N., Zhang X., *Rapid Commun. Mass Spectrom.* 18 25 (**2004**) 58–2564
41. Myung S.W., Kim M., Min H.K., Yoo E.A., Kim K.R., *J Chromatogr. B* 727 (**1999**)1–8
42. Kaspar H., Dettmer K., Chan Q., Daniels S., Nimkar S., Daviglus M.L., Stamler J., Elliott P., Oefner P.J., *J. Chromatogr. B* 877 (**2009**)1838–1846
43. Monteleone M., Naccarato A., Sindona G., Tagarelli A., *Anal. Chim. Acta* (**2012**) doi:10.1016/j.aca.2012.11.017

44. Monsaingeon M., Perel Y., Simonnet G., Corcuff J.B., *Eur. J. Pediatr.* 162 (**2003**) 397-402.
45. Hanai J., Kawai T., Sato Y., Tagasuki N., Nishi M., Takeda T., *Clin. Chem.* 33 (**1987**) 2043-2046.
46. Tuchman M., Auray-Blais C., Ramnaraine M.L.R., Negla J., Krivit W., Lemieux B., *Clin. Biochem.* 20 (**1987**) 173-177.
47. Kerbl R., Urban C.E., Ladenstein R., Ambros I.M., Spuller E., Mutz I., Amann G., Kovar, H., *Principles of Internal Medicine,* Vol. 1, McGraw-Hill, **1998**, p. 585.
48. Joy T., Walsh G., Tokmakejian S., Van Uum S.H., *Can. J. Gastroenterol.* 22 (**2008**) 49-53.
49. Mashige F., Ohkubo A., Matsushima Y., Takano M., Tsuchiya E., Kanazawa H., Nagata Y., Takai N., Shinozuka N., Sakuma I., *J. Chromatogr. B Biomed. Appl.* 658 (**1994**) 63-68.
50. Krstulovic A.M., *J. Chromatogr.* 229 (**1982**) 1-34.
51. Seegal R.F., Brosch K.O., Bush B., *J. Chromatogr.* 377 (**1986**) 131-144.
52. Bonfigli A.R., Coppa G., Testa R., Testa I., De Sio G., *Eur. J. Clin. Chem. Clin. Biochem.* 35 (**1997**) 57-61.
53. Manickum T., *J. Chromatogr. B* 877 (**2009**) 4140-4146.
54. Gironi A., Seghieri G., Niccolai M., Mammini P., *Clin. Chem.* 34 (**1988**) 2504-2506.
55. Kawaguchi S., Hirachi N., Fukamachi M., *J. Chromatogr.* 567 (1) (**1991**) 11-19.
56. Manini P., Andreoli R., Cavazzini S., Bergamaschi E., Mutti A., Niessen W.M.A., *J. Chromatogr. B* 744 (**2000**) 423-431.
57. Lionetto L., Lostia A.M., Stigliano A., Cardelli P., Simmaco M., *Clin. Chim. Acta* 398 (**2008**) 53-56.

58. Wadman S.K., Ketting D., Voute P.A., *Clin. Chim. Acta* 72 (**1976**) 49-68.
59. Fauler G., Leis H.J., Huber E., Schellauf C., Kerbl R., Urban C., Gleispach H., *J. Mass Spectrom.* 32 (**1997**) 507-514.
60. Adami A., Guarnieri V., Margesin B., Mulloni V., Vincenzi D., *Biosens. Bioelectron.* 20 (**2005**) 1968-1976.
61. Kałużna-Czaplińska J., *Crit. Rev. Anal. Chem.* 41 (**2011**) 114-123.
62. Hušek P., *J. Chromatogr. B* 717 (**1998**) 57-91.
63. Arthur C.L., Pawliszyn J., *Anal. Chem.* 62 (**1990**) 2145-2148.
64. Lord H., J. Pawliszyn, *J. Chromatogr. A* 885 (**2000**) 153-193.
65. Citová I., Sladkovský R., Solich P., *Anal. Chim. Acta* 573-574 (**2006**) 231-241.
66. Cavaliere B., Monteleone M., Naccarato A., Sindona G., Tagarelli A., *J. Chromatogr. A* 1257 (**2012**) 149– 157
67. Box G.E.P., Hunter W.G., Hunter J.S., *Statistics for Experimenters,* John Wiley, **1978**.
68. Li Q.X., Hammock B.D., Seiber J.N., *J. Agric. Food Chem.* 39 (**1991**) 1537.
69. Soriano M., Jiménez B., Font G., Moltó J.C., *Crit. Rev. Anal. Chem.* 31 (**2001**) 19.
70. Vandecasteele K., Gaus I., Debreuck W., Walraevens K., *Anal. Chem.* 72 (**2000**) 3093.
71. Slobodnik J., Groenewegen M.G.M., Brouwer E.R., Lingeman H., Brinkman U.A.Th., *J. Chromatogr.* 642 (**1993)** 359.
72. Sun L., Lee H.K., *J. Chromatogr. A* 1014 (**2003**) 165.
73. Ozhan G., Ozden S., Alpertunga B., *J. Environ. Sci. Health B* 40 (**2005**) 827.

74. Ahmad N., Guo L., Mandarakas P., Farah V., Appleby S., Gibson T., *J. AOAC .Int.* 79 (**1996**) 1417.
75. Nunes G.S., Ribeiro M.L., Polese L., *J. Chromatogr. A* 795 (**1998**) 43.
76. Pérez-Ruiz T., Martínez-Lozano C., García M.D., *J. Chromatogr. A* 1164 (**2007**) 174.
77. Mayer-Helm B., Hofbauer L., Muller J., *Rapid Commun. Mass Spectrom.* 20 (**2006**) 529.
78. Lee J.M., Chesney D.J., *Anal. Chim. Acta* 389 (**1999**) 53.
79. Fillion J., Hindle R., Lacroix M., Selwyn J., *J. AOAC Int.* 78 (**1995**) 1252.
80. Prados-Rosales R.C., Herrera M.C., Luque-García J.L., Luque de Castro M.D., *J.Chromatogr. A* 953 (**2002**) 133.
81. Sagratini G., Manes J., Giardiná D., Damiani P., Picó Y., *J. Chromatogr. A* 1147 (**2007**) 135.
82. Goulart S.M., Alves R.D., Nenes A.A., de Queiroz J.H., de Assis T.C., de Queiroz M.E.L.R., *Anal. Chim. Acta* 671 (*2010*) 41.
83. Mayer-Helm B., Hofbauer L., Müller J., *Rapid Commun. Mass Spectrom.* 20 (**2006**) 529.
84. Basheer C., Alnedhary A.A., Madhava Rao B.S., Lee H.K., *J. Chromatogr. A* 1216 (**2009**) 211.
85. Y. Gou, R. Eisert, J. Pawliszyn, J. Chromatogr. A 873 (2000) 137.
86. Gou Y., Pawliszyn J., *Anal. Chem*. 72 (**2000**) 2774.
87. Wu Q., Zhou X., Li Y., Zang X., Wang C., Wang Z., *Anal. Bioanal. Chem.* 393 (**2009**) 1755.
88. Zhou Q., Pang L., Xiao J., *Mikrochim. Acta* 173 (**2011**) 477.
89. Wu Q., Chang Q., Wu C., Rao H., Zeng X., Wang, C., Wang Z., *J. Chromatogr. A* 1217 (**2011**) 1773.

90. Wu Q., Zhao G., Feng C., Wang C., Wang Z., *J. Chromatogr. A* 1218 (**2011**) 7936.
91. Chen H., Chen R., Li S., *J. Chromatogr. A* 1217 (**2011**) 1244.
92. Zhang J., Lee H.K, *J. Chromatogr. A* 1117 (**2006**) 31.
93. López-Blanco M.C., Gómez-Álvarez S., Rey-Garrote M., Cancho-Grande B., Simal-Gándara J., *Anal. Bioanal. Chem*. 383 (**2005**) 557.
94. Lee J., Lee H.K., *Anal. Chem.* 83 (**2011**) 6856.
95. Portolés T., Pitarch E., López F.J., Hernández F., *J. Chromatogr. A* 1218 (**2011**) 303.
96. Chen H., Chen R., Feng R., Li S., *Chromatographia* 70 (**2009**) 165.
97. Saraji M., Esteki N., *Anal. Bioanal. Chem.* 391 (**2008**) 1091.
98. Tostado G., Polo-Díez M.L., J. Chromatogr. A 921 (2001) 287.
99. E. Crespo-Corral, M.J. Santos-Delgado, M.L. Polo-Díez, Soria A.C., *J. Chromatogr.A* 1209 (**2008**) 22.
100. E. Crespo-Corral, M.J. Santos-Delgado, M.L. Polo-Díez, A.C. Soria, *J. Chromatogr.A* 1209 (**2008**) 22.
101. R. Carabias-Martinez, C. García-Hermida, E. Rodríguez-Gonzalo, L. Ruano-Miguel, J. Sep. Sci. 28 (2005) 2130.
102. Crespo-Corral E., Santos-Delgado M.J., Polo-Díez M.L., Sanz-Perucha J., *J. Chromatogr. A* 1132 (**2006**) 241.
103. Molina M., Pérez-Bendito D., Silva M., *Electrophoresis* 20 (**1999**) 3439.
104. Wu Y.S., Lee H.K., Li S.F.Y., *J. Microcolumn Sep.* 10 (**1998**) 239.
105. Hildebrandt A., Bragós R., Lacorte S., Marty J.L., *Sens. Actuators B: Chem.* 13(**2008)** 195.

106. Stan H.J., Klaffenbach P., Fresenius J., *Anal. Chem.* 339 (**1991**) 151.

107. Okumura T., Imamura K., Nishikawa Y., *Analyst* 120 (**1995**) 2675.

108. Ballesteros E., Gallego M., Valcarcel M., *Anal. Chem.* 65 (**1993**) 1773.

109. King J.W., Zhang Z., *Anal. Bioanal. Chem.* 374 (**2002**) 88.

110. Przybylski C., Bonnet V., *J. Chromatogr. A* 1216 (**2009**) 4787.

111. Marchio F., *Tesi-Determinazione di carbammati in acqua tramite microestrazione in fase solida (SPME), analisi GC-MS e ottimizzazione dei parametri cromatografici mediante "Experimental design"*., A.A, **2008-2009**.

112. Monteleone M., Naccarato A., Sindona G., Tagarelli A., J. *Chromatogr. A* 1251 (**2012**) 160-8

113. Kissa E., *Fluorinated Surfactants and Repellents*, 2nd ed., Marcel Dekker, , **2001**

114. Houde M., Martin J.W., Letcher R.J., Solomon K.R., Muir D.C.G., *Environ. Sci. Technol.* 40 (**2006**) 3463.

115. de Voogt P., Sáez M., *Trends Anal. Chem.* 25 (**2006**) 326-342

116. Tittlemier S.A., Braekevelt E., *Anal. Bioanal. Chem.* 399 (**2011**) 221.

117. Tseng C.L., Liu L.L., Chen C.M., Ding W.H., *J. Chromatogr. A* 1105 (**2006**) 119.

118. Key B.L., Howell R.D., Criddle C.S., *Environ. Sci. Technol.* 31 (**1997**) 2445

119. Hekster F.M., Laane R.W., de Voogt P., *Rev. Environ. Contam. Toxicol.* 179 (**2003**) 99.

120. Kovarova J., Svobodova Z., *Neuroendocrinol. Lett.* 29 (**2008**) 599.

121. Mawn M.P., McKay R.G., Ryan T.W., Szostek B., Powley C.R., Buck R.C., *Analyst* 130 (**2005**) 670.

122. Martin J.W., Muir D.C.G., Moody C.A., Ellis D.A., Kwan W.C., Solomon K.R., Mabury S.A., *Anal. Chem.* 74 (**2002**) 584.

123. González-Barreiro C., Martínez-Carballo E., Sitka A., Scharf S., Gans O., *Anal. Bioanal. Chem.* 386 (**2006**) 2123

124. Ericson I., Nadal M., van Bavel B., Lindström G., Domingo J.L., *Environ. Sci. Pollut. Res. Int.* 15 (**2008**) 614.

125. Del Gobbo L., Tittlemier S., Diamond M., Pepper K., Tague B., Yeudall F., Vanderlinden L., *J. Agric. Food Chem.* 56 (**2008**) 7551.

126. Kärrman A., Ericson I., van Bavel B., Darnerud P.O., Aune M., Glynn A., Lignell S., Lindström G., *Environ. Health Perspect.* 115 (**2007**) 226.

127. Upham B.L., Deocampo N.D., Wurl B., Trosko J.E., *Int. J. Cancer* 78 (**1998**) 491.

128. Renner R., *Environ. Sci. Technol.* 35 (**2001**) 154A.

129. Hu W., Jones P.D., Upham B.L., Trosko J.E., Lau C., Giesy J.P., *Toxicol. Sci.* 68 (**2002**)429.

130. Melzer D., Rice N., Depledge M.H., Henley W.E., Galloway T.S., *Environ. Health Perspect.* 118 (**2010**) 686.

131. Weremiuk A.M., Gerstmann S., Frank H., *J. Sep. Sci.* 29 (**2006**) 2251.

132. Zhao X., Li J., Shi Y., Cai Y., Mou S., Jiang G., *J. Chromatogr. A* 1154 (**2007**) 52.

133. Loos R., Wollgast J., Huber T., Hanke G., *Anal. Bioanal. Chem.* 387 (**2007**) 1469.

134. Wojcik L., Korczak K., Szostek B., Trojanowicz M., *J. Chromatogr. A* 1128 (**2006**) 290.

135. Furdui V.I., Crozier P.W., Reiner E.J., Mabury S.A., *Chemosphere* 73 (**2008**) S24.

136. Nakata H., Kannan K., Nasu T., Cho H.S., Sinclair E., Takemurai A., *Environ. Sci. Technol.* 406 (**2006**) 4916.

137. Miyake Y., Yamashita N., Rostkowski P., So M.K., Taniyasu S., Lam P.K., Kannan K., *J. Chromatogr. A* 1143 (**2007**) 98.

138. Murakami M., Kuroda K., Sato N., Fukushi T., Takizawa S., Takada H., *Environ. Sci. Technol.* 43 (**2009**) 3480.

139. Takagi S., Adachi F., Miyano K., Koizumi Y., Tanaka H., Mimura M., Watanabe I., Tanabe S., Kannan K., *Chemosphere* 72 (**2008**) 1409.

140. Mak Y.L., Taniyasu S., Yeung L.W., Lu G., Jin L., Yang Y., Lam P.K., Kannan K., Yamashita N., *Environ. Sci. Technol.* 43 (**2009**) 4824.

141. Loganathan B.G., Sajwan K.S., Sinclair E., Senthil Kumar K., Kannan K., *Water Res.* 41 (**2007**) 4611.

142. Wilhelm M., Bergmann S., Dieter H.H., *Int. J. Hyg. Environ. Health* 213 (**2010**) 224.

143. Becker A.M., Gerstmann S., Frank H., *Chemosphere* 72 (2008) 115.

144. Murakami M., Shinohara H., Takada H., *Chemosphere* 74 (2009) 487.

145. Yu J., Hu J., Tanaka S., Fujii S., *Water Res.* 43 (2009) 2399.

146. Becker A.M., Suchan M., Gerstmann S., Frank H., *Environ. Sci. Pollut. Res.* 17 (**2010**) 1502.
147. Renner R., *Environ. Sci. Technol.* 42 (**2008**) 648
148. Stadalius M., Connolly P., L'Empereur K., Flaherty J.M., Isemura T., Kaiser M.A., Knaup W., Noguchi M., *J. Chromatogr. A* 1123 (**2006**) 10.
149. Moody C.A., Field J.A., *Environ. Sci. Technol.* 33 (**1999**) 2800.
150. Alzaga R., Bayona J.M., *J. Chromatogr. A* 1042 (**2004**) 155.
151. Scott B.F., Moody C.A., Spencer C., Small J.M., Muir D.C.G., Mabury S.A., *Environ. Sci. Technol.* 40 (**2006**) 6405.
152. Scott B.F., Spencer C., Mabury S.A., Muir D.C.G, *Environ. Sci. Technol*. 40 (**2006**) 7167.
153. Dufková V., Čabala R., Maradová D., Štícha M., *J. Chromatogr. A* 1216 (**2009**) 8659.

CHAPTER 5

EXPERIMENTAL SECTION

5.1 Sarcosine as a Marker in Prostate Cancer Progression: a Rapid and Simple Method for its Quantification in Human Urine by Solid-Phase Microextraction–Gas Chromatography–Triple Quadrupole Mass Spectrometry.

5.1.1 Chemicals and Reagents

Sarcosine standard, pyridine, and sodium chloride were purchased from Sigma-Aldrich (Milan, Italy). Sarcosine-d3 was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Ethyl chloroformate and ethanol were obtained from Fluka (Milan, Italy). The SPME fibers tested were purchased from Supelco (Bellefonte, PA, USA) and conditioned as recommended by the manufacturer. Synthetic urine (negative urine control) was obtained from Cerilliant (Round Rock, TE, USA).

5.1.2 Instrumentation and Apparatus

GC-MS analyses for the optimization of SPME variables were performed using a Varian (Walnut Creek, CA, USA) Saturn 2000 GC-MS ion-trap system in electron ionization mode, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector. The ion trap temperature was set at 210°C with an ionization time of 25 ms, an emission current of 10 μA, and a scan rate of 1,000 ms. The capillary column was a 30 m×0.25-mm inner diameter, 0.25-μm film thickness Zebron GC ZB-5 ms

[95% polydimethylsiloxane (PDMS), 5% polydiphenylsiloxane]. The gas chromatograph oven temperature was initially held at 70°C for 5 min, then ramped at 8° C/min to 210°C, held at this temperature for 2 min, and finally ramped at 60°C/min to 280°C and held at this temperature for 2 min. The carrier gas was helium (purità 99.999%) at a flow rate of 1 ml/min. For SPME analyses, a narrow-bore Supelco 0.8-mm inner diameter gas chromatograph inlet liner was used. Analyses were performed in splitless mode and spectra were acquired in full-scan mode in a mass range of m/z 40–200. GC-MS analyses for calibration and quantification of real samples were carried out using a TSQ Quantum GC (Thermo Fischer Scientific) system constituted by a Quantum triple quadrupole (QqQ) mass spectrometer and a TRACE GC Ultra equipped with an TriPlus autosampler. The capillary column was a 30 m×0.25-mm inner diameter, 0.25-μm film thickness Thermo TR-5MS. The injector temperature was set at 270°C and the gas chromatograph oven temperature was programmed in the same way as for the Saturn 2000 GC-MS system. Helium at a constant flow rate of 1 ml/min was used as the carrier gas; argon at a pressure of about 1.0 mTorr was used as the collision gas. The QqQ mass spectrometer was operated in electron ionization and SRM mode. The transfer line and ionization source temperatures were both set at 250°C. A filament multiplier delay of 16 min was set to prevent instrument damage. The emission current was set at 50 μA. The scan width and the scan time were set at 0.1m/z and 0.1 s. The peak width of the first quadruple was fixed at 0.7 amu.

5.1.3 Samples

The urine samples were taken as aliquots from the 24-h urine specimens collected from ten healthy male volunteers between the ages of 24 and 35 years.

5.1.4 Analytical Procedure

A 60-µl volume of sarcosine-d_3 solution at 10 mg/l was added to 1.44 ml of urine. Afterwards, 750 µl of an ethanol and pyridine mixture (3:1 v/v) was added, and the mixture was magnetically stirred for 10 min. Then, 156 µl of ethyl chloroformate was added, and the sample was shaken for 2 min. Finally, 6 ml of NaCl solution at 0.14 g/ml was added. SPME was performed with a 50/30 µm divinylbenzene (DVB)/ Carboxen (CAR)/PDMS fiber. Equal amounts of urine and derivatization mixture were placed in each septumclosed vial, and the extraction was performed in immersion mode for 20 min. The adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 270°C for 10 min. A blank analysis of the fiber did not display any peak due to the analyte under investigation.

5.1.5 Optimization of SPME Variables

The experimentalmatrix designs were carried out and evaluate using Statistica 8.0 (2007 edition, StatSoft, Tulsa, USA).

5.2 A Reliable and Simple Method for the Assay of Meuroendocrine Tumor Markers in Human Urine by Solid-Phase Microextraction-Gas Chromatography-Triple Quadrupole Mass Spectrometry

5.2.1 Chemicals and Reagents

Homovanillic acid (HVA), vanylmandelic acid (VMA) and 5-hydroxyindoleacetic acid (5-HIAA) were purchased from Sigma-Aldrich as analytical standards (Milan, Italy). Homovanillic acid-d5 (HVA-d5),

vanylmandelic acid-d3 (VMA-d3) and 5-hydroxyindoleacetic acid-d5 (5-HIAA-d5), used as internal standards, were bought from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile, pyridine and sodium chloride were purchased from Sigma-Aldrich (Milan, Italy). Alkyl chloroformates and the corresponding alcohols were obtained from Fluka (Milan, Italy). The tested solid phase microextraction fibers were purchased 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) was obtained from Cerilliant (Round Rock, TE, USA).

5.2.2 Instrumentation and Apparatus

GC-MS analyses were carried out using 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 30m×0.25mm i.d., 0.25 µm film thickness Thermo TR-5MS (95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 100 °C for 2 min, and then ramped at 10 °C min-1 to 200 °C, ramped again at 5 °C min-1 to 280 °C and finally held at this temperature for 4 min. The carrier gas was helium at 1 mL min-1 of purity 99.999% and argon at a pressure of 2.3 mTorr was used as collision gas. For SPME analyses, a Thermo PTV straight Liner 0.75×2.75×105 mm was used as GC inlet liner. Analyses were performed in splitless mode and by setting the injector temperature at 290 °C. The QqQ mass spectrometer was operated in electron ionization (EI) in multiple reaction monitoring (MRM) mode. The transfer line and ionization source temperatures were set both at 250 °C. The emission current was set at 25 µA. The scan width and scan time were set at 0.1 m/z and 0.15 s for all segments. Peak width of Q1 was fixed at 0.7 amu.

5.2.3 Samples

The urine samples were taken as aliquots from the 24-h urine specimens collected from ten healthy volunteers (five female and five male) between the ages of 25 and 38 years.

5.2.4 Analytical Procedure

50 µL of urine and a 50-µL volume of internal standards solution at 10 mg/1 were added to 75 µL of reaction medium (acetonitrile, ethanol and pyridine in the ratio 2:2:2, respectively) and mixed for 1 min. Afterwards, an amount of 18.75 µL of ethyl chloroformate was added, the sample was shaken for 1 min and finally 8 mL of NaCl solution at 9.5 % was added. The vial was then crimped and SPME extraction was performed with a 85 µm polyacrylate (PA) fiber in immersion mode for 25.8 min at room temperature and the adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 290 °C for 10 min. A blank analysis of the fiber did not display any peak due to the analyte under investigation.

5.2.5 Optimization of SPME Variables

The experimentalmatrix designs were carried out and evaluate using Statistica 8.0 (2007 edition, StatSoft, Tulsa, USA).

5.3 A solid-phase Microextraction-Gas Chromatographic Approach Combined with Triple Quadrupole Mass Spectrometry for the Assay of Carbamate Pesticides in Water Samples

5.3.1 Chemicals and Reagents

Carbaryl, aldicarb, carbofuran, methiocarb, propoxur, pirimicarb and carbetamide were purchased from Fluka (Milan, Italy) as analytical standards

(PESTANAL©). 4-Bromo-3,5-dimethylphenyln- methylcarbamate (BDMC) and carbaryl-d7, used as internal standards, were bought from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2,3,5-Trimethacarb, used as internal standard, and sodium chloride were purchased from Sigma–Aldrich (Milan, Italy). The tested solid phase microextraction fibers were obtained 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).

5.3.2 Instrumentation and Apparatus

GC–MS analyses were carried out using 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 30 m ×0.25 mm i.d., 0.25 µm film thickness Thermo TR-5MS (95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 40 ◦C for 7 min, then ramped at 30 ◦C min−1 to 140 ◦C and held at this temperature for 1 min and finally ramped at 40 ◦C min−1 to 270 ◦C and held at this temperature for 5 min. The carrier gas was helium at 1.7 ml/minof purity 99.999% and argon at a pressure of 2.3 mTorr was used as collision gas. For SPME analyses, a Thermo PTV straight Liner 0.75 × 2.75 × 105 (highly deactivated), was used as GC inlet liner. Analyses were performed in splitless mode and by setting the injector temperature at 270 ◦C. The QqQ mass spectrometer was operated in electron ionization (EI) in multiple reaction monitoring (MRM) mode. The transfer line and ionization source temperatures were set both at 250 ◦C. The emission current was set at 25 _A. The scan width and scan time were set at 0.1 m/z and 0.15 s for all segments

except for those containing carbaryl and carbaryl-d7 (0.1 m/z and 0.075 s, respectively). Peak width of Q1 was fixed at 0.7 amu.

5.3.3 Samples

The real samples of water were collected from public water supply of Rende (Italy) and from three bottled mineral waters (different brands).

5.3.4 Analytical Procedure

1 g of NaCl was directly weighted in the vial used for autosampler and then 10 ml of aqueous sample was added. Afterwards, 10 µl of carbaryl-d7 solution at 100 mg /l and 20 µl of 2,3,5-trimethacarb solution at 50 mg /l were added, the vial was crimped and the solution was stirred to dissolve the salt.SPME extraction was performed with a 65 µm polydimethylsiloxane/ divinylbenzene (PDMS/DVB) fiber in immersion mode for 45 min at room temperature and the adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 270 ◦C for 6.5 min.

5.3.5 Optimization of SPME Variables

The experimentalmatrix designs were carried out and evaluate using Statistica 8.0 (2007 edition, StatSoft, Tulsa, USA).

5.4 A Rapid and Sensitive Assay of Perfluorocarboxylic Acids in Aqueous Matrices by Headspace Solid Phase Microextraction–Gas Chromatography–Triple Quadrupole Mass Spectrometry

5.4.1 Chemicals and Reagents

Perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA) and

perfluorododecanoic acid (PFDoA) were pur-chased from Wellington Laboratories as analytical standards (Guelph, Ontario, Canada). Also perfluoro [1,2,3,4-^{13}C4]octanoic acid (PF^{13}C4OA), used as internal standard, was bought from Wellington Laboratories. Acetonitrile, phosphoric acid, sodium hydroxide, pyridine and sodium chloride were purchased from Sigma–Aldrich (Milan, Italy). Alkyl chloroformates and the cor-responding alcohols were obtained from Fluka (Milan, Italy). The tested solid phase microextraction fibers were purchased 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). Prior to use, all glassware were soaked for 24 h in 0.1 M HCl, washed with five volumes of MilliQ water and baked at 300 ◦C for 16 h.

5.4.2 Instrumentation and Apparatus

GC–MS analyses for the optimization of SPME variables were performed using a Varian (Walnut Creek, CA, USA) Saturn 2000 GC-MS ion-trap system in electron ionization mode, coupled to a Varian 3400 gas chromatograph (GC) equipped with a Varian 8200 autoinjector. The ion trap temperature was set at 210 ◦C with an ionization time of 25 ms, emission current at 10 µA and scan rate at 1000 ms. The capillary column was a 30 m × 0.25 mm i.d., 0.25 µm film thickness Varian VF-5ms (95% polydimethylsiloxane, 5% polydiphenylsiloxane). The GC oven temperature was initially held at 40 ◦C for 3 min, then ramped at 10 ◦C min−1 to 170 ◦C and held at this temperature for 3 min. The carrier gas was helium at 1 ml/min of purity 99.999%. For SPME analyses, a narrow-bore Supelco 0.8 mm i.d. GC inlet liner was used. Analyses were per-formed in splitless mode for 1 min and then in split mode (split ratio 1/40) and acquired in electron ionization and in full scan mode in a mass range of m/z 40–650. GC–MS analyses for calibration procedure and quantification of real samples were

carried out using 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 30 m × 0.25 mm i.d., 0.25 µm film thickness Thermo TR-5MS. The injector tem-perature was set at 290 ◦C and the GC oven temperature was programmed the same way as Saturn GC–MS. Helium at a constant flow rate of 1 ml min−1 was used as carrier gas; argon at a pressure of 2.3 mTorr was used as collision gas. The QqQ mass spectrometer was operated in negative ion chemical ionization (NCI, ammonia as reagent gas) in multiple reaction monitoring (MRM) mode. The transfer line and ionization source temperatures were set at 210 ◦C and 167 ◦C, respectively. A filament multiplier delay of 3 min was fixed in order to prevent instrument damages. The emission current was set at 100 µA. The scan width and scan time were set at 0.1 m/z and 0.3 s for all segments except for those containing PFOA and PF13C4OA (0.1 m/z and 0.15 s, respectively). Peak width of Q1 was fixed at 0.7 amu

5.4.3 Samples

The real samples of water were collected at six rivers located in Calabria in January 2012. In particular, Campagnano (in the city of Cosenza), Mavigliano (Montalto Uffugo, (CS)), Amusa (Caulonia, (RC)), Allaro (Caulonia, (RC)), Crati (Cosenza) and Busento (Cosenza) were considered. The samples were collected 50 cm under the water level into 0.5 l PE bottles. Samples were stored under refrigerated conditions (4 ◦C) and then analyzed without any previous treatment.

5.4.4 Analytical Procedure

A 50 µl volume of $PF_{13}C4OA$ solution at 1 mg/l was added to 10 ml of aqueous sample. Afterwards, 600 µl of this sample was put in a vial, 4.83 ml

of phosphate buffer, 30 µl of pyridine, 240 µl of propanol and 300 µl of propyl chloroformate were added. The vial was then crimped and let to equilibrate for 30 min. SPME extrac-tion was performed with a 85 µm carboxen/polydimethylsiloxane (CAR/PDMS) fiber in headspace mode for 10 min at roomtemperature and the adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 290 ◦C for 10 min. A blank analysis of the fiber did not display any peak due to the analyte under investigation.

5.4.5 Optimization of SPME Variables

The experimentalmatrix designs were carried out and evaluate using Statistica 8.0 (2007 edition, StatSoft, Tulsa, USA).

List of Publications

1. Monteleone M., Naccarato A., Sindona G., Tagarelli A., *Anal. Chim. Acta* (**2012**) In Press. doi:10.1016/j.aca.2012.11.017

2. Cavaliere B., Monteleone M., Naccarato A., Sindona G., Tagarelli A., *J. Chromatogr. A* 1257 (**2012**) 149– 157

3. Monteleone M., Naccarato A., Sindona G., Tagarelli A., J. *Chromatogr. A* 1251 (**2012**) 160-8

4. Cavaliere B., Macchione B., Monteleone M., Naccarato A., Sindona G., Tagarelli A., *Anal. Bioanal. Chem.* 400 (**2011**) 2903–2912

ACKNOWLEDGMENTS

The person who played the biggest part in the success of this thesis is without doubt my supervisor ***Dott. Antonio Tagarelli****. Thank you very much for his guidance and support during these three years. I am very grateful for his patience, discussions and comments.*

A special thanks to ***Prof. Giovanni Sindona*** *for his availability during my PhD period.*

Many thanks to my wife ***Anna*** *for her support and presence in all important moments.*

Last but not least, many thanks to ***My Parents****, who through their sacrifices, help me in this and other important goals.*