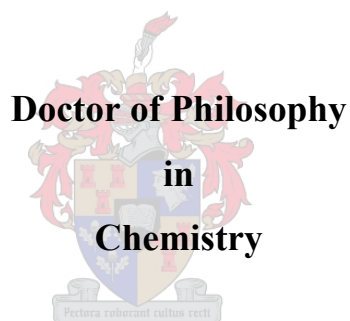


Stir Bar Sorptive Extraction and Gas Chromatography- Mass Spectrometry for the Analysis of Biological Matrices.

A. Stopforth

Dissertation submitted to the Faculty of Science at the University of Stellenbosch
in partial fulfillment of the requirements for the degree of



Prof. Dr. P. J. F. Sandra (supervisor)
Prof. Dr. A. M. Crouch (co-supervisor)
Prof. Dr. B. V. Burger (co-supervisor)

Stellenbosch

July 2007

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: _____

Date: _____

Copyright ©2007 University of Stellenbosch
All rights reserved.

Summary

This study describes the development of simplified analytical methods for the analysis of trace quantities of selected naturally occurring target compounds in complex biological matrices by stir bar sorptive extraction (SBSE) and gas chromatography/mass spectrometry (GC/MS). SBSE facilitates the direct extraction of organic compounds from aqueous samples by allowing the solutes to partition between the aqueous phase and a glass stir bar that is coated with a layer of polydimethylsiloxane (PDMS). The partitioning of polar compounds into the PDMS coating was enhanced by using different derivatization techniques in combination with SBSE. The derivatization of polar functional groups was performed with ethyl chloroformate, acetic acid anhydride, and *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine directly in the aqueous samples. Headspace derivatization of compounds containing a secondary alcohol group was performed directly on the stir bar coating in the presence of acetic acid anhydride vapors. The derivatized compounds were thermally desorbed (TD) and analyzed on-line by GC/MS. A number of experimental parameters, including salt addition, temperature and time were optimized to improve the recovery of the derivatized compounds by SBSE. The optimized methods were validated in terms of linearity, precision, and detection and quantitation limits prior to performing the quantification. Trace levels of tuberculostearic acid, a marker of tuberculosis, was detected in sputum samples that were decontaminated and concentrated before being analyzed by SBSE-TD-GC/MS. The method is sufficiently sensitive to detect the marker without the need to culture the organisms, namely *M. Tuberculosis*. The analysis of 4-hydroxynonenal has also been demonstrated by detecting trace levels of this oxidative stress marker in urine samples obtained from healthy volunteers. Furthermore, abnormally low testosterone/epitestosterone ratios were detected in a group of HIV positive patients by means of SBSE-TD-GC/MS. Further research is required to determine the clinical significance of this finding in the context of HIV infection. Finally, the excessive urinary excretion of estrone and 17 β -estradiol following the administration of a high dose of the conjugated equine estrogens to a female volunteer has also been demonstrated.

Opsomming

Hierdie studie beskryf die ontwikkeling van vereenvoudigde analitiese metodes vir die analise van spoorhoeveelhede geselekteerd-natuurlikvoorkomende teikenverbindinge in komplekse biologiese matrikse deur roerstaaf sorptiewe ekstraksie (SBSE, 'Stir bar sorptive extraction') en gaschromatografie/massaspektrometrie (GC/MS). SBSE vergemaklik die direkte ekstraksie van organiese verbindinge vanuit waterige monsters deur toe te laat dat opgeloste verbindinge geskei word tussen die waterige fase en 'n glasroerstaaf wat bedek is met 'n laag polidimetielsiloksaan (PDMS). Die verdeling van polêre verbindinge in die PDMS bedekking in, was verhoog deur gebruik te maak van verskillende derivatiseringstegnieke tesame met SBSE. Die derivatisering van polêre funksionele groepe is uitgevoer met etielchloroformaat, asynsuuranhidried, en *O*-(2,3,4,5,6-pentafluorobensiel)hidroksielamien direk in die waterige monsters. Bospasiederivatisering van verbindinge wat 'n sekondêre alkoholgroep bevat is uitgevoer direk op die roerstaafbedekking in die teenwoordigheid van asynsuuranhidrieddampe. Die gederivatiseerde verbindinge is termies gedesorbeer (TD) en aanlyn geanaliseer deur GC/MS. 'n Aantal eksperimentele parameters, insluitende souttoevoeging, temperatuur en tyd is geoptimaliseer om die herwinning van die gederivatiseerde verbindinge deur SBSE te verbeter. Die geoptimaliseerde metodes is gevalideer in terme van lineariteit, akkuraatheid, en deteksie- en kwantifiseringslimiete voor die uitvoering van die kwantifisering. Spoorvlakke van tuberkulosteariensuur, 'n merker van tuberkulose, is opgemerk in speekselmonsters wat ontsmet en gekonsentreer is voor hul analisering deur SBSE-TD-GC/MS. Die metode is genoegsaam sensitief om die merker te meet sonder die noodsaaklikheid om die organismes, naamlik *M. Tuberculosis*, te kweek. Die analise van 4-hidroksielnonenaal is ook aangetoon deur lae vlakke van hierdie oksidatiewe stresmerker te meet in uriene monsters wat verkry is van gesonde vrywilligers. Verder is abnormaal lae testosteroon/epitestosteroon verhoudings waargeneem in 'n groep MIV positiewe pasiënte deur middel van SBSE-TD-GC/MS. Verdere navorsing word benodig om vas te stel wat die kliniese belang van hierdie bevinding is in die konteks van HIV infeksie. Om mee af te sluit, is die oormatige urinêre uitskeiding van estroon en 17 β -estradiool ook aangedui na die toediening van 'n hoë dosis van die gekonjugeerde ekwilde estrogene aan 'n vroulike vrywilliger.

Acknowledgements

I am privileged to extend my deepest appreciation to friends and colleagues who assisted me in various ways during the course of this study:

I am indebted to Prof. Pat Sandra for giving me the opportunity to complete this study and for his guidance and encouragement, which is gratefully appreciated.

My deepest gratitude also goes to Prof. Andrew Crouch for his willingness to support this project and for his friendship and moral support during this period.

To Prof. Henk Lauer, who initially accepted my application to participate in this study.

Sincere gratitude is also extended to Prof. Ben Burger for his friendship and scientific input.

I gratefully acknowledge the help given by Prof. Paul van Helden in obtaining ethics approval from the Committee for Human Research and for supplying the TB samples.

Mr Dries van Vuuren is thanked for providing prompt and highly professional instrumental support.

Sincere appreciation is further extended to Prof. Elmarie Terblanche for providing the control urine samples, without which the development of the methods in this study would not have been possible.

Mr Rodney Wright, for graciously assisting me with a large number of diverse problems.

Staff at the NHLS, for their willingness to prepare the sputum samples in-between very busy schedules.

Dr. Nelis Grobbelaar, co-workers and nursing staff at the Idas Valley Infectious Diseases Clinic, for their help in obtaining the HIV positive urine samples. The willingness of patients who attended the clinic to participate in the HIV study is gratefully acknowledged.

Mr Eric Ward, for his immense patience in preparing some of the glass vessels used in this study.

Dr Carine Smith is thanked for her valuable input in obtaining approval from the Human Research Committee to conduct the HIV study.

Mr Glen de Jongh, for his willingness to provide a range of chemical substances.

Mr Jakkie Blom and co-workers at the Department of Mechanical Engineering are thanked for constructing the magnetic stirrer and other apparatus used in this study.

I also extend my sincere appreciation to my colleagues at the university, Astrid, Andreas, André, Estella, Jessie, Liliana, Marlene, Nagaraju and Stefan for their continued friendship and support.

To Daniel, Johannes, Mary, Mubarick and Rodger, for their friendship and invaluable assistance with a host of menial tasks.

The National Research Foundation and the Eskom Tertiary Education Support Program are thanked for financial support.

Finally, I sincerely thank my friends and family for their love and encouragement throughout the years of this study.

“Do what you can, with what you have, where
you are.”

- Theodore Roosevelt

Contents

Publications	xii
Abbreviations	xiii

PART I: General overview

1. Quantitative analysis in clinical chemistry	1
1.1 Gas chromatography and mass spectrometry in clinical chemistry.....	2
1.2 Sample preparation techniques.....	3
1.3 Quantitative analysis.....	6
1.3.1 Specificity.....	6
1.3.2 Calibration.....	7
1.4 Method evaluation.....	10
1.4.1 Accuracy and precision	10
1.4.2 Sensitivity and detection limit.....	11
1.5 Conclusion.....	12
References.....	13
2. Stir bar sorptive extraction	15
2.1 Historical background.....	16
2.2 Fundamental principles of SBSE.....	21
2.3 Surface characteristics of polydimethylsiloxane.....	25
References.....	31
3. Derivatization	34
3.1 Introduction.....	35
3.2 Ethyl chloroformate.....	37
3.3 Acetic acid anhydride	41
3.4 Pentafluorobenzyl hydroxylamine	44
References.....	46

4.	Instrumentation	47
4.1	Introduction.....	48
4.2	Gas chromatograph.....	48
4.3	Cooled injection system.....	48
4.4	Thermal desorption system.....	50
4.5	Mass spectrometer.....	51
4.5.1	MS inlet.....	51
4.5.2	Ion source.....	51
4.5.3	Mass analyzer.....	52
4.5.4	Detector.....	53
4.6	Ways to improve sample throughput during SBSE analysis.....	54
	References.....	58
5.	Drug-profiling by SBSE in biological fluids	59
	Abstract and key words.....	60
5.1	Introduction.....	61
5.2	Experimental.....	62
5.2.1	Sample preparation.....	62
5.2.2	Instrumentation.....	63
5.2.3	Comprehensive presentation of GC/MS data.....	63
5.3	Results and discussion.....	64
5.4	Conclusion.....	83
	References.....	84

PART II: SBSE applications

6.	Rapid diagnosis of pulmonary TB	86
	Abstract and key words	87
6.1	Introduction.....	88
6.2	Experimental.....	89
6.2.1	Chemicals and materials.....	89
6.2.2	Sputum samples.....	89

6.2.3	Sample preparation and SBSE procedure.....	90
6.2.4	Instrumental conditions.....	91
6.2.5	Figures of merit.....	91
6.3	Results and discussion.....	92
6.4	Conclusion.....	95
	References.....	97
7.	Urinalysis of 4-hydroxynonenal.....	98
	Abstract and key words	99
7.1	Introduction.....	100
7.2	Experimental.....	102
7.2.1	Chemicals and materials.....	102
7.2.2	Solutions.....	103
7.2.3	Urine samples.....	103
7.2.4	Sample preparation, in-situ derivatization and SBSE procedure.....	104
7.2.5	Headspace derivatization.....	105
7.2.6	Thermal desorption–gas chromatography–mass spectrometry.....	105
7.3	Results and discussion.....	106
7.3.1	SBSE-HD-TD-GC/MS determination of 4HNE.....	106
7.3.2	Method optimization.....	110
7.3.3	Figures of merit.....	112
7.3.4	Measurement of 4HNE in urine samples.....	113
7.4	Conclusion.....	115
	References.....	116
8.	Measurement of the T/ET ratio in HIV infection.....	118
	Abstract and key words	119
8.1	Introduction.....	120
8.2	Experimental.....	122
8.2.1	Chemicals and materials.....	122
8.2.2	Solutions.....	122
8.2.3	Urine samples.....	122

8.2.4	Enzymatic hydrolysis, SBSE, and HD procedure.....	123
8.2.5	Thermal desorption – GC/MS.....	124
8.3	Results and discussion.....	124
8.3.1	Analysis of T and ET by SBSE-HD-TD-GC/MS.....	124
8.3.2	Method optimization.....	127
8.3.3	Figures of merit.....	130
8.3.4	Analysis of urine samples.....	132
8.3.5	Considerations on the measurement of the T/ET ratio in patients with HIV infection.....	133
8.4	Conclusion.....	135
	References.....	137
9.	Detection of E₁ and 17β-E₂ in hormone replacement therapy.....	140
	Abstract and key words	141
9.1	Introduction.....	142
9.2	Experimental.....	144
9.2.1	Materials, standard solutions and urine samples.....	144
9.2.2	Enzymatic hydrolysis, SBSE and derivatization procedure.....	146
9.2.3	Thermal desorption – gas chromatography/mass spectrometry (TD-GC/MS).....	147
9.3	Results and discussion.....	148
9.3.1	Mass spectrometry.....	148
9.3.2	Method optimization.....	150
9.3.3	Quantification of E ₁ and 17β-E ₂ in human urine samples.....	153
9.3.4	Increased urinary excretion of E ₁ and 17β-E ₂ following the oral administration of Premarin.....	154
9.4	Conclusion.....	159
	References.....	161
10.	Concluding comments.....	164

Publications

This dissertation is based on the four publications listed below. The corresponding chapters have been assigned with the appropriate numerical symbol in the text.

- I. A. Stopforth, A. Tredoux, A. Crouch, P. van Helden, P. Sandra. A rapid method of diagnosing pulmonary tuberculosis using stir bar sorptive extraction – thermal desorption – gas chromatography-mass spectrometry. *Journal of Chromatography A*, 1071 (2005) 135-139.
- II. A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra. Urinalysis of 4-hydroxynonenal, a marker of oxidative stress, using stir bar sorptive extraction – thermal desorption – gas chromatography/mass spectrometry. *Journal of Chromatography B*, 834 (2006) 134-140.
- III. A. Stopforth, C.J. Grobbelaar, A.M. Crouch, P. Sandra. Quantification of testosterone and epitestosterone in human urine samples by stir bar sorptive extraction – thermal desorption – gas chromatography/mass spectrometry: Application to HIV-positive urine samples. *Journal of Separation Science*, 30 (2007) 257-265.
- IV. A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra. The analysis of estrone and 17 β -estradiol by stir bar sorptive extraction – thermal desorption – gas chromatography/mass spectrometry: Application to urine samples after oral administration of conjugated equine estrogens. *Journal of Chromatography B*, 856 (2007) 156-164.

A more general publication on the use of SBSE is included in Part I: General overview.

- V. B. Tienpont, F. David, A. Stopforth, P. Sandra. Comprehensive profiling of drugs of abuse in biological fluids by stir bar sorptive extraction – thermal desorption – capillary gas chromatography – mass spectrometry. *LCGC Europe* 16 (12A) (2003) 5-13 Sp. Iss SI DEC.

Abbreviations

AFB	Acid-fast bacilli	MS	Mass spectrometry
AFM	Atomic force microscopy	MSD	Mass selective detector
AIDS	Acquired immunodeficiency syndrome	NHLS	National Health Laboratory Service
BHT	Butylated hydroxytoluene	OTT	Open tubular trap
CIS	Cooled injection system	PAH	Polyaromatic hydrocarbon
Creat	Creatinine	PCB	Polychlorinated biphenyl
DHBA	Dihydroxy benzaldehyde	PDMS	Polydimethylsiloxane
E₁	Estrone	PFB	Pentafluorobenzyl
E₂	Estradiol	PFBHA	O-(2,3,4,5,6,-pentafluorobenzyl) hydroxylamine
ECF	Ethyl chloroformate	PG	Progesterone
EDC	Endocrine disrupting chemical	PTV	Programmed temperature vaporization
Eq	Equilin	ROS	Reactive oxygen species
Eqn	Equilenin	RSD	Relative standard deviation
ET	Epitestosterone	S/N	Signal-to-noise
GC	Gas chromatography	SBSE	Stir bar sorptive extraction
GC/MS	Gas chromatography/mass spectrometry	SEM	Scanning electron microscopy
HD	Headspace derivatization	SG	Specific gravity
HIV	Human immunodeficiency virus	SIM	Selected ion monitoring
HNE	Hydroxynonenal	SPE	Solid phase extraction
HP	Hewlett-Packard	SPME	Solid phase microextraction
HPG	Hypothalamic-pituitary-gonadal	STP	Sewage treatment plant
HPLC	High performance liquid chromatography	T	Testosterone
HRT	Hormone replacement therapy	T/ET	Testosterone/epitestosterone
ID	Internal diameter	TB	Tuberculosis
IS	Internal standard	TBSA	Tuberculostearic acid
LC	Liquid chromatography	TD	Thermal desorption
LD	Liquid desorption	TDS	Thermal desorption system
LLE	Liquid-liquid extraction	TDU	Thermal desorption unit
LOD	Limit of detection	UV	Ultraviolet
LOQ	Limit of quantitation	WHO	World Health Organization
MODA	17-Methyloctadecanoic acid		

Chapter 1

Quantitative GC/MS analysis in clinical chemistry

1.1 Gas chromatography and mass spectrometry in clinical chemistry

A vast number of applications for gas chromatography (GC) and mass spectrometry (MS) have been reported in the literature in recent years. The versatility of GC as an analytical tool was first realized in the early 1950's [1], where after scientists rapidly developed the technique to include the analysis of a wide variety of organic compounds. Initially it was believed that only volatile compounds could be analyzed by GC, but following the introduction of various chemical derivatizing reagents, which improved the volatility of polar, high molecular weight substances, it was possible to analyze an array of compounds that also had specific importance in the biochemical and medical fields.

In contrast to the range of analyses that could be performed by GC, the standalone applications of MS were limited by the fact that pure samples were required for analysis. To overcome this limitation, the combination of the two techniques was suggested in 1959 [2, 3] which created a system that had superior analytical capabilities. GC/MS combines the high resolving power of GC with the unique identification capabilities of MS, which led to the widespread use of this technique in the fields of industry and agriculture, in environmental science and law enforcement, as well as in drug detection. Nowadays, GC/MS has also become an indispensable tool in the field of medical research and clinical chemistry.

Although GC/MS has steadily gained importance in the clinical chemistry laboratory, the routine application of this technique has been limited for a number of reasons. The majority of frequently ordered biochemical tests (e.g. glucose, urea, and bilirubin) are performed using fully automated electrochemical, immunochemical, and spectrophotometric methods [4]. This situation considerably improves sample throughput, with over 20 components that can be analyzed in more than 100 samples per hour [5]. However, there are a number of substances for which standard chemical procedures do not exist, or where more sensitive and specific assays are required. Compounds that are frequently analyzed by GC/MS include toxicologic agents, steroids, fatty acids and alcohols [6], while other procedures may need to be validated such as those utilized for creatinine [4, 7], cortisol [8],

triglycerides [9] and cholesterol [10]. Several reviews on the potential applications of GC/MS in clinical chemistry have been reported previously [11 - 13].

Another reason that hampers the routine use of GC/MS in clinical laboratories is the fact that extensive sample preparation may be required prior to the analysis of biological specimens. These samples (e.g. blood and urine) are highly complex mixtures from which specific compounds, or classes of compounds, need to be isolated to ensure adequate separation by GC as well as proper identification by MS. A typical sample preparation scheme may consist of the initial extraction of the sample, followed by purification and concentration of the analytes, as well as a derivatization step to improve the volatility of the extracted compounds. Most of these actions are performed manually, and very often it is the most difficult and rate-limiting part of the whole analysis. Analytical chemists recognize these limitations and are constantly trying to develop faster and simpler analytical methods that will improve sample turnover.

The main part of this study involves the development of simplified analytical methods for the measurement of frequently (and less frequently) analyzed biochemical compounds. In each case, a novel sample enrichment technique known as stir bar sorptive extraction (SBSE) was utilized in conjunction with GC/MS to detect four distinct biomarkers in human bodily fluids. This work also demonstrates the value of utilizing GC/MS to identify potentially new diagnostic applications for already frequently monitored biochemical compounds. The individual methods are discussed in detail in Chapters 6, 7, 8 and 9.

1.2 Sample preparation techniques

Biological fluids are some of the most complex mixtures currently recognized [6]. These samples are rarely suitable for direct analysis by GC or GC/MS. Therefore, some form of sample preparation will be required to remove the interfering substances and to isolate the compounds of interest. For liquid samples, the most popular extraction techniques include liquid-liquid extraction (LLE), solid phase extraction (SPE), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE). Each method will be discussed briefly within this section.

LLE: In spite of the availability of more modern extraction techniques, liquid-liquid extraction is still frequently used in environmental, clinical and industrial laboratories [14]. In its simplest form, it involves the partitioning of a solute between two immiscible phases, one being an aqueous phase and the other an organic solvent such as chloroform. The technique generally requires the use of a large amount of organic solvent, which is a major reason for the development of new miniaturized methods such as micro-LLE [15]. In spite of its simplicity, LLE requires several steps, lacks specificity and is fairly time consuming [16].

SPE: Solid phase extraction was developed in the late 1970's and has to a large extent replaced LLE because it accomplishes faster extractions, requires less organic solvent and improves the ability to concentrate analytes [14]. This technique is a miniaturization of column chromatography, where an exhaustive, non-equilibrium extraction of the analytes is achieved by allowing an aqueous sample to percolate through a bed of adsorbent material contained in short polypropylene tubes. The speed of the extraction can be improved by applying pressure to the top, or a vacuum at the lower end of the tube. Different adsorbents based on silica or synthetic resins are available for the removal of analytes from liquid samples. The separation is optimized through careful consideration of the different physicochemical interactions that occur between the solute, the solvent and the adsorbent material. After the compounds have been retained on the adsorbent bed, the analytes are desorbed by passing a suitable organic solvent through the tube. Several companies have developed mechanized and robotic SPE systems for complete automation of the extraction process [16].

SPME: Although SPE requires less organic solvent as compared to LLE, it still consumes large quantities in relation to the new sorptive extraction techniques that are essentially solvent-free. Solid phase microextraction is an example of such a technique that was initially developed to analyze micropollutants in water. Several new applications have been reported in the literature since it was first invented by Pawliszyn and coworkers in 1990 [17]. Analytes are extracted from a sample by exposing a polymer-coated fiber to an aqueous solution. SPME is a non-exhaustive, equilibrium procedure where analytes diffuse

from the aqueous phase to the polymer layer when the sample is agitated (i.e. stirred). The amount of analytes extracted may be controlled accurately by optimizing the stirring rate, the extraction time, temperature, pH and ionic strength of the solution [18]. The equipment used in SPME, also known as the fiber assembly, consists of four major parts. The coated fiber is attached to a stainless steel plunger, which is housed inside a septum-piercing needle. The needle is joined to a modified syringe holder. A number of fiber coatings have been developed for SPME, including polydimethylsiloxane (PDMS), polyacrylate and mixed coatings of PDMS with Carbowax or divinylbenzene that are now commercially available. Once the analytes have been extracted, the SPME fiber is transferred directly to a GC instrument. Thermal desorption is accomplished after the fiber has been inserted into the heated injector port of the gas chromatograph. Some automated on-line applications for SPME have already been reported in the literature [18, 19].

SBSE: A disadvantage to using SPME is that the required detection limits for a wide range of analytes cannot be reached because of the small amount of extraction phase coated onto the SPME fiber (i.e. 0.5 μl). In 1999, Baltussen et al. [20] introduced a new technique known as stir bar sorptive extraction, which significantly improved the detection of various compounds because of the large amount of polydimethylsiloxane (PDMS) coated onto a glass stir bar. In SBSE, the sample is stirred for a predetermined time until equilibrium is reached. Thereafter, the stir bar is transferred to an empty glass tube and desorbed at high temperatures to release the compounds from the PDMS coating. A specially designed thermal desorption unit (TDU) is required to transfer the analytes from the stir bar to the injector of a GC instrument (see Section 4.4). PDMS is the only stationary phase currently used in SBSE, but this situation will probably not limit the applicability of the technique because several new derivatization methods have been developed to enhance the extraction of polar compounds by the stir bar coating. Furthermore, the newly developed composite extraction phases, such as those used in SPME, often result in competitive adsorption and matrix effects because the fibers no longer contain pure polymeric sorbents [21]. A more detailed discussion of the fundamental principles of SBSE is given in Chapter 2.

1.3 Quantitative analysis

1.3.1 Specificity

After separating the sample on a suitable GC column, the compounds are introduced into the MS ion source where they are fragmented and ionized (see Section 4.5.2). The fragments have a specific mass-to-charge ratio (m/z), and are recorded either by repetitive scanning of all the fragments to produce a total ion chromatogram, or by the recording of selected masses, which is known as selected ion monitoring (SIM).

SIM is particularly useful for the analysis of trace biological compounds in complex matrices because of the sensitivity that can be achieved by using this mode of detection. The selection of specific masses that are characteristic for the analyzed compounds assists in eliminating background interferences that are always part of complex matrices. In general, it is preferred to select ions in the higher mass range, as there is less chance of encountering a signal that originates from the background (i.e. from column bleed) or from the sample matrix. A disadvantage to using SIM however is the risk of lowering the specificity of the analysis, which may compromise the accuracy of the results obtained.

Specificity is lost when two to three ions are monitored as opposed to the recording of a full mass spectrum that provides a specific fragmentation pattern by which a compound can be identified. One option is to improve the specificity of the MS itself by using high resolution selected ion monitoring, selected reaction monitoring or different chemical ionization techniques [22]. However, these instruments are highly sophisticated and not always available to the clinical chemist. Therefore, non-mass spectrometric methods should be considered first when aiming to improve specificity.

As mentioned previously, the use of an appropriate sample preparation procedure should eliminate the majority of interferences, while optimization of the GC oven temperature program will further assist in resolving out impurities. One factor that needs to be considered when using the sorptive extraction techniques (i.e. SBSE or SPME), is that only partial sample clean-up is provided due to the large number of compounds extracted by the

polymer coating. Careful consideration should be given to the selection of ions that are characteristic for the compounds being measured, while ensuring that this selection does not affect the sensitivity of the method. Unfortunately, the ion that is most specific for a compound often occurs at low intensity and the use of such an ion will result in decreased sensitivity [12]. Therefore, it is often preferred to monitor ions of high intensity, although a loss in sensitivity can be offset by using an enrichment technique such as SBSE if the compound is amenable for extraction by this method. Thus in SBSE, it is often more important to optimize the specificity of the method because the use of this technique already leads to a significant improvement in analytical sensitivity. In general, a great deal of attention should be given to verifying the specificity of SIM analyses, because there is no purpose in quantifying a compound when there is little confidence in the accuracy of the results obtained.

1.3.2 Calibration

Internal standard calibration: The visual output of a mass spectrometer is similar to that of any single channel GC detector (e.g. flame ionization detector). The peaks correspond to the separated components of the sample that can be integrated to determine the concentration of each compound. The integration can be performed by using the interactive software that is usually part of the GC/MS system. A frequently used quantification method in mass spectrometry involves the use of an internal standard, which is often a chemical analogue or homologue of the compound being analyzed. A stable isotope labeled analogue results in the highest precision, but this type of standard may not always be available. An internal standard is often used to correct for variations that occur during the analysis. Sources of variation include the numerous manipulations that are required for the extraction and derivatization of the compounds, as well as alterations that may occur in the operating conditions of the GC/MS system.

To determine the relationship between the instrument's signal (i.e. peak area) and the concentration of the analyte, a series of dilutions containing known amounts of the analyte and internal standard are analyzed. The resulting graph is obtained by plotting the ion abundance ratios of the analyte and the internal standard against the concentration of the

compounds. For linear data, the calibration curve is constructed by means of simple regression analysis that minimizes the sum of the squares of the distance between each data point and the straight line [23]. This method was used to determine the concentration of tuberculostearic acid (TBSA) in sputum samples (Chapter 6) and 4-hydroxynonenal (4HNE) in human urine samples (Chapter 7). The calibration curve obtained for 4HNE is shown in Figure 1.1. A correlation coefficient (r^2) of 0.997 was obtained using the least squares method, demonstrating that 99% of the variation in the peak area can be explained by the linear equation [23].

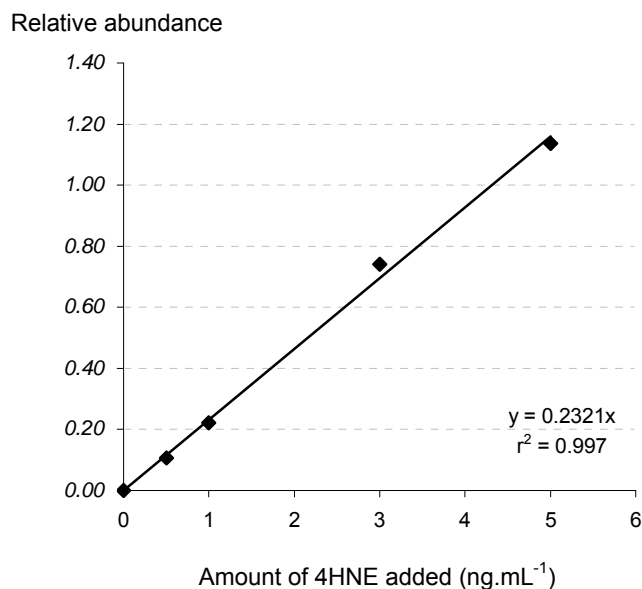


Figure 1.1 Calibration curve used for the quantification of 4HNE by electron impact GC/MS in SIM mode. The internal standard was 2,5-dihydroxybenzaldehyde.

Standard addition: The standard addition technique was used in the latter part of this study for the quantification of the androgens (Chapter 8) and the estrogens (Chapter 9). In this technique, the calibration curve is obtained by analyzing a series of spiked samples containing increasing amounts of the analyte. The curve is constructed in the same way as described for the internal standard calibration method, except that a calibration curve is obtained for each sample. One of the main limitations in using standard addition is that each of the spiked levels should be analyzed in triplicate for the results to be statistically valid. For this reason, standard addition is not recommended for routine applications, particularly in laboratories where a quick turnaround time is required [14].

As mentioned previously, an internal standard can correct for errors that occur during sample work-up and analysis because the analyte and standard undergo similar variations during the procedure. If an internal standard is included during the standard addition method, the precision may improve to such an extent that a single analysis at each of the spiked levels may be sufficient. Furthermore, a manner of quality control is provided by the fact that each sample requires its own calibration curve. Therefore, the use of the standard addition technique may be considered during the development of a new method for the following reasons:

1. Standard addition is one of the most efficient ways of determining if the compound that one wants to analyze is in fact the compound being analyzed. The incremental increase in peak area that is observed following the addition of known amounts of the analyte provides confirmatory data that the selected ions (i.e. during SIM analysis) are specific for the compound being measured.
2. As demonstrated in Chapter 8 (Section 8.3.3), the standard addition method corrects for matrix effects that may affect the individual sorption of the compounds by the stir bar coating.
3. By using this technique, the compound being determined is also the compound being added to the sample. Therefore, any variations in the ion abundance ratios that occur due to alterations in the operating conditions of the MS or changes in sensitivity that result from a build-up of contamination in the ion source [22], will automatically be corrected because the response factors remain the same.
4. Standard addition may also correct for alterations occurring on the stir bar coating as a result of systematic degradation of the polymer surface. Degradation of the stir bar coating can alter the extraction of individual compounds. Therefore, frequent recalibration of the method may be required when the internal standard technique is used, especially when an isotopically labeled analog is not available.

1.4 Method evaluation

1.4.1 Accuracy and precision

Accuracy and precision measure the quality and efficacy of an analytical procedure. Accuracy determines the closeness of the mean result to the true value, which is usually unknown. Errors that produce inaccurate results lead to a systematic deviation from the true value [23]. In other words, all the results will either be too high or too low. For example, when there is a co-eluting compound that also gives a signal at the m/z value being monitored, then a net determinate error will be introduced which causes bias [22]. In this respect, accuracy is quite difficult to determine and a correct estimate can only be obtained if the sample is analyzed using a method that is known to produce accurate results [14]. In this study, however, no inter-laboratory comparisons were made and therefore the accuracy of the developed methods have not been determined.

Precision on the other hand, measures the scatter of individual measurements about their mean value, which is frequently expressed as the standard deviation, variance and coefficient of variation. In analytical chemistry, precision is usually reported as the relative standard deviation (RSD), which is calculated by dividing the standard deviation (s) by the average value (\bar{x}) of the data set, expressed as a percentage [23].

$$\%RSD = \frac{s}{\bar{x}} \times 100\%$$

Repeatability and reproducibility are the two main components of precision. Repeatability is defined as the closeness of individual results obtained by the same analyst, using the same method during a single period of laboratory work [14], while reproducibility is defined as the closeness of individual results obtained by the same or a different analyst using the same method under different conditions. Errors that affect precision occur randomly during an experiment and cannot be eliminated, although their collective effect can be minimized. For example, if the analyte and internal standard have different sorption kinetics or different octanol-water distribution coefficients, then several factors can influence the precision of SBSE. Small changes in sample pH, temperature and ionic strength can alter the relative amount of analytes extracted by the stir bar coating. The

effect of these errors can be minimized by carefully controlling the aforementioned experimental conditions, or they might be entirely eliminated by using an isotopically labeled internal standard.

1.4.2 Sensitivity and detection limit

In SIM analysis, the sensitivity of the mass spectrometer is significantly increased due to the monitoring of a limited number of ions that allow a greater portion of the ion beam to reach the detector. For example, in a scanning experiment during total ion monitoring about one-thousandth of the total time is spent at any one mass, whereas half of the total time is available when only two ions are monitored [22]. The sensitivity of a procedure is often regarded as being equal to its detection limit, which is the lowest concentration that can be detected with a certain level of confidence [23]. In analytical chemistry, the sensitivity of an instrument can be defined as the magnitude of the response derived from a specific concentration. Thus, the sensitivity (S) can be estimated by dividing the strength of the signal (i.e. peak height (X)) by the concentration of the analyte (C), as determined by the entire analytical procedure, which includes sample preparation and analysis:

$$S = \frac{X}{C}$$

This estimate may be used to calculate the detection limit of the analyte. The detection limit is usually measured at three times the baseline variability (i.e. noise). In other words, the signal produced by the analyte should exceed the average baseline noise by this value, which is commonly referred to as the signal to noise (S/N). The following equation can be used to determine the limit of detection (LOD) by using the sensitivity (S) determination that was calculated in the previous equation:

$$\text{LOD} = \frac{3 \times S/N}{S}$$

1.5 Conclusion

Today, GC/MS is used in almost every field of analytical chemistry due to its high resolving power and unique identification capabilities. Its use in the clinical chemistry laboratory is also steadily increasing because of its ability to detect trace amounts of biologically important compounds in complex matrices. A drawback to using GC/MS however is the fact that extensive sample preparation may be required prior to the analysis of complex biological samples. Multi-step sample preparation procedures are often required to isolate the compounds of interest, which add to the difficulty of applying GC/MS to routine clinical analyses. The use of a novel sample enrichment technique, namely stir bar sorptive extraction, has greatly simplified the isolation of target compounds from complex biological matrices. SBSE may be compared to liquid-liquid extraction except that the compounds partition into the polydimethylsiloxane layer covering the stir bar. A large number of compounds may be extracted by the stir bar coating; therefore the specificity of the method should be established before commencing with the actual quantification of the analytes. The quantification may be performed by internal standard or standard addition calibration. The use of the standard addition technique in combination with an internal standard often results in greater accuracy, especially when a suitable isotopically labeled standard is not available. Finally, the method should fulfill certain criteria in terms of accuracy, precision, sensitivity and detection limit in order to determine its applicability in the clinical setting.

References

- [1] A.T. James, A.J.P. Martin, *Analyst* 77 (1952) 915.
- [2] R.S. Gohlke, *Anal. Chem.* 31 (1959) 535.
- [3] R.S. Gohlke, F.W. McLafferty, *J. Am. Soc. Mass Spectrom.* 4 (1993) 367.
- [4] R.L. Bertholf, *Gas chromatography and mass spectrometry in clinical chemistry*, In: R.A. Meyers (Ed.), *Encyclopedia of analytical chemistry*, John Wiley & Sons Ltd., Chichester, 2000, pp. 1314-1336.
- [5] L.A. Kaplan, A.J. Pesce (Ed's), *Clinical chemistry – Theory, analysis, and correlation*, The C.V. Mosby Company, St. Louis, 1989.
- [6] B.M. Mitruka, *Gas chromatographic applications in microbiology and medicine*, John Wiley & Sons, New York, 1975.
- [7] L.M. Thienpont, K.G. Van Landuyt, D. Stockl, A.P. De Leenheer, *Clin. Chem.* 41 (1995) 995.
- [8] V.I. De Brabandere, L.M. Thienpont, D. Stockl, A.P. De Leenheer, *Clin. Chem.* 41 (1995) 1718.
- [9] P. Ellerbe, L.T. Sniegowski, M.J. Welch, *Clin. Chem.* 41 (1995) 397.
- [10] L.M. Thienpont, K.G. Van Landuyt, D. Stockl, A.P. De Leenheer, *Clin. Chem.* 42 (1996) 531.
- [11] J. Roboz, *Adv. Clin. Chem.* 17 (1975) 109.
- [12] I. Björkhem, *CRC Crit. Rev. Clin. Lab. Sci.* 11 (1979) 53.
- [13] R.E. Hill, D.T. Whelan, *Clin. Chim. Acta* 139 (1984) 231.
- [14] D. Harvey, *Modern analytical chemistry*, McGraw-Hill Companies Inc., New York, 2000.
- [15] R.J. Flanagan, G. Withers, *J. Clin. Path.* 25 (1972) 899.
- [16] F.M. Lanças, *J. Braz. Chem. Soc.* 14 (2003) 183.
- [17] C.L. Arthur, J. Pawliszyn, *Anal. Chem.* 62 (1990) 2145.
- [18] J. Pawliszyn, *Solid phase microextraction – Theory and practice*, Wiley-VCH, New York, 1997.
- [19] D. Louch, S. Motlagh, J. Pawliszyn, *Anal. Chem.* 64 (1992) 1187.
- [20] E. Baltussen, P. Sandra, F. David, C.A. Cramers, *J. Microcolumn. Sep.* 11 (1999) 737.
- [21] E. Baltussen, C.A. Cramers, P.J.F. Sandra, *Anal. Bioanal. Chem.* 373 (2002) 3.

- [22] J.R. Chapman, *Practical organic mass spectrometry – A guide for chemical and biochemical analysis*, Wiley & Sons Ltd., West Sussex, England, 1995.
- [23] D.A. Skoog, D.M. West, F.J. Holler, S.R. Crouch, *Fundamentals of analytical chemistry*, Brooks/Cole-Thomson Learning, Belmont, CA, U.S.A., 2004.

Chapter 2

Stir Bar Sorptive Extraction

2.1 Historical background

The current trend in analytical chemistry is to minimize the use of organic solvents that are potentially harmful to humans and the environment. In sorptive extraction, organic solvents are avoided because the analytes partition between the aqueous sample and a water-immiscible polymeric phase such as polydimethylsiloxane. The polymer layer may be attached to the walls of an open tubular trap (OTT; [1]), to a silica fiber (SPME; [2]), or to a magnetic stir bar (SBSE; [3]). Also, particles containing 100 % of the polymer material may be packed into a short tube to enable the preconcentration of liquids and gases, which is currently known as gum-phase extraction [4, 5]. SBSE is the most recent addition to a number of existing sorptive extraction methods that have gained wide acceptance in various fields of analytical chemistry. SBSE is becoming more popular because it addresses several of the shortcomings associated with previously developed methods. Compared to OTT, SBSE has an improved sample capacity, provides greater sensitivity than can be achieved with SPME, and prevents the loss of volatile compounds encountered during gum-phase extraction. David and Sandra [21] have recently published a comprehensive review on the different applications of SBSE in analytical chemistry. The majority of SBSE methods involve the analysis of environmental contaminants in water [6-10], the measurement of aroma compounds and pesticides in food [11-15], as well as the detection of a wide range of compounds in wine samples [16-20]. In this study, the focus is solely on analyzing target compounds in biological specimens; therefore only recently developed SBSE applications in the biomedical field will be highlighted.

SBSE is frequently used in combination with gas chromatography because improved analytical sensitivities can be achieved when the entire extract on the stir bar coating is transferred to the capillary column by means of thermal desorption. For thermally labile or high molecular weight compounds, the combination of SBSE and liquid chromatography (LC) is also a possibility. Liquid desorption of the extracted analytes is accomplished by placing the stir bar in a vial or an insert containing a small amount of organic solvent. Following sonication, a portion of the solvent is transferred to the LC system. Detection can be accomplished by means of fluorescence or ultraviolet (UV) detectors or by mass spectroscopy (MS). Fernandes et al. recently reported the analysis of fluoxetine in plasma

samples using SBSE-liquid desorption (LD)-LC/MS [22]. A limit of quantitation (LOQ) of 10 ng.ml^{-1} could be achieved, which allows the routine monitoring of this antidepressant drug directly in clinical samples. In a similar study reported by Chaves et al. [23], the measurement of a wide range of antidepressants, including fluoxetine, by SBSE-LD-LC/UV has also been demonstrated. The authors showed that back extraction of the compounds by magnetic stirring resulted in higher desorbed amounts of the analytes as compared to using sonication. The achieved sensitivities were high enough to allow the routine therapeutic monitoring of various frequently administered antidepressant drugs directly in plasma. Almeida and Nogueira [24] recently developed the first method for the analysis of naturally occurring compounds by SBSE and liquid chromatography. A range of natural and synthetic estrogens were analyzed in urine samples using SBSE-LD-HPLC with diode array detection. The detection limits (LOD) obtained for estrone and 17β -estradiol were 50 ng.ml^{-1} , in comparison to 0.02 ng.ml^{-1} and 0.03 ng.ml^{-1} obtained for the same compounds analyzed by SBSE-TD-GC/MS in SIM mode [25].

SBSE has mainly been used for the measurement of environmental contaminants (e.g. PAHs, PCBs, pesticides, and phenols) in water samples. The detection of these substances in the environment is of great importance because most chemicals find their way back to the food chain where they are ingested, metabolized and excreted in the form of glucuronide and sulfate conjugates. The measurement of specific metabolites in biological fluids may be used as indicators of human exposure to potentially harmful chemicals. For the analysis of urinary metabolites by SBSE, a deconjugation step is often required that may be accomplished by adding β -glucuronidase to the sample matrix. Furthermore, a derivatization step may also be necessary since the conjugation process renders the compounds hydrophilic and therefore less amenable to gas chromatographic separation.

Desmet et al. [26] reported the exposure of firefighters to polyaromatic hydrocarbons (PAHs) by measuring 1-hydroxypyrene in the urine of a healthy volunteer. Following enzymatic hydrolysis with β -glucuronidase and derivatization of the liberated compounds with acetic acid anhydride, the acetate derivatives were extracted by SBSE and analyzed on-line by TD-GC/MS. Three reference urine samples (Clin-CheckTM) containing known amounts of 1-hydroxypyrene were analyzed to assess the accuracy of the developed

method. The SBSE results were in excellent agreement with the approved concentrations. Polychlorinated biphenyls (PCBs) are another class of potentially toxic compounds that may be linked to various reproductive problems. Benijts et al. [27] developed an SBSE method for the analysis of PCBs in human sperm, and also demonstrated the advantage of adding small amounts of organic solvent (i.e. methanol) to the sample matrix to prevent the adsorption of highly apolar compounds to glass surfaces in the sample container. A variety of environmental contaminants, including the PCBs, also act as endocrine disrupting chemicals (EDCs) that have been shown to affect the growth and development of humans and wildlife [28]. The alkylphenoxyethoxylates (APEs) are well known xenoestrogens for which a large number of breakdown products, including 4-nonylphenol have been detected in the environment. Kawaguchi et al. recently developed a number of methods for the detection of different APEs by SBSE in various biological matrices, including urine [29], plasma and saliva [30]. Trace levels of these contaminants were detected using different derivatization techniques, namely *in situ* derivatization with acetic acid anhydride [31] and in-tube silylation with BSTFA [32]. In addition, the detection of pesticides (another class of EDCs) in breast milk using SBSE-TD-GC/MS has also recently been demonstrated [33].

Apart from measuring environmental contaminants in biological fluids, the detection of pharmaceutical drugs and other biochemical compounds in forensic and clinical samples by SBSE is also becoming more important. Measurement of biological substances may be used to detect therapeutic or lethal drug doses in blood or plasma, or to monitor specific biomarkers during health and disease. Various methods have been developed for the detection of theophylline [34], drugs of abuse [35] and different pharmaceuticals [36] by SBSE in conjunction with GC/MS. Tienpont et al. recently demonstrated the possibility of extracting several compounds containing phenolic and acidic functional groups following derivatization with ethyl chloroformate [36]. Furthermore, a number of specially designed extraction devices based on SBSE have recently been developed to detect target analytes in sample matrices that are difficult to analyze. The analysis of plasma by SBSE for example, results in fouling of the sorbent layer due to the high protein content of these samples. Protein fouling shortens the life of the stir bar and limits the number of reproducible extractions that can be performed by SBSE. Lambert et al. [37] demonstrated the coating of a glass stir bar with restricted access materials to allow low molecular weight compounds

to partition into the phase's interior, while excluding macromolecules with a molecular weight in excess of 15 000 (i.e. proteins). This device was used to extract caffeine and its metabolites directly from plasma, allowing more than 50 analyses to be performed with a single stir bar when used in combination with HPLC/UV. In a study reported by Buettner and Welle [38], certain odors were also detected intra-orally using a modified SBSE device. The stir bars were placed inside small perforated glass containers to allow the penetration of air and saliva into the holder shortly after swallowing food and beverages. Extractions were performed directly in the oral cavity, after which the stir bars were removed from the holders and analyzed by TD-GC-olfactometry.

Of the numerous SBSE applications reported in the literature thus far, less than one fifth involves the analysis of biological matrices, and only a handful of these biological applications include the detection of trace amounts of naturally occurring organic compounds in bodily fluids. Soini and coworkers [39-41] recently reported the analysis of volatile and semi-volatile components in animal urine and glandular tissues using SBSE. The obtained chromatographic profiles showed that quantitative differences detected for certain compounds may be associated with individual or gender recognition. These authors also introduced a new SBSE sampling device that may be rolled over biological surfaces to detect a variety of volatile organic compounds [42].

Currently, only a limited number of SBSE methods address the analysis of naturally occurring compounds in human bodily fluids. The detection of selected steroids in urine samples by SBSE has previously been demonstrated by Almeida and Noguiera [24] and by Tienpont et al. [36]. More recently, the trace analysis of different biological markers by SBSE have been reported by Stopforth et al. who demonstrated the analysis of tuberculostearic acid in sputum samples [43]; the detection of 4-hydroxynonanal as an oxidative stress marker in urine [44]; measurement of the testosterone/epitestosterone ratio in HIV infection [45], as well as the measurement of a high urinary excretion of estrone and 17 β -estradiol during hormone replacement therapy [46]. The low detection limits achieved in these studies were due to the development of a new headspace derivatization technique [44] that was used in conjunction with SBSE. Several potentially useful developments in SBSE, particularly in the biomedical field, have been hampered by the lack of a suitable

derivatization method for compounds containing polar functional groups that are difficult to derivatize in aqueous samples. This limitation has been overcome by the development of two new headspace derivatization procedures that were recently reported by Kawaguchi et al. [32] and Stopforth et al. [44]. A variety of polar compounds that previously lacked sufficient volatility and thermal stability may now be analyzed by SBSE-TD-GC/MS. Consequently, the potential to develop several new SBSE methods for application in the clinical and biomedical fields now exists.

2.2 Fundamental principles of SBSE

Stir bar sorptive extraction was developed as a means to enhance the low extraction efficiencies of compounds analyzed by SPME. The volume of extraction phase coated onto a 100 μm SPME fiber corresponds to approximately 0.5 μl PDMS, whereas a stir bar (1 cm x 0.5 mm d_f) contains at least 25 μl of the stationary phase. Although both techniques follow the same principles, the amount of stationary phase used accounts for the majority of differences observed between the two techniques. The main differences are the rate at which equilibrium is achieved within the stationary phase coating, as well as the total amount of analytes extracted by the sorbent phase. Although SPME achieves faster extractions, it suffers from low sensitivity because of the small amount of stationary phase used.

In sorptive extraction, the compounds diffuse from the aqueous sample to the PDMS coating and migrate through the sorbent phase. The rate of diffusion is determined by the coating thickness and the distribution constant of a particular analyte. The amount of analytes sorbed by the stationary phase coating increases rapidly after the device (i.e. stir bar) is placed in the sample matrix and then slows as the system reaches equilibrium. This relationship is demonstrated by the extraction-time profile obtained for estrone and 17 β -estradiol as shown in Figure 2.1. Generally, the shortest equilibration time that corresponds to the extraction of approximately 95 % of the equilibrium amount of the analyte is selected for routine analysis (i.e. 60 minutes for the estrogens as shown in Figure 2.1). Non-equilibrium extraction conditions may also be used, providing that the sorption procedure is precisely time-controlled [47].

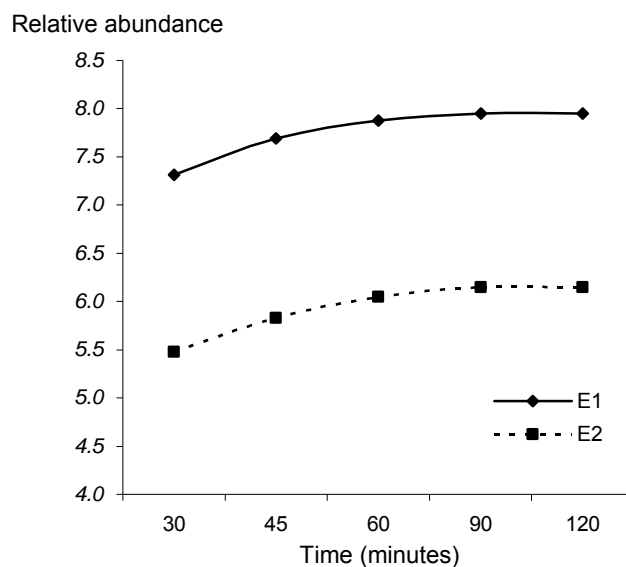


Figure 2.1 Effect of extraction time on the recovery of estrone (E1) and 17 β -estradiol (E2) by SBSE-TD-GC/MS. The experimental conditions are given in Chapter 9.

The total equilibrium amount of analytes extracted by the PDMS coating also depends on the amount of analytes present in other phases of the sample system. The headspace above the sample solution, as well as the sample matrix may have a capacity to retain the analytes. Under ambient conditions, semi-volatile compounds do not readily partition into the gas phase that is present above the sample solution, therefore the amount extracted by the stir bar coating will not be affected. The analytes may, however, be retained by suspended particles and organic solvents present in the sample matrix. This situation lowers the mass transfer of analytes to other phases in the system and decreases the overall kinetics of the extraction process [47].

The kinetics of sorptive extraction are mainly affected by the affinity of the analytes for the stationary phase coating. Affinity of the compounds for the PDMS layer is estimated from the distribution constant ($K_{\text{PDMS/W}}$) of an analyte which is proportional to its octanol-water partitioning coefficient ($K_{\text{O/W}}$). The distribution constant is used to predict the sensitivity of SBSE and estimates the total amount of analytes extracted by the stir bar coating. A characteristic feature that differentiates sorptive extraction from adsorptive methods (e.g. SPE) is that the analytes partition into the bulk of the polymer where they diffuse

throughout the sorbent layer. Consequently, the total recovery of analytes is affected also by the phase ratio (β) between the aqueous sample and the amount of PDMS used [48]. The relationship between $K_{\text{PDMS/W}}$ and β is demonstrated in Eq. 1:

$$K_{\text{O/W}} \approx K_{\text{PDMS/W}} = \frac{C_{\text{PDMS}}}{C_{\text{W}}} = \frac{m_{\text{PDMS}}}{m_{\text{W}}} \times \frac{V_{\text{W}}}{V_{\text{PDMS}}} = \frac{m_{\text{PDMS}}}{m_{\text{W}}} \beta \quad (1)$$

The distribution constant between PDMS and water ($K_{\text{PDMS/W}}$) is defined as the ratio between the concentration of the analyte in the PDMS phase (C_{PDMS}) over its concentration in water (C_{W}) under equilibrium conditions. This ratio is equal to the ratio of the mass of the analyte in the PDMS phase (m_{PDMS}) over the mass of the analyte in water (m_{W}) multiplied by the phase ratio β , where $\beta = V_{\text{W}}/V_{\text{PDMS}}$ [48]. The theoretical recovery can therefore be calculated based on the distribution constant of the analyte and a known phase ratio, which is expressed as the ratio of the extracted amount of analyte (m_{PDMS}) over the original amount of analyte in the sample matrix ($m_0 = m_{\text{PDMS}} + m_{\text{W}}$) as shown in Eq. 2.

$$\frac{m_{\text{PDMS}}}{m_0} = \frac{K_{\text{PDMS/W}} / \beta}{1 + (K_{\text{PDMS/W}} / \beta)} \quad (2)$$

Eq. 2 demonstrates that the extraction efficiency will increase with increasing $K_{\text{PDMS/W}}$ and decreasing β . The phase ratio is basically a fixed parameter because a specific sample volume and coating thickness can be selected. The distribution constant on the other hand, is influenced by a variety of conditions, including changes in temperature and pH, salt addition, derivatization of polar functional groups, as well as the presence of organic solvents in the aqueous sample.

Temperature has a significant effect on the extraction equilibrium. If the temperature is raised, two opposing effects will be observed. Firstly, the diffusion coefficients of the analytes are increased so that the time required to reach equilibrium is decreased [49]. Secondly, the distribution constants are decreased so that potentially lower amounts of the compounds will be extracted. The ideal extraction temperature ranges from 40°C – 50°C for most semi-volatile compounds. However, the optimal extraction temperature should be

determined experimentally, which is usually accomplished by constructing a temperature profile for the extracted analytes.

Natural sample matrices, such as urine, are influenced by variations in pH and ionic strength due to the homeostatic concentration effects produced by the kidneys. The addition of salt not only improves the extraction efficiency of compounds analyzed by SBSE, but also normalizes these random salt concentrations that may affect the reproducibility of the method. Depending on the amount of salt added, the extracted quantity may either increase or decrease [47]. Lower salt concentrations generally result in higher extractions due to the salting out effect, whereas high salt concentrations lead to lower extractions caused by electrostatic interactions at the molecular level [48]. Sample pH is an important parameter for compounds that possess a pH-dependent functional group. The extraction efficiency will increase at a pH level that produces more of the undissociated form of the compound, since it is this form that will partition into the stir bar coating.

Other parameters that may lead to an improved extraction efficiency for the compounds include, stirring speed which increases the rate of diffusion by decreasing the static aqueous layer surrounding the stir bar (i.e. Prandtl boundary layer), and derivatization which replaces polar functional groups with less polar units to enhance the compound's affinity for the sorbent layer. Reproducible extractions can only be obtained when each parameter that affects $K_{\text{PDMS/W}}$, namely stirring speed, temperature, pH, ionic strength etc., is held constant to ensure equal distribution constants in all experiments.

2.3 Surface characteristics of polydimethylsiloxane

Polymers have become indispensable in daily life. They are essentially made up of macromolecular structures that are formed by linking a large number of smaller molecules together through strong covalent bonds. Polymers are commonly referred to as plastics, but this term is slightly misleading because it refers to a class of polymers known as *thermoplastics*. As their name suggests, they have the ability to melt when exposed to heat. Thermoplastics consist of linear or branched polymer chains which allow the material to change shape when heated. *Thermosets* on the other hand, do not have this ability, but rather have a tendency to decompose when exposed to high temperatures. Thermosets are cross-linked, and this rigid structure prevents them from melting [49]. Some cross-linked polymers are soft and flexible and these are known as elastomers. The best well-known silicon elastomer is polydimethylsiloxane (PDMS).

PDMS is extensively used as a stationary phase in analytical chemistry because of its unique properties. It is thermally stable and allows the diffusion of compounds into the bulk of the material as opposed to being bonded to active sites on the surface. PDMS also has excellent water-repellent properties owing to its structure, which consists of repeat units of silicon and oxygen that are 100% covered and end-capped with non-polar methyl groups (Figure 2.1). Although PDMS is durable, this polymer is not completely resistant to degradation. The polymer layer degrades in the presence of oxygen and water, or from deposits of impurities and organic material on the surface [50].

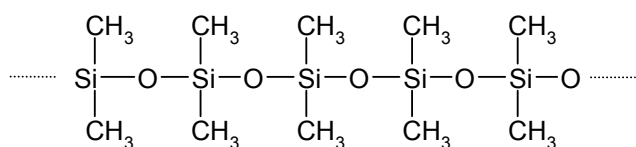


Figure 2.1 Structure of PDMS

Thermal oxidation of PDMS results in the formation of cyclic oligomers that are vaporized into the carrier gas stream and are carried through to the mass spectrometer [50, 51]. Specific masses can be detected in full scan mode that correspond to the breakdown

products of these volatile components, namely m/z 207 for hexamethylcyclotrisiloxane (HCTS), m/z 281 for octamethylcyclotetrasiloxane and m/z 267 and 355 for decamethylcyclopentasiloxane. HCTS is the smallest of the cyclic dimethylsiloxanes that may be released from the surface of the stir bar coating or from the stationary phase in the GC column (i.e. column bleed). A schematic illustration of the breakdown process of PDMS is shown in Figure 2.2.

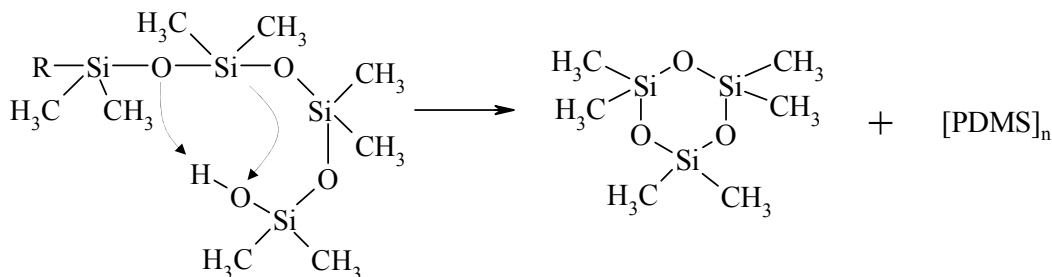


Figure 2.2 Formation of hexamethylcyclotrisiloxane during PDMS degradation [51].

An additional degradation step that occurs at high temperatures is the onset of a free radical mechanism that leads to cross-linking of the polymer [52]. This oxidative reaction stabilizes the surface of the polymer by preventing further volatilization of cyclic oligomers. Cross-linking of the PDMS surface leads to the formation of a brittle silica layer that is hydrophilic, and the systematic build-up of this layer over time prevents the recovery of the polymer surface [50, 53].

PDMS has a remarkable ability to repair initial damage caused by thermal or oxidative degradation. If the damaged surface is left to age in ambient air, the surface hydrophobicity is recovered [54, 55]. Two mechanisms have been proposed by which this process occurs, namely reorientation of newly formed hydroxyl groups into the bulk of the material, and migration of low-molecular-weight siloxanes from the bulk to the surface [56]. Tóth et al. suggested that about one third of this recovery is due to the reorientation of the main chain, whereas two thirds results from diffusion of hydrophobic groups to the surface [57]. As mentioned previously, the oxidation of the polymer surface greatly impedes this recovery process.

Several analytical techniques can be used to study the surface properties of different polymers. In this study, 3 techniques were used to characterize the surface of the PDMS-coated stir bars, namely atomic force microscopy (AFM), scanning electron microscopy (SEM) and contact angle measurements.

Contact angles: Contact angles provide a measure of the hydrophobic recovery of a polymer layer. The measurements are obtained by recording digital images of small drops of deionized water (i.e. $\pm 20 \mu\text{l}$) placed on the surface of a PDMS sample. In this study, the reported values were obtained from an average of four measurements made on different parts of the stir bar coating. A static contact angle instrument, which consisted of a Nikon SMZ-2T optical microscope combined with a camera attachment, was used to perform the different measurements. Figure 2.3 illustrates the shape of a water droplet placed on one of the stir bar samples.

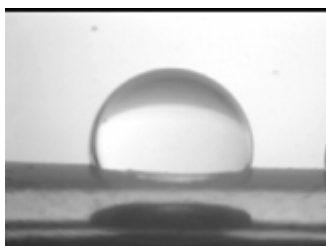


Figure 2.3 A 20 μl water drop placed on the PDMS coating of a stir bar.

SEM: Scanning electron microscopy produces an image of a polymer sample that has greater depth than that obtained by an optical microscope. A three-dimensional image is obtained from which the surface roughness of the polymer can be assessed. A fine electron beam is scanned across the polymer surface which is coated with a thin layer of conducting material. In this study, the stir bars were sputtered with a 10 nm pure gold layer and placed in a Leo 1430VP scanning electron microscope. The images were acquired using a magnification of 68 and 500, respectively.

AFM: Atomic force microscopy provides an image of the polymer surface at the angstrom level, thus allowing the topography of the surface to be studied. The recorded images in this study were obtained using an Explorer atomic force microscope (TopoMetrix, Darmstadt,

Germany) that was operated in the non-contact mode. The cantilevers used for image acquisition were terminated with silicon tips (NanoSensors, Santa Clara, CA, U.S.A.) and had a resonance frequency of ~ 160 kHz. Recording of the images was performed at a scan rate of 1 – 3 Hz with a 100 μm scanner.

The surface characteristics of four different stir bars (Gerstel GmbH, Müllheim a/d Ruhr, Germany) were determined using the abovementioned techniques. A summary of the contact angle measurements, scanning electron micrographs and AFM images are shown in Table 2.1. The imaging data indicate that:

1. The stir bars are systematically degraded during routine use. The AFM and SEM images show that progressive smoothing of the polymer surface takes place following exposure of the stir bars to a number of unfavorable conditions such as water, high thermal desorption temperatures (stir bars 2, 3 and 4), and derivatizing reagents such as trifluoroacetic acid anhydride (stir bar 2) and ethyl chloroformate (stir bars 3 and 4).
2. The contact angle measurements obtained for the different stir bars demonstrate that their surface hydrophobicity is maintained, in spite of the extreme conditions the stir bars are exposed to. However, the stir bars never regain their initial surface hydrophobicity as shown by the contact angle measurements obtained for the different stir bars.
3. Eventually the surface layer decomposes to such an extent (i.e. stir bar 4) that the stir bar is no longer suitable to be used. The contact angle, scanning electron micrograph and AFM image obtained for stir bar 4, show that after prolonged use the stationary phase becomes hydrophilic. Generally, up to 40 extractions can be performed with a single stir bar before the reproducibility of the experiment is compromised. The number of extractions performed usually depends on the conditions the stir bar is exposed to.

Table 2.1 Summary of surface data obtained by SEM, AFM and contact angle measurements

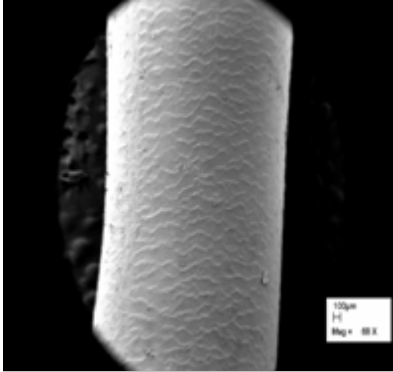
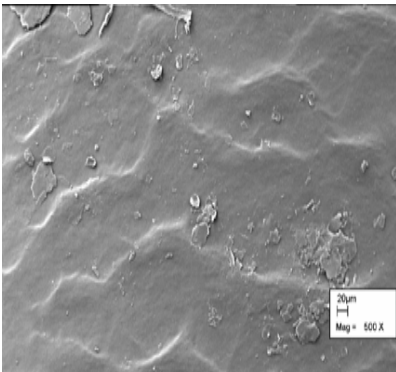
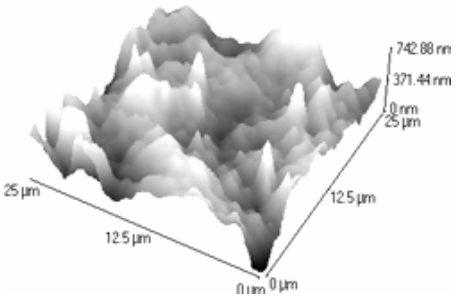
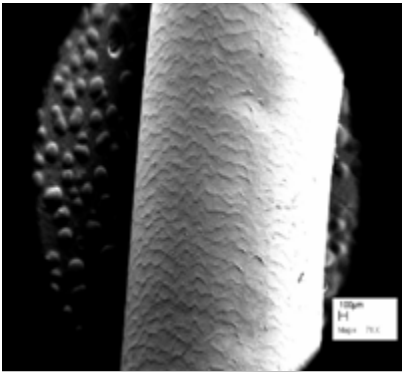
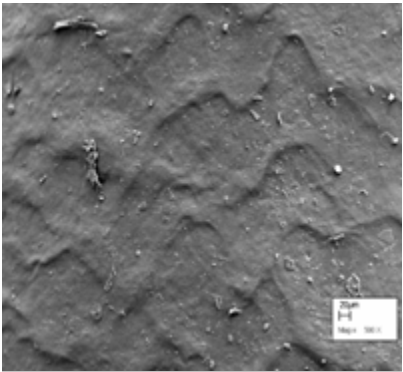
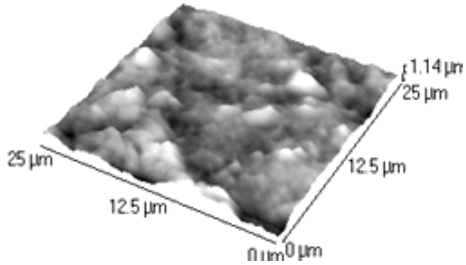
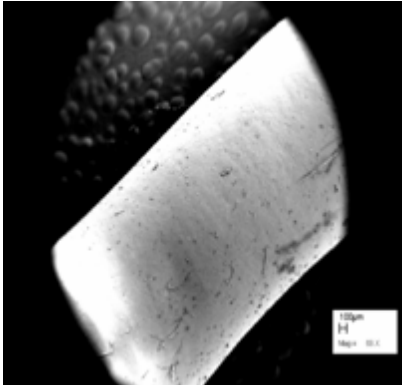
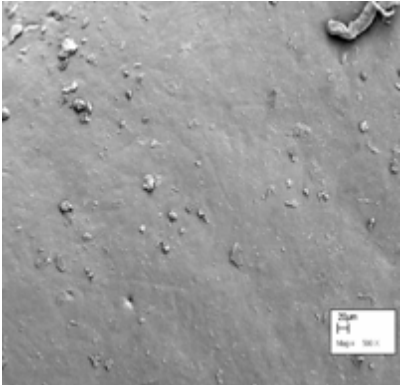
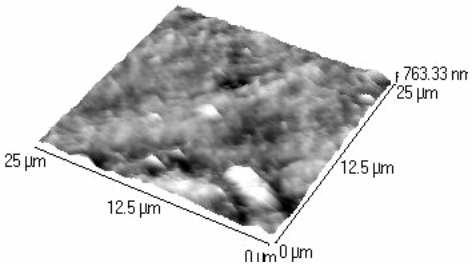
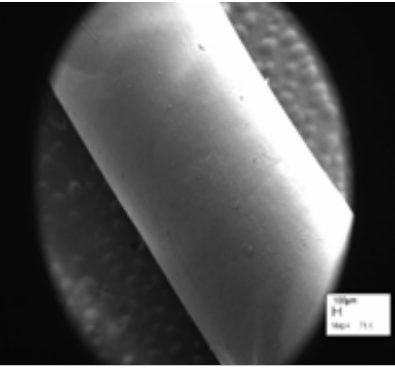
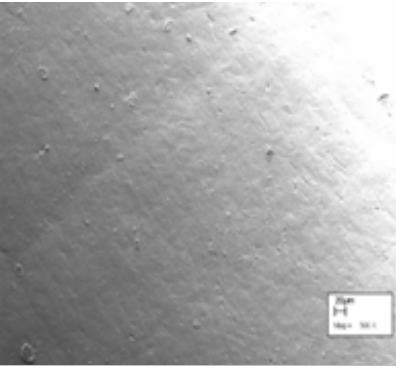
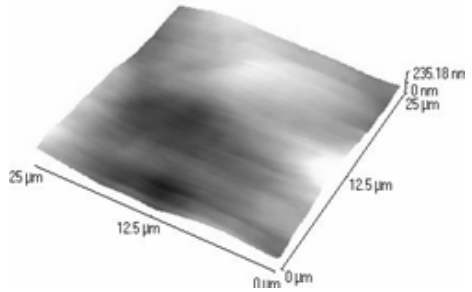
Stir bar no.	SEM: X 68	X 500	AFM
<p><u>Stir bar 1:</u> A new, unused stir bar. Contact angle: 95.1°</p>			
<p><u>Stir bar 2:</u> A new, unused stir bar exposed to trifluoroacetic anhydride at 70°C for 20 minutes; and desorbed under helium at 300°C for 10 minutes. Contact angle: 93.2°</p>			

Table 2.1 Cont.

Stir bar no.	SEM: X 68	X 500	AFM
<p><u>Stir bar 3:</u> Used for ± 20 extractions in aqueous media containing ethyl chloroformate. Contact angle: 94.1°</p>			
<p><u>Stir bar 4:</u> Used for > 40 extractions in aqueous media containing ethyl chloroformate. Contact angle: 90.6°</p>			

References

- [1] K. Grob, A. Habich, *J. Chromatogr.* 321 (1985) 45.
- [2] C.L. Arthur, J. Pawliszyn, *Anal. Chem.* 62 (1990) 2145.
- [3] E. Baltussen, P. Sandra, F. David, C.A. Cramers, *J. Microcolumn. Sep.* 11 (1999) 737.
- [4] E. Baltussen, H.-G. Janssen, P. Sandra, C.A. Cramers, *J. High Resolut. Chromatogr.* 20 (1997) 385.
- [5] E. Baltussen, H.-G. Janssen, P. Sandra, C.A. Cramers, *J. High Resolut. Chromatogr.* 20 (1997) 395.
- [6] B. Kolahgar, A. Hoffmann, A.C. Heiden, *J. Chromatogr. A* 963 (2002) 225.
- [7] P. Popp, C. Bauer, L. Weinrich, *Anal. Chim. Acta* 436 (2001) 1.
- [8] P. Popp, C. Bauer, B. Hauser, P. Keil, L. Weinrich, *J. Sep. Sci.* 26 (2003) 961.
- [9] C. Bicchi, C. Cordero, P. Rubiolo, P. Sandra, *J. Sep. Sci.* 26 (2003) 1650.
- [10] L. Montero, P. Popp, A. Paschke, J. Pawliszyn, *J. Chromatogr. A* 1025 (2004) 17.
- [11] C. Bicchi, C. Cordero, E. Liberto, P. Rubiolo, B. Sgorbini, F. David, P. Sandra, *J. Chromatogr. A* 1094 (2005) 9.
- [12] V. Gomez Zuin, M. Schellin, L. Montero, J.K. Yariwake, F. Augusto, P. Popp, *J. Chromatogr. A* 1114 (2006) 180.
- [13] L. Wennrich, P. Popp, J. Breuste, *Chromatographia* 53 (2001) S380.
- [14] M. Kreck, A. Scharrer, S. Bilke, A. Mosandl, *Eur. Food Res. Technol.* 213 (2001) 389.
- [15] C. Blasco, M. Fernandez, Y. Pico, G. Font, *J. Chromatogr. A* 1030 (2004) 77.
- [16] Y. Hayasaka, K. Macnamara, G.A. Baldock, R.L. Taylor, A.P. Pollnitz, *Anal. Bioanal. Chem.* 375 (2003) 948.
- [17] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, *J. Chromatogr. A* 928 (2001) 117.
- [18] R.F. Alves, A.M.D. Nascimento, J.M.F. Nogueira, *Anal. Chim. Acta* 546 (2005) 11.
- [19] J. Diez, C. Dominguez, D.A. Guillen, R. Veas, C.G. Barroso, *J. Chromatogr. A* 1025 (2004) 263.
- [20] A. Buettner, *J. Agric. Food Chem.* 52 (2004) 2339.
- [21] F. David, P. Sandra, *J. Chromatogr. A* 1152 (2007) 54.
- [22] C. Fernandes, P. Jiayu, P. Sandra, F.M. Lanças, *Chromatographia* 64 (2006) 517.

- [23] A.R. Chaves, S.M. Silva, R.H. Costa Quieroz, F.M. Lanças, M.E. Costa Quieroz, *J Chromatogr. B* 850 (2007) 295.
- [24] C. Almeida, J.M.F. Nogueira, *J. Pharm. Biomed. Anal.* 41 (2006) 1303.
- [25] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, *J. Chromatogr. B* 856 (2007) 156.
- [26] K. Desmet, B. Tienpont, P. Sandra, *Chromatographia* 57 (2003) 681.
- [27] T. Benijts, J. Vercammen, R. Dams, H.P. Tuan, W. Lambert, P. Sandra, *J. Chromatogr. B* 755 (2001) 137.
- [28] T. Colburn, F. Vom Saal, A. Soto, *Environ. Health Perspect.* 101 (1993) 378.
- [29] M. Kawaguchi, R. Ito, Y. Hayatsu, H. Nakata, N. Sakui, N. Okanouchi, K. Saito, H. Yokota, S. Izumi, T. Makino, H. Nakazawa, *J. Pharm. Biomed. Anal.* 40 (2006) 82.
- [30] M. Kawaguchi, K. Inoue, M. Yoshimura, R. Ito, N. Sakui, N. Okanouchi, H. Nakazawa, *J. Chromatogr. B* 805 (2004) 41.
- [31] M. Kawaguchi, N. Sakui, N. Okanouchi, R. Ito, K. Saito, S. Izumi, T. Makino, H. Nakazawa, *J. Chromatogr. B* 820 (2005) 49.
- [32] M. Kawaguchi, R. Ito, N. Sakui, N. Okanouchi, K. Saito, S. Yasuo, H. Nakazawa, *Anal. Bioanal. Chem.* 388 (2007) 391.
- [33] B.A. Rocha-Gutierrez, W.-Y. Lee, *Abstracts of Papers, 229th ACS National Meeting*, San Diego, CA, United States, March 13-17, 2005.
- [34] T. Kumazawa, X.-P. Lee, M. Takano, H. Seno, T. Arinobu, A. Ishii, O. Suzuki, K. Sato, *Jpn. J. Forensic Toxicol.* 20 (2002) 295.
- [35] B. Tienpont, F. David, A. Stopforth, P. Sandra, *LCGC Eur.* 16 (2003) 5.
- [36] B. Tienpont, F. David, T. Benijts, P. Sandra, *J. Pharm. Biomed. Anal.* 32 (2003) 569.
- [37] J.-P. Lambert, W.M. Mullet, E. Kwong, D. Lubda, *J. Chromatogr. A* 1075 (2005) 43.
- [38] A. Buettner, F. Welle, *Flavour Fragr. J.* 19 (2004) 505.
- [39] H.A. Soini, K.E. Bruce, D. Wiesler, F. David, P. Sandra, M.V. Novotny, *J. Chem. Ecol.* 31 (2005) 377.
- [40] H.A. Soini, S.E. Schrock, K.E. Bruce, D. Wiesler, E.D. Ketterson, M.V. Novotny, *J. Chem. Ecol.* 33 (2007) 183.
- [41] J.X. Zhang, H.A. Soini, K.E. Bruce, D. Wiesler, S.K. Woodley, M.J. Baum, M.V. Novotny, *Chem. Senses* 30 (2005) 727.
- [42] H.A. Soini, K.E. Bruce, I. Klouckova, R.G. Brereton, D.J. Penn, M.V. Novotny, *Anal. Chem.* 78 (2006) 7161.

- [43] A. Stopforth, A. Tredoux, A. Crouch, P. van Helden, P. Sandra, *J. Chromatogr. A* 1071 (2005) 135.
- [44] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, *J. Chromatogr. B* 834 (2006) 134.
- [45] A. Stopforth, C.J. Grobbelaar, A.M. Crouch, P. Sandra, *J. Sep. Sci.* 30 (2007) 257.
- [46] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, *J. Chromatogr. B* 856 (2007) 156.
- [47] J. Pawliszyn, *Solid phase microextraction – Theory and practice*, Wiley-VCH, New York, 1997.
- [48] H. Lord, J. Pawliszyn, *J. Chromatogr. A* 902 (2000) 17.
- [49] D. Walton, P. Lorimer (Ed's), *Oxford chemistry primers – Polymers*, Oxford University Press, Bath, England, 2000.
- [50] H. Hillborg, S. Karlsson, U.W. Gedde, *Polymer* 42 (2001) 8883.
- [51] G. Camino, S.M. Lomakin, M. Lazzari, *Polymer* 42 (2001) 2395.
- [52] G. Camino, S.M. Lomakin, M. Lageard, *Polymer* 43 (2002) 2011.
- [53] J. Kim, M.K. Chaudhury, M.J. Owen, *J. Colloid Interface Sci.* 226 (2000) 231.
- [54] M. Morra, E. Occhiello, R. Marola, F. Garbassi, P. Humphrey, D. Johnson, *J. Colloid Interface Sci.* 137 (1990) 11.
- [55] B.T. Ginn, O. Steinbock, *Langmuir* 19 (2003) 8117.
- [56] E. Vallés, C. Sarmoria, M. Villar, M. Lazzari, O. Chiantore, *Polym. Degrad. Stabil.* 69 (2000) 67.
- [57] A. Tóth, I. Bertóli, M. Blaszó, G. Bánhegyi, A. Bogнар, P. Szaplanczay, *Appl. Polym. Sci.* 52 (1994) 1293.

Chapter 3

Derivatization

3.1 Introduction

Most biological samples are not suitable for direct analysis by GC/MS even after an extraction step has been performed. The target compounds often contain one or more polar functional groups that limit their volatility and thermal stability. One way to improve the chromatography of polar analytes is to include a derivatization step during the sample work-up procedure. Derivatization is basically a micro-scale synthetic reaction that replaces active hydrogens in functional groups such as $-\text{COOH}$, $-\text{OH}$ and $-\text{NH}_2$ with less polar, thermally stable groups. These reactions are frequently employed to prevent hydrogen bonding between different molecules, thereby decreasing their interaction with active sites in the GC system. Therefore, derivatized compounds often demonstrate improved chromatographic behavior because peak tailing or broadening is minimized, the compounds are better resolved and more symmetrical, which in turn enhances their detectability.

Chemical reactions that are frequently used to modify polar compounds in gas chromatography include silylation, alkylation, acylation and different condensation reactions. Silylation is probably the most frequently used technique, which involves the use of highly reactive trimethylsilylating reagents (e.g. bistrimethylsilyltrifluoroacetamide - BSTFA) that are capable of derivatizing most functional groups. A drawback of using this method, however, is that the reagents are sensitive to moisture, and in SBSE it is often necessary to perform the derivatization step directly in the aqueous sample. One way to overcome this limitation is to derivatize the compounds directly in the PDMS coating by exposing the stir bar [1] or SPME fiber [2] to vapors of the reagent in a sealed container (i.e. vial). In SBSE, a technique known as in-tube silylation has been developed where a small drop of reagent is placed on a piece of glass wool next to the stir bar inside the thermal desorption tube [3]. The compounds are derivatized during the thermal desorption step, which involves heating the tube to a desired temperature to release the compounds from the PDMS coating. This mode of derivatization is very efficient, but the tubes utilized in SBSE are not entirely sealed off, which means that the reagent will be released into the atmosphere when the stir bar is transferred to the thermal desorption unit (TDU). Silylation reagents are known to be hazardous and potentially harmful; therefore one of the objectives

in this study was to investigate the use of a more environmentally friendly reagent for the headspace derivatization of compounds enriched by SBSE (see section 3.3).

Not all compounds are suitable for extraction by SBSE due to their high polarity. It is often necessary to perform an *in situ* derivatization step to improve the recovery of polar analytes by the stir bar. Currently, only a small number of chemical reactions can be used successfully in aqueous matrices. One such reaction involves the replacement of an acidic proton (i.e. $-\text{COOH}$) with an aliphatic group, also known as alkylation. Ethyl chloroformate (ECF) is a well known alkylating reagent that is frequently used to derivatize free fatty acids in biological samples [4]. The synthesized ethyl esters are sufficiently stable to be extracted directly from an aqueous sample using SBSE [5]. Another reagent that is frequently used for direct derivatization purposes is acetic acid anhydride. This versatile reagent replaces even non-acidic protons (e.g. $-\text{OH}$) with stable acetate groups. The target analytes can be derivatized directly in the aqueous phase prior to SBSE, and/or in the headspace after the extraction step has been performed [6]. The resulting acyl derivatives demonstrate improved chromatographic behavior as well as enhanced recovery by the stir bar coating.

The amount of analytes extracted by the stir bar is determined by the affinity of the compounds for the various phases in the sample [7]. Polar analytes have a greater affinity for the aqueous phase as compared to the relatively non-polar PDMS extraction phase. For this reason, the extraction of highly hydrophilic compounds by SBSE will be poor, unless a hydrophobic entity is attached to the analyte. *O*-(2,3,4,5,6,-pentafluorobenzyl) hydroxylamine (PFBHA) is a highly reactive chemical reagent that converts polar, low molecular weight carbonyl compounds (e.g. aldehydes) to their corresponding oxime derivatives directly in aqueous matrices. The reaction involves the replacement of two protons between the analyte and the reagent to form a double bond by means of condensation [8]. The partition coefficient or $\log K_{o/w}$ value of the derivatives is markedly higher than that of the parent compounds, which favors the distribution of the derivatives into the PDMS coating and enhances the total amount of analytes extracted by the stir bar [1].

In this chapter, the chemical reactions used to improve the gas chromatography of various polar analytes in biological samples will be discussed. Several factors affecting the yield of the individual reactions will also be highlighted.

3.2 Ethyl chloroformate

In 1990, it was discovered that the chloroformates are potent esterification reagents of carboxylic acids in aqueous samples [9]. A range of organic acids can be converted to their corresponding esters within minutes when the reagent is added directly to an aqueous medium [10]. If the reaction is performed in a mixture that combines the reagent together with pyridine and an alcohol, then two products are formed, namely a major product that results from the reaction of the alcohol and a mixed anhydride, and a minor product that is formed by decarboxylation of the mixed anhydride [8]. A single derivative can be obtained in high yield, however, if the chloroformate is combined with its corresponding alcohol; that is, if ethyl chloroformate (ECF) is used together with ethanol. The reaction scheme for the derivatization of organic acids with the alkyl chloroformates is shown in Fig. 3.1.

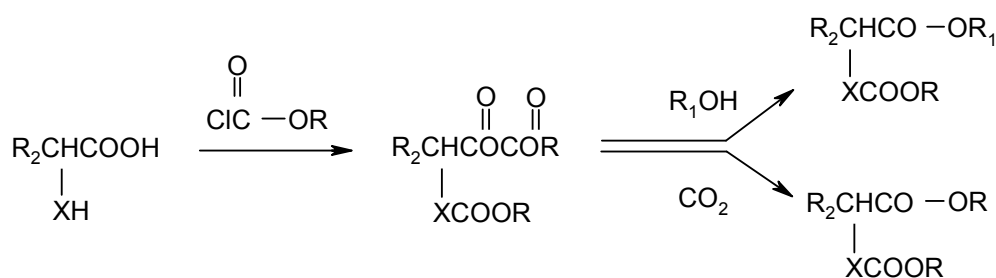


Figure 3.1 Reaction pathways for the alkylation of acids with chloroformates (reproduced from Ref. 8)

It is known that free fatty acids can be converted to their corresponding ethyl esters by adding ECF directly to an aqueous sample. However, the final yield of the derivatives depends on the composition of the aqueous medium [10] and the reactivity of ECF is greatly determined by the amount of ethanol added. [11]. Therefore, initial experiments were performed to investigate whether the composition of the reaction medium would affect the final yield of the derivatized compounds. The studied samples included a number

of free fatty acids ranging from decanoic acid (C_{10}) to octadecanoic acid (C_{18}), and special attention was given to optimizing the yield of the longer chain fatty acids such as hexadecanoic acid (C_{16}) and C_{18} . The objective was to establish the optimal ratios between the different components in the sample, namely ECF, pyridine, ethanol and water to improve the recovery of C_{16} and C_{18} by SBSE. The optimized method would then be used to detect trace amounts of tuberculostearic acid in sputum samples as described in Chapter 6.

At first, it was found that by decreasing the water to ethanol ratio, a much higher yield of the long chain fatty acids could be achieved (results not shown). Large quantities of water delay the reaction kinetics of the reagent and this situation is avoided by increasing the amount of ethanol added to the sample. In subsequent experiments it was found that by decreasing the ratio between the reagent and the catalyst (i.e. pyridine), the yield of C_{16} and C_{18} was significantly increased. ECF starts to decompose when it is added to the reaction medium producing carbon dioxide, alcohol and hydrochloric acid that block the catalytic effect of pyridine [12]. Therefore, pyridine should always be present in molar excess as demonstrated in Figure 3.2.

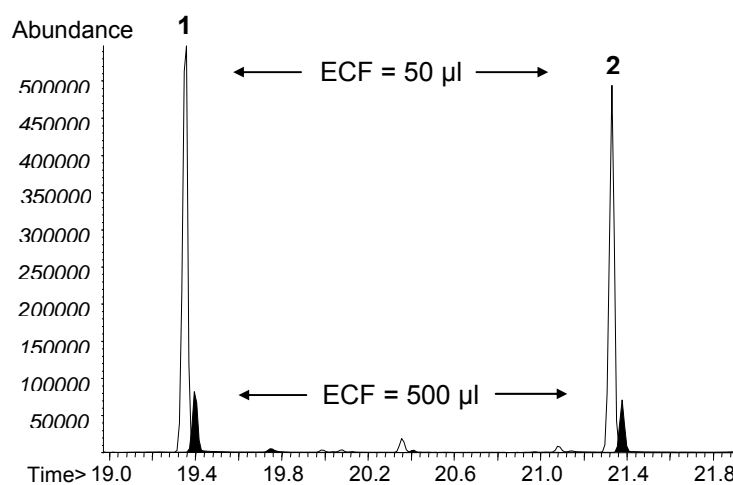


Figure 3.2 SIM chromatograms (m/z 88) of 1) C_{16} and 2) C_{18} following the addition of different quantities of ECF to 1 ml aqueous samples containing 0.33 ml ethanol and 0.17 ml pyridine. The extraction and instrumental conditions are described in Chapter 6.

Apart from decreasing the sensitivity of the analysis, a major disadvantage to using larger quantities of ECF is that an excess amount of reagent will be absorbed by the stir bar coating. This situation affects the reproducibility of the method because ECF is corrosive, and its presence in the stir bar coating may alter the surface characteristics of the PDMS layer. At high temperatures (e.g. during thermal desorption) an excess amount of reagent leads to varying peak ratios, decreased sensitivity (e.g. for the longer chain fatty acids) and variations in the retention times of the analytes as demonstrated in Figure 3.3.

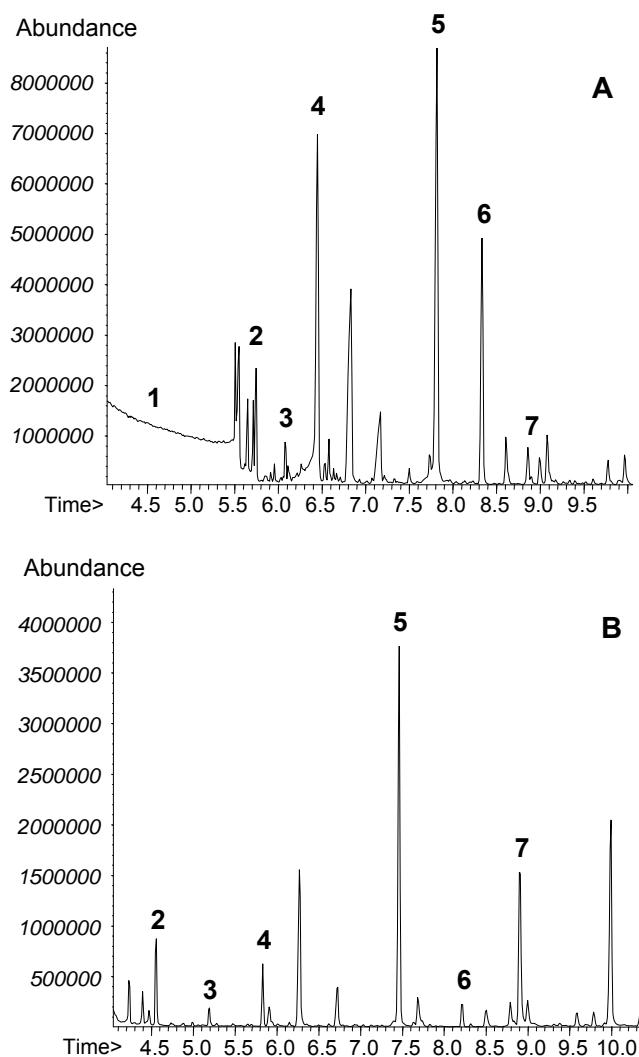


Figure 3.3 Total ion chromatograms of A) of a urine sample containing 500 µl ECF and B) 50 µl ECF. The upper trace (A) demonstrates a shift in retention time for the early eluting compounds due to the presence of an ECF reagent peak. The compounds are 1) ECF; 2) dimethylcarbamic acid; 3) 2-chloro-1,1-diethoxyethane; 4) octamethylcyclotetrasiloxane; 5) hexachloroethane; 6) decamethylcyclopentasiloxane; 7) benzoic acid. The extraction and instrumental conditions are given in Chapter 6.

It has previously been reported that the conversion of organic acids to their corresponding alkyl esters occurs instantaneously [10]. However, in our experience it is advantageous to prolong the ECF reaction time in order to increase the derivatization yield of the longer chain fatty acids. In this study, the samples were routinely sonicated for 15 minutes after the addition of the catalyst, reagent and solvent to the sample matrix. The effect of time on the derivatization yield of the fatty acids was investigated by sonicating the samples for various lengths of time. The optimal yield was obtained when the samples were sonicated for 15 minutes, whereas shorter reaction times significantly decreased the recovery of C₁₆ and C₁₈ (Figure 3.4).

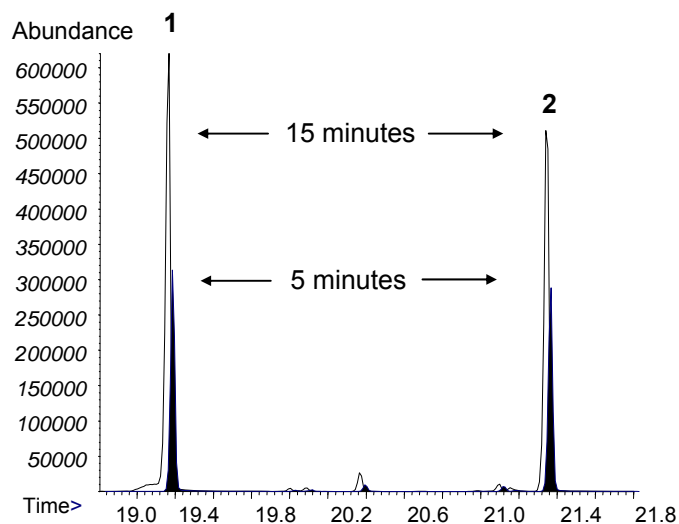


Figure 3.4 SIM chromatograms (m/z 88) of 1) C₁₆ and 2) C₁₈ in 1 ml aqueous samples containing 0.33 ml ethanol, 0.17 ml pyridine and 0.05 ml ECF that were sonicated for 5 min and 15 min, respectively. The extraction and instrumental conditions are given in Chapter 6.

Using the optimized reaction conditions, the ideal ratio between ethanol and pyridine were investigated as a way of increasing the recovery of tuberculostearic acid (TBSA) and 17-methyloctadecanoic acid (MODA) from aqueous samples. The investigated ratios are presented in Table 3.1. The optimal ethanol:pyridine ratio was obtained for sample composition C (Figure 3.5), which consisted of 1 ml water, 0.88 ml ethanol, 0.16 ml pyridine and 0.08 ml ECF. These reaction conditions were applied to accomplish the trace analysis of TBSA in decontaminated sputum samples (Chapter 6).

Table 3.1 Optimization of the ethanol:pyridine ratio

Ethanol : Pyridine Ratio (Volume)	ECF (μl)
A) 4 : 1 (0.5 ml)	50
B) 10 : 1 (1.1 ml)	50
C) 5.5 : 1 (1.04 ml)	80
D) 6.25 : 1 (1.16 ml)	160
E) 8.33 : 1 (2.24 ml)	160
F) 6.25 : 1 (2.32 ml)	160

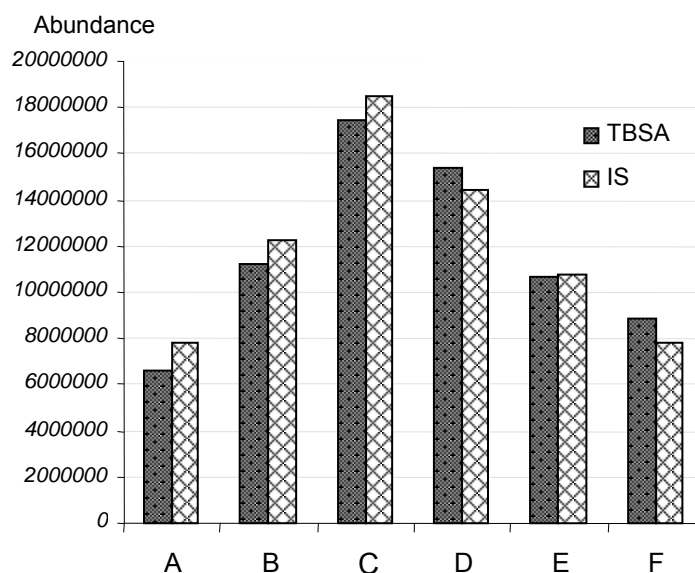


Figure 3.5 Graphic representation of the derivatization yields obtained for TBSA and MODA (IS) using the ethanol:pyridine ratios presented in Table 3.1. The extraction and instrumental conditions are given in Chapter 6.

3.3 Acetic acid anhydride

Acetic acid anhydride is frequently used as a derivatizing reagent in analytical chemistry because of its low cost and ease of use. It hydrolyzes slowly in water and can therefore be used to derivatize compounds directly in aqueous matrices. Several methods have been reported where acetic acid anhydride was used in combination with SBSE to improve the

extraction and gas chromatographic separation of phenols [13], hydroxylated-PAHs, [14] and estrogens [6] in environmental and biological samples. The versatility of this reagent is due to the fact that both phenolic and aliphatic hydroxyl groups can be derivatized using either direct (i.e. *in situ*) derivatization or headspace (i.e. post-extraction) derivatization. The *in situ* derivatization step proceeds rapidly in an aqueous medium in the presence of a base such as NaHCO_3 , which is often added to drive the reaction to completion. The acylation of hydroxyl-containing compounds with acetic acid anhydride is illustrated in Figure 3.6.

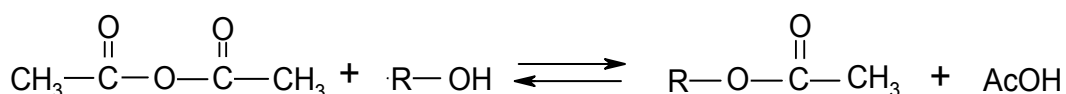


Figure 3.6 Reaction pathway for the acylation of hydroxyl-containing compounds

Derivatization of phenolic hydroxyl groups: Phenolic hydroxyl groups can be derivatized directly in an aqueous medium following the addition of acetic acid anhydride to the sample matrix. The reaction rate is increased when a base, such as K_2CO_3 , NaHCO_3 , Na_2CO_3 or pyridine, is added to the sample. The appropriate base should be selected because the yield of the derivatives is determined by the final pH of the reaction medium. Phenolic hydroxyl groups dissociate at higher pH levels and it is this anionic form of the compound that is able to react with the reagent in aqueous media. The pKa value of the phenolic functional group is approximately 9.8, which means that at neutral pH the majority of the compound will exist in its non-ionic form [15]. Higher pH levels, however, produce the phenolate anion which increases the rate of the reaction due to its high solubility in water. Thus, the overall yield of the derivatives can be improved when increased amounts of the phenolate anion are made available. The reaction should be performed at a pH that is high enough to increase the available amount of the phenolate anion, but low enough to avoid destruction of the reagent before the reaction is completed [15]. The pH of the sample matrix can be adjusted to a suitable level by selecting an appropriate base to catalyze the reaction. For acylation of the estrogens in urine samples,

the use of Na_2CO_3 resulted in higher yields as compared to K_2CO_3 and NaHCO_3 (results not shown). The analysis of estrone and 17β -estradiol is described in detail in Chapter 9.

Derivatization of aliphatic hydroxyl groups: Most of the reagents available for the derivatization of aliphatic hydroxyl groups are sensitive to moisture. To overcome this limitation, Okeyo et al. [16] developed a novel SPME method which involves the direct derivatization of compounds in the fiber coating. The analytes are first enriched from the aqueous sample, where after the fiber is exposed to vapors of the reagent in a sealed vial to allow the chemical conversion of the compounds directly in the polymer coating. A modification of this technique involves the simultaneous derivatization and extraction of the compounds in the fiber coating. In this technique, the fiber is first doped with the reagent by exposing the SPME device to vapors of the reagent in a sealed container, where after the fiber is placed in the sample matrix to extract and derivatize the compounds [17]. For the analysis of polar compounds by SBSE, a slightly different approach has been used as demonstrated by two recently developed derivatization techniques that were reported by Kawaguchi et al. [3] and Stopforth et al. [1]. The first method involves the in-tube silylation of polar compounds by BSTFA, whereas the second method involves exposure of the stir bars to acetic acid anhydride vapors in modified headspace vials. This last approach has been used to derivatize the hydroxyl-containing compounds investigated in this study, namely 4-hydroxynonenal (Chapter 7), testosterone and epitestosterone (Chapter 8), as well as estrone and 17β -estradiol (Chapter 9).

The in-vial headspace derivatization technique was developed by investigating the acylation of two long chain fatty alcohols, namely 1-eicosanol and 1-docosanol. In a preliminary experiment, the fatty alcohols were derivatized directly in a 1 ml aqueous sample containing 50 μl acetic acid anhydride and 100 μl pyridine. The sample was spiked with 2 $\mu\text{g ml}^{-1}$ of each compound and was sonicated for 15 minutes. Extraction of the derivatized compounds was performed by SBSE at 1000 rpm for 1 hour, and thermal desorption and GC/MS was performed using the instrumental conditions described in Chapter 9. Figure 3.7-A demonstrates that only a small amount of the compounds were converted to their corresponding acetates using the direct derivatization approach. In subsequent experiments the post-extraction derivatization of the fatty alcohols was

investigated using the two techniques mentioned previously, namely doping of the PDMS stationary phase with the reagent prior to extracting the compounds (i.e. simultaneous extraction and derivatization), and conversion of the fatty alcohols to their corresponding acetates in the PDMS coating after the compounds have been extracted from the sample (i.e. headspace derivatization). For the simultaneous extraction and derivatization of the compounds, the stir bar was first placed in a 2 ml modified autosampler vial containing 50 μl acetic acid anhydride and 50 μl pyridine. A detailed description of the vial used for the headspace derivatization of the extracted compounds is given in Chapter 7 (Section 7.2.5). The vial was heated at 90°C for 30 minutes to load the reagent onto the stir bar coating. Thereafter, the extraction was performed as described for the direct derivatization procedure. Figure 3.7-B shows that the stir bar doping method resulted in low yields of the fatty alcohol derivatives due to the poor diffusion coefficient of acetic acid anhydride into the PDMS coating ($\log K_{o/w} = -0.12$). Thus, the stir bar cannot be loaded successfully using this reagent. Finally, headspace derivatization of the fatty alcohols was investigated by exposing the stir bar to acetic acid anhydride vapors in a modified headspace vial after the compounds were extracted from the aqueous sample. Almost complete derivatization of the fatty alcohols was obtained using the headspace derivatization method. Approximately 98.8% of 1-eicosanol and 97.8% of 1-docosanol were converted to their corresponding acetates as shown in Figure 3.7-C. The estimated detection limits for the compounds were 0.2 ng mL^{-1} for 1-eicosanol and 0.37 ng mL^{-1} for 1-docosanol.

3.4 Pentafluorobenzyl hydroxylamine

The oximation of 4-hydroxynonenal (4HNE) can be accomplished by adding PFBHA directly to an aqueous sample in the presence of a catalyst such as pyridine. The reaction is straightforward and proceeds rapidly following the addition of the reagent to the sample matrix. However, each step of the reaction should be optimized to improve the yield of the oxime derivatives and this process is described in detail in Chapter 7 (Section 7.3.2).

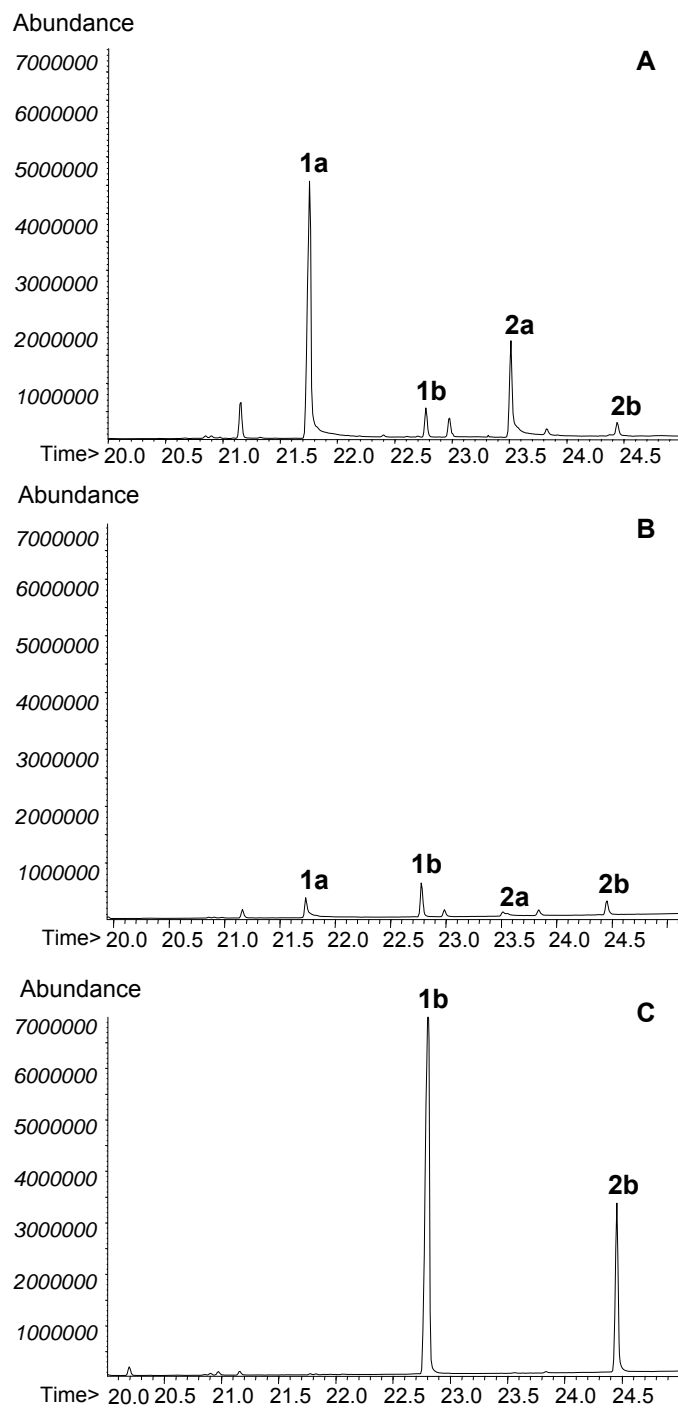


Figure 3.7 Total ion chromatograms of 1a) eicosanol; 1b) eicosanol-acetate; 2a) docosanol; and 2b) docosanol-acetate as obtained by SBSE-TD-GC/MS. The upper trace (A) corresponds to the *in situ* derivatization of the compounds; the center trace (B) to the simultaneous derivatization and extraction of the compounds; and the lower trace (C) to the headspace derivatization of the extracted alcohols. The instrumental conditions are described in Chapter 9.

References

- [1] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, *J. Chromatogr. B* 834 (2006) 134.
- [2] L. Pan, M. Adams, J. Pawliszyn, *Anal. Chem.* 67 (1995) 4396.
- [3] M. Kawaguchi, R. Ito, N. Sakui, N. Okanouchi, K. Saito, H. Nakazawa, *J. Chromatogr. A* 1105 (2006) 140.
- [4] P. Hušek, *Clin. Chem.* 43 (1997) 1999.
- [5] B. Tienpont, F. David, T. Benijts, P. Sandra, *J. Pharm. Biomed. Anal.* 32 (2003) 569.
- [6] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, *J. Chromatogr. B* 856 (2007) 156.
- [7] H. Lord, J. Pawliszyn, *J. Chromatogr. A* 902 (2000) 17.
- [8] R.J. Wells, *J. Chromatogr. A* 843 (1999) 1.
- [9] P. Hušek, J.A. Rijks, P.A. Leclercq, C.A. Cramers, *J. High Resolut. Chromatogr.* 13 (1990) 633.
- [10] P. Hušek, H.M. Liebich, *J. Chromatogr. B* 656 (1994) 37.
- [11] P. Hušek, *J. Chromatogr. B* 717 (1998) 57.
- [12] P. Hušek, *J. Chromatogr.* 552 (1991) 289.
- [13] M. Kawaguchi, K. Inoue, M. Yoshimura, N. Sakui, N. Okanouchi, R. Ito, Y. Yoshimura, H. Nakazawa, *J. Chromatogr. A* 1041 (2004) 19.
- [14] N. Itoh, H. Tao, T. Ibusuki, *Anal. Chim. Acta* 555 (2006) 201.
- [15] L. Renberg, K. Lindström, *J. Chromatogr.* 214 (1981) 327.
- [16] P. Okeyo, S.M. Rentz, N.H. Snow, *J. High Resolut. Chromatogr.* 20 (1997) 171.
- [17] E.H.M. Koster, C.H.P. Bruins, G.J. de Jong, *Analyst* 127 (2002) 598.

Chapter 4

Instrumentation

4.1 Introduction

In this chapter, the analytical instrumentation used to detect trace amounts of various biological markers in human bodily fluids is presented. The system is made up of four main components, namely a gas chromatograph (GC), a thermal desorption system (TDS), a cooled injection system (CIS), and a mass selective detector (MSD). The specifications of the system, including instrument model and make, column dimensions and operating conditions are described in detail in Part II of this study (i.e. Chapters 6 – 9). The different components of the system have been constructed of various parts, and the function and operation of these units is discussed in detail in the following section.

4.2 Gas chromatograph

The fundamental principles of gas chromatography are based on the partitioning of a vaporized sample between a gaseous mobile phase and a liquid or solid stationary phase. The sample is first introduced into the inlet of the instrument, where after the vaporized compounds are carried through a capillary column by a stream of inert gas (i.e. helium). The compounds diffuse between the carrier gas and the stationary phase that is immobilized on the inner walls of the capillary tubing. The stationary phase retains the compounds to varying degrees, depending on the chemical and physical properties of each compound. Separation of the different sample components can be optimized by carefully selecting the carrier gas velocity, oven temperature program, and type of stationary phase used. The compounds that elute at the end of the column are recorded electronically using different detectors, such as a single channel detector (i.e. flame ionization detector), or a multi-channel detector such as a mass spectrometer [1].

4.3 Cooled injection system

Conventional injectors are operated at high temperatures to aid the immediate volatilization of sample constituents introduced into the injector body. The entire sample (i.e. splitless injection) or a portion of the sample (i.e. split injection) can be transferred to the head of the capillary column. In trace analysis however, it is often necessary to inject the entire

sample to improve analytical sensitivity. Classical hot split/splitless injection involves the injection of only a few microliters of the sample to avoid overloading the column and detector. To allow the injection of much larger sample volumes (i.e. up to 250 μl), Vogt et al. [2, 3] developed a programmed temperature vaporization (PTV) inlet in 1979, which improved the detection of trace amounts of analytes in biomedical and environmental samples. During temperature-programmed sample introduction, the sample is deposited in the inlet at a temperature slightly below the boiling point of the solvent. The solvent is continuously evaporated through the split vent, while the analytes remain in the liner. After a certain time, the split vent is closed and the analytes are transferred to the column in splitless mode by rapidly increasing the temperature of the PTV liner (i.e. at $12\text{ }^{\circ}\text{C}\cdot\text{s}^{-1}$). This procedure has several advantages, in that sample degradation is decreased, fewer contaminants are transferred to the column, and the vaporization of higher boiling compounds is significantly improved [4]. In SBSE, the PTV inlet may also be used as a cryogenic trap to focus the desorbed compounds in the injector liner prior to being transferred to the capillary column. The temperature of the liner may be lowered to sub-ambient temperatures using CO_2 or liquid nitrogen. A schematic drawing of the cooled injection system (CIS) developed by Gerstel is shown in Figure 4.1 [5].

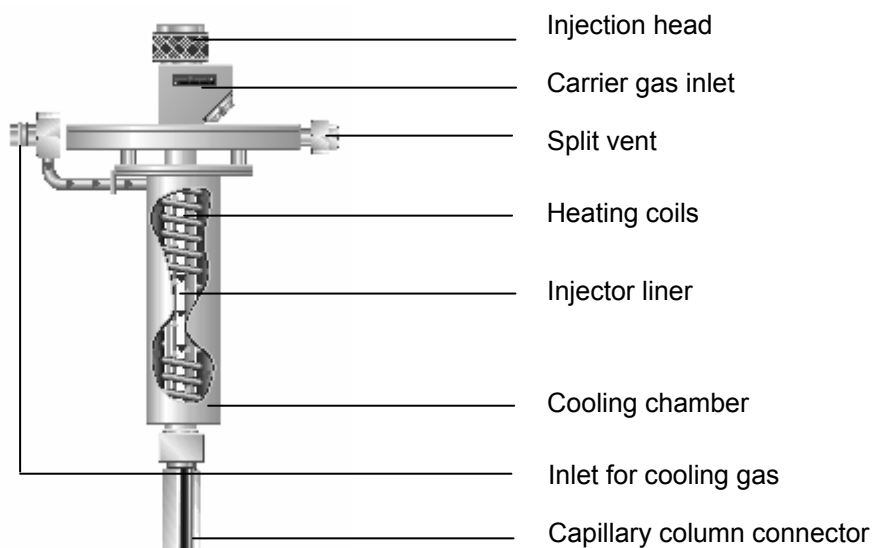


Figure 4.1 The Gerstel CIS-4 PTV injector [5].

4.4 Thermal desorption system

The CIS injector is connected to a thermal desorption system (TDS) by means of a short temperature controlled capillary column. The transfer capillary is maintained at a constant high temperature that facilitates the transfer of vaporized compounds to the PTV liner. Prior to performing the thermal desorption step, the oven chamber is cooled to ambient temperature, the GC column head pressure is reduced to zero, and the column temperature is lowered to below 90°C to prevent oxidative damage of the stationary phase in the capillary column. A glass tube containing the stir bar is transferred to the TDS desorption chamber and the air is purged out of the system. The TDS oven is heated to 300°C using a preset temperature program, and the vaporized compounds are swept toward the PTV liner by a constant flow of carrier gas that is maintained through the tube. The thermal desorption step may be performed in split, splitless or solvent vent mode. Traces of moisture in the TDS can be removed in the solvent venting mode, which prevents potential water damage to the GC column. Figure 4.2 illustrates the different components of the Gerstel thermal desorption system [6].

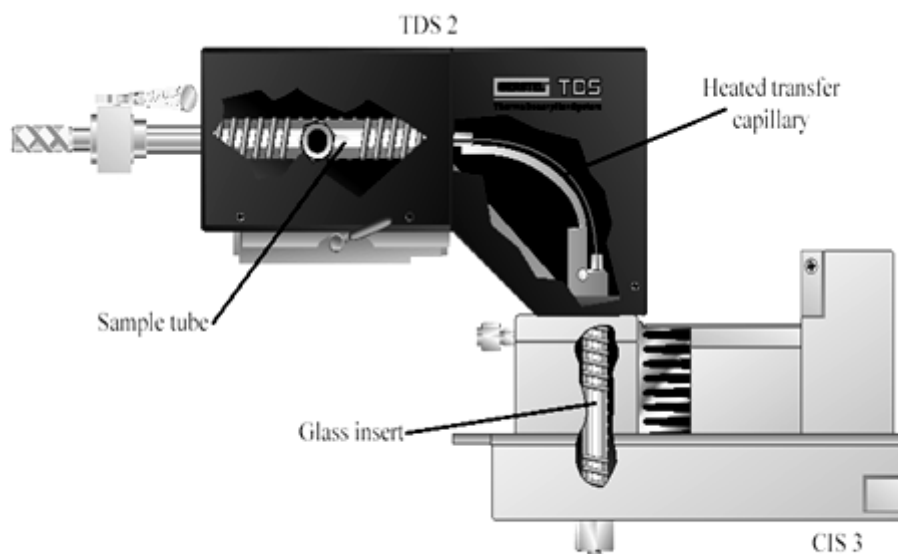


Figure 4.2 The Gerstel Thermal desorption system (TDS 2) linked with a CIS-3 PTV injector [6].

4.5 The mass spectrometer

The mass spectrometer (MS) consists of 5 main components, namely a) an inlet system that interfaces the MS with the gas chromatograph; b) an ion source, which generates the electrons required to ionize and fragment the different sample components; c) a mass analyzer that separates the ions according to their mass-to-charge ratio; d) a detector and e) a vacuum system that maintains the low pressure environments required for mass spectrometric measurements.

4.5.1 MS inlet

A mass spectrometer can be interfaced directly to a GC instrument when high capacity pumping systems are utilized to remove the constant inflow of carrier gas into the MS ion source [7]. Helium is usually chosen as the carrier gas for GC/MS analysis because of its low molecular weight and negligible ionization potential. Helium is therefore easily removed from the system without producing ions that will interfere with the ionization of other materials. The flow rate of the carrier gas that is maintained through the GC capillary column is usually in the order of 1 – 25 ml min⁻¹ [8]. In GC/MS, however, this flow rate is restricted to 1 – 2 ml min⁻¹ to facilitate the direct coupling of the capillary column to the MS ion source and to ensure that the sensitivity of the analysis is maintained. The main advantage of direct coupling is that the entire sample deposited into the GC column is transferred to the mass spectrometer, which avoids any losses due to the presence of dead volumes.

4.5.2 Ion source

A schematic diagram of an electron impact ion source is shown in Figure 4.3 [9]. The compounds that elute from the GC column are bombarded with a stream of electrons emitted by a metal filament. The electrons pass through a slit in the ionization chamber and are accelerated toward an anode using a specific energy potential (i.e. 5 - 100 V) [7]. Two permanent magnets, placed on either side of the ion source, focus the emitted electrons into a narrow beam before it collides with the neutral gas molecules eluting from the GC

column. The energy acquired by the electron beam allows a certain percentage of molecules to be ionized, and also determines the fragmentation pattern of the ionized compounds. Most electron impact mass spectra are recorded using 70 eV electrons. All positive ions formed by collision with the electron beam are drawn out of the ion source by applying a small accelerating voltage (i.e. 20 V) to the repeller plate [10]. Electron impact ionization leads to substantial fragmentation of the analyzed compounds, which provide considerable information about the structure of the analytes. In addition, the fragmentation patterns and mass spectra obtained are highly reproducible.

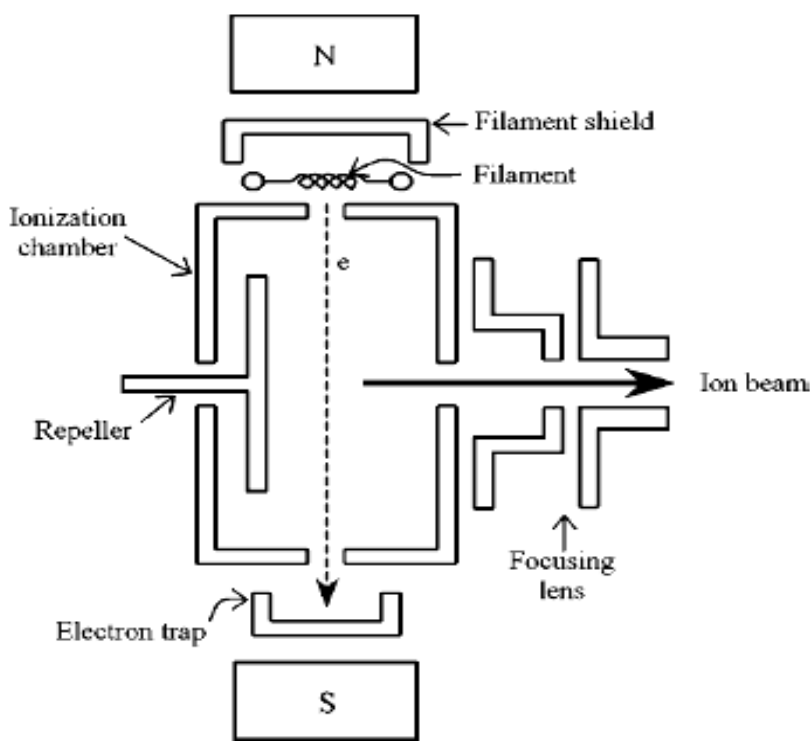


Figure 4.3 A schematic diagram of an electron impact ion source [9].

4.5.3 Mass analyzer

Mass analyzers are categorized into 3 main groups, namely magnetic, quadrupole and time-of-flight mass analyzers. The quadrupole mass filter used in this study consists of four symmetrically aligned metal rods to which different combinations of dc and ac voltages are applied. The rods are diagonally paired and this arrangement creates an oscillating field along the axis of the rods that affects the flight path of ions entering the

quadrupole. At certain combinations of the applied voltages, ions of a specific m/z value maintain stable oscillations between the rods which allow them to reach the detector. All other ions undergo unstable oscillations and collide with the rod assembly [11]. A complete mass spectrum can be scanned by continuously varying the dc and ac voltages between the rods, while keeping their ratio constant (e.g. full scan mode). In contrast, selected ions are monitored by applying preset voltages to the rods, which allows more ions of a specific m/z value to pass through the quadrupole and to be detected (e.g. SIM) [12]. A schematic overview of the quadrupole mass spectrometer is given in Figure 4.4 [13].

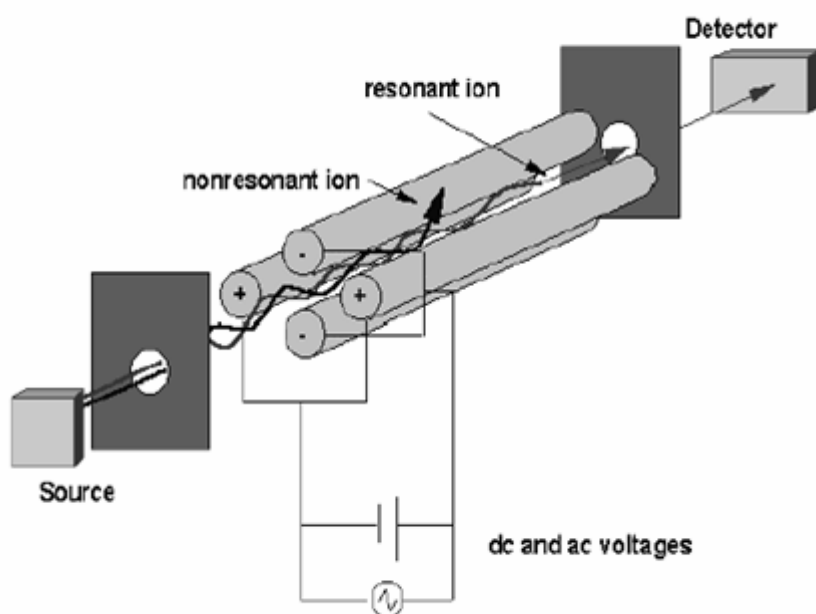


Figure 4.4 Schematic overview of the quadrupole mass spectrometer.

4.5.4 Detector

Electron multipliers are the most frequently used detector devices in mass spectrometers. Ions that pass through the quadrupole filter are accelerated and strike a conversion dynode which releases secondary electrons. These electrons are focused onto a second dynode, which again releases a greater number of electrons. Many stages are used in these instruments to produce a gain in the order of 10^6 . The final current is connected to a pre-amplifier that converts it to a digital signal [10, 13].

4.6 Ways to improve sample throughput during SBSE analysis

One factor that limits the application of GC/MS in clinical chemistry is the elaborate sample preparation steps required to analyze complex matrices. The new sorptive extraction methods (e.g. SBSE) are becoming increasingly popular because they limit the time required to prepare samples. A significant amount of analyst time can be saved by using an enrichment technique that combines the extraction, concentration and purification of a sample in a single step. However, in SBSE it is still not possible to automate the entire analytical procedure, therefore a number of techniques may be used to improve sample throughput. One way to increase the rate of analysis is to use an automated sample tray (Figure 4.5) which facilitates the on-line analysis of samples enriched by SBSE. The tray transfers each desorption tube to the TDU by mechanical means. In addition, other techniques are available that may improve sample throughput and these have been illustrated in more detail in the following section.



Figure 4.5 The TDS on-line rack

Sonication: A number of derivatization reactions were used in this study to prepare thermally stable, volatile derivatives for GC/MS analysis. A sonication step was often included in the derivatization procedure to increase the overall yield of the derivatives. A specially designed sample holder (Figure 4.6) was constructed by J Blom and coworkers at the Department of Mechanical Engineering (University of Stellenbosch). The holder is lowered into the sonicator bath, which allows up to 10 samples to be sonicated simultaneously.



Figure 4.6 Sample holder used during sonication

Stir bar conditioning: All stir bars were thermally conditioned prior to being used for SBSE. The stir bars were placed in a modified glass desorption tube and heated at 280°C for 15 minutes under a constant flow of carrier gas (i.e. 50 ml min⁻¹). A schematic drawing of the conditioning tube is shown in Figure 4.7-A. Instead of placing one stir bar in the tube, four stir bars were routinely conditioned during a single run (Figure 4.7-B).



Figure 4.7-A Schematic drawing of a glass conditioning tube.



Figure 4.7-B Experimental set up for the conditioning of the stir bars.

Magnetic stirring: Magnetic stirring in SBSE is required to enhance the partitioning of solutes between the aqueous sample phase and the stir bar coating. The magnetic stirrer shown in Figure 4.8-A and -B was constructed by J Blom and coworkers (Department of Mechanical Engineering, University of Stellenbosch). The stirrer essentially consists of a base plate that is combined with a convection oven. The base plate (Figure 4.8-A) houses an electrical motor, a digital display device, and 10 magnets that are connected and rotated by means of a chain system. The sample vials are held in place by a stainless steel plate holder. Prior to extracting the samples, the oven (Figure 4.8-B) is placed on top of the base plate and heated to temperatures ranging between 40°C and 100°C. The oven provides precise temperature control (e.g. up to 0.2°C).

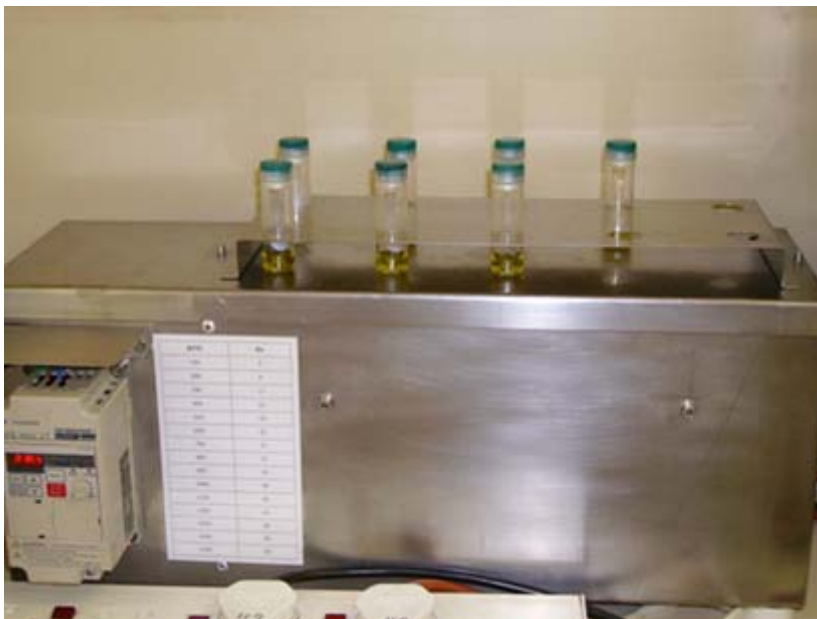


Figure 4.8-A Magnetic stirrer used during SBSE (without the oven).



Figure 4.8-B Magnetic stirrer with the oven placed on top of the base plate.

References

- [1] C.F Poole, S.K. Poole, *Chromatography today*, Elsevier, 1991.
- [2] W. Vogt, K. Jacob, H.W. Obwexer, *J. Chromatogr.* 174 (1979) 437.
- [3] W. Vogt, K. Jacob, A.-B. Ohnesorge, H.W. Obwexer, *J. Chromatogr.* 186 (1979) 179.
- [4] J.G.J. Mol, *Trace analysis with gas chromatography using on-line enrichment and large volume injection*, Technische Universiteit Eindhoven, 1995.
- [5] Gerstel GmbH, Products, Gerstel website, 2007.
- [6] A. Hoffmann, J. Kames, J. Steinhanses, *Application note 3/1996*, Gerstel GmbH, Germany, 1996.
- [7] J.R. Chapman, *Practical organic mass spectrometry – A guide for chemical and biochemical analysis*, Wiley & Sons Ltd., West Sussex, England, 1995.
- [8] D. Harvey, *Modern analytical chemistry*, McGraw-Hill Companies Inc., New York, 2000.
- [9] C.J. Park, J.R. Ahn, *Rev. Sci. Instrum.* 76 (2005) 044101.
- [10] J. Roboz, *Adv. Clin. Chem.* 17 (1975) 109.
- [11] P. Sandra, *Environmental analysis*, University of Stellenbosch, 1999.
- [12] I. Björkhem, *CRC Crit. Rev. Clin. Lab. Sci.* 11 (1979) 53.
- [13] R.E. Hill, D.T. Whelan, *Clin Chim. Acta* 139 (1984) 231.

Chapter 5

Drug-profiling by SBSE in biological fluids^V

Abstract

A comprehensive approach of capillary GC/MS data handling and mapping of specific target analytes is presented and illustrated with the detection of drugs of abuse in biological fluids. The word “comprehensive” is used here in *sensu stricto* i.e. that includes everything one wants to detect. Enrichment of the target solutes is performed by stir bar sorptive extraction (SBSE) followed by thermal desorption-capillary gas chromatography/mass spectrometry (TD-CGC/MS) analysis. The high sensitivity that can be reached with the SBSE-TD-CGC/MS technique allows the use of the MS in the scan mode. The GC/MS data are plotted in a contour plot with locked retention times in the x-axis and the ion traces in the y-axis. Target solutes are detected as a spot at specific positions in the plot and the color of the spots is related to the peak abundances. Semi-quantitative information can readily be obtained from the contour plots while precise quantitation needs the conventional calibration procedures. The graphical representation of CGC/MS data provides an easy way to elucidate samples positive on drugs of abuse by non-skilled personnel in forensic and medical laboratories.

Key words:

Drugs of abuse, biological fluids, stir bar sorptive extraction, thermal desorption, capillary gas chromatography-mass spectrometry, comprehensive profiling.

5.1 Introduction

The increasing availability of drugs of abuse [1] results in a growing demand for rapid and ‘universal’ screening methods for their determination in biological fluids in combination with fast and easily accessible data interpretation procedures. Immunoassay tests suffer from a limited range of substances detectable at low concentrations [2]. In recent years, several groups made efforts to develop chromatographic methods that cover a wide range of target drug solutes [3-6]. Liquid chromatography (LC) especially in combination with atmospheric pressure ionization mass spectrometry (API-MS) [7, 8] became increasingly important in clinical and forensic sciences. Still, capillary gas chromatography (CGC) is the method of choice [9], preferentially in combination with mass selective detection.

Despite the development of analytical methodologies, interpretation of the data is mostly performed manually which is a time-consuming process, especially when a large amount of compounds has to be screened. Data interpretation can be automated using chemometric interpretation or principal component analysis (PCA) of LC, GC and GC/MS data [10]. However, these tools are less useful when specific target drug compounds and their metabolites have to be elucidated in the complex matrix of biological fluids. For multi-target analysis by CGC/MS important improvements have been made in recent years. Through the features of electronic pneumatic control (EPC), retention time locked libraries (RTL) can be constructed and by linking the locked retention times to the mass spectral data obtained in the scan mode, hardly any target that is in the library can escape detection and elucidation [11]. An RTL-MS method has recently been described to monitor GC-amenable pesticides in different matrices [12].

In this contribution, a “comprehensive profiling” method is described for the interpretation of CGC/MS data. The method is based on graphical presentation of the locked retention times and mass spectral data in a three-dimensional plot. The method is generally applicable and will be illustrated by the multi-component screening of drugs of abuse in biological fluids. Enrichment of the target analytes from the biological fluids is performed by stir bar sorptive extraction (SBSE) [13]. The features of SBSE for drug analysis have recently been described [14, 15].

5.2 Experimental

5.2.1 Sample Preparation

The sample preparation procedures using SBSE for different biological fluids are detailed in ref. 14 and 15. An overview is presented in Figure 5.1. Five mL urine sample is transferred into a 20 mL headspace vial and 1 mL ammonium acetate buffer (1M, pH 6.5) is added. Ten μL β -glucuronidase of *Escherichia coli K12* (Roche Molecular Biochemical, Mannheim, Germany) is added and the mixture is thermostated at 37°C during 90 min for enzymatic hydrolysis. A Twister™ (Gerstel GmbH, Müllheim a/d Ruhr, Germany) of 1 cm x 0.5 mm d_f (25 μL) PDMS is stirred in the sample for 60 min at 1000 rpm. After sampling, the stir bar is taken out of the vial with tweezers, rinsed with a few mL pure water and dipped on clean paper tissue to remove residual water droplets. The Twister is placed in an empty glass thermal desorption tube of 187 mm L, 6 mm O.D. and 4 mm I.D. for thermal desorption.

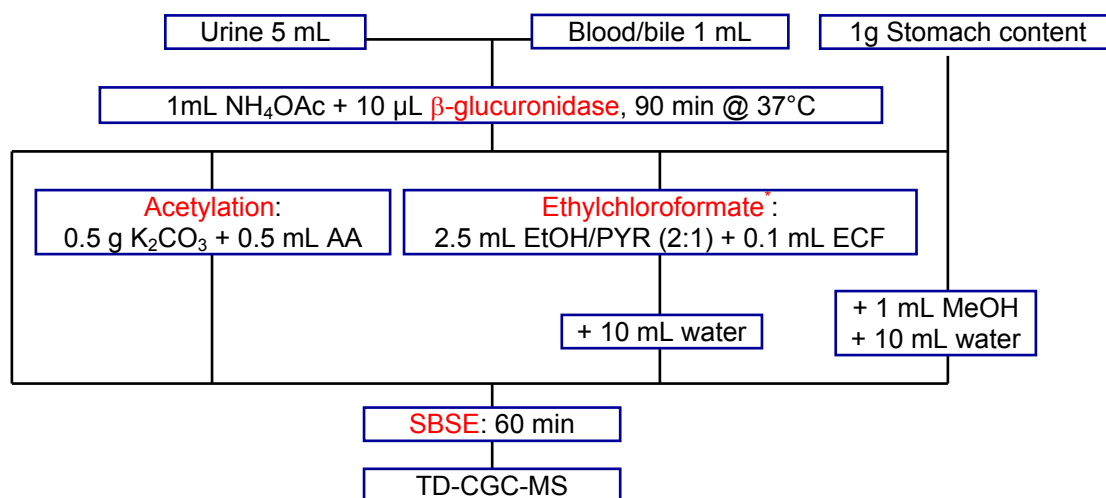


Figure 5.1 SBSE sample preparation procedure for biological fluids. (*) Other alkylchloroformates may be used. Abbreviations: AA, acetic acid anhydride; ECF, ethylchloroformate.

In situ acylation of urine samples is performed as follows. After hydrolysis with β -glucuronidase, 0.75 g potassium carbonate (Sigma-Aldrich, Bornem, Belgium) and 0.5 mL acetic acid anhydride (Sigma-Aldrich) are added. SBSE sampling followed immediately as described above. One mL blood or bile fluid sample is placed into a 20 mL headspace vial and 1 mL of ammonium acetate buffer (1M, pH 6.5) is added. Ten μ L β -glucuronidase of *Escherichia coli* K12 is added and the mixture is equilibrated at 37°C for 90 min. One mL of methanol is added and the mixture is placed in an ultrasonic bath for 15 min. The mixture is diluted with 10 mL of bi-distilled water and SBSE sampling is performed as for the urine samples. One g stomach content is mixed with 1 mL methanol and 10 mL water and SBSE is carried out as described above.

5.2.2 Instrumentation

Analyses were performed on a TDS-A thermodesorption unit (Gerstel) mounted on a 6890 Agilent GC (Agilent Technologies, Little Falls, DE, USA). The thermally desorbed analytes were cryo-focused in a programmed temperature vaporization injector (PTV, CIS-4, Gerstel) at -100 °C with liquid nitrogen prior to injection. An empty baffled liner was used. The TDS was operated in the splitless mode and ramped from 30 °C to 300 °C at a rate of 60 °C.min⁻¹ and the upper temperature was held for 10 min. After splitless thermal desorption, splitless injection (2 min) was performed by ramping the PTV injector from -100 to 300 °C at a rate of 600 °C.min⁻¹. Capillary GC analyses were performed on a 30 m L x 0.25 mm I.D., 0.25 μ m d_f HP-5MS column (Agilent Technologies). The oven was programmed from 50 °C (1 min) to 320 °C at a rate of 10 °C.min⁻¹. Helium was used as carrier gas and the head pressure was calculated using the retention time locking (RTL) software [11]. Ethyl palmitate was used as RT locking standard (retention time of 17.68 min). The Agilent 5973 mass spectrometric detector was operated in the scan mode (*m/z* 50-500).

5.2.3 Comprehensive presentation of GC-MS data

For GC/MS data acquisition and analysis the Enhanced Chemstation G1701CA software, Version C.00.00 (Agilent Technologies) was used. Three-dimensional GC/MS

data were automatically exported into ‘comma separated values’ (csv) files using the ‘export 3D data’ option. The procedure was repeated twice exporting from m/z 50 to 300 firstly, followed by the export from m/z 301 to 500. This double procedure is necessary because of limited data space in the csv files. The exported files were opened in Microsoft[®] Excel[™] resulting in spreadsheets with the GC/MS data file header in the first four rows. The fifth row contained the m/z values and the scan numbers were in the first column. The remaining data were the abundances at every scan number and at all ion traces. The latter data were copied and pasted into a matrix in MicroCal[®] Origin[™] software (Originlab, Northhampton, MA, USA). The matrix coordinates were adjusted and a three-dimensional or contour plot was created.

5.3 Results and discussion

Stir bar sorptive extraction (SBSE) results in strong enrichment for solutes with octanol-water distribution coefficients higher than 100 from aqueous samples. A typical profile of the SBSE-TD-CGC/MS analysis of a urine sample of a drug addict is shown in Figure 5.2.

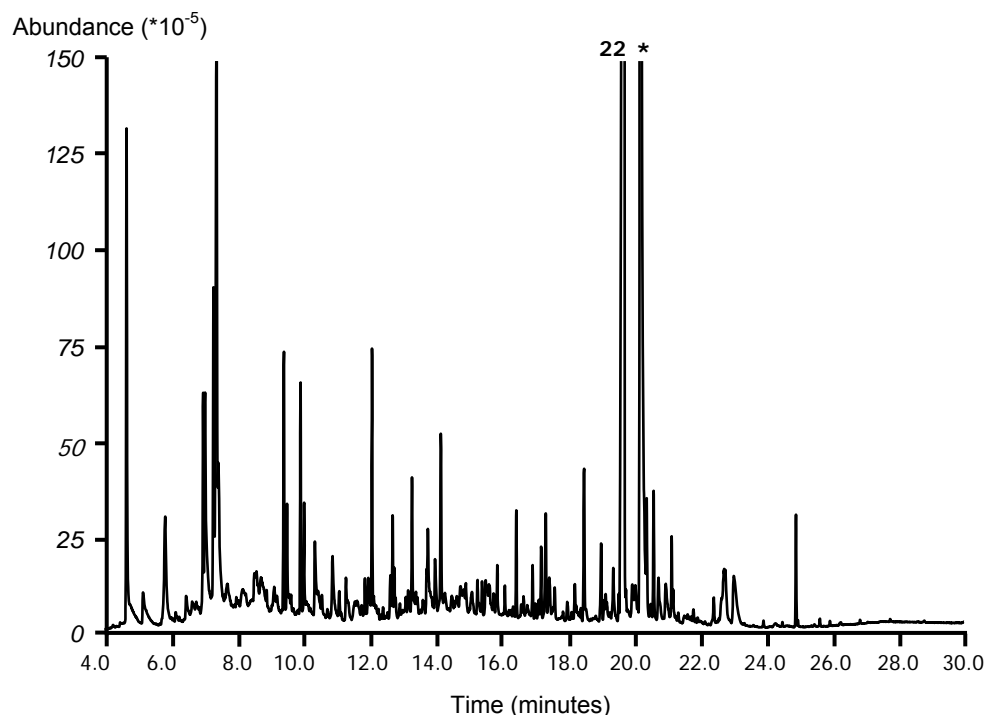


Figure 5.2 Total ion chromatogram of the SBSE-TD-CGC/MS analysis of 5 mL urine of a drug addict; peak 22: methadone; peak *: androst-16-en-3-ol.

The chromatogram represents a collection of more than 200 compounds illustrating the versatility and sensitivity of the technique. Most of the major peaks originate from food and/or biological processes [14]. In the profile the drug methadone (19.72 min), a potent analgesic and sedative widely used in the treatment of heroin addiction [16], could be readily identified through its mass spectrum. Methadone and its catabolic metabolites show very high affinity for PDMS ($\log K_{o/w} > 4.2$) and are quantitatively extracted with SBSE. Screening of other drugs or metabolites out of the complex matrix in Figure 5.2 is very time consuming. This can be drastically simplified using a retention time locked (RTL) CGC method in combination with the automatic analyte search software program 'RTL result screener' from Agilent Technologies [11]. The GC method was locked using ethyl palmitate (RTL 17.68 min) and a RTL library was created by the import of the retention times and mass spectral data of several groups of compounds: cocaine and metabolites, opiates, methadone and analogues, cannabinoids, amphetamines, benzodiazepines and barbiturates (Table 5.1).

Table 5.1 Locked retention times (t_R), target ions (Tion) and qualifier ions (Q1-Q3) of drugs introduced in the library. Other drugs can easily be incorporated in the library; Chromatographic conditions see text.

Number	Compound Name	t_R (min)	Tion	Q1	Q2	Q3
1	3-monoacetylmorphine	23.08	327	285	162	215
2	7-hydroxycannabidiol	21.78	312	244	284	187
3	alprazolam	26.67	279	308	204	273
4	amobarbital	14.93	156	141	157	142
5	beta-codeine	22.52	299	285	291	300
6	bromazepam	22.08	315	58	317	236
7	brotizolam	24.73	394	392	207	393
8	cannabichromene	17.67	231	174	232	246
9	cannabidiol	21.85	231	238	223	195
10	cannabielsoin	22.45	205	247	147	148
11	cannabielsoin isomer	22.62	205	247	147	148
12	cannabinol	23.23	295	296	238	310
13	clonazepam	25.92	280	314	315	288
14	cocaine	20.27	182	303	304	198
15	delorazepam	22.25	275	304	303	305
16	diazepam	22.53	283	256	284	285
17	ethylbenzoylecgonine (cocaethylene)	20.77	196	82	96	317
18	flunitrazepam	24.00	312	285	313	286
19	flurazepam	25.20	86	99	87	387
20	heroin	24.15	327	369	310	268
21	hexobarbital	16.68	221	157	155	79
22	methadone	19.72	72	73	57	91
23	methadone impurity	20.17	72	73	193	165
24	methadone metabolite I	18.55	277	276	262	220
25	methadone metabolite (1,4-diphenyl-6-methyl-1,4-dihydropyrimidine-5-carbonitrile)	19.82	273	196	197	230
26	methylenedioxyamphetamine (MDMA,XTC)	12.82	177	58	186	-
27	N-1-methylclonazepam, IS	25.25	329	328	302	294
28	N-acetyl-p-methoxyamphetamine	15.38	121	148	105	86
29	nordazepam	23.13	242	241	270	269
30	papaverine	25.62	338	324	339	308
31	pentobarbital	15.23	156	141	157	155
32	secobarbital	15.80	168	167	195	97
33	temazepam	23.83	271	273	272	300
34	triazolam	27.42	313	315	342	238

The library can easily be extended if there is a need to. During the screening procedure, the GC/MS data are automatically monitored to elucidate similarities in retention and mass spectral information between the library and the detected compounds. As an example, Figure 5.3 shows a positive elucidation of the result screener in the profile of Figure 5.2 confirming the presence of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, a metabolite of methadone.

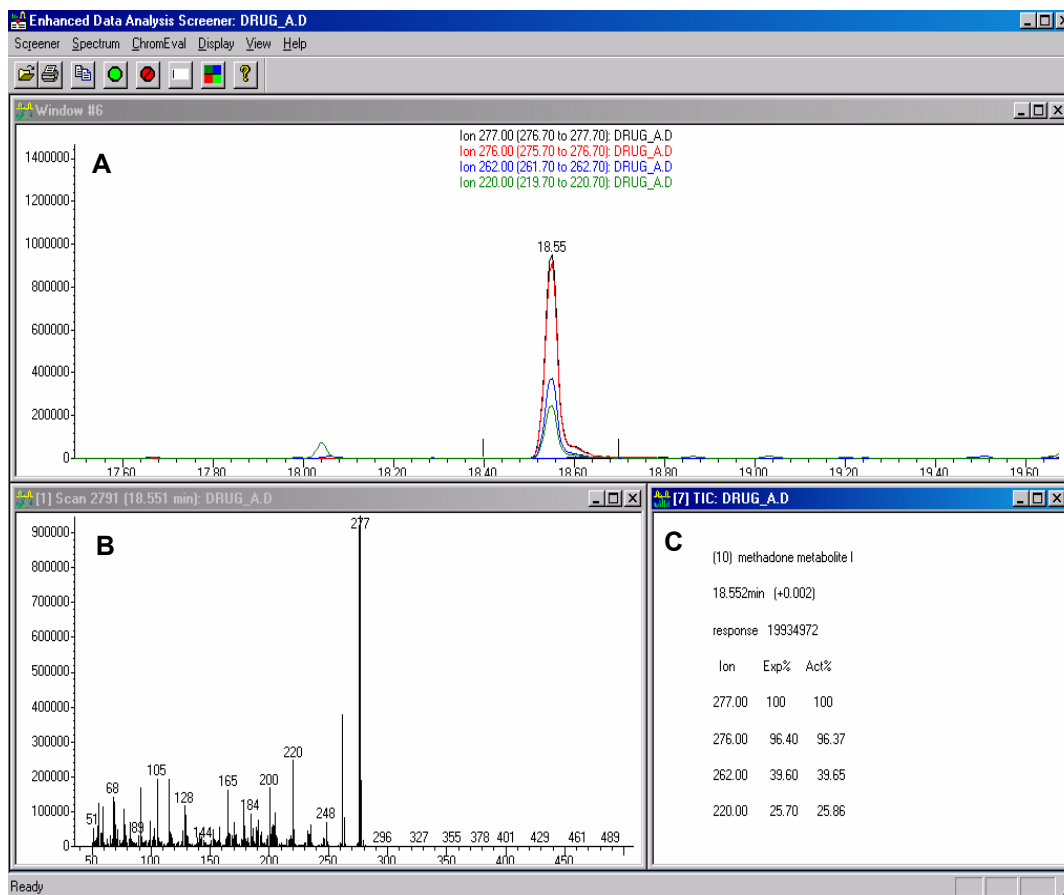


Figure 5.3 Result screener windows for the detection of methadone metabolite I. Window A: Extracted ion chromatograms at m/z 277 (Tion), 276 (Q1), 262 (Q2) and 220 (Q3); window B: mass spectrum; window C: retention time data, peak response and expected (Exp%) and measured (Act%) ion ratios of the mass spectrum.

Based on all data of the result screener, specific ions of target solutes can be extracted to show the presence of those solutes in a biological fluid under investigation. The selective ion traces at m/z 72, 273 and 277 for methadone (peak 22), the primary metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (2-EDDP, peak 24), 1,4-diphenyl-6-methyl-1,4-dihydropyrimidine-5-carbonitrile (peak 25), and a methadone impurity (peak 23) are shown in Figure 5.4-A for the blood sample and in Figure 5.4-B for the urine sample of the same person.

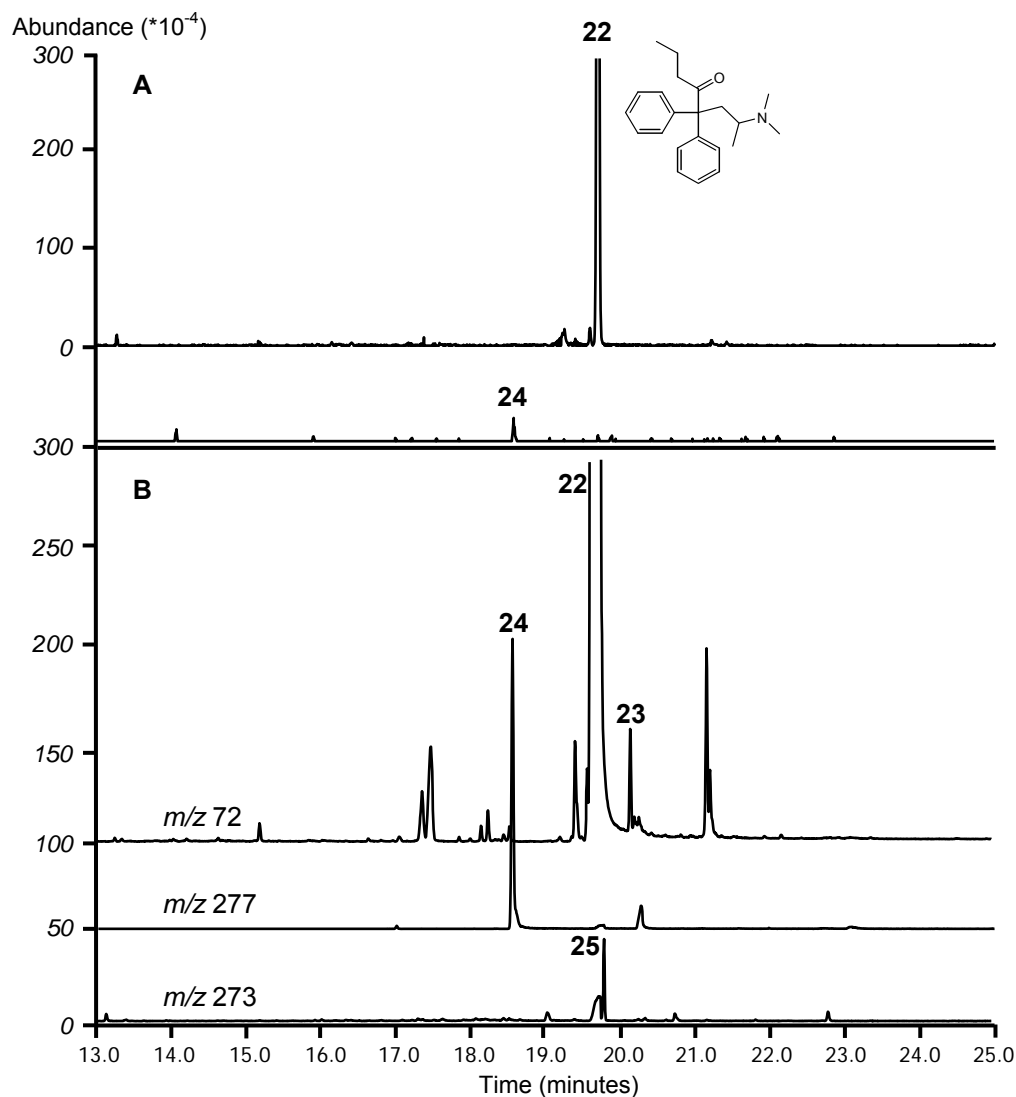


Figure 5.4 Extracted ion chromatograms at m/z 72, 273, 277 of the SBSE-TD-CGC/MS analysis of methadone (22), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (metabolite I, 24), 1,4-diphenyl-6-methyl-1,4-dihydropyrimidine-5-carbonitrile (25) and a methadone impurity (23), respectively in the blood (A) and urine (B) of a drug addict.

In the blood sample mainly methadone and a small fraction of 2-EDDP are detected while, as expected, more methadone related compounds are found in the urine sample. Like most drug compounds, a distinctive fraction of methadone is phase II metabolized to its glucuronic acid conjugate and should be liberated before analysis. All samples are therefore enzymatically hydrolysed by β -glucuronidase from *Escherichia coli* K12. Note that the enzyme does not interfere in the SBSE-TD-CGC/MS analysis since it is not absorbed into the PDMS coating.

Several samples were analyzed applying the same methodology and some representative examples are given. Figure 5.5-B shows the detection of cannabis related compounds and metabolites in the urine sample of a drug addict by extracted ion chromatography (EIC) selecting m/z 205, 231 and 295 for cannabichromene (peak 8), cannabidiol (peak 9), cannabielsoin (peak 10) and cannabinol (peak 12), respectively. All detected compounds possess high $\log K_{o/w}$ (>5) values and consequently show high affinity for the PDMS coated stir bar. Theoretical recoveries can be calculated from the solute specific $\log K_{o/w}$ values using the Twister Calculator software package (Gerstel), that is based on the software program KowWIN [17] and are all higher than 99 %. The versatility of SBSE-TD-CGC/MS is demonstrated by detection of two of the cannabinoids in the bile fluid of the same person (Figure 5.5-A). Next to cannabidiol, which is one of the primary substances of the marijuana plant, cannabichromene is detected. Turbid biological fluids like bile fluid or blood samples are mixed with methanol and placed in an ultrasonic bath for 15 min before SBSE. This causes lyses of the cells and better release of the drug compounds from the matrix. The mixture is then diluted with 10 mL water to increase the extraction efficiencies of SBSE.

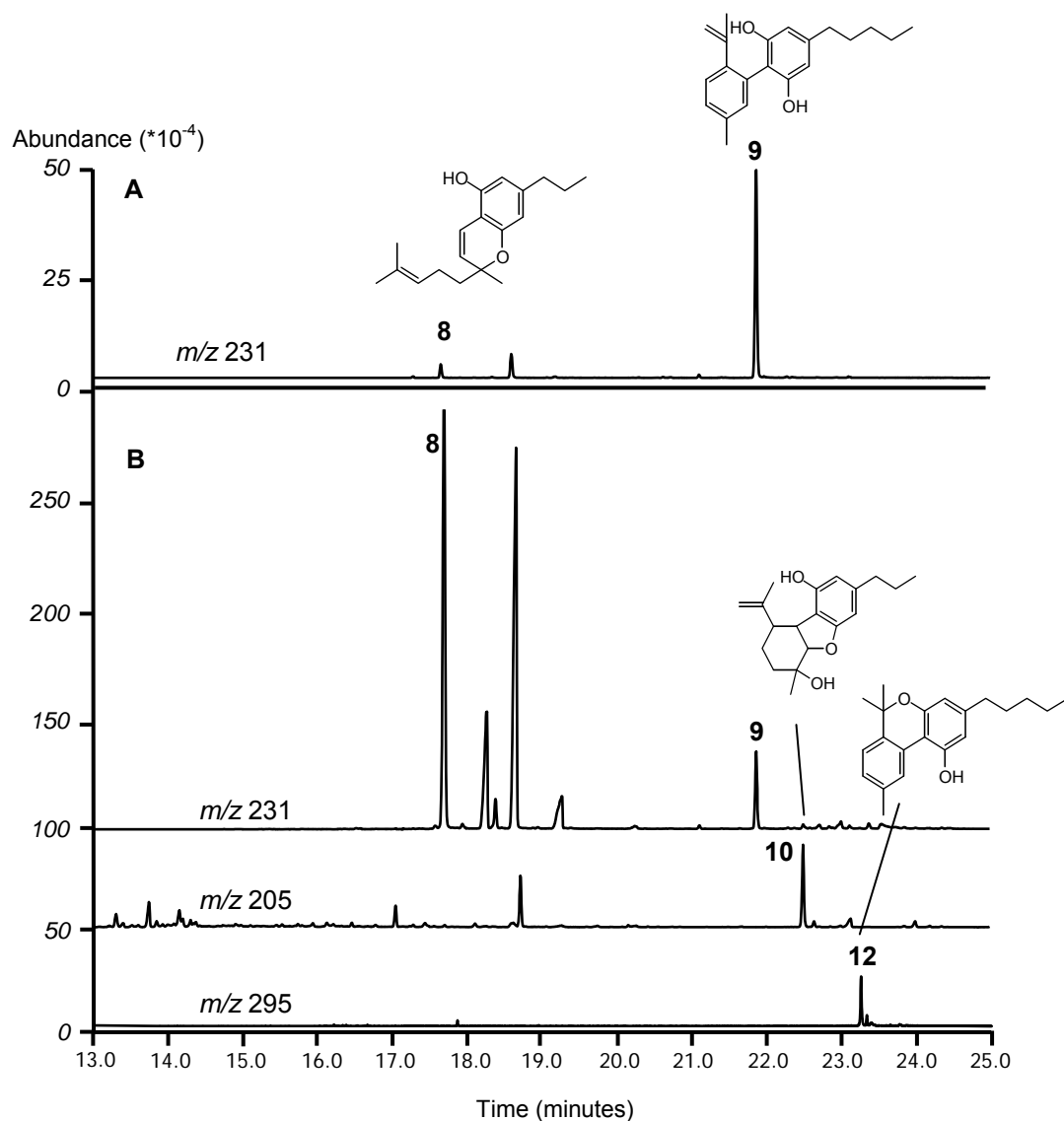


Figure 5.5 Extracted ion chromatograms at m/z 205, 231, 295 of the SBSE-TD-CGC/MS analysis of cannabichromene (8), cannabidiol (9), cannabielsoin (10) and cannabinol (12), respectively in the bile (A) and urine (B) of a cannabis user.

Also polar compounds showing less affinity for the PDMS phase can be properly enriched. Cocaine (benzoylecgonine methyl ester, $\log K_{o/w} = 2.2$) is enriched for ca. 40 % from an aqueous sample of 5 mL while the ethylbenzoylecgonine analogue (cocaethylene, $\log K_{o/w} = 2.7$) is recovered for ca. 65%. The latter compound is formed in the human body when cocaine is administered in combination with alcoholic (ethanol) drinks [18, 19]. Both compounds could easily and automatically be detected in the urine of a cocaine addict (Figure 5.6).

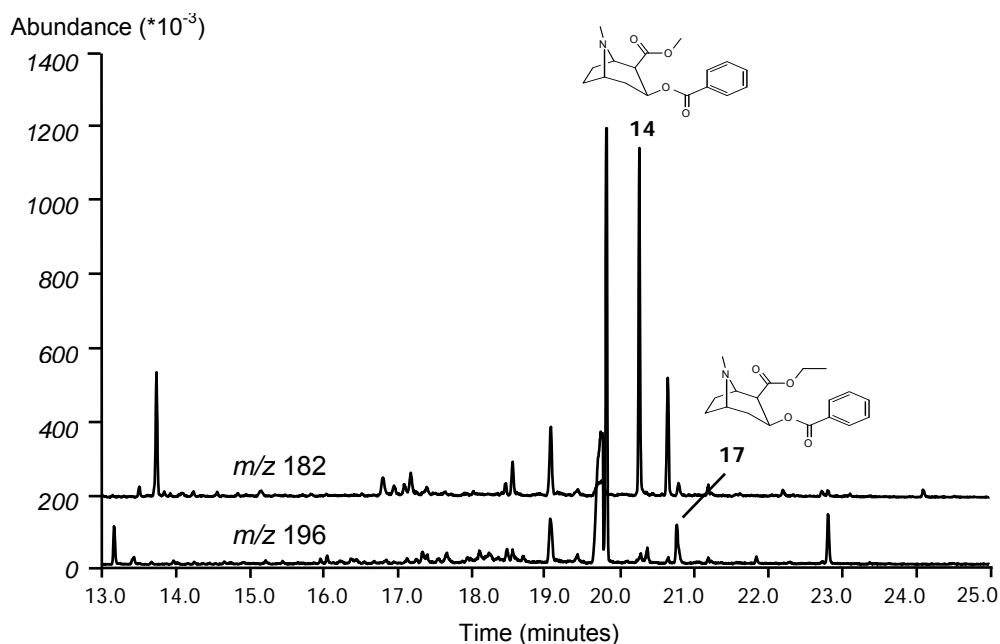


Figure 5.6 Extracted ion chromatograms at m/z 182 and 196 of the SBSE-TD-CGC/MS analysis of cocaine (14) and ethylbenzoylecgonine (cocaethylene, 17) in a urine sample.

Another example concerns the determination of methylenedioxymetamphetamine (MDMA, ecstasy) in the urine sample of a recreational drug user. This relative polar compound ($\log K_{o/w} = 2.3$) was traced in EIC at m/z 177 (Figure 5.7).

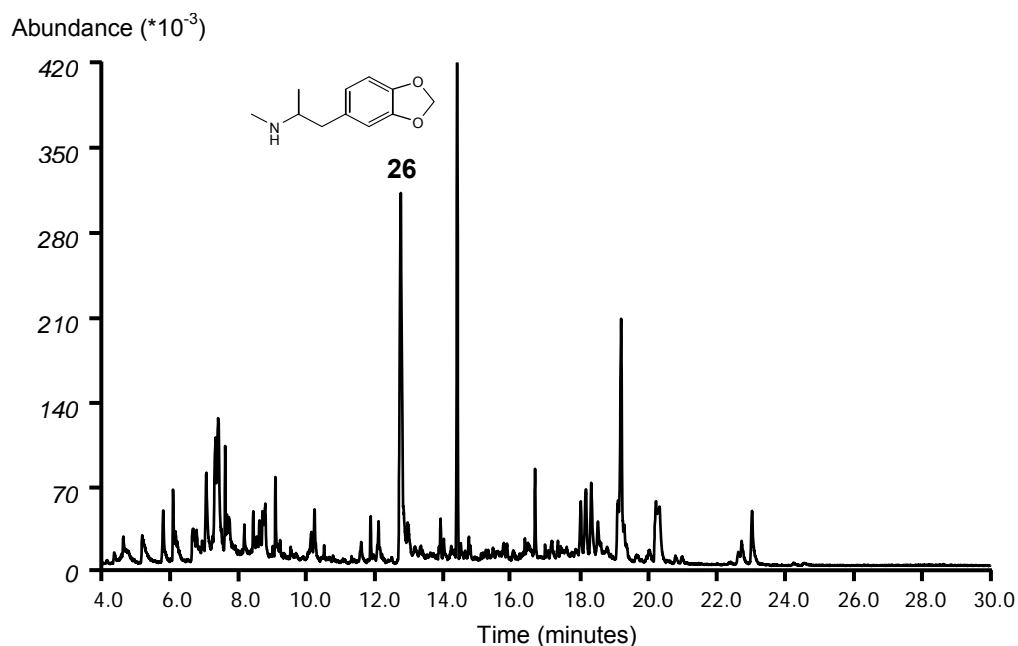


Figure 5.7 Extracted ion chromatogram at m/z 177 of the SBSE-TD-CGC/MS analysis of methylenedioxymetamphetamine (MDMA, ecstasy) in the urine of a recreational drug user.

SBSE in combination with TD-CGC/MS has shown its good performance in terms of quantitation for biological markers [14], pharmaceutical drug compounds [15], pollutants like PCBs [20] and PAH metabolites [21] in biological fluids. Precise and accurate quantification using SBSE-TD-CGC/MS is illustrated with the analysis of benzodiazepines and p-methoxyamphetamine (PMA). Semi-quantitation which often is sufficient for diagnostic purposes will be discussed in the comprehensive profiling.

Benzodiazepines are prescribed in treatment of stress, anxiety, sleep disorders and seizures but are often abused by drug addicts [22]. This group of drugs is therefore frequently present in forensic cases. Diazepam, flunitrazepam and flurazepam were spiked from methanol standard solutions (10 μL) into blank urine samples (5 mL) to individual concentrations between 5 and 500 $\mu\text{g.L}^{-1}$. N-methylclonazepam was added as internal standard (IS) to every sample at a constant level of 50 $\mu\text{g.L}^{-1}$. Similar to the real samples, 1 mL of ammonium acetate buffer solution (pH 6.5) was added and incubation with β -glucuronidase was performed. Figure 5.8-A shows the EIC at m/z 86, 283, 312, and 329 of a blank urine sample spiked to individual concentrations of 50 $\mu\text{g.L}^{-1}$. Quantitation was performed at these selected ion traces. Because of the relative differences in SBSE recoveries and the MS ionization process, equal concentrations of the solutes result in diverse abundances. Table 5.2 shows the $\log K_{o/w}$, the calculated theoretical recoveries and the relative response factors (RRF) for several benzodiazepines. The last values are calculated as the ratios of the peak areas of the analyte over the IS at equal concentrations. Linear regression was performed on the relative peak areas (A_X/A_{IS}) versus the spiked concentrations (Figure 5.8-B) and correlation coefficients all exceeded 0.99, independent of the analyte recoveries. In a urine sample of a drug user diazepam was detected in a concentration of 21 $\mu\text{g.L}^{-1}$. Nordazepam and temazepam, which are diazepam metabolites, were also elucidated in concentrations of 61 and 17 $\mu\text{g.L}^{-1}$, respectively.

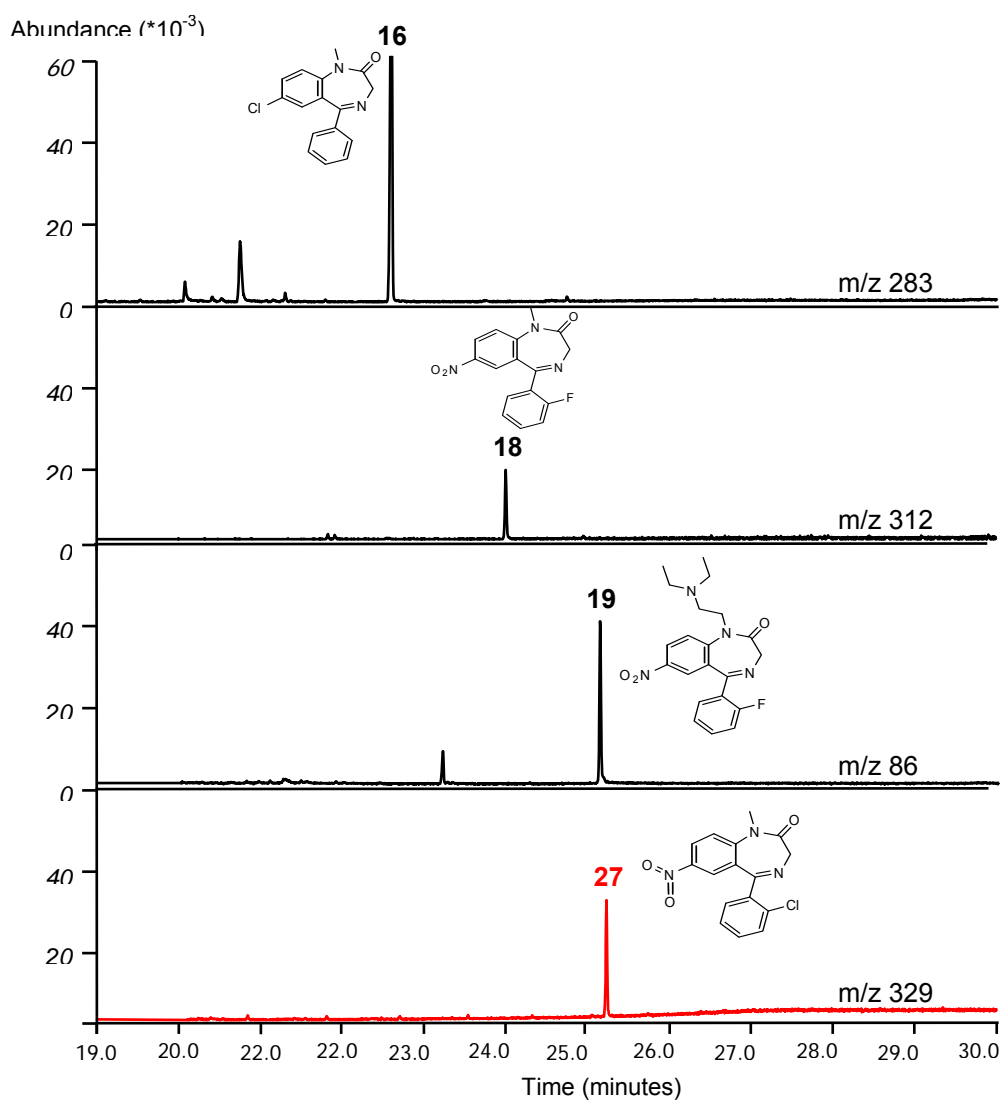


Figure 5.8-A Extracted ion chromatograms at m/z 86, 283, 312, 329 of the SBSE-TD-CGC/MS analysis of diazepam (16), flunitrazepam (18), flurazepam (19) and N-methylclonazepam (IS, 27) spiked into a blank urine sample (5 mL) at a concentration level of $50 \mu\text{g}\cdot\text{L}^{-1}$.

Table 5.2 Log $K_{o/w}$ values, theoretical SBSE recoveries (Twister™ with 24 μL PDMS, 5 mL sample) and relative response factors (RRF) with N-methylclonazepam as I.S.

Compound	Log $K_{o/w}$	SBSE recovery (%)	RRF
Diazepam	2.7	71	2.4
Flunitrazepam	1.9	28	0.7
Flurazepam	3.0	83	1.7
Nordazepam	2.9	80	2.7 ^(*)
Temazepam	2.2	43	1.4 ^(*)
N-methylclonazepam (IS)	2.4	52	-

^(*) Estimated from diazepam

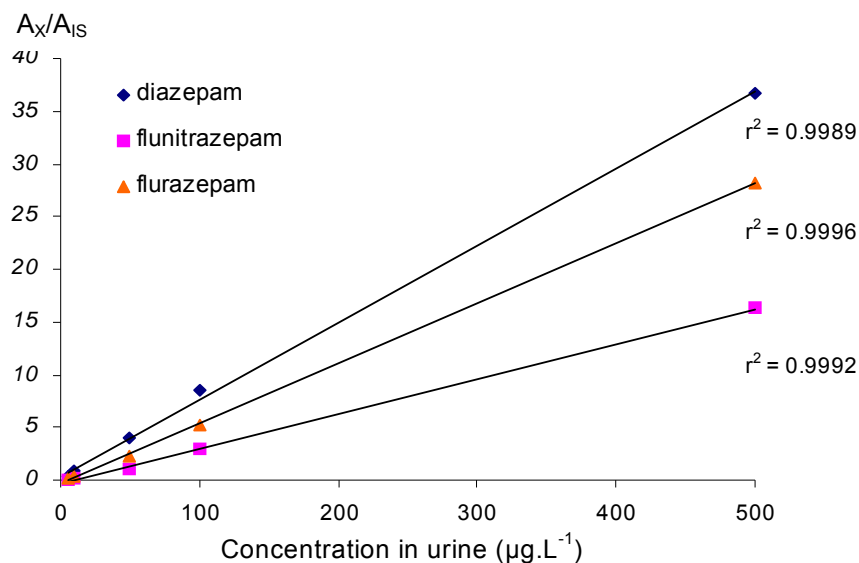


Figure 5.8-B Individual calibrations curves between concentrations of 5 and 500 $\mu\text{g.L}^{-1}$. For calibration, the internal standard was spiked to 50 $\mu\text{g.L}^{-1}$ in all cases.

p-Methoxyamphetamine (PMA) is a relative new amphetamine-like ‘designer’ drug that is often sold as ecstasy or MDMA [23]. Since hallucinating effects are retarded in comparison with ecstasy and the compound is more toxic, intake of several tablets has led to several lethal intoxications. For CGC/MS analysis, the amphetamine is preferentially reacted with an anhydride to obtain the N-acetyl derivative [24, 25], which shows improved chromatographic behavior on apolar capillary columns. In the case of SBSE enrichment, acylation also drastically increases the enrichment. Blank urine samples (5 mL) were spiked to concentration levels between 5 and 500 $\mu\text{g.L}^{-1}$, enzymatically hydrolyzed and 0.75 g potassium carbonate was added. After addition of 0.5 mL acetic anhydride, the mixture was immediately stirred with a Twister™. N-acetyl-p-methoxyamphetamine was recovered for ca. 32% and could be selectively extracted at m/z 121. Figure 5.9-A shows a urine sample spiked to a concentration of 50 $\mu\text{g.L}^{-1}$. Linear regression of the N-acetyl PMA peak area versus the spiked PMA concentrations revealed good linearity (correlation coefficient > 0.99) of the in-situ derivatisation-SBSE-TD-CGC/MS procedure (Figure 5.9-B).

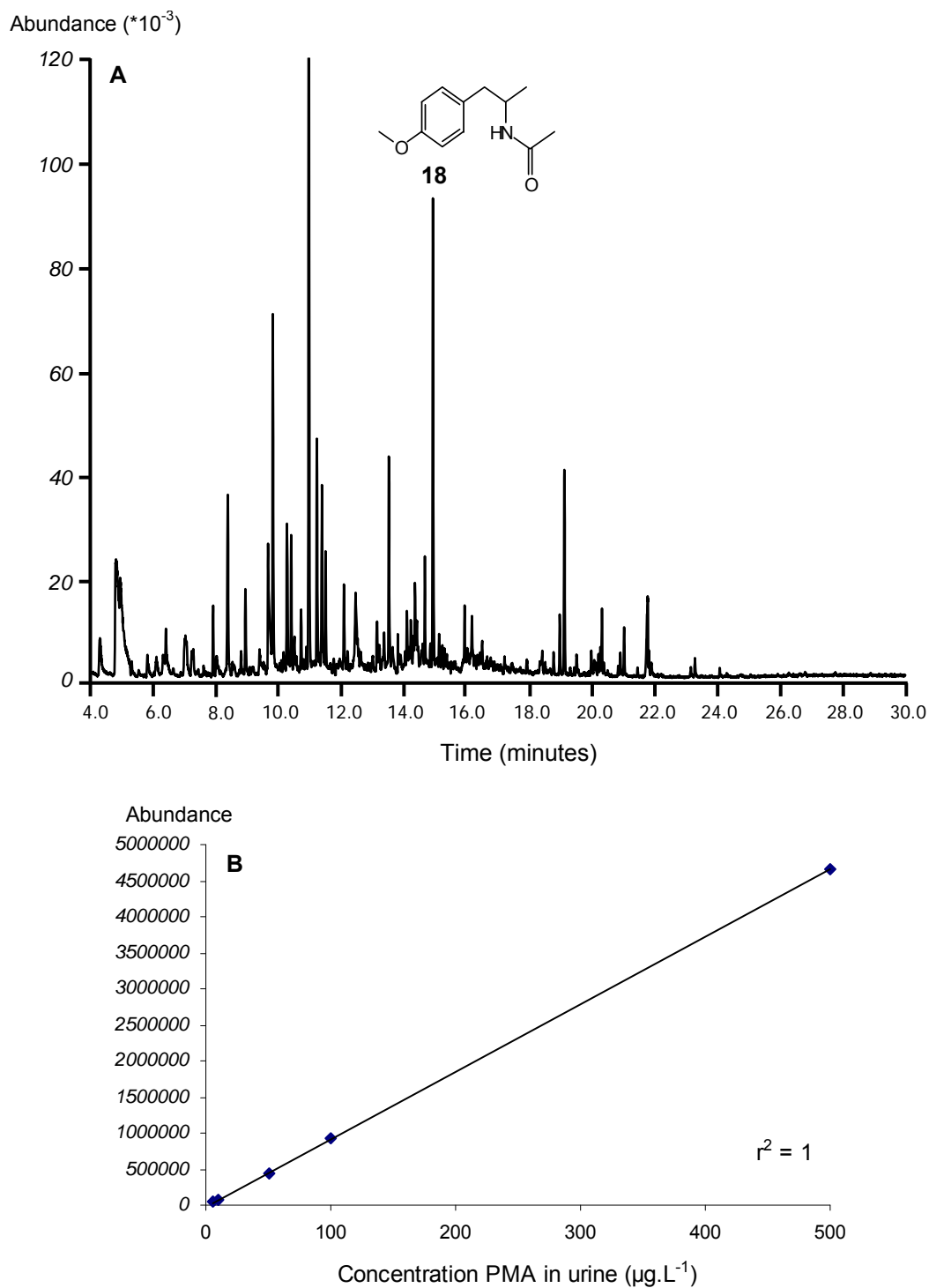


Figure 5.9 Extracted ion chromatogram at m/z 121 (A) for N-acetyl-p-methoxyamphetamine (N-acetyl PMA) of the *in situ*-derivatisation-SBSE-TD-CGC/MS analysis of PMA spiked into a blank urine sample (5 mL) at a concentration level of $50 \mu\text{g}\cdot\text{L}^{-1}$; calibration curve of PMA (B) spiked in urine between concentrations of 5 and $500 \mu\text{g}\cdot\text{L}^{-1}$.

In medical and forensic laboratories, the main question is “what kind of drugs are present in a given biological sample and in what order of magnitude”. The faster the data are provided in a simple format, the faster actions can be taken. From an analytical point of view, this means a fast multi-residue method with semi-quantitative features and presentation of data in a simple format. An alternative to the RTL result screener method that still is to be interpreted by specialized personnel is comprehensive mapping of GC/MS data.

Comprehensive profiling implies the graphical presentation of all acquired or pre-selected m/z traces in a three-dimensional (3D) plot. This is performed by exporting the scan data into a graphical interface like the MicroCal[®] Origin[™] software. Figure 5.10-A shows a simplified plot of a SBSE-TD-CGC/MS analysis of a urine profile of a drug addict. Presenting all extracted ion chromatograms in a 3D plot results in an extremely complex figure and for reasons of clarity, only a selected amount of ion traces are presented. From this graph, a contour plot is created at a pre-selected specific cut-off abundance. This gives a graph in which the x-axis represents the retention times, the y-axis the mass spectral data (m/z) and the color intensity reflects the quantity. The latter strongly depends on the selected cut-off. Figure 5.10-B and 5.10-C show the total ion contour plot (all extracted ion chromatograms are presented) of the SBSE-TD-CGC/MS analysis of the urine profile shown in Figure 5.2 at two different cut-offs. In both cases, the presence of a compound can be verified locating a spot at its specific elution time (e.g. methadone at 19.72 min) and at one (e.g. methadone at m/z 72) or more of its solute specific m/z values. The analyses are performed under retention time locked conditions allowing direct comparison of contour plots originating from different samples.

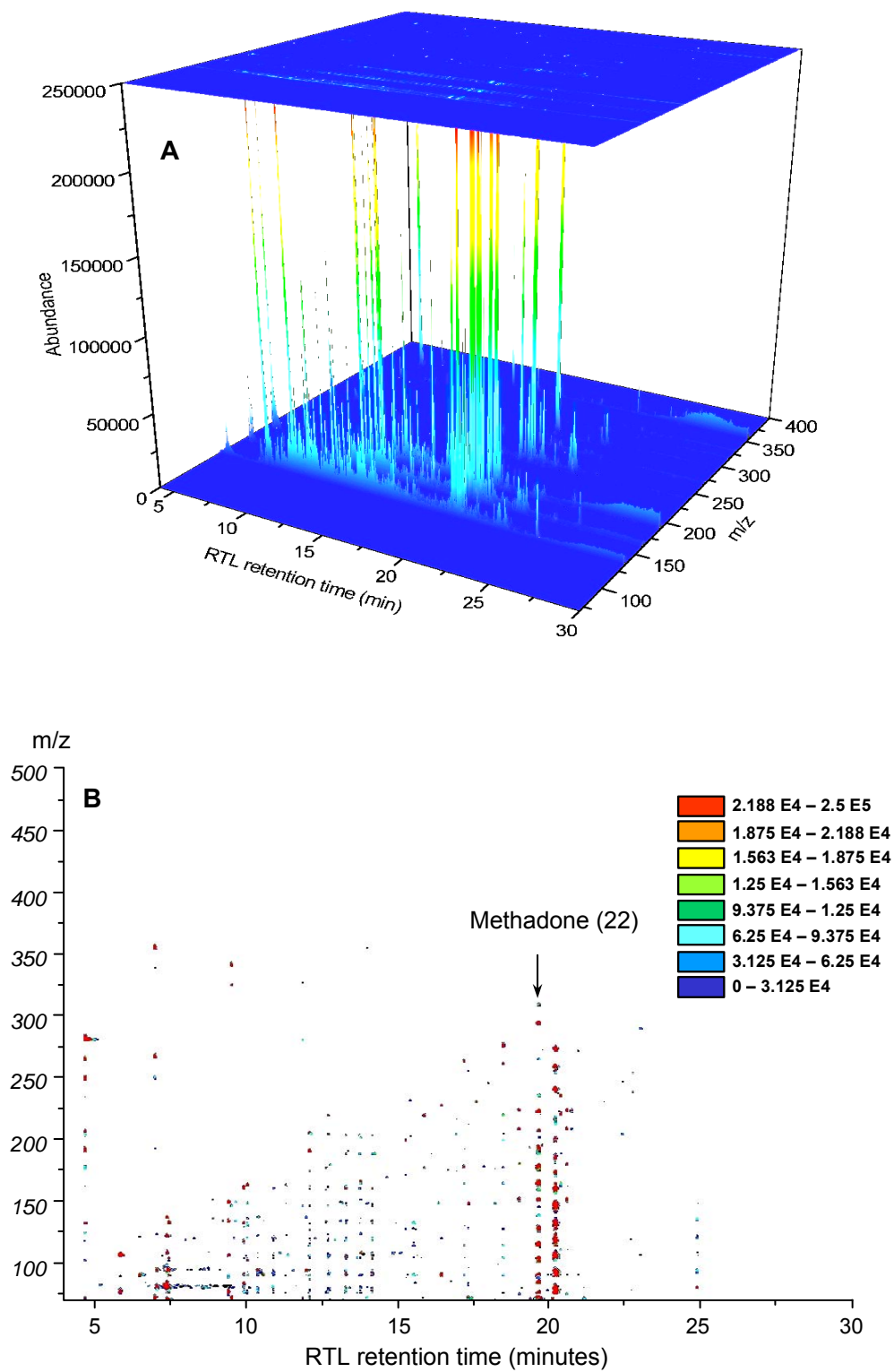


Figure 5.10-A and -B 3D-CGC/MS (A) and a CGC/MS contour plot at a cut-off of 2.5×10^5 (B) of the urine of a drug addict. For experimental and graphical conditions see text.

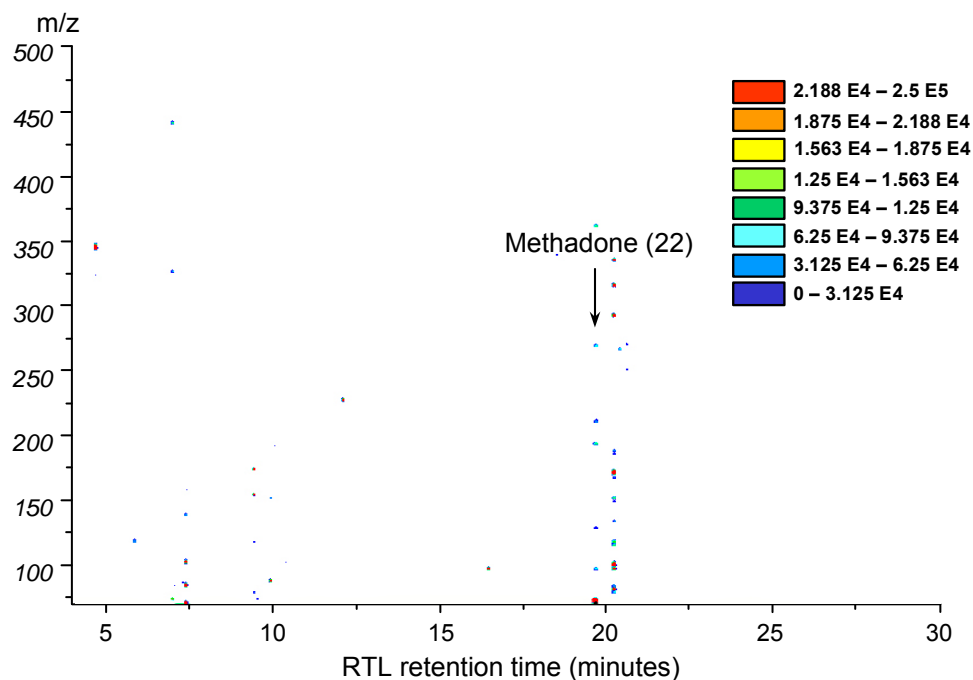


Figure 5.10-C CGC/MS contour plot at a cut-off of 2.5E6 of the urine of a drug addict. Experimental and graphical conditions see text.

For the compounds in the home-made RTL drug library (Table 5.1) and selecting the Tion, the contour plot shown in Figure 5.11 is obtained.

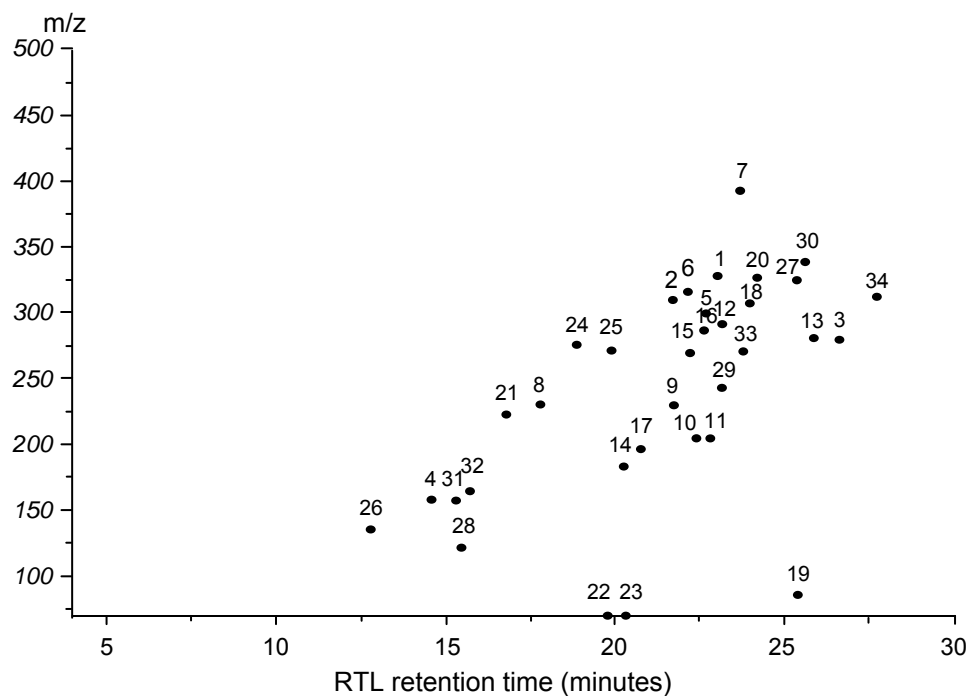


Figure 5.11 Expected spots in the CGC/MS contour plot of drugs analysed by SBSE-RTL-CGC/MS. The numbered spots refer to the compounds listed in Table 5.1.

This target compound contour plot is drastically simplified compared to the plot in Figure 5.10-B and 5.10-C. Fine-tuning can thus be done by restricting the data import into the graphical interface through selection of target compound specific ion(s) and through focusing on specific retention time windows. Moreover, the height at which the contour plot is taken can be varied hereby choosing the amount of details one wants to obtain. This is illustrated by the selective CGC/MS contour plots at a retention window between 17 and 30 min and an ion window between m/z 180 and 400 of a urine (Figure 5.12-A), blood (Figure 5.12-B) and stomach content sample (Figure 5.12-C) of the same drug addict. The main ion traces of cannabinoids, the methadone group and the benzodiazepines were selected. Cannabichromene (spot 8), 7-hydroxycannabidiol (spot 2), cannabidiol (spot 9), α -cannabielsoin (spot 10), an α -cannabielsoin analogue (spot 11), methadone metabolite I (spot 24), diazepam (spot 16) and temazepam (spot 33) are easily elucidated in the urine contour plot (Figure 5.12-A). The presence of these compounds was verified by checking the mass spectra at the given retention times in the RTL 'conventional' chromatogram. The presence of several of the spots was also confirmed in the blood sample (spots 2,8,9,10,16) (Figure 5.12-B). The series of spots at 27.3 min (spots a) indicate the presence of a large amount of cholesterol in the blood sample. α -Cannabielsoin (spot 10) and diazepam (spot 16) were also found in the stomach content (Figure 5.12-C) which is also characterized by high amounts of co-eluting free fatty acids like linoleic and oleic acids (spots b). Note that in the cases of the blood and stomach content samples, the contour plots were taken at lower cut-off abundances (abundance = $2E4$) than for the urine sample (abundance = $1E5$). This allowed detection of lower concentrations of the selected compounds.

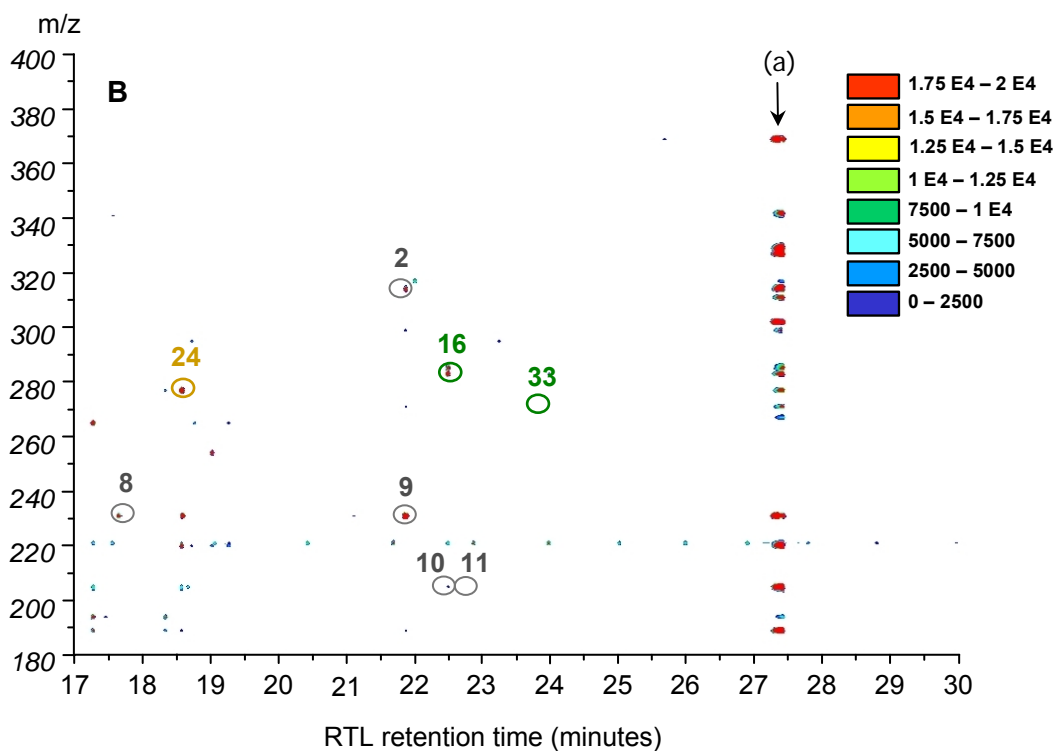
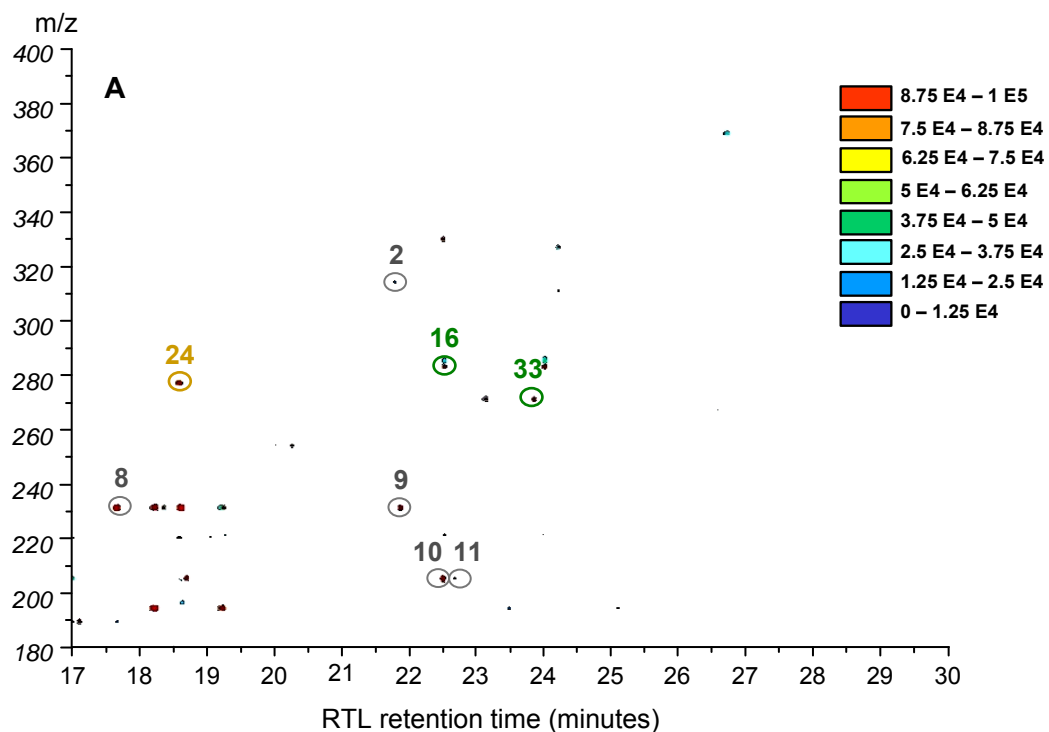


Figure 5.12-A and -B Ion selective contour plots of urine (A) and blood (B) of a drug addict; Compounds: cannabichromene (8), 7-hydroxycannabidiol (2), cannabidiol (9), α -cannabielsoin (10), α -cannabielsoin analogue (11), methadone metabolite I (24), diazepam (16), temazepam (33). The numbers refer to Table 5.1.

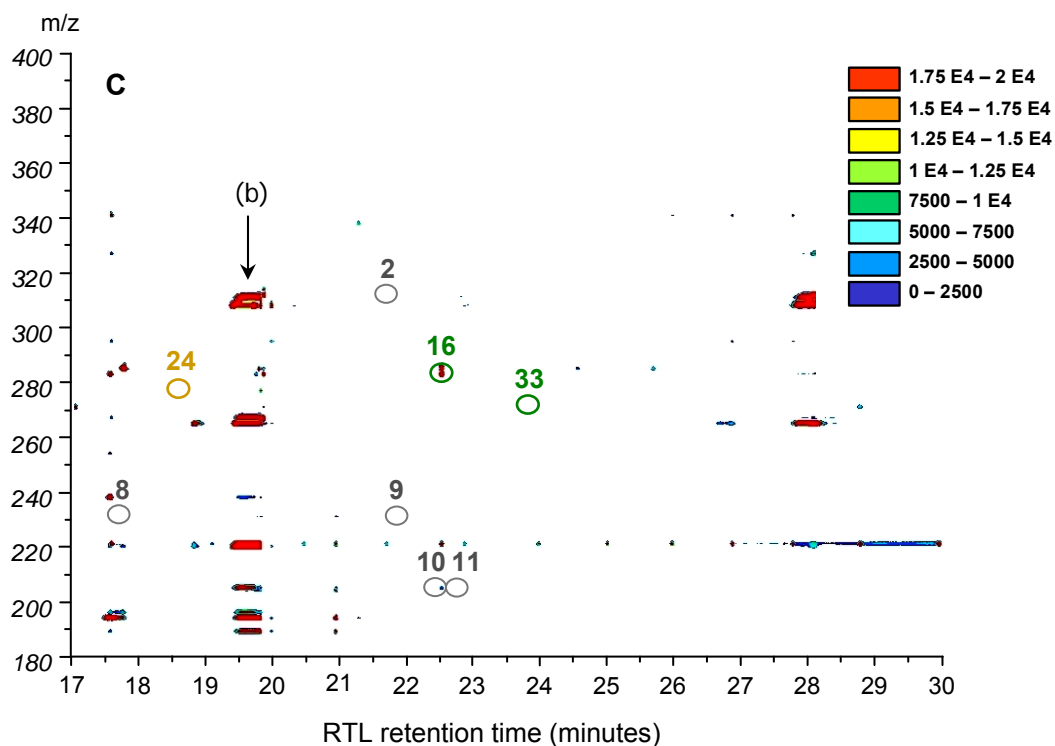


Figure 5.12-C Ion selective contour plot of bile fluid of a drug addict; Compounds: cannabichromene (8), 7-hydroxycannabidiol (2), cannabidiol (9), α -cannabielsoin (10), α -cannabielsoin analogue (11), methadone metabolite I (24), diazepam (16), temazepam (33). The numbers refer to Table 5.1

Apart from this example, in which only a selected amount of analytes is targeted, the procedure can easily be expanded to a wider variety of solutes. Moreover, if needed, comprehensive GC/MS profiling can be simplified by using the MS in the chemical ionization mode instead of electron impact ionization. Application of the soft chemical ionization technique resulting in no or less fragmentation, often provides simpler contour plots.

GC/MS contour maps can also be semi-quantitatively interpreted since the colors of the spots refer to specific peak height intervals and give a rough estimate of the solute concentration. The screening of street drugs in biological fluids in most cases involves detection of a specific compound around or above a pre-defined cut-off concentration. The SBSE-TD-GC/MS technique therefore suits well for this purpose. However, some critical aspects regarding quantitation should be discussed. Firstly, when calibrating MSD signals, the response factors of different compounds, even within the same classes of analytes,

should be encountered. For the same compounds, responses can shift between MSD tunes. Secondly, SBSE is based on the equilibrium distribution of the analyte between the PDMS extraction phase and the sample matrix and responses of specific analytes are highly influenced by their PDMS-sample distribution coefficient ($K_{\text{PDMS/sample}}$). In the case of e.g. cannabinoids, recoveries are all around 100% and quantitation is relatively easy. However, Table 5.2 shows that benzodiazepines give different relative response factors in SBSE-TD-CGC/MS analysis and quantitative estimates should therefore encounter these values. As an example, Figure 5.13 shows the detailed ion-selective GC/MS contour plot of the urine sample of a drug user. The same ion traces as in Figure 5.12 were selected. N-methylclonazepam was added as internal standard to a cut-off concentration of $50 \mu\text{g.L}^{-1}$ and the contour plot was created at the peak height of this solute giving a small red colored spot (spot 27). Diazepam (spot 16) has a similar spot, but because of its RRF of 2.4, the concentration is estimated at ca. $20 \mu\text{g.L}^{-1}$. Temazepam (spot 33) is detected as a dark green colored spot, indicating a peak height of between 18750 and 25000 (average 21875). The RRF of the solute (1.44) was estimated from the theoretical recovery taking diazepam as a reference (both compounds result in similar MSD responses). This corresponds to an estimated average concentration of approximately $18 \mu\text{g.L}^{-1}$. The concentration of nordazepam largely exceeds the cut-off concentration of $50 \mu\text{g.L}^{-1}$. These values are similar to those reported earlier for the same sample with in depth quantitation (Figure 5.8-B). When isotope-labeled standards are available, the comprehensive profiling gives exact quantitative data.

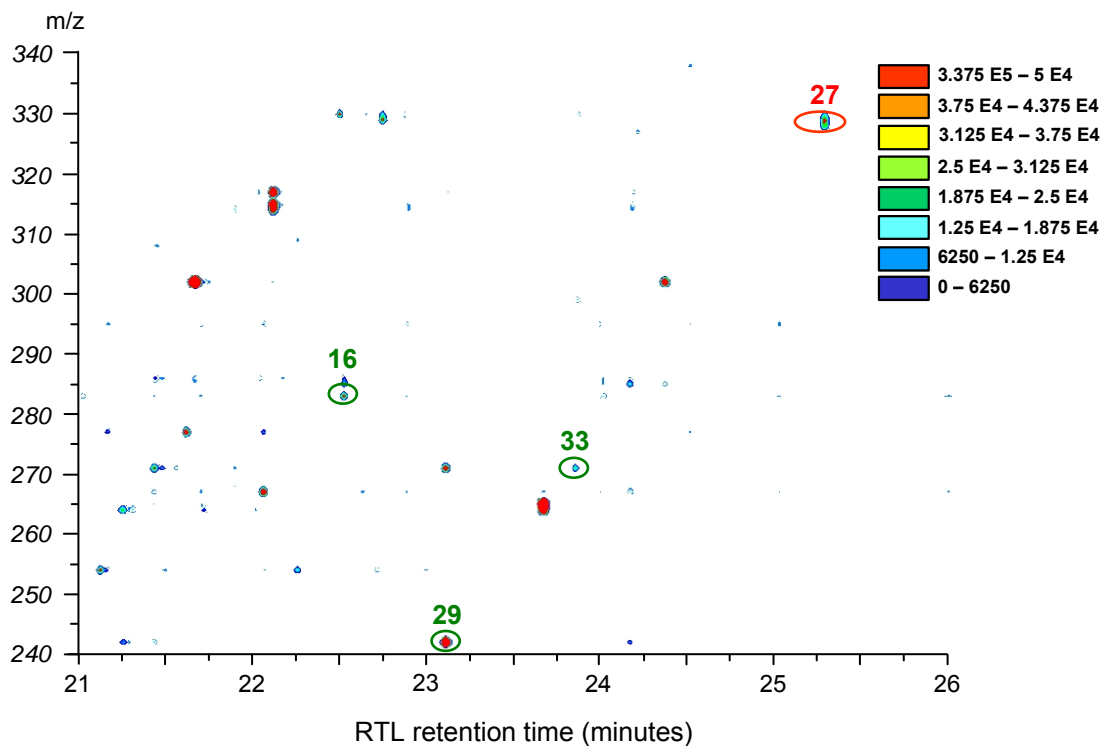


Figure 5.13 Semi-quantitation of benzodiazepines in the ion selective CGC/MS contour plot of a urine sample. Compounds: diazepam (16), temazepam (33), nordazepam (29) and N-methylclonazepam (IS, 27). The numbers refer to Table 5.1.

5.4 Conclusion

Stir bar sorptive extraction-thermal desorption-capillary gas chromatography/mass spectrometry (SBSE-TD-CGC/MS) provides a versatile tool for the analysis of drugs of abuse in biological fluids. The high sensitivity of SBSE allows to use the mass spectrometer in the scan mode. In combination with retention time locking (RTL), identification of the analytes is accurate. Interpretation of CGC/MS data is simplified by comprehensive profiling. The contour plots representing retention times in the x-axis and solute specific m/z -traces in the y-axis are easily interpretable, also by non-skilled personnel, and provide semi-quantitative information.

References

- [1] EMCDDA, 2001 *Annual Report on the State of the Drugs Problem in the European Union*, European Monitoring Centre for Drugs and Drug Addiction, Office for Official Publications of the European Communities, ISBN 92-9168-114-8, 2001.
- [2] I.B. Collison, V.R. Spiehler, S. Guluzian, P.R. Sedgwick, *J. Forensic Sci.* 43 (1998) 390.
- [3] B.J. Buchan, J.M. Walsh, P.E. Leaverton, *J. Forensic Sci.* 43 (1998) 395.
- [4] Y. Gaillard, G. Pepin, *J. Chromatogr. A* 762 (1997) 251.
- [5] P. Kintz, P. Mangin, *Forensic Sci. Int.* 73 (1995) 93.
- [6] R. Kronstrand, R. Grundin, J. Jonsson, *Forensic Sci. Int.* 92 (1998) 29.
- [7] P. Marquet, *Therap. Drug Monit.* 24 (2002) 255.
- [8] F.E. Dussy, C.H. Staubli, T.A. Briellmann, *Chimia* 56 (2002) 53.
- [9] Editorial, Chromatography market profile – Testing drugs of abuse with GC-MS, *LCGC North America* 20 (2002) 328.
- [10] F. Brakstad, *Chemometr. Intell. Lab.* 29 (1995) 157.
- [11] L. Blumberg, M. Klee, *Anal. Chem.* 70 (1998) 3828.
- [12] P. Sandra, B. Tienpont, F. David, *J. Chromatogr. A*, 1000 (2003) 299.
- [13] E. Baltussen, P. Sandra, F. David, C.A. Cramers, *J. Microcolumn Sep.* 11 (1999) 737.
- [14] B. Tienpont, F. David, K. Desmet, P. Sandra, *Anal. Bioanal. Chem.* 373 (2002) 46.
- [15] B. Tienpont, F. David, T. Benijts, P. Sandra, *J. Pharm. Biomed. Anal.* 32 (2003) 569.
- [16] C. Moore, F. Guzaldo, M.J. Hussain, D. Lewis, *Forensic. Sci. Int.* 119 (2001) 155.
- [17] W.M. Meylan, Software KowWIN Version 1.66, SRC-LOGKOW, SRC-ESC, Syracuse, USA, (2000).
- [18] C.S. Boyer, D.R. Petersen, *J. Pharmacol. Exp. Ther.* 260 (1992) 939.
- [19] L. Antonilli, C. Suriano, M.C. Grassi, P. Nencini, *J. Chromatogr. B* 751 (2001) 19.
- [20] T. Benijts, J. Vercammen, R. Dams, H.P. Tuan, W. Lambert, P. Sandra, *J. Chromatogr. B* 755 (2001) 137.
- [21] K. Desmet, B. Tienpont, P. Sandra, *Chromatographia* 57 (2003) 681.
- [22] D. Borrey, E. Meyer, W. Lambert, C. Van Peteghem, A.P. De Leenheer, *J. Chromatogr. B* 765 (2001) 187.
- [23] S. Voorspoels, V. Coucke, P. Schepens, *TIAFT Bull.* 31 (2001) 12.

- [24] J.C. Kraner, D.J. McCoy, M.A. Evans, L.E. Evans, B.J. Sweeney, *J. Anal. Toxicol.* 25 (2001) 645.
- [25] T.L. Martin, *J. Anal. Toxicol.* 25 (2001) 649.

Chapter 6

Rapid diagnosis of pulmonary TB^I

Abstract

A fast method for detection of tuberculostearic acid (TBSA) in sputum samples is described. The samples obtained from patients with known or suspected pulmonary tuberculosis, were decontaminated and concentrated before being analyzed by stir bar sorptive extraction – thermal desorption – gas chromatography/mass spectrometry (SBSE-TD-GC/MS). Prior to extraction, the mycobacterial lipids were hydrolyzed and then derivatized with ethyl chloroformate to increase the sorption of the compounds by the polydimethylsiloxane (PDMS) stir bar coating. The limit of detection (LOD) is 0.2 ng.mL^{-1} . Four sputum samples that were classified by direct microscopy as smear-positive or negative were analyzed by GC/MS. TBSA was detected at concentrations ranging from $0.47 - 2.2 \text{ ng.mL}^{-1}$. The method is sufficiently sensitive to detect TBSA directly in clinical samples without the need to culture the organisms.

Key words:

Stir bar sorptive extraction (SBSE), capillary GC/MS, tuberculostearic acid, pulmonary tuberculosis, sputum samples.

6.1 Introduction

There is an increased demand for the rapid detection and identification of *Mycobacterium tuberculosis* due to the re-emergence of diseases associated with this organism. *M. tuberculosis* indeed is frequently associated with the human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS), which has led to the increased prevalence of pulmonary tuberculosis (TB) globally, but particularly in sub-Saharan Africa [1].

Traditional methods used for the identification of mycobacteria include direct microscopy and culturing, which form the basis of present-day laboratory diagnosis of TB. Culturing is often the only decisive test of mycobacterial disease, but since the organisms are slow-growing it may take up to 8 weeks to obtain results [2]. More recently, new diagnostic methods such as the BACTEC radiometric system and the polymerase chain reaction (PCR) have decreased the time required to diagnose tuberculous infections (i.e. 10 - 14 days) [3]. However, most laboratories in developing countries are ill equipped to successfully utilize these techniques because of inadequate facilities and lack of funding [4].

Gas chromatography was first used as a tool in diagnosing TB in the late 1970's [5]. However, the use of chromatographic methods for the identification of mycobacteria has been restricted to larger reference and research laboratories [6]. Reasons include the cost and maintenance of equipment, the time required to prepare samples, and the fact that most gas chromatographic methods require a culture-step prior to the analysis of clinical samples [7-10].

Stir bar sorptive extraction (SBSE) was developed to facilitate the direct extraction of organic trace compounds from aqueous samples [11]. Rapid, solventless extraction is achieved by using a stir bar coated with a layer of polydimethylsiloxane (PDMS). Target analytes are enriched in a reproducible way by a partitioning mechanism based on the water-octanol distribution constants ($K_{o/w}$) of the solutes [12] when extrinsic factors such as time, pH, temperature, and magnetic stirring speed are held constant. For polar solutes, the introduction of a derivatization-step prior to extraction enhances their sorption by the stir

bar coating. Finally, the stir bar is desorbed using a thermal desorption (TD) device and analyzed by gas chromatography/mass spectrometry (GC/MS). The aim of this investigation was to demonstrate the direct measurement of a chemical marker of tuberculosis, namely tuberculostearic acid (TBSA), in sputum samples using SBSE-thermal desorption-GC/MS.

6.2 Experimental

6.2.1 Chemicals and materials

Ethyl chloroformate, pyridine, sodium hydroxide, 17-methyloctadecanoic acid (MODA – internal standard) and 10-methyloctadecanoic acid (tuberculostearic acid - TBSA) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Ethanol and chloroform were obtained from Merck (Darmstadt, Germany). Hydrochloric acid was supplied by Acros (Geel, Belgium). The sonicator was a Branson model 3510 obtained from LASEC (Cape Town, South Africa). The 15 ml screw cap vials were from Supelco (Sigma-Aldrich, Johannesburg, South Africa). Stir bars (10 mm × 0.5 mm) with a 25 µl PDMS coating were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany).

6.2.2 Sputum samples

Sputum samples were obtained from routine clinical specimens that were sent to the National Health Laboratory Service (NHLS, Cape Town, South Africa). Approximately 5 to 10 ml of a first-morning sputum specimen was collected from patients with known or suspected pulmonary tuberculosis. Each sample was classified as 1+, 2+, or 3+ positive based on the results obtained by direct microscopy. One sample was analyzed in each category, including a sample that was smear-negative. Five samples, obtained from patients with nonmycobacterial pneumonia that had no previous history of pulmonary tuberculosis, were used as controls. All samples were concentrated and decontaminated according to a standard procedure used by the NHLS [13]. The procedure was carried out with slight modification to the approved WHO method. Briefly, an equal volume of Sputagest was added to each sample and placed in an orbital shaker/incubator at 37°C for 10 min at 330

rpm. One ml aliquots were removed and reserved as spare samples. The remainder of the specimens was decontaminated with equal volumes of 1 M sodium hydroxide and 0.1 M sodium citrate, such that the final volume was twice that of the sample volume. The samples were again placed in the orbital shaker/incubator for 20 min using the same settings. Two volumes of phosphate buffer (pH 6.8) were added and the samples were concentrated by centrifugation at 3000 rpm for 20 min. After removal of the supernatant, the sample pellets were re-suspended in 1.5 ml phosphate buffered saline and mixed gently. A 0.5 ml aliquot was used for culturing and direct microscopy while the remainder was concentrated by centrifugation and re-suspended in 0.5 ml phosphate buffered saline for GC/MS analysis. The samples were autoclaved at 180°C for 20 min before being dispatched to the Chemistry Department at Stellenbosch University. All samples were stored at -20°C until analyzed.

6.2.3 Sample preparation and SBSE procedure

The samples were transferred to 2 ml autosampler vials and 120 µl of concentrated hydrochloric acid (36%) was added to hydrolyze the bacterial lipids. The vials were capped and heated at 80°C for 30 min. After cooling to room temperature, 0.5 ml of a 0.5 M sodium hydroxide solution was added to the vial and vortexed gently. The content was transferred to 15 ml glass vials with Teflon-lined screw caps and spiked with 2.6 ng of the internal standard, namely 17-methyloctadecanoic acid. The fatty acids were derivatized using ethyl chloroformate according to a previously described procedure [12]: 1.04 ml of a mixture of ethanol and pyridine (5.5:1) were added to the vial followed by 80 µl of ethyl chloroformate (ECF) that was added under sonication in a fume hood. The open vial was sonicated for 15 min before placing a conditioned stir bar in the sample. The stir bars were conditioned at 280°C under a nitrogen flow in a separate GC oven prior to SBSE. The samples were stirred at 1000 rpm for 30 min and extraction took place at 40°C. After extraction of the analytes, the stir bar was removed from the sample, washed with distilled water and briefly dried with lint-free tissue paper to remove residual water droplets. The stir bars were then placed in a glass TDS tube for thermal desorption and analysis by GC/MS.

6.2.4 Instrumental conditions

The stir bars were thermally desorbed using a TDS-2 thermal desorption unit (Gerstel) operated in the solvent-venting mode. Thermal desorption was accomplished by increasing the temperature from 50 to 150°C (held for 1 min) at 60°C.min⁻¹ using a helium flow-rate of 60 ml.min⁻¹. After 2 min, the TDS split-valve was closed followed by a final temperature increase to 300°C (held for 10 min) at 60°C.min⁻¹. The desorbed compounds were transferred to a programmable temperature vaporization (PTV) inlet (CIS 4, Gerstel) through a heated transfer line (325°C). Cryofocussing of the compounds took place at 40°C in a baffled liner using liquid nitrogen. Sample injection was performed in the splitless mode by programming the CIS 4 from 40 to 300°C (held for 5 min) at 12°C.s⁻¹. The split-valve was opened after 2.5 min. GC/MS analyses were performed on a Hewlett-Packard 5890 gas chromatograph interfaced with a model 5972 mass spectrometric detector (MSD). Chromatographic separations were carried out on a HP-5MS fused-silica capillary column (Agilent Technologies; 30 m x 0.25 mm I.D. x 0.5 µm film thickness) using helium as the carrier gas at a flow rate of 1.4 ml.min⁻¹. The oven temperature was programmed from 90°C (held for 6 min) to 300°C at 15°C.min⁻¹. The final temperature was held for 10 min. The transfer line temperature was at 280°C. The MSD was operated in both the scan and the selected ion monitoring (SIM) modes with an electron ionization voltage of 70 V. For SIM, two ions characteristic for the fatty acid-ethyl esters (*m/z* 88, 101) were monitored at 100 m/s for both ions.

6.2.5 Figures of merit

The linearity of the method was evaluated by setting up an internal standard calibration curve in control sputum samples that were obtained from patients with nonmycobacterial pulmonary infections. Standard stock solutions were prepared in chloroform at a final concentration of 0.7 µg.mL⁻¹ for TBSA and 1 µg.mL⁻¹ for the internal standard MODA. The samples were spiked with different concentrations of TBSA at 0.4, 0.7, 1.5 and 2.2 ng.mL⁻¹ to construct the calibration curve.

6.3 Results and discussion

Preliminary experiments performed on aqueous solutions of TBSA and the internal standard MODA, showed that extraction recoveries of ca. 90% were obtained using the conditions described in the experimental part. Moreover, because of the structural similarity between TBSA and the internal standard MODA, no differences in recovery between both solutes were noted. Calibration was performed in control sputum samples to determine the effect of the sample matrix on the limit of detection of TBSA. A four point calibration curve using internal standard calibration ($0.4 - 2.2 \text{ ng.mL}^{-1}$) showed good linearity over this range with a correlation coefficient of 0.9965. The LOD for TBSA was calculated at a signal to noise level of 3 and was determined at 0.2 ng.mL^{-1} for the control sputum samples. The spiked sputum sample at 1.5 ng.mL^{-1} was analyzed 3 times and the RSD% was 4.8.

Five sputum samples containing different bacterial loads were analyzed by SBSE-TD-GC/MS, after the decontamination/concentration procedure was carried out by the NHLS. One sample was from a patient being treated for pulmonary infection caused by *Streptococcus pneumoniae* (Sample A) while the other samples were classified by direct microscopy as 1+ (Sample B), 2+ (Sample C), 3+ (Sample D) and one negative for the mycobacteria (Sample E).

In sample A, no TBSA was detected by SBSE-TD-GC/MS. Sample B was classified as 1+ by direct microscopy. Smears stained for acid-fast bacilli (AFB) provide important preliminary information in the diagnosis of TB. It is also the least expensive method used in the detection of the mycobacteria, but the technique is known to lack specificity, which ranges from 25 to 80% [14]. No culture results were available for sample B but no TBSA was detected by GC/MS. The result obtained is therefore inconclusive because of the outstanding culture results and the fact that no other clinical information with regard to the patient's condition was provided.

Sample C was categorized as 2+ by direct microscopy and a clinical diagnosis of pulmonary TB was previously documented. At the time of the study, the patient was being re-treated for a previously acquired TB infection. The amount of TBSA detected by GC/MS

was 0.47 ng.mL^{-1} (Figure 6.1 – Sample C). No information with regard to the type of medication used or the duration of treatment was available. The result was accepted as a ‘true positive’ because a diagnosis of pulmonary TB had previously been established and the results obtained by direct microscopy and GC/MS were both positive for the presence of the mycobacteria. Sample D was obtained from a patient diagnosed with disseminated tuberculosis. This is a severe form of the disease, which spreads to other parts of the body. The sample was classified as 3+ by direct microscopy and the microbial culture result was also positive. The concentration of TBSA determined by GC/MS was 2.3 ng.mL^{-1} (Figure 6.1 – Sample D). This sample is definitely a ‘true positive’ because all three tests performed were positive for the mycobacteria. Note that the two main peaks eluting before TBSA namely octadecenoic and octadecanoic acid are strongly fluctuating. The reason for this phenomenon is unclear, although it may be attributed to the clinical course of the disease that is still not well understood.

For sample E, a diagnosis of pulmonary TB was documented following clinical assessment of the patient’s condition. However, the AFB smear result was negative and no TBSA was detected by GC/MS (Figure 6.1E). Also, the culture result obtained from the MGIT system was still negative after a 6 week incubation period. The results obtained for direct microscopy, culturing and GC/MS are summarized in Table 6.1.

Table 6.1 Comparison of data for GC/MS, direct microscopy and culturing.

Number	Diagnosis	Direct microscopy	Mycobacterial culture	GC-MS (ng mL^{-1})
A	Sample (<i>Streptococcus pneumoniae</i>)	np	np	nd
B	New case (diagnosis to be confirmed)	1+ positive	np	nd
C	Pulmonary tuberculosis (retreatment)	2+ positive	–	0.47
D	Disseminated tuberculosis	3+ positive	+	2.27
E	Pulmonary tuberculosis (?)	Negative	–	nd

np: not performed; nd: not detected.

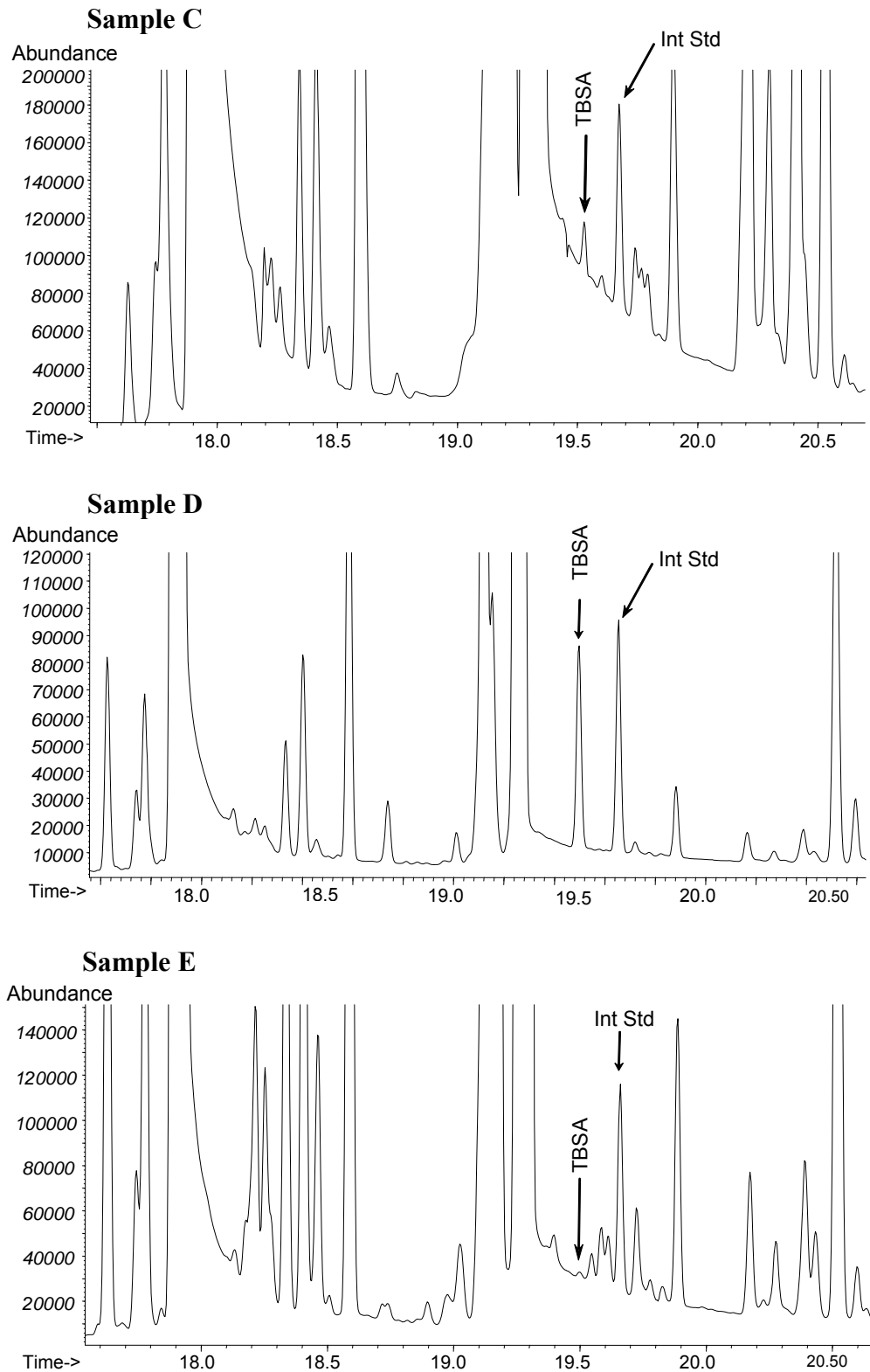


Figure 6.1 SIM chromatograms (m/z 88,101) of sputum samples obtained by SBSE-TD-GC/MS. Sample C. 2+ positive, TBSA detected at 0.47 ng.mL^{-1} (pulmonary TB); Sample D. 3+ positive, TBSA detected at 2.3 ng.mL^{-1} (disseminated TB); Sample E. Smear negative, culture negative, no TBSA detected by GC/MS.

From the results obtained, it is clear that the GC/MS data follow the same trend as the conclusions derived from the direct microscopy and microbacterial culture tests. However, more samples have to be analyzed to be able to determine the diagnostic sensitivity and specificity of the method. The results should also be compared to the clinical diagnosis given to each patient as well as the results obtained by standard culturing techniques to establish the positive and negative predictive values of the method. The type and duration of drug treatment should be considered and how it affects the detectable levels of TBSA. There is a significant advantage in being able to quantify the amount of TBSA in sputum samples. It may be used for example, to determine the progression of treatment and to establish whether a correlation exists between the concentration of TBSA and the extent of the disease. Moreover, the rapidity with which the method can be performed significantly reduces the time required to obtain clinically useful results without the need to culture the samples prior to analysis.

The use of TBSA alone for the diagnosis of pulmonary TB has been criticized in the past. TBSA is regarded as not specific enough due to its presence in other organisms such as the Actinomycetes, Nocardia and Rhodococcus species. However, the incidence of infections caused by these organisms as opposed to the mycobacteria should be considered. A study by Jones et al. [15] estimated that one case of pulmonary Nocardial infection occurred to 90 cases of pulmonary TB in a population of HIV infected patients. On the other hand, it would also be possible to increase the specificity of the TBSA method by identifying other markers that are specific for *M tuberculosis*. A recent report by Alugupalli S et al. [16] showed that certain 3-hydroxy fatty acids may be used to distinguish *M tuberculosis* from other organisms. The interesting possibility that these compounds also provide a measure of the virulence of the mycobacteria deserves further investigation.

6.4 Conclusion

A rapid technique for the detection of TBSA in sputum samples was developed. Traditional decontamination and concentration methods were combined with SBSE-TD-GC/MS for the detection of TBSA at trace levels in clinical samples. The technique was sufficiently

sensitive to detect TBSA without the need to culture the samples, thereby reducing the time required to obtain results. The future application of the method in the routine identification of the mycobacteria should be established by comparing the diagnostic accuracy of the technique against standard biochemical tests used to detect pulmonary tuberculosis.

References

- [1] A Herz, M Leichsenring, M Felten, OJ Oosthuizen, E Mayatepek, W Haas and HJ Bremer. *Eur. J. Clin. Invest.* 24 (1994) 114.
- [2] AF Yassin, H Brzezinka and KP Schaal. *Zbl. Bakt.* 279 (1993) 316.
- [3] GD Roberts, EC Böttger and L Stockman. *Clin. Lab. Med.* 16 (1996) 603.
- [4] PA Jenkins. *Commun. Dis. Rep. CDR Rev.* 2 (1992) R101.
- [5] G Odham, L Larsson, P Mardh. *J. Clin. Invest.* 63 (1979) 813.
- [6] L Larsson. *Acta Path. Microbiol. Immunol. Scand. Sect. B* 91 (1983) 235.
- [7] S Alugupalli, MK Sikka, L Larsson, DC White. *J. Microbiol. Meth.* 31 (1998) 143.
- [8] GL French, CY Chan, D Poon, SW Cheung, AFB Cheng. *J. Med. Microbiol.* 31 (1990) 21.
- [9] BC Mayall. *Pathology* 17 (1985) 24.
- [10] L Larsson, PA Mårdh, G Odham, G Westerdahl. *Acta Path. Microbiol. Immunol. Scand. Sect. B* 89 (1981) 245.
- [11] E Baltussen, P Sandra, F. David and CA Cramers *J. Microcolumn. Sep.* 11 (1999) 737.
- [12] B Tienpont, F David, T Benijts and P Sandra. *J. Pharm. Biomed. Anal.* 32 (2003) 569.
- [13] K Weyer et al. "Laboratory services in tuberculosis control". Part III: Culture p 40. World Health Organization, Geneva, Switzerland (1998). http://www.who.int/gtb/publications/whodo_c/who_tb-98-258/en/98.258_culture-3.pdf.
- [14] GL Woods, E Pentony, MJ Boxley, AM Gatson. *J. Clin. Microbiol.* 33(1995) 1915.
- [15] N Jones, M Khoosal, M Louw, A Karsteadt. *J. Infection* 41 (2000) 232.
- [16] S Alugupalli, F Portaels and L Larsson. *J. Bacteriol.* 176 (1994) 2962.

Chapter 7

Urinalysis of 4-Hydroxy- nonenal^{II}

Abstract

A simple and fast method for the measurement of 4-hydroxynonenal (4HNE), a highly toxic end product of lipid peroxidation, in urine samples is described. The method combines stir bar sorptive extraction (SBSE) with two derivatization steps, followed by thermal desorption and GC/MS. 4HNE is derivatized in-situ with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine and the oxime is extracted from the aqueous phase with SBSE. The 4HNE-oxime is further acylated by headspace derivatization prior to thermal desorption. Derivatization reactions and extraction were optimized in terms of reagent quantities, temperature and time. The method is linear over a concentration range of 0.5 – 5 ng.mL⁻¹ with a correlation coefficient of 0.997. The limit of detection and limit of quantitation are 22 pg.mL⁻¹ and 75 pg.mL⁻¹ urine, respectively. The high sensitivity of the method allows the measurement of physiological concentrations of 4HNE in urine samples

Keywords:

Stir bar sorptive extraction, gas chromatography – mass spectrometry, 4-hydroxynonenal, oxidative stress, urine samples.

7.1 Introduction

The measurement of aldehydes in biological samples has become increasingly important due to their significance as markers of oxidant injury. Aldehydes are primarily formed *in vivo* by a free radical-mediated mechanism that is initiated when reactive oxygen species (ROS) such as O_2^- and H_2O_2 interact with cellular membranes [1]. Oxidation of the phospholipid components of these membranes results in the formation of hydroperoxide intermediates that rapidly breakdown to a variety of stable aldehydes, including alkanals, 2-alkenals, 2,4-alkadienals and 4-hydroxyalkenals [2]. One of the most important end-products formed following the oxidation of lipid membranes is the α,β -unsaturated aldehyde, 4-hydroxynonenal (4HNE) [3, 4].

4HNE has been studied intensively for many years and found to be highly toxic to mammalian cells [3]. Several adverse effects have been observed in biological tissues when this compound increases above physiological concentrations (i.e. $> 1 \mu M$). 4HNE has been shown to inhibit DNA, RNA and protein synthesis [5, 6], initiate lipid peroxidation [7], rapidly deplete intracellular glutathione [8], and inhibit respiration and glycolysis [9]. These harmful effects have been attributed to the chemical structure of 4HNE, which may explain its high reactivity toward biological substrates [10]. This aldehyde readily reacts with target proteins in biological membranes, forming toxic substances that may lead to the development of chronic diseases. For this reason, 4HNE has been implicated in the pathogenesis of diseases such as atherosclerosis [11], Alzheimer's disease [12], and cancer [13].

Due to the difficulty of measuring ROS directly *in vivo*, most estimates of oxidative stress rely on the measurement of the breakdown products of lipid peroxidation (e.g. 4HNE). Several analytical methods have been developed for the measurement of 4HNE in biological tissues and fluids, most of which rely on spectrophotometric detection of stable chromophore derivatives. One of the most widely used methods involves the derivatization of 4HNE with 2,4-dinitrophenylhydrazine prior to analysis by HPLC [14, 15]. This method offers relatively facile measurement of a number of aldehydes, but its selectivity has been

questioned, especially when analyzing complex biological matrices where 4HNE occurs at trace levels [16].

The most sensitive analytical methods currently available for the measurement of 4HNE are based on the formation of an oxime derivative using *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) followed by silylation of the hydroxyl group prior to GC/MS analysis [17, 18, 19]. The method recently developed by Meagher et al. [20] is sufficiently sensitive to measure physiological concentrations of 4HNE in urine samples. This method was adapted from a procedure that was previously developed for the measurement of 4-hydroxyalkenals in oxidized LDL [21]. All of the previously mentioned methods, however, share a common disadvantage, namely that they require tedious sample preparation procedures which involve several extraction and cleanup steps before the derivatives can be analyzed.

New solventless sample-enrichment techniques that allow the direct extraction of solutes from aqueous matrices have recently been introduced such as stir bar sorptive extraction (SBSE) and solid-phase microextraction (SPME) [22, 23]. Both techniques combine extraction and concentration of the analytes in a single step, thereby reducing the time required to prepare the samples. A number of reports have already been published for the analysis of carbonyl compounds using SBSE [24] and SPME [25, 26]. SBSE has the advantage that higher analytical sensitivities (i.e. > 100 fold) can be reached as compared to SPME [22, 27], thus favoring this technique for the analysis of trace solutes such as 4HNE. No reports have so far been published on the analysis of hydroxylated-carbonyl compounds using either of these techniques.

One of the limitations in the analysis of compounds containing hydroxyl groups by aqueous SBSE has been the lack of a suitable derivatization method for these analytes. A headspace derivatization technique was recently described by Kawaguchi et al. [28], where BSTFA was added in the thermal desorption tube for in-situ silylation of 17 β -estradiol. This method has been based on a concept originally developed by Okeyo et al. [29], in which SPME fibers were exposed to BSTFA vapors to derivatize steroids that were extracted from urine samples. Shao et al. [30] later extended this technique by replacing BSTFA with

acetic acid anhydride for the headspace derivatization of trans-resveratrol in wine. In this contribution, a new SBSE derivatization technique is presented that utilizes acetic acid anhydride catalyzed by pyridine for the headspace derivatization of extracted hydroxycarbonyl-oximes. The developed method has been applied for the measurement of 4HNE in urine samples.

7.2 Experimental

7.2.1 Chemicals and materials

Potassium hydrogen phthalate, butylated hydroxytoluene (BHT), *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA), pyridine and 2,5-dihydroxybenzaldehyde (2,5-DHBA) were purchased from Sigma Aldrich (Johannesburg, South Africa). Acetic acid anhydride and concentrated sulfuric acid were obtained from Merck (Darmstadt, Germany). 4-hydroxynonenal (4HNE) was supplied by Cayman Chemical (Kat Medical, Cape Town, South Africa). Methanol was obtained from Riedel-de Haën (Sigma Aldrich, Johannesburg, South Africa). Five mL glass screw-cap vials (34 mm × 20 mm) and 2 mL glass headspace vials were prepared by E. Ward (University of Stellenbosch). The 5 mL vials were modified from 15 mL screw cap vials obtained from Supelco (Sigma Aldrich, Johannesburg, South Africa). The 2 mL headspace vials were adapted from 2 mL autosampler vials purchased from Agilent Technologies (Chemetrix, Johannesburg, South Africa). A 10 position magnetic stirrer combined with a convection oven was designed and built by J. Blom and colleagues (Department of Mechanical Engineering, University of Stellenbosch). Twister stir bars (10 mm × 0.5 mm d_f PDMS) were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany). The stir bars were pre-conditioned by sonication in a 1:1 mixture of dichloromethane:methanol for 5 min after which they were heated at 280°C for 15 min under a nitrogen flow of 50 mL.min⁻¹. The sonicator (Branson 3510) and sterile 50 mL centrifuge tubes were from LASEC (Cape Town, South Africa).

7.2.2 Solutions

Concentrated solutions of 4HNE (0.1 mg.mL⁻¹) and of the internal standard 2,5-DHBA (1 mg. mL⁻¹) were prepared in methanol. The solutions were stored at -80°C and dilutions were made weekly. Standard working solutions were prepared in methanol at concentrations of 1 µg.mL⁻¹ and 10 µg.mL⁻¹ for both compounds. Each solution contained 100 µM BHT and was stored at -20°C. PFBHA was prepared in pyridine at a concentration of 10 mg.mL⁻¹. A fresh solution was prepared weekly that was stored at -20°C.

7.2.3 Urine samples

Spot urine samples were obtained from 10 volunteers (7 males and 3 females) that were recruited from students and staff at the University of Stellenbosch. The samples were collected between 9h00 and 12h00 each day using 50 mL sterile centrifuge tubes that were immediately placed on dry ice before being stored at -80°C. The volunteers were asked to fill out a short questionnaire to obtain information about their general health and to record their demographic data. Some of the questions that were asked included whether they smoked; were using anti-oxidants; or suffered from any chronic diseases such as asthma or diabetes. The creatinine content in each urine sample was determined by a modification of the Jaffé method [31, 32] and the specific gravity (SG) measurements were estimated using an Abbe refractometer (Atago, USA).

The measured 4HNE concentrations were normalized using the corresponding creatinine levels in each sample, and the adjusted concentrations were compared with normalized values that were calculated using a modification of the method of Vij and Howell [33]. These authors recently introduced a new equation for normalizing the excretion of xenobiotic biomarkers in spot urine samples [34]. Using a slight modification of their method the SG-normalized concentrations of 4HNE were calculated as follows:

$$[4\text{HNE}]_{sz} = \frac{[4\text{HNE}]}{\left(\frac{1.020-1}{\text{SG}-1}\right)}(Z) \quad (1)$$

where $[4\text{HNE}]_{\text{sz}}$ was the SG- and Z-normalized urine samples. The value 1.020 was defined as the mean SG of normal human urine and the respective Z-values were calculated by plotting the $\log[4\text{HNE}]$ values against the $\log(\text{SG}-1)$ of the individual samples as shown below:

$$Z = \frac{\Delta \log [4\text{HNE}]}{\Delta \log (\text{SG} - 1)} \quad (2)$$

where Z corresponded to the slope of the graph. The SG-normalized values of 4HNE were adjusted using the corresponding creatinine concentration in each sample (Eq. 3):

$$[4\text{HNE} - \text{Creat}]_{\text{sz}} = \frac{[4\text{HNE}]_{\text{sz}}}{[\text{Creat}]} (113.12) \quad (3)$$

where $[4\text{HNE}-\text{Creat}]_{\text{sz}}$ was the creatinine, SG and Z-normalized urine samples. The symbol $[\text{Creat}]$ corresponded to the creatinine concentration (mmol.L^{-1}) in the sample and the value 113.12 is the molecular weight of creatinine.

7.2.4 Sample preparation, in-situ derivatization and SBSE procedure

Urine samples were allowed to thaw overnight at 4°C after which 1 mL aliquots were transferred to 5 mL glass screw-cap vials containing 1 mL 1 M potassium hydrogen phthalate and 0.52 mL $1 \mu\text{M}$ BHT in methanol. The BHT was added to the samples to prevent artificial formation of 4HNE during the sample work-up procedure. Each sample was spiked with 1 ng of the internal standard 2,5-DHBA, corresponding to a final concentration of 0.18 ng.mL^{-1} in the sample mixture. The pentafluorobenzyl-oxime derivatives of 4HNE and of the IS were synthesized by adding 150 μL of 40 mM PFBHA in pyridine. The vials were capped, vortexed gently for 10 s and placed in a sonicator bath for 15 min. Following sonication, 3 mL de-ionized water and 20 μL concentrated sulfuric acid was added before placing a conditioned stir bar in the sample solution. The pH of the solution was ca. 5.5 prior to the extraction of the oxime derivatives. The samples were

stirred at 1100 rpm for 50 min using a home-built magnetic stirrer/oven that was heated up to 42°C.

7.2.5 Headspace derivatization

Following SBSE extraction of the pentafluorobenzyl (PFB)-oxime derivatives, the stir bars were removed from the sample vials, washed with distilled water and dried with tissue paper. The stir bars were transferred to cups attached inside 2 mL headspace vials (Figure 7.1) and 20 μL acetic acid anhydride and 20 μL pyridine were added. The vials were incubated at 70°C for 20 min to form the acetate derivatives of the extracted compounds.

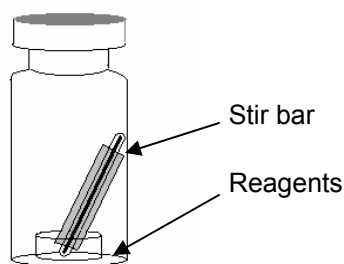


Figure 7.1 Modified autosampler vial (2 mL) used for headspace acylation of the SBSE extracted 4HNE-oxime derivatives.

7.2.6 Thermal desorption – gas chromatography – mass spectrometry

The stir bars were removed from the headspace vials, washed and dried, and placed in glass desorption tubes of a TDS A2 autosampler (Gerstel). Thermal desorption was accomplished in the solvent-venting mode using helium at a flow rate of 60 $\text{mL}\cdot\text{min}^{-1}$. The temperature of the TDS 2 was increased from 50°C to 270°C (held for 10 min) at a rate of 60°C $\cdot\text{min}^{-1}$. The split-valve was closed after 2 min. The desorbed compounds were transferred to a programmable temperature vaporization (PTV) inlet (CIS 4, Gerstel), and were cryofocussed at 5°C using liquid nitrogen. The TDS transfer line was held at 280°C. Sample injection onto the capillary column was accomplished by programming the PTV from 5°C to 280°C (held for 5 min) at a rate of 12°C $\cdot\text{s}^{-1}$.

GC/MS analyses were carried out on a Agilent 6890 gas chromatograph that was interfaced with a 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA). The derivatives were separated on a HP-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent) using helium as carrier gas at a flow rate of 1 mL.min⁻¹. The oven temperature was programmed from 70°C (held for 1 min) to 145°C at 20°C.min⁻¹, and from 145°C to 300°C at 8°C.min⁻¹. The solvent delay was 9.5 min and the transfer line temperature was maintained at 280°C. Mass spectra of the derivatized compounds were first recorded in full scan, whereas quantification was performed in the selected ion monitoring (SIM) mode. The electron ionization voltage was 70 V. For SIM, three ions characteristic for the analytes were monitored at 100 m.s⁻¹ each, namely *m/z* 322, 351 and 393 for 4HNE and *m/z* 316, 333 and 375 for 2,5-DHBA (internal standard). The underlined values are the ions used for quantification.

7.3 Results and discussion

7.3.1 SBSE-HD-TD-GC/MS determination of 4HNE

The presented method is based on the analysis of 4HNE in urine samples using SBSE – headspace derivatization – thermal desorption – GC/MS (SBSE-HD-TD-GC/MS). The 4HNE-oxime derivatives were prepared by a reaction with PFBHA in the aqueous sample matrix, followed by extraction of the derivatives using SBSE. Thereafter, the acetate derivatives of the extracted compounds were formed in the polydimethylsiloxane phase by exposing the stir bars to acetic acid anhydride vapors at 70°C. Pyridine was used as a catalyst in both reaction steps as shown in Figure 7.2. The PFB-oxime-acetate derivatives were thermally desorbed and analyzed on-line by GC/MS.

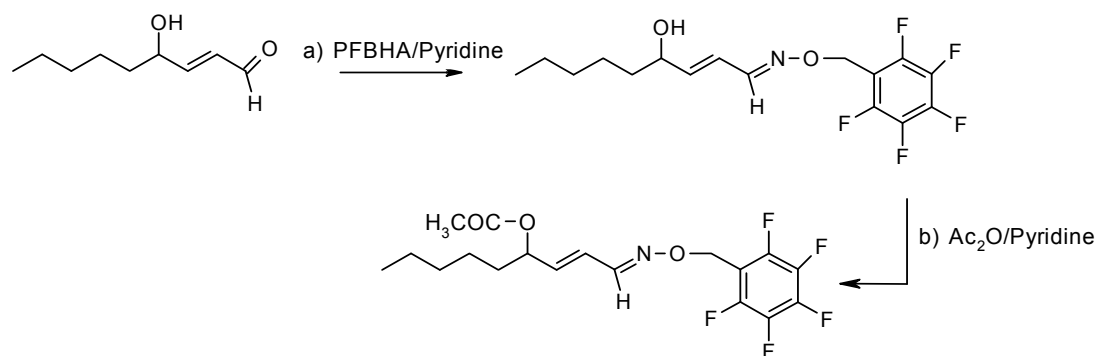


Figure 7.2 Reaction scheme of the two-step derivatization method for 4HNE using a) PFBHA in the aqueous sample matrix and b) acetic acid anhydride in the headspace to form the 4HNE-oxime-acetate derivatives. Conditions are given in the experimental section.

Initial experiments were performed with pure water samples containing 4HNE and the internal standard at a concentration of $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ to determine the retention times and mass spectra of the respective PFB-oxime-acetate derivatives. Electron impact mass spectra of 4HNE and 2,5-DHBA (Figure 7.3) show the molecular ion of 4HNE (m/z 393), and that of the IS (m/z 417). The spectra of both compounds are characterized by the initial loss of $[\text{CH}_2=\text{C}=\text{O}]$, resulting in m/z 351 for 4HNE and m/z 375 for 2,5-DHBA. The loss of a second $[\text{CH}_2=\text{C}=\text{O}]$ group from 2,5-DHBA results in ion m/z 333, which is the base peak of this derivative. The peak of highest intensity for 4HNE is ion m/z 181, which is characteristic for PFB-derivatized compounds analyzed by electron impact mass spectrometry. Using this ion for SIM analyses of aldehyde-oxime derivatives is believed to lack specificity because many other compounds, including ketones, are present in biological fluids [16]. Ions were selected that are highly characteristic for 4HNE to ensure adequate separation and specific detection of this aldehyde in complex matrices such as urine. Figure 7.4 shows the SIM chromatograms of the derivatized compounds in water (A) and in a urine sample (B). For the water sample $1 \text{ ng}\cdot\text{mL}^{-1}$ of 4HNE and IS were added while only $1 \text{ ng}\cdot\text{mL}^{-1}$ IS was added to the urine sample. The figures indicate that the selected ions are highly specific and that the urine matrix does not disturb the quantification. Note that 4HNE consists of a *syn* and *anti*-isomer peak while a single peak is observed for the IS [18]. Quantification of 4HNE was performed using the second isomer peak, as the first peak was not always separated when different urine samples were analyzed.

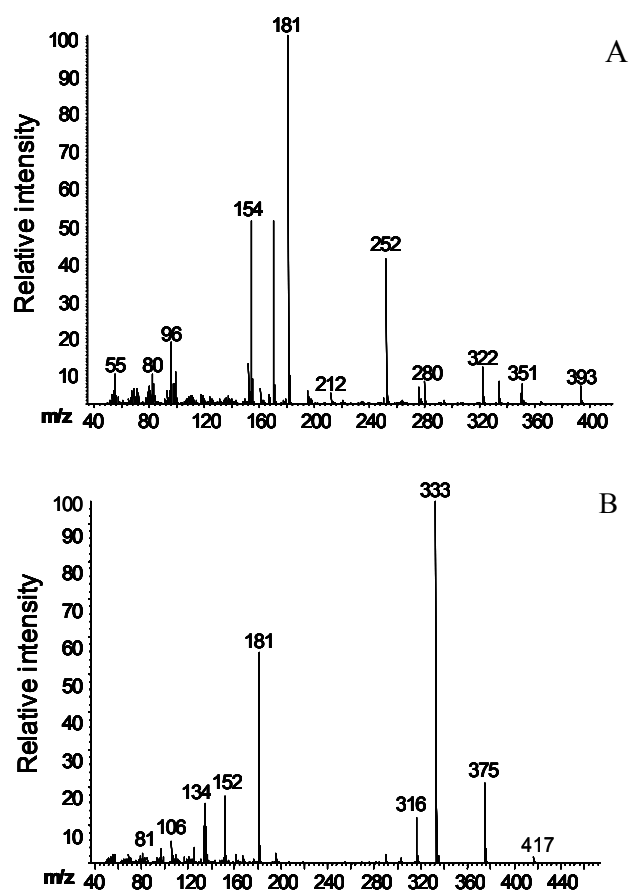


Figure 7.3 Mass spectra of the PFB-oxime-acetate derivatives of A) 4HNE and B) 2,5-DHBA (IS). The selected ions used to construct the chromatograms in Figure 7.4 were m/z 322, 351 and 393 for 4HNE and m/z 316, 333 and 375 for 2,5-DHBA.

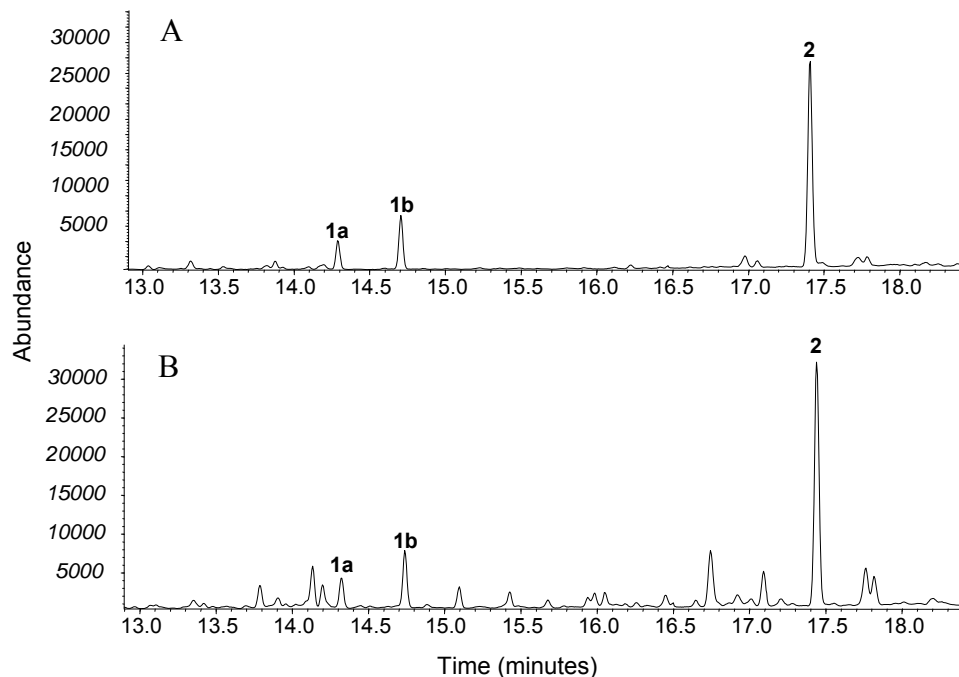


Figure 7.4 SIM chromatograms by SBSE-HD-TD-GC/MS of the PFB-oxime-acetate derivatives of 1a,1b) the *syn*- and *anti*-isomers of 4HNE and 2) 2,5-DHBA (IS). The upper trace (A) corresponds to the analysis of a 1 mL water sample containing 1 ng of 4HNE and 1 ng of the IS; and the lower trace (B) corresponds to the analysis of a 1 mL urine sample spiked with 1 ng of the IS. Extraction, derivatization and chromatographic conditions are described in the experimental section.

The sorption of compounds by the stir bar depends largely on the partition coefficient of the solutes between the polydimethylsiloxane (PDMS) layer covering the stir bar and the aqueous phase. By using the octanol-water distribution coefficient ($K_{o/w}$), which is comparable to the partition coefficient ($K_{PDMS/w}$), it is possible to predict the enrichment factor for a given compound from an aqueous solution [35]. The $\log K_{o/w}$ values of 4HNE (non-oximated and oximated) were calculated using the Log P predictor which is available from Interactive Analysis Inc. (Bedford, MA, USA) and are 1.35 and 2.48, respectively. The enrichment factors at equilibrium estimated with the TwisterCalculator available from RIC (Kortrijk, Belgium) are 8.6 and 56 %, respectively for the 0.5 mm PDMS layer. Enrichment of 4HNE from aqueous matrices is thus significantly enhanced when the 4HNE-oxime derivative is formed prior to performing SBSE.

7.3.2 Method optimization

The different steps of the derivatization reactions and SBSE extraction were optimized in order to obtain the highest yields and this combined with good reproducibility. All experiments were conducted in urine samples to which an extra 1 ng.mL^{-1} of 4HNE and 1 ng.mL^{-1} of the IS standard were spiked. The samples were analyzed by SBSE-HD-TD-GC/MS using the conditions described in the experimental section.

Firstly, the PFBHA reaction was optimized and compared with two PFBHA derivatization methods that were recently published [20, 26]. Different amounts of the reagent were added to the sample matrix, namely 0.35, 0.56, 0.70 and 1.05 mM PFBHA and the reaction was allowed to proceed as described in the experimental section. SBSE was performed at 42°C during 50 min. The results are shown in Figure 7.5.

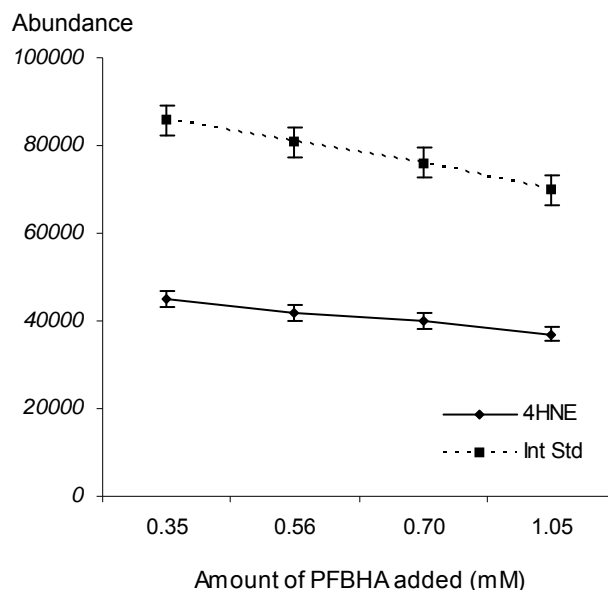


Figure 7.5 Amount of PFBHA used to form the oxime-derivatives of 4HNE and the IS. A 1 mL urine sample spiked with 1 ng of both compounds was analyzed by SBSE-HD-TD-GC/MS. Conditions are given in the experimental section.

The use of 0.35 mM PFBHA resulted in higher yields of the PFB-oxime derivatives as compared to using 1.05 mM PFBHA. Using lower amounts of the reagent seemed

advantageous because fewer by-products of the PFBHA reaction were extracted by the stir bar. However, it was noticed that the reproducibility of the method was influenced by the amount of reagent used. Repeatability ($n=3$) was improved at higher concentrations of PFBHA (3 % for 1.05 mM PFBHA vs. 8 % for 0.35 mM PFBHA). Therefore, 1.05 mM PFBHA was chosen for the derivatization of 4HNE. Using these conditions, the effect of time on the reaction yield of the PFB-oxime derivatives was assessed. Samples were sonicated for 15 min, placed in the dark for 30 min and left in the dark for 2 h. Comparable yields of the 4HNE-oxime derivatives were obtained using the three methods.

The time required for the PFB-oxime derivatives to reach extraction equilibrium was studied by varying the extraction time from 30 – 120 min. The peak areas for 4HNE and the IS reached an optimum at ca. 50-60 min. While the peak area of the IS slightly declined in the time interval 60 to 120 min, a sharp rise was observed in the extracted amounts of 4HNE between 90 and 120 min, indicating that 4HNE could be artificially formed in the urine sample. This was further investigated by varying the extraction temperature from 40-70°C. This experiment was also performed using pure water samples to control for the surplus formation of 4HNE in urine samples. The extraction of the IS followed the same decreasing trend in both sample matrices, but an increased amount of 4HNE was extracted from the urine sample in the temperature interval 50 to 70°C. Therefore it was concluded that higher extraction temperatures, as well as prolonged extraction times, may result in artificial formation of 4HNE. For this reason, an extraction temperature of 42°C and a stirring time of 50 min were chosen to prevent the artificial increase of 4HNE during the extraction process.

Secondly, parameters that affect the headspace acylation of the extracted PFB-oxime derivatives namely temperature and time were evaluated. The stir bars were exposed to acetic acid anhydride vapors at various temperatures (i.e. 60 to 90°C) to determine the efficiency of the derivatization reaction at 20 min exposure time. Figure 7.6 illustrates that 70°C resulted in the highest conversion of the oxime derivatives to the corresponding oxime-acetates. The optimum time required to form the acetate derivatives of the extracted compounds was investigated by exposing the stir bars to the acetic acid anhydride vapors for various lengths of time i.e. 10 to 60 min. An optimal yield was obtained at 20 min.

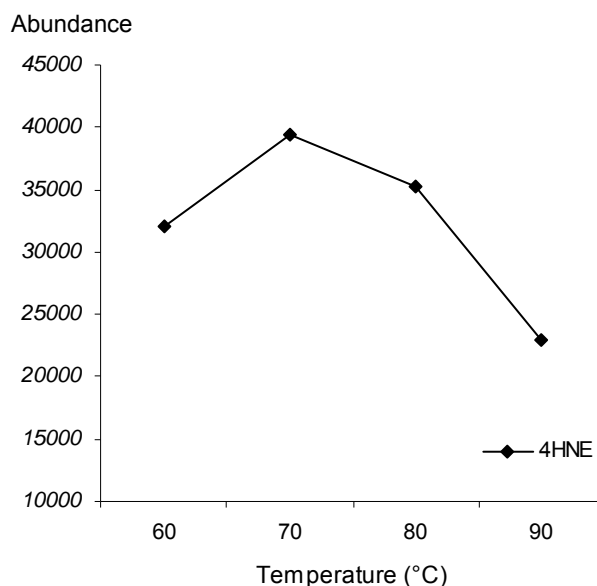


Figure 7.6 Headspace derivatization temperature. A 1 mL urine sample containing 1 ng of 4HNE was analyzed by SBSE-HD-TD-GC/MS. Conditions are given in the experimental section.

7.3.3 Figures of merit

The optimized SBSE-HD-TD-GC/MS procedure was validated with regard to linearity, detection and quantitation limit, precision and relative (versus IS) recovery. Calibration curves were obtained by spiking 1 mL urine samples with increasing amounts of 4HNE, namely 0.5, 1, 3 and 5 ng.mL⁻¹. A fixed amount of the internal standard was added (i.e. 1 ng.mL⁻¹). The slope of the curve was obtained by plotting the peak area ratios of 4HNE corrected for the IS against the concentration of the analytes. The method was linear over the chosen concentration range with a correlation coefficient (r^2) of 0.997. The detection and quantitation limits were calculated using the peak areas of 4HNE in urine samples of known concentration. The LOD corresponded to 22 pg.mL⁻¹ urine at a signal-to-noise level (S/N) of 3, whereas the LOQ was estimated at 75 pg.mL⁻¹ urine (S/N = 10). The precision of the SBSE method was determined by calculating the relative standard deviation (RSD %) of 5 replicate analyses that were made on 3 different days in urine samples containing 1 ng.mL⁻¹ 4HNE. The intra-day repeatability was 3.3% and the inter-day repeatability was 2.8%, 5.6% and 7.2%, respectively. The recovery was calculated by adding 4HNE to urine samples at two different concentration levels namely 0.8 ng.mL⁻¹ and 2 ng.mL⁻¹. The results were obtained by subtracting the peak areas obtained for the

spiked urine samples from those obtained for the non-spiked samples. The calculated recoveries were 95% and 104% for each of the spiked levels. A summary of the figures of merit is shown in Table 7.1.

Table 7.1 Figures of merit obtained for 4HNE analyzed by SBSE-HD-TD-GC/MS.

Parameter	Result
^a LOD (pg.mL ⁻¹)	22.5
^b LOQ (pg.mL ⁻¹)	75
Correlation coefficient (r ²)	0.997
Recovery (%)	
• 0.8 ng.mL ⁻¹	95
• 2 ng.mL ⁻¹	104
Intra-day repeatability (RSD%)	3.3
Inter-day repeatability (RSD%)	7.2

^a Limit of detection; ^b Limit of quantitation

7.3.4 Measurement of 4HNE in urine samples

Urine samples obtained from 10 volunteers were analyzed by the developed SBSE-HD-TD-GC/MS method. The measured 4HNE concentrations in most of the samples were below 1 ng.mg⁻¹ creatinine, but two samples showed highly elevated levels of 4HNE (Table 7.2). One of the volunteers suffered from asthma, whereas the other had smoked heavily for a number of years. Both conditions have previously been associated with increased levels of oxidative stress [36, 37]. However, it is not known whether these specific concentrations could be attributed to asthma or smoking alone. More in-depth and broader investigations are required. No apparent agreement could be made between the use of antioxidants and the reported 4HNE concentrations, but no information was given regarding the type of antioxidants used or the frequency of their use. The mean \pm SD for the seemingly healthy volunteers (i.e. those that had 4HNE levels below 1 ng) were 0.39 ± 0.11 ng.mg⁻¹ creatinine. These concentrations are in agreement with previously published results obtained for healthy controls [20].

This study describes the analysis of 4HNE in random-collected spot urine samples. The measured amounts were normalized to reflect the overall 24 h excretion of 4HNE and to control for possible urine concentration effects. A number of normalization techniques were considered. The most commonly used method involves adjustment of the measured concentration against the creatinine content in the sample. However, this method has been criticized because creatinine excretion has been shown to vary considerably over short intervals [38]. The modified specific gravity (SG) normalization technique of Vij and Howell [33] appeared to be the most promising method. This technique incorporates SG and creatinine, and has been shown to result in the lowest between-subject variability for xenobiotic biomarkers measured in spot urine samples of smokers [34]. However, the values calculated by this method did not always compare well with 4HNE values obtained by the creatinine normalization technique (results not shown). Therefore, we investigated using a modification of the equation introduced by Vij and Howell (Section 7.2.3), which gave much improved results. It was also observed that the values calculated by this modified equation were highly correlated with the actual measured concentrations of 4HNE, as compared to values that were obtained by the creatinine normalization technique (see Table 7.2). Nevertheless, the application of this equation for the normalization of 4HNE in spot urine samples should be validated.

Table 7.2 Summary of data obtained for the analysis of urine samples by SBSE-HD-TD-GC/MS. Some of the responses to the questionnaires are included.

No.	Age	Gender	4HNE (ng.mL ⁻¹) ^a	4HNE-Creat ^b	4HNE-SG-Creat ^c	Antioxidants
1.	19	M	0.31	0.20	0.31	+
2.	20	F	0.39	0.29	0.41 ^d	-
3.	19	M	0.35	1.08	0.46	+
4.	21	M	1.41	1.07	1.37 ^e	+
5.	19	M	0.62	0.35	0.52	-
6.	19	M	0.25	0.14	0.25	+
7.	23	F	0.32	0.19	0.30	-
8.	28	M	0.36	0.52	0.33	+
9.	39	M	2.48	1.73	2.45 ^f	+
10.	20	F	0.68	0.42	0.54	-

^aActual 4HNE concentrations measured by SBSE-HD-TD-GC/MS; ^b4HNE normalized for the creatinine concentration in each sample (ng.mg⁻¹ creatinine); ^c4HNE normalized for urine specific gravity using Eq. 1 and 2 in Section 7.2.3. These values were then corrected for the creatinine content in the samples using Eq. 3 (ng.mg⁻¹ creatinine); ^dSmoked occasionally; ^ePerson suffering from asthma; ^fDaily smoker.

7.4 Conclusion

4HNE is one of the most cytotoxic end-products of lipid peroxidation. Reliable, non-invasive measurements of this aldehyde are required to further elucidate the role of free radicals in the pathophysiology of human diseases. In this investigation, a highly sensitive analytical method was developed for the trace measurement of 4HNE in urine samples. The method combines a solventless sample enrichment technique, namely SBSE, with two derivatization steps, followed by thermal desorption and GC/MS. Detection limits of 22 pg.mL⁻¹ urine could be achieved, which is much lower than the physiological quantities of 4HNE excreted daily. Furthermore, the measurement of 4HNE in random-collected spot urine samples has several advantages, such as convenience, preservation of sample integrity and greater compliance among participants. These advantages and the simplicity of the analytical technique highlight the potential that this method could be applied to larger clinical trials.

References

- [1] Yang Y, Sharma R, Sharma A, Awasthi S, Awasthi YC. *Acta Biochim. Pol.* 50 (2003) 319.
- [2] Esterbauer H, Cheeseman KH, Dianzani MU, Poli G, Slater TF. *Biochem. J.* 208 (1982) 129.
- [3] Benedetti A, Comporti M, Esterbauer H. *Biochim. Biophys. Acta* 620 (1980) 281.
- [4] Esterbauer H, Schaur RJ, Zollner H. *Free Radic. Biol. Med.* 11 (1991) 81.
- [5] Eckl PM. *Mol. Aspects Med.* 24 (2003) 161.
- [6] Benedetti A, Barbieri L, Ferrali M, Casini AF, Fulceri R, Comporti M. *Chem. Biol. Interact.* 35 (1981) 331.
- [7] Haenen GR, Tai Tin Tsoi JN, Vermeulen NP, Timmerman H, Bast A. *Arch. Biochem. Biophys.* 259 (1987) 449.
- [8] Poot M, Verkerk A, Koster JF, Esterbauer H, Jongkind JF. *Eur. J. Biochem.* 162 (1987) 287.
- [9] Benedetti A, Casini AF, Ferrali M, Comporti M. *Biochem. J.* 180 (1979) 303.
- [10] Schaur RJ. *Mol. Aspects Med.* 24 (2003) 149.
- [11] Rosenfeld ME, Khoo JC, Miller E, Pathasarathy S, Palinski W, Witztum JL. *J. Clin. Invest.* 87 (1991) 90.
- [12] Liu Q, Raina AK, Smith MA, Sayre LM, Perry G. *Mol. Aspects Med.* 24 (2003) 305.
- [13] Toyokuni S, Uchida K, Okamoto K, Hattori-Nakakuki Y, Hiai H, Stadtman ER. *Proc. Natl. Acad. Sci. U.S.A.* 91 (1994) 2616.
- [14] Kim S, Gallaher DD, Csallany AS. *Lipids* 34 (1999) 489.
- [15] Goldring C, Casini AF, Maellaro E, Del Bello B, Comporti M. *Lipids* 28 (1993) 141.
- [16] Bruenner BA, Jones AD, German JB. *Anal. Biochem.* 241 (1996) 212.
- [17] Luo XP, Yazdanpanah M, Bhooi N, Lehotay DC. *Anal. Biochem.* 228 (1995) 294.
- [18] Rauli S, Del Puppo M, Magni F, Galli Kienle M. *J. Biochem.* 123 (1998) 918.
- [19] Sullivan S, Roberts RJ, Spitz D. *J. Chromatogr.* 578 (1992) 9.
- [20] Meagher EA, Barry OP, Burke A, Lucey MR, Lawson JA, Rokach J, FitzGerald GA. *J. Clin. Invest.* 104 (1999) 805.
- [21] Van Kuijk FJGM, Siakotos AN, Fong LG, Stephens RJ, Thomas DW. *Anal. Biochem.* 224 (1995) 420.

- [22] Baltussen E, Sandra P, David F, Cramers CA. *J. Micro. Sep.* 11 (1999) 737.
- [23] Arthur CL, Pawliszyn J. *Anal Chem* 62 (1990) 2145.
- [24] Ochiai N, Sasamoto K, Daishima S, Heiden AC, Hoffman A. *J. Chromatogr. A* 986 (2003) 101.
- [25] Cancho B, Ventura F, Galceran MT. *J. Chromatogr. A* 943 (2001) 1.
- [26] Bao M, Pantani F, Griffini O, Burrini D, Santianni D, Barbieri K. *J. Chromatogr. A* 809 (1998) 75.
- [27] Sandra P, Tienpont B, Vercammen J, Tredoux A, Sandra T, David F. *J. Chromatogr. A* 928 (2001) 117.
- [28] Kawaguchi M, Ito R, Sakui N, Okanouchi N, Saito K, Nakazawa H. *J. Chromatogr. A* 1105 (2006) 140.
- [29] Okeyo PD, Snow NH. *J. Micro. Sep.* 10 (1998) 551.
- [30] Shao Y, Marriott P, Hügel H. *Chromatographia* 57 (2003) S349.
- [31] Vasiliades J. *Clin. Chem.* 22 (1976) 1664.
- [32] Heinegard D, Tiderstrom G. *Clin. Chim. Acta* 43 (1973) 305.
- [33] Vij HS, Howell S. *Am. Ind. Hyg. Assoc. J.* 59 (1998) 375.
- [34] Heavner DL, Morgan WT, Sears SB, Richardson JD, Byrd GD, Ogden MW. *J. Pharm. Biomed. Anal.* 40 (2006) 928.
- [35] David F, Tienpont B, Sandra P. *LCGC N. Am.* 21 (2003) 1.
- [36] Burke A, FitzGerald GA. *Prog. Cardiovasc. Dis.* 46 (2003) 79.
- [37] Bowler RP, Crapo JD. *J. Allergy Clin. Immunol.* 110 (2002) 349.
- [38] Curtis G, Fogel M. *Psychosom. Med.* 32 (1970) 337.

Chapter 8

Measurement of the T/ET ratio in HIV infection^{III}

Abstract

A simple method is described for the measurement of testosterone (T) and epitestosterone (ET) in human urine samples. The deconjugated steroids were extracted directly from the samples by stir bar sorptive extraction (SBSE) and derivatized *in situ* on the stir bar by headspace acylation prior to thermal desorption and gas chromatography/mass spectrometry (GC/MS). Extraction and derivatization parameters, namely salt addition, temperature and time, were optimized to improve the recovery of T and ET by SBSE. The limits of quantification (S/N 10) were 0.9 ng.mL^{-1} for T and 2.8 ng.mL^{-1} for ET. Quantification of the steroids in urine samples was performed using standard addition to avoid the influence of matrix effects. The method was applied for the measurement of urinary T and ET in a group of healthy volunteers and HIV+ patients. Decreased levels of T were detected in the HIV+ group, whereas the excretion of ET was comparable for the two groups. Further clinical research is required to elucidate the biomarker significance of the T/ET ratio in HIV infection.

Keywords:

Gas chromatography/mass spectrometry, stir bar sorptive extraction, urine samples, testosterone, epitestosterone, human immunodeficiency virus.

8.1 Introduction

Testosterone is the main androgenic hormone in humans. The detection of this steroid in biological samples is commonly employed to identify hypogonadism in men, hyperandrogenic disorders in women, and virilizing adrenocortical conditions in children [1]. Testosterone (T) is frequently measured in blood, but analysis of the glucuronide-conjugate in urine samples has been preferred due to the daily cyclic secretion of T. Urine levels of T are commonly utilized as an index of androgen production, since it is essentially free of the short-term fluctuations observed in blood samples [2].

The measurement of T has gained a lot of interest over the past few years due to the illicit use of synthetic T in competitive sport. The official method approved by the World Anti-Doping Agency to detect the misuse of this substance by athletes, is based on the measurement of the testosterone:epitestosterone ratio. Epitestosterone (ET) is the 17α -epimer of T that was first reported by Clark and Kochakian [3] in 1947. Compared to T, little is known about the metabolism and physiological significance of ET. It has been reported that the production rate of ET is only 3% of T, but that the urinary excretion rate is about 1/3 of that of T in adult males [4]. ET is also not metabolized to or from T [5]. Furthermore, it has been demonstrated that ET may have some antiandrogenic effects [6, 7] and that it could be a regulator of androgenic hormone action [8].

So far, a large number of analytical methods have been developed for the measurement of natural steroids in biological samples. An extensive review on this subject was recently published by Shimada et al. [9]. The most frequently used techniques include liquid chromatography (LC) with UV detection and gas chromatography combined with mass spectrometry (GC/MS). Both techniques require extensive sample preparation procedures before the compounds can be analyzed. A typical sample preparation scheme for the analysis of T and ET by GC/MS includes: concentration of the analytes by solid-phase extraction (SPE); enzymatic hydrolysis with β -glucuronidase; and extraction with diethyl ether [10, 11]. A derivatization step is also included to improve the volatility and thermal stability of the extracted analytes. Several derivatization reagents have been used [12]. *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) in combination with a catalyst is

most frequently used to form the *O*-trimethylsilyl ethers of the deconjugated steroids [13, 14].

Recently, there has been an increased interest in the development of simple, cost-effective and solvent-free sample preparation procedures. Sorptive extraction techniques, such as stir bar sorptive extraction (SBSE) and solid phase microextraction (SPME), have been developed for the direct extraction of solutes from aqueous matrices [15, 16]. By using these techniques, organic solvents that are often toxic and harmful to the environment can be avoided. The principle of sorptive extraction is based on the diffusion of analytes between an aqueous sample phase and a polymeric partitioning phase such as polydimethylsiloxane (PDMS). Enrichment of the analytes by the PDMS layer is determined by the PDMS-water partition coefficient ($K_{\text{PDMS/w}}$) or the octanol-water distribution coefficient ($K_{\text{o/w}}$) of the analytes. High recoveries (i.e. nearly 100%) can be achieved from aqueous solutions when the $\log K_{\text{o/w}}$ value exceeds 2.7 [17]. Therefore, androgenic hormones are highly suitable for extraction by SBSE or SPME (i.e. $\log K_{\text{o/w}}$ values > 3 ; K_{ow} [18]).

Only a few analytical methods that include sorptive extraction techniques have been developed for the detection of T in humans [19, 20]. To date, no reports have been published on the measurement of the T/ET ratio using SBSE or SPME. Okeyo and Snow [19] developed the first SPME method for the detection of T in urine. These authors also introduced a headspace derivatization technique in which silylation of the extracted steroids was performed in situ on the SPME fiber [21]. Different variations of this technique have been used for the headspace derivatization of compounds extracted by SBSE. For example, 17 β -estradiol was derivatized by in-tube silylation using BSTFA [22], whereas 4-hydroxynonanal was derivatized directly on the stir bar in a specially modified headspace vial with acetic acid anhydride [23]. This contribution describes the development of a SBSE method to determine the T/ET ratio in human urine samples. The extraction of T and ET is accomplished by aqueous SBSE, followed by headspace acylation of the extracted steroids directly on the stir bar coating. The method has been applied for investigation of the T/ET ratio in patients infected with the human immunodeficiency virus (HIV).

8.2 Experimental

8.2.1 Chemicals and materials

Anhydrous sodium acetate, glacial acetic acid, sodium chloride, testosterone, epitestosterone, progesterone, β -glucuronidase from *Helix Pomatia* (Type H-2), and pyridine were purchased from Sigma Aldrich (Johannesburg, South Africa). Acetic acid anhydride and dichloromethane were obtained from Merck (Darmstadt, Germany). Methanol was supplied by Riedel-de Haën (Sigma Aldrich, Johannesburg, South Africa). Fifteen mL screw cap vials were obtained from Supelco (Sigma Aldrich, Johannesburg, South Africa). The 2 mL headspace vials were prepared by E Ward (University of Stellenbosch) from 2 mL autosampler vials that were purchased from Agilent Technologies (Chemetrix, Johannesburg, South Africa) [23]. Sterile 50 mL centrifuge tubes were supplied by LASEC (Cape Town, South Africa). A 10 position magnetic stirrer combined with a convection oven was designed and built by J. Blom and colleagues (Department of Mechanical Engineering, University of Stellenbosch). Twister stir bars (10 mm \times 0.5 mm d_f PDMS) were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany). The stir bars were pre-conditioned by sonication in a 1:1 mixture of dichloromethane/methanol for 5 min after which they were heated at 280°C for 10 min under a nitrogen flow of 50 mL.min⁻¹.

8.2.2 Solutions

Concentrated solutions of T and ET (4 mg.mL⁻¹) were prepared in methanol. The solutions were diluted to prepare the quality control standards at concentrations of 5 μ g.mL⁻¹ and 50 μ g.mL⁻¹, respectively. Progesterone was used as internal standard and a solution of 10 μ g.mL⁻¹ was prepared in methanol. All solutions were stored at -20°C until used.

8.2.3 Urine samples

Urine samples were obtained from 7 HIV positive patients (3 females and 4 males) who attended an HIV Clinic at a local Day Hospital in Stellenbosch (South Africa). The patients were previously diagnosed as HIV+ and had CD4⁺ T lymphocyte counts ranging

from 66 – 283 cells.mm⁻³. All CD4⁺ measurements were obtained > 1 month prior to the study. The control samples were obtained from 7 healthy volunteers (3 females and 4 males) who were not asked to reveal their HIV status. Written consent was obtained from all the participants before enrollment in the study. The urine samples were randomly collected between 9h00 and 12h00 each day and were immediately placed on dry ice before being stored at -25°C. Urine osmolality and creatinine levels were determined by Pathcare (Cape Town, South Africa). Approval for the study was obtained from the Committee for Human Research at the University of Stellenbosch.

8.2.4 Enzymatic hydrolysis, SBSE and headspace derivatization procedure

The urine samples were thawed overnight at 4°C and 1.4 mL aliquots were transferred to 15 mL screw cap vials containing 1.4 mL 2 M sodium acetate buffer (pH 4.6). Twenty µL of a crude solution of *Helix Pomatia* was added to the sample mixtures and the vials were incubated at 55°C for 3 hours [24]. Following hydrolysis of the conjugated steroids, the vials were briefly vortexed and allowed to cool to room temperature. Two mL of the sample mixture corresponding to 1 mL urine were transferred to clean 15 mL vials, and 0.2 mL methanol and 4 mL 5.2 M sodium chloride were added. The pH of the solution was ca. 4.5 prior to the extraction of the deconjugated steroids. A conditioned stir bar was placed in each vial and the samples were stirred at 1100 rpm for 60 min using a home-built multiposition magnetic stirrer/oven that was heated to 50°C. After SBSE, the stir bars were removed from the sample vials, washed with distilled water and dried with tissue paper. The stir bars were placed inside 2 mL headspace vials as described previously [23], and 20 µL acetic acid anhydride and 20 µL pyridine were added. The vials were incubated at 90°C for 30 min to form the acetate derivatives of the extracted compounds. The stir bars were removed from the headspace vials, washed and dried, and placed in the glass desorption tubes of a TDS A2 autosampler (Gerstel).

8.2.5 Thermal desorption – gas chromatography/mass spectrometry

Thermal desorption was accomplished in the solvent-venting mode using helium at a flow rate of 60 mL.min⁻¹. The temperature of the TDS 2 was increased from 50°C to 150°C (held for 1 min), and from 150°C to 300°C (held for 10 min) at a rate of 60°C.min⁻¹. The split-valve was closed after 2 min. The desorbed compounds were transferred to a programmable temperature vaporization (PTV) inlet (CIS 4, Gerstel), and were cryofocussed at 10°C using liquid nitrogen. The TDS transfer line was held at 320°C. Sample injection onto the capillary column was accomplished by programming the PTV from 10°C to 300°C (held for 2 min), and from 300°C to 320°C (held for 3 min) at a rate of 12°C.s⁻¹. The splitless time was 2.5 min.

GC/MS analyses were carried out on an Agilent 6890 gas chromatograph that was interfaced with a 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA). The derivatives were separated on a HP-5MS fused silica capillary column (30 m × 0.25 mm ID × 0.25 μm, Agilent) using helium as carrier gas at a flow rate of 1.25 mL.min⁻¹. The oven temperature was programmed from 70°C (held for 2 min) to 240°C (held for 10 min) at 10° C.min⁻¹, and from 240°C to 300°C (held for 8 min) at 20°C.min⁻¹. The total run time was 40 min. Mass spectra of the derivatized compounds were recorded in full scan, whereas quantification was performed in the selected ion monitoring (SIM) mode. For SIM, two or three ions characteristic for the analytes were monitored at 100 ms each, namely *m/z* 228 and 288 for T, *m/z* 270 and 330 for ET and *m/z* 229, 272 and 314 for progesterone (PG), the internal standard. The underlined values are the ions used for quantification.

8.3 Results and discussion

8.3.1 Analysis of T and ET by SBSE-HD-TD-GC/MS

The mass spectra of T and ET were obtained in full scan by analyzing pure water samples spiked at a concentration of 0.5 μg.mL⁻¹. The compounds were extracted directly from the aqueous samples by SBSE, after which the stir bars were placed inside modified autosampler vials [23] to prepare the acetate derivatives of the extracted compounds.

Headspace derivatization (HD) of T and ET was accomplished by exposing the PDMS stationary phase to acetic acid anhydride and pyridine vapors at 90°C. The derivatives were thermally desorbed (TD) and finally analyzed on-line by GC/MS.

The mass spectra of the derivatives (Figure 8.1) show that most of the fragment ion peaks in the higher mass range are formed by the loss of different combinations of [CH₃COOH], [CH₂=C=O] and [CH₃]. The formation of ions in the low mass range was reported previously [25]. For SIM analysis, two characteristic ions were selected for each compound, namely *m/z* 228 and 288 for T, and *m/z* 270 and 330 for ET. The first ion was used to identify the presence of co-eluting compounds during the analysis, whereas the second ion was used for the quantification of T and ET. Some representative SIM chromatograms are shown in Figure 8.2.

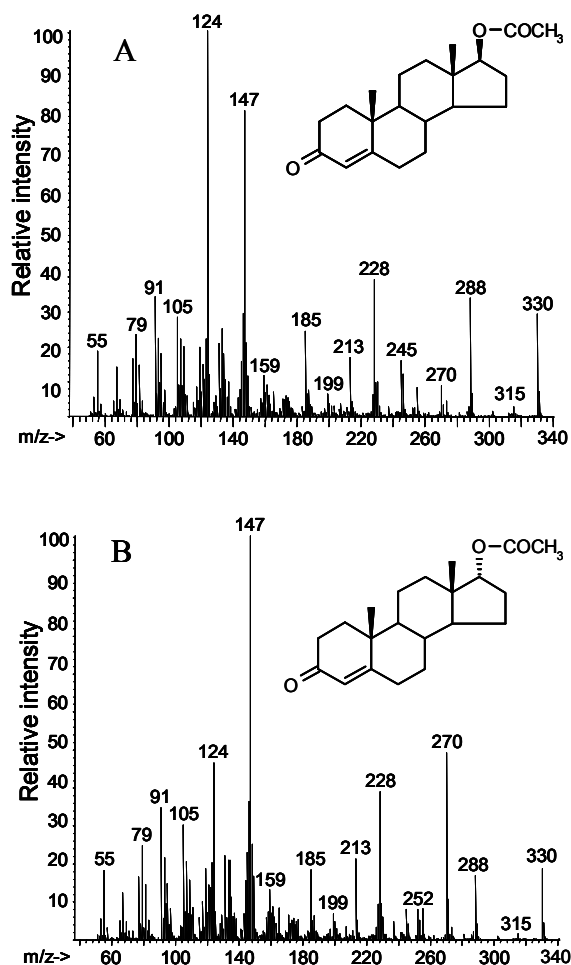


Figure 8.1 Mass spectra of the acetate derivatives of A) testosterone and B) epitestosterone.

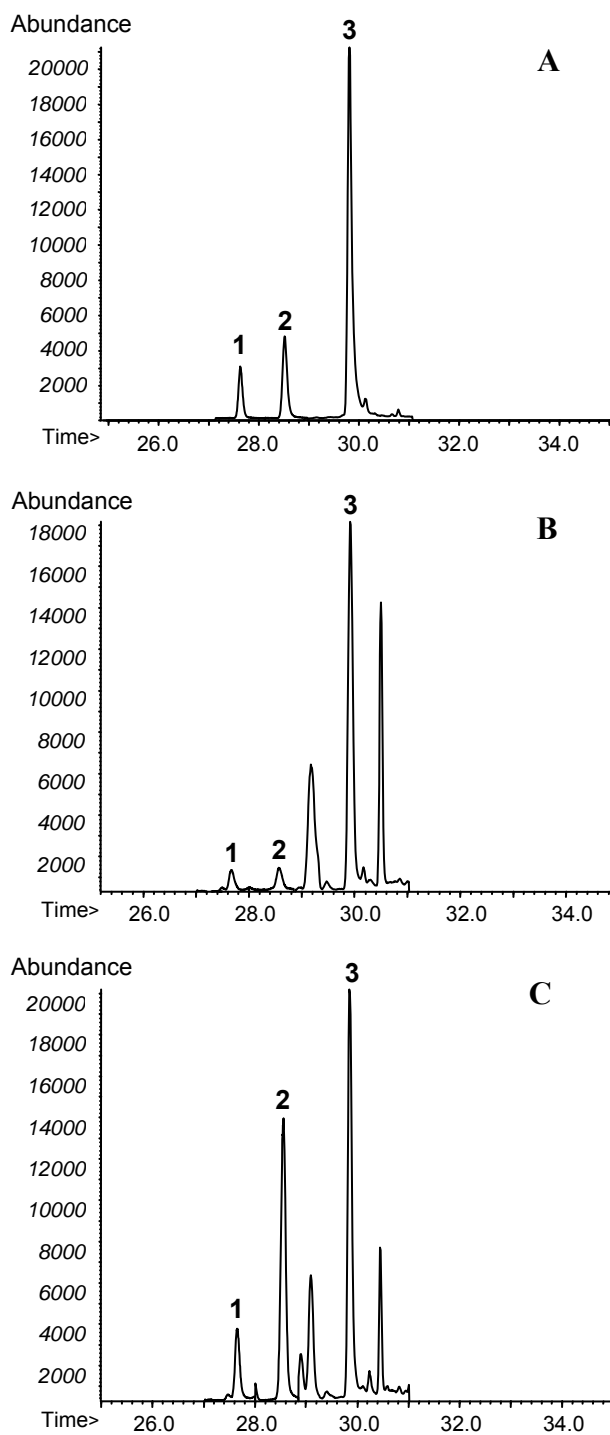


Figure 8.2 SIM chromatograms of the acetate derivatives of 1) ET, 2) T and 3) the internal standard PG. The upper trace (A) corresponds to the analysis of a water sample spiked with 10 ng.mL⁻¹ of T, ET and PG; the center trace (B) and the lower trace (C) correspond to the analyses of urine samples obtained from a female and a male volunteer, respectively. Both (B) and (C) contain 10 ng.mL⁻¹ of the IS. The SIM ions were m/z 330 for ET, m/z 288 for T and m/z 314 for PG. Analytical conditions are given in the experimental section.

Accurate identification of the compounds was based on the retention times of the respective analytes as well as on the compound-specific ions. Figure 8.2-A was obtained after analyzing a water sample spiked with 10 ng of T and ET, and 10 ng of the internal standard (PG) per mL. The retention times are 27.6, 28.5, and 29.8 min for ET, T and PG, respectively. Further experiments performed in urine samples containing only 10 ng.mL⁻¹ of the internal standard (PG), show that the selected ions are specific for the detection of T and ET in complex biological matrices. The samples were obtained from a female (Figure 8.2-B) and from a male (Figure 8.2-C) volunteer.

8.3.2 Method optimization

Experimental parameters that affect the sensitivity of the SBSE procedure were investigated to increase the overall yield of the developed method. Urine samples (female), spiked with 50 ng.mL⁻¹ of T and ET and 50 ng.mL⁻¹ of the IS were used for method optimization. It is well known that the extraction efficiencies for a large number of compounds can be enhanced by increasing the ionic strength of the sample solution [26]. Figure 8.3 demonstrates that the addition of 2.5 M and 5.2 M NaCl to the samples increased the sorptive extraction of T and ET due to the decreased solubility of the compounds in aqueous solution (i.e. the ‘salting out’ effect).

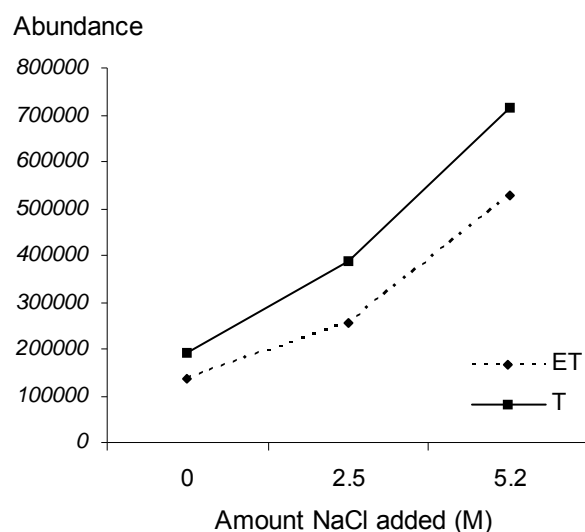


Figure 8.3 SBSE optimization. Effect of salt concentration on the extraction efficiency of T and ET. Urine samples spiked with 50 ng.mL⁻¹ of both compounds were analyzed by SBSE-HD-TD-GC/MS.

Other parameters that may affect the amount of analytes extracted by the stir bar include the time of extraction and the temperature of the sample solution. The effect of temperature on the extraction of T and ET was investigated at 40, 50, 60, 70 and 80°C while the extraction time was held constant at 60 min (Figure 8.4-A). The peak areas of T increased slightly up to 80°C, whereas the peak areas of ET started to decline after 50°C. Therefore, 50°C was chosen as the optimal temperature for the extraction of the compounds. The extraction-time profiles of T and ET were obtained by stirring the samples for various lengths of time which ranged from 30 – 120 min (Figure 8.4-B). The compounds appeared to reach extraction equilibrium after approximately 45 min, but further evaluation of the method using different extraction times showed that the repeatability ($n = 4$) of T and ET improved when the samples were stirred for a longer period (i.e. 11.8% for T and 15% for ET at 45 min versus 2% for T and 5.3% for ET at 60 min). Therefore, 60 min was chosen for the extraction of the compounds.

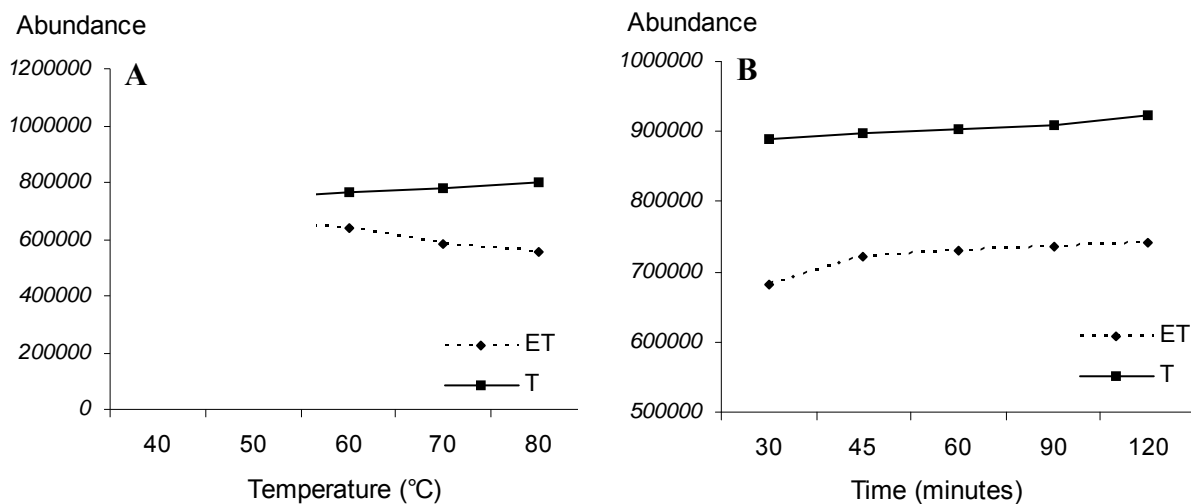


Figure 8.4 SBSE optimization. A. Effect of temperature; B. Effect of time on the extraction efficiency of T and ET. Urine samples spiked with 50 ng.mL⁻¹ of both compounds were analyzed by SBSE-HD-TD-GC/MS.

Lastly, parameters that affect the headspace acylation of the extracted compounds, namely temperature and time were evaluated. The stir bars were exposed to acetic anhydride and pyridine vapors for 30 min at various temperatures ranging from 60°C to 90°C. Figure 8.5-A demonstrates that the extracted compounds were almost completely derivatized at 80°C. A small increase in the peak areas of T and ET was observed when the stir bars were incubated at a higher temperature (i.e. 90°C). In order to select the most efficient headspace derivatization temperature, the reproducibility of the method was evaluated at 80°C and 90°C, respectively. Repeatability ($n = 4$) for ET was slightly improved when a higher headspace derivatization temperature was used (i.e. 2.8% for T and 5.8% for ET at 80°C versus 3.2% for T and 4% for ET at 90°C). Therefore, 90°C was selected as the optimal temperature for the formation of the acetate derivatives of T and ET. The optimum time required for acylation of the extracted compounds was investigated by exposing the stir bars to acetic anhydride and pyridine vapors for various lengths of time (i.e. 15-60 min) at 90°C. As shown in Figure 8.5-B, the optimal yield of the acetate derivatives was obtained after 30 min.

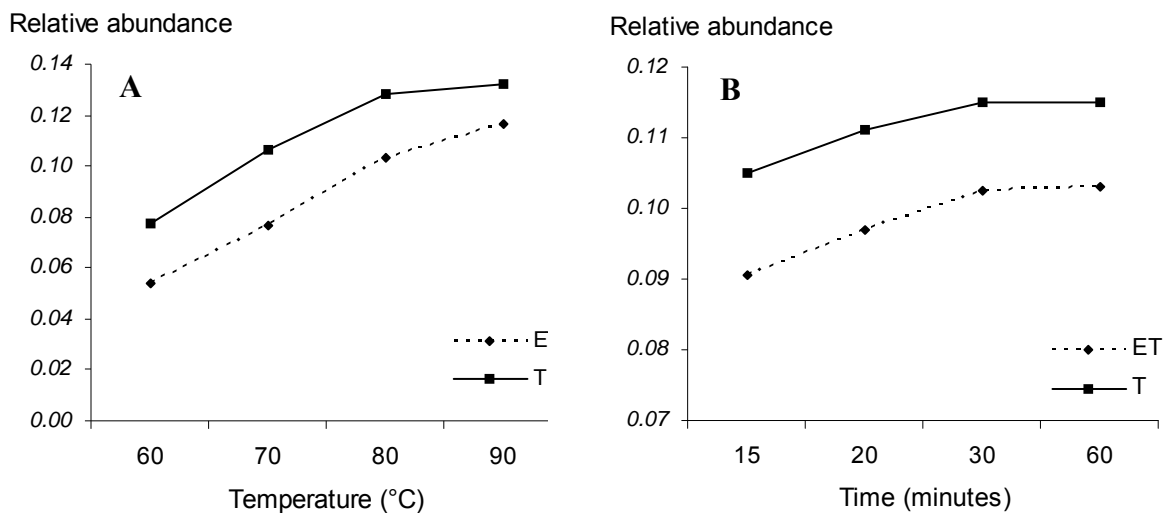


Figure 8.5 SBSE headspace derivatization optimization. A. Temperature; B. Time. For other experimental conditions see Figure 8.3.

8.3.3 Figures of merit

The linearity of the SBSE-HD-TD-GC/MS method was determined by internal standard calibration using blank steroid-free urine samples that were spiked at 4 different concentration levels of T and ET, namely 2.5, 7.5, 15 and 30 ng.mL⁻¹ for females and 25, 75, 150 and 300 ng.mL⁻¹ for males. A fixed amount of the internal standard (PG) was added (i.e. 10 ng.mL⁻¹ urine). Steroid-free urine was obtained by passing three 5 mL portions of a urine sample through a C₁₈-SPE cartridge. The fractions were combined and used as a sample matrix to construct the calibration curves. The curves were obtained by plotting the abundance of *m/z* 288 (T) and *m/z* 330 (ET) corrected for the IS (*m/z* 314) against the concentration of the analytes. The correlation coefficients (*r*²) obtained for T and ET were > 0.99.

To investigate if the sample matrix would have an effect on the extraction efficiency of the compounds from authentic urine, the calibration curves were set up in samples donated by a male and a female volunteer. The samples were spiked at the same concentration levels as described for the steroid-free urine samples. To construct the curves, the background concentrations of T and ET were subtracted before the peak area ratios of the compounds were plotted against the concentration of the analytes. Although the calibration curves were linear over the measured concentration range (*r*² > 0.99), the slopes of the curves differed significantly from those obtained for the steroid-free urine matrix (Figure 8.6). Dissolved compounds in biological samples may interfere with the sorptive extraction of analytes, either by direct binding of the compounds or by competitive interaction with the PDMS extraction phase. Furthermore, the composition of urine samples may vary due to the concentrating ability of the kidneys. Thus, it would be difficult to ensure reproducible extractions for T and ET when different urine samples were analyzed.

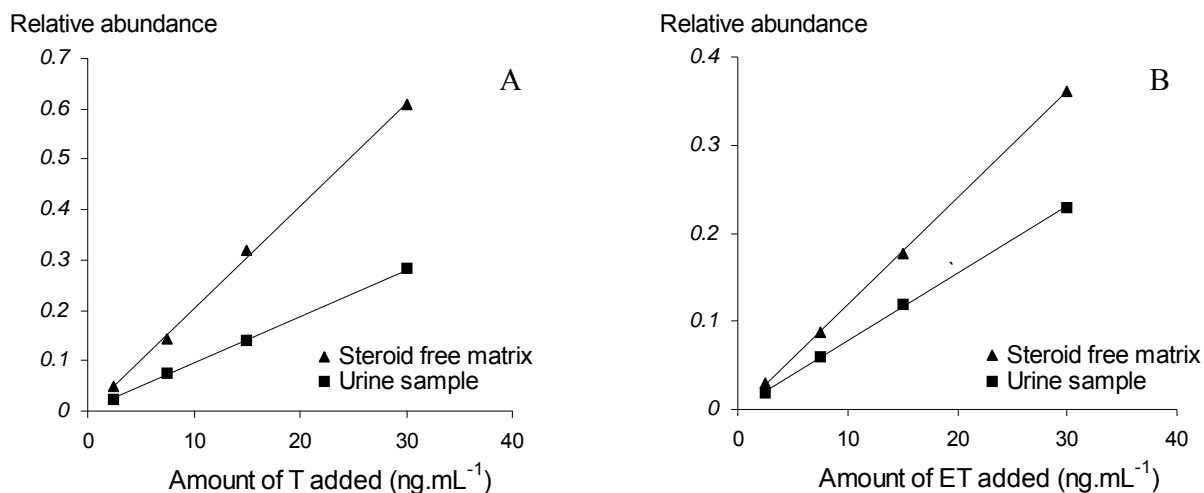


Figure 8.6 Effect of sample matrix on the extraction efficiency of T and ET. The samples were spiked with different concentrations of A) T and B) ET to construct the curves. A fixed amount of IS (10 ng.mL⁻¹) was added to the samples. The extraction, derivatization and chromatographic conditions are described in the experimental section.

To improve the accuracy of the results, the quantification of T and ET in urine samples was performed using the standard addition method. The samples were spiked at two concentration levels, namely 5 ng.mL⁻¹ and 10 ng.mL⁻¹ for female samples and 50 ng.mL⁻¹ and 100 ng.mL⁻¹ for male urine samples. The linear plots that were obtained after analyzing the spiked and non-spiked samples were extrapolated to the negative axis to calculate the original steroid concentrations. The detection and quantitation limits of T and ET were calculated in a female urine sample containing known amounts of the compounds. The limits of detection for T (*m/z* 288) and ET (*m/z* 330) were calculated at a signal to noise level (S/N) of 3 and were 0.3 ng.mL⁻¹ and 0.9 ng.mL⁻¹ urine, respectively. The limits of quantitation were calculated at a S/N of 10 and were 0.9 ng.mL⁻¹ for T and 2.8 ng.mL⁻¹ for ET. The precision of the SBSE method was estimated from the relative standard deviation of 12 replicate analyses (4 replicates were analyzed on 3 different days). The intra-day repeatability was 2.3% for T and 4.6% for ET, whereas the inter-day repeatability was 2.1%, 5.8% and 6.0% for T and 3.2%, 4.4% and 7.6% for ET.

8.3.4 Analysis of urine samples

To assess the performance of the technique, the SBSE-HD-TD-GC/MS method was used to detect low levels of urinary T and ET in a group of healthy volunteers and HIV+ patients (Table 8.1). T and ET for the healthy controls were within normal range [27], but decreased levels of T were found in most of the HIV+ patients. Significant differences in the T/ET ratios were observed between the two groups.

Table 8.1 Summary of data obtained for the controls and the HIV+ patients after analyzing the urine samples by SBSE-HD-TD-GC/MS.

No.	Age (years)	T (ng.mL ⁻¹) ^a	T (ng.mOsm.kg ⁻¹) ^b	ET (ng.mL ⁻¹)	ET (ng.mOsm.kg ⁻¹)	T/ET
<u>Females</u>						
<i>Controls:</i>						
1.	20	13.5	17.2	21.4	27.3	0.63
2.	23	6.5	7.0	8.6	9.3	0.75
3.	20	11.2	12.8	6.4	7.3	1.75
<i>HIV+:</i>						
4.	25	0.58	1.1	2.48	4.8	0.23
5.	29	0.47	2.8	5.5	33.0	0.08
6.	37	5.0	7.2	8.2	11.8	0.61
<u>Males</u>						
<i>Controls:</i>						
1.	28	25.0	56.1	11.7	26.2	2.14
2.	19	86.0	92.2	43.7	46.8	1.97
3.	19	38.1	39.7	65.8	68.5	0.58
4.	19	65.3	53.1	36.1	29.3	1.81
<i>HIV+:</i>						
5.	32	40.0	74.1	143.2	224.2	0.33
6.	26	3.0	7.8	23.7	61.6	0.13
7.	43	3.8	6.0	13.0	20.6	0.29
8.	32	2.1	6.1	14.7	43.0	0.14

^aActual concentrations of T and ET.

^bConcentrations of T and ET were corrected for urine osmolality.

Hypogonadism is frequently encountered in patients with HIV/AIDS, although the clinical significance of this abnormality is still unknown [28]. In a study by Dobbs et al. [29], it was shown that the incidence of hypogonadism is associated with the stage of the disease. The number of patients shown to be hypogonadal was 6% of asymptomatic HIV+ men, 44% of symptomatic HIV+ men and 50% of men with AIDS.

Apart from these endocrine abnormalities, other complications of HIV infection such as glomerular disease and renal failure may occur [30, 31]. The results obtained from urinary measurements should be interpreted cautiously due to the fact that impaired renal function may be present in these patients. We noticed that a lower amount of creatinine was excreted in 3 out of 7 patients in the HIV+ group (i.e. $\leq 5.4 \text{ mmol.L}^{-1}$). Factors such as malnutrition and low body mass may also contribute to the low amount of creatinine excreted [32]. To correct for any variations in the concentration/dilution of the samples, urine osmolality (see Table 8.1) was selected as a reference to bypass the problems associated with urine creatinine measurements [32-34].

8.3.5 Considerations on the measurement of the T/ET ratio in patients with HIV infection

Many severe illnesses are associated with a significant decrease in the circulating levels of T, i.e. a condition that is also known as hypogonadotropic hypogonadism [35-37]. This temporary change in endocrine function results from altered functioning of the hypothalamic-pituitary-gonadal (HPG) axis during critical illness [38]. Similar, but more lasting endocrine changes have been observed in patients with HIV/AIDS, which may occur due to direct infection of the adrenal glands with the virus [39] or as a result of nonspecific dysfunction of the HPG axis [40] as seen in other acute illnesses such as burns [41], myocardial infarction [42], brain injury [43] and major surgery [44]. Low levels of T in HIV+ patients can lead to a wide range of symptoms such as fatigue, depression, anemia, impaired sexual function [45] and muscle wasting [46]. Due to the considerable variation of T levels between individuals, a number of screening questionnaires have been developed to assist clinicians in diagnosing androgen deficiency [47]. In contrast, the problem of varying T levels in athletes has been overcome by measuring the T/ET ratio, which is based on the

nearly constant urinary excretion of T relative to ET in healthy individuals [48]. The T/ET ratio is widely used as an anti-doping control measure, but it has found relatively few applications in the clinical setting. Furthermore, very little scientific information is available regarding the excretion of T and ET during hypogonadism [49]. In one study by Hubl et al. [50], it was shown that patients with hypogonadism excreted submolar amounts of both T and ET. They also demonstrated that following the administration of human chorionic gonadotropin (hCG) it was possible to distinguish between patients with primary or secondary hypogonadism using the T/ET ratio.

In this study, the male and female HIV+ patients excreted much lower concentrations of T in comparison to levels obtained for the controls (Table 8.1). The results also demonstrate that the excretion of ET in the HIV+ group is comparable to concentrations obtained for the control subjects. Therefore, in this study population it appears that the urinary excretion of ET remained relatively unchanged during HIV infection. To illustrate, the excretion of T and ET for a healthy male volunteer (Males, No. 1 in Table 8.1) and for an HIV+ male patient (Males, No. 7 in Table 8.1) is shown in Figure 8.7. It should be mentioned that none of the patients in the study group received antiretroviral therapy or any other medications known to influence steroid production. Furthermore, it is not known whether these patients experienced hypogonadism because they were not specifically tested for this abnormality and the presence of symptoms usually associated with low T levels was not recorded. Only one other study reported the measurement of the T/ET ratio during HIV infection. Strawford et al. [51] showed that, for a group of eugonadal HIV+ men ($n = 24$) who received anti-retroviral therapy for at least 3 months, the baseline T/ET ratios were similar to published normal values (i.e. median, 1.1). More research is required to establish the clinical significance of the T/ET ratio in the context of HIV/AIDS.

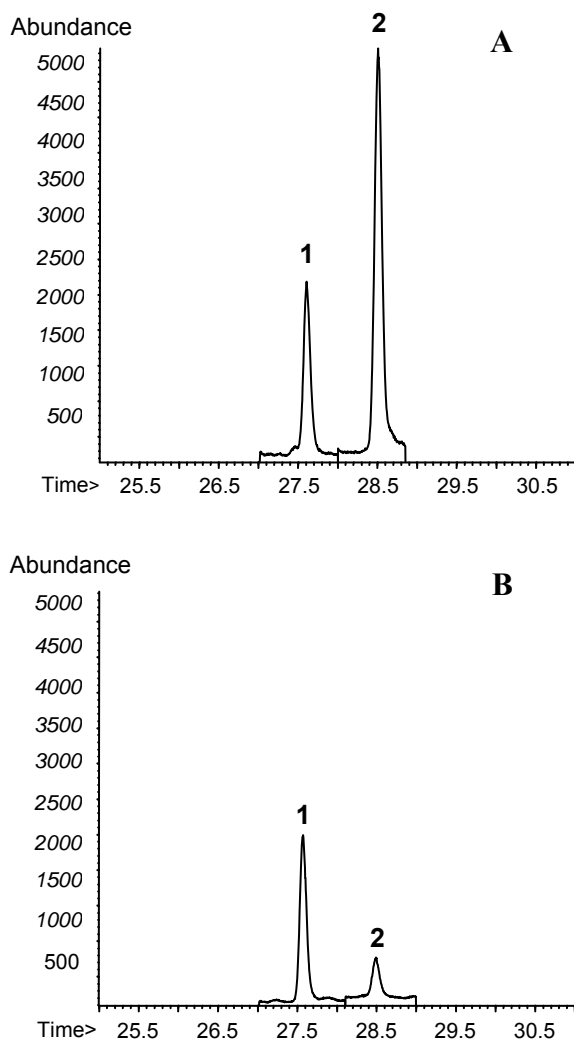


Figure 8.7 The excretion of 1) ET and 2) T in the urine of two males. The upper trace (A) corresponds to the analysis of a urine sample obtained from a healthy male volunteer (Males, No.1, Table 8.1); and the lower trace (B) corresponds to the analysis of a urine sample obtained from an HIV+ male patient (Males, No.7, Table 8.1). Conditions are given in the experimental section.

8.4 Conclusion

The method described for the measurement of T and ET in human urine samples is simple, rapid and avoids the use of organic solvents due to the direct extraction of the deconjugated steroids by aqueous SBSE. Headspace derivatization of the extracted compounds was performed to improve the volatility and thermal stability of the steroids prior to analysis by TD-GC/MS. The method was optimized, validated and specifically applied for the

measurement of the T/ET ratio in a group of healthy controls and HIV+ patients. Significant differences in the T/ET ratios between the two groups were detected. Decreases in the T/ET ratios were mainly due to the lower excretion of T in the HIV+ group, as compared to the excretion of ET that was comparable to levels obtained for the controls. Future work in this area should aim to clarify the clinical significance of the T/ET ratio in HIV infection and determine if the ratio can be used to detect hypogonadism during HIV/AIDS.

References

- [1] Pal S.B., *Clin. Chim. Acta* 33 (1971) 215.
- [2] Venturelli E., Cavalleri A., Secreto G., *J. Chromatogr. B* 671 (1995) 363.
- [3] Clark L.C., Kochakian C.D., *J. Biol. Chem.* 170 (1947) 23.
- [4] Wilson H., Lipsett M.B., *J. Clin. Endocrinol.* 26 (1966) 902.
- [5] Korenman S.G., Wilson H., Lipsett M.B., *J. Biol. Chem.* 239 (1964) 1004.
- [6] Nuck B.A., Lucky A.W., *J. Invest. Dermatol.* 89 (1987) 209.
- [7] Stárka L., Bičíková M., Hampl R., *J. Steroid Biochem.* 33 (1989) 1019.
- [8] Stárka L., *J. Steroid Biochem. Mol. Biol.* 87 (2003) 27.
- [9] Shimada K., Mitamura K., Higashi T., *J. Chromatogr. A* 935 (2001) 141.
- [10] Aguilera R., Becchi M., Grenot C., Casabianca H., Hatton C.K., *J. Chromatogr. B* 687 (1996) 43.
- [11] Saugy M., Cardis C., Robinson N., Schweizer C., *Baillieres Best Pract. Res. Clin. Endocrinol. Metab.* 14 (2000) 111.
- [12] Knapp D.R., *Handbook of analytical derivatization reactions*, Wiley, New York 1979.
- [13] Aguilera R., Hatton C. K., Catlin D.H., *Clin. Chem.* 48 (2002) 629.
- [14] Dehennin L., Lafarge P., Dailly P., Bailloux D., Lafarge J.P., *J. Chromatogr. B* 687 (1996) 85.
- [15] Baltussen E., Sandra P., David F., Cramers C.A., *J. Microcolumn Sep.* 11 (1999) 737.
- [16] Arthur C.L., Pawliszyn J., *Anal. Chem.* 62 (1990) 2145.
- [17] David F., Tienpont B., Sandra P., *LCGC N. Am.* 21 (2003) 1.
- [18] Meylan W.M., *Software Kowwin Version 1.66, SRC-LOGKOW*, SRC-ESC, Syracuse, USA 2000.
- [19] Okeyo P.D., Snow N.H., *J. Microcolumn Sep.* 10 (1998) 551.
- [20] Yang L., Luan T., Lan C., *J. Chromatogr. A* 1104 (2006) 23.
- [21] Okeyo P., Rentz S.M., Snow N.H., *J. High Resol. Chromatogr.* 20 (1997) 171.
- [22] Kawaguchi M., Ito R., Sakui N., Okanouchi N., Saito K., Nakazawa H., *J. Chromatogr. A* 1105 (2006) 140.
- [23] Stopforth A., Burger B.V., Crouch A.M., Sandra P., *J. Chromatogr. B* 834 (2006) 134.
- [24] Shackleton C.H., *J. Chromatogr.* 379 (1986) 91.
- [25] Zaretskii V.I., Wulfson N.S., Sadovskaya V.L., *Tetrahedron Lett.* 32 (1966) 3879.

- [26] Pawliszyn J., *Solid-Phase Microextraction: Theory and Practice*. Wiley-VHC, New York 1997.
- [27] Karila T., Kosunen V., Leinonen A., Tähtelä R., Seppälä T., *J. Chromatogr. B* 687 (1996) 109.
- [28] Poretsky L., Can S., Zumoff B., *Metabolism* 44 (1995) 946.
- [29] Dobs A.S., Dempsey M.A., Ladenson P.W., Polk B.F., *Am. J. Med.* 84 (1988) 611.
- [30] Szczech L.A., Gange S.J., van der Horst C., Bartlett J.A., Young M., Cohen M.H., Anastos K., Klassen P.S., Svetkey L.P., *Kidney Int.* 61 (2002) 195.
- [31] Lu T.C., Ross M., *Mt. Sinai J. Med.* 72 (2005) 193.
- [32] Miller R.C., Brindle E., Holman D.J., Shofer J., Klein N.A., Soules M.R., O'Connor K.A., *Clin. Chem.* 50 (2004) 924.
- [33] Chadha V., Garg U., Alon U.S., *Pediatr. Nephrol.* 16 (2001) 374.
- [34] Noormohamed S.E., Katseres J.K., Stapleton J.T., *Ren. Fail.* 20 (1998) 627.
- [35] Woolf P.D., Hamill R.W., McDonald J.V., Lee L.A., Kelly M., *J. Clin. Endocrinol. Metab.* 60 (1985) 444.
- [36] Spratt D.I., Cox P., Orav J., Moloney J., Bigos T., *J. Clin. Endocrinol. Metab.* 76 (1993) 1548.
- [37] Spratt D.I., *Best Pract. Res. Clin. Endocrinol. Metab.* 15 (2001) 479.
- [38] Van den Berghe G., Weekers F., Baxter R. C., Wouters P., Iranmanesh A., Bouillon R., Veldhuis J.D., *J. Clin. Endocrinol. Metab.* 86 (2001) 3217.
- [39] Reichert C.M., O'Leary T.J., Levens D.L., Simrell C.R., Macher A.M., *Am. J. Pathol.* 112 (1983) 357.
- [40] Sellmeyer D.E., Grunfeld C., *Endocr. Rev.* 17 (1996) 518.
- [41] Vogel A.V., Peake G. T., Rada R.T., 60 (1985) 658.
- [42] Wang C., Chan V., Tse T.F., Yeung R.T., *Clin. Endocrinol. (Oxf.)* 9 (1978) 249.
- [43] Rudman D., Fleischer A.S., Kutner M.H., Raggio J.F., *J. Clin. Endocrinol. Metab.* 45 (1977) 747.
- [44] Aono T., Kurachi K., Mizutani S., Hamanaka Y., Uozumi T., Nakasima A., Koshiyama K., *J. Clin. Endocrinol. Metab.* 35 (1972) 535.
- [45] Highleyman L., *BETA.* 16 (2004) 34.

-
- [46] Grinspoon S., Corcoran C., Miller K., Biller B.M., Askari H., Wang E., Hubbard J., Anderson E.J., Basgoz N., Heller H.M., Klibanski A., *J. Clin. Endocrinol. Metab.* 82 (1997) 1332.
- [47] Morley J.E., Perry H.M. III, Kevorkian R.T., Patrick P., *Maturitas* 53 (2006) 424.
- [48] Catlin D.H., Hatton C.K., Starcevic S.H., *Clin. Chem.* 43 (1997) 1280.
- [49] Van de Kerkhof D.H., de Boer D., Thijssen J.H., Maes R.A., *J. Anal. Toxicol.* 24 (2000) 102.
- [50] Hubl W., Siebert W., Büchner M., Hering H., *Endokrinologie* 58 (1971) S62.
- [51] Strawford A., Barbieri T., Van Loan M., Parks E., Catlin D., Barton N., Neese R., Christiansen M., King J., Hellerstein M.K., *JAMA* 281 (1999) 1282.

Chapter 9

Detection of E_1 and 17β - E_2 in hormone replacement therapy^{IV}

Abstract

The development of a sensitive and solvent-free method for the measurement of estrone (E₁) and 17 β -estradiol (17 β -E₂) in human urine samples is described. The deconjugated estrogens were derivatized *in situ* with acetic acid anhydride and the derivatives were extracted directly from the aqueous samples using stir bar sorptive extraction (SBSE). The compounds containing a secondary alcohol function are further derivatized by headspace acylation prior to thermal desorption and gas chromatography/mass spectrometry (GC/MS). A number of experimental parameters, including salt addition, temperature and time, were optimized to increase the recovery of E₁ and 17 β -E₂ by SBSE. The derivatization reactions were also optimized to obtain the highest yields of the acylated estrogens. Detection limits of 0.02 ng.mL⁻¹ and 0.03 ng.mL⁻¹ were obtained for E₁ and 17 β -E₂, respectively. The method was applied to determine the effect of conjugated equine estrogen intake on the excretion of E₁ and 17 β -E₂ in human urine samples. Increased levels of the endogenous estrogens were detected after administering a standard dose of Premarin to a female volunteer. Routine monitoring of estrogen levels is recommended to avoid a high urinary excretion of E₁ and 17 β -E₂, nowadays enlisted as endocrine disrupting chemicals (EDCs), during hormone replacement therapy.

Keywords:

Stir bar sorptive extraction, gas chromatography/mass spectrometry, estrone, 17 β -estradiol, conjugated equine estrogens, urine samples.

9.1 Introduction

The accurate and sensitive measurement of various estrogenic hormones is becoming more and more important due to an increased understanding of the significance of these steroids in health and disease. Not only are the estrogens essential for maintenance of the female reproductive system, they are also important regulators of growth and bone metabolism [1]. Measurement of the main biologically active estrogens, namely estrone (E_1) and 17 β -estradiol (17 β - E_2) in blood and urine can be used to identify inborn errors of steroid metabolism; to monitor hormone replacement therapy; and to detect early puberty [2, 3, 4]. Highly sensitive assays are also required to study the role of these hormones in Alzheimer's disease and breast cancer [5, 6].

Evidence that estrogens may promote breast and ovarian cancer has recently received considerable attention. Epidemiological studies have indicated that women exposed to high serum and urinary levels of the estrogens are at increased risk of developing cancer [7, 8]. Postmenopausal women who receive hormone replacement therapy (HRT) are also at risk, especially when the combined estrogen-progestogen formulations are used [9]. HRT preparations that contain endogenous estrogens or a mixture of the conjugated equine estrogens are frequently prescribed to treat menopausal symptoms such as hot flashes and excessive sweating. The most frequently used drug is a formulation obtained from pregnant mares' urine called Premarin (Wyeth) [9]. It has been estimated that during 1998 more than 46 million prescriptions for Premarin were issued in the United States alone [10]. The high use of this and other types of estrogen formulations (including the oral contraceptives) are regarded as possible sources for levels of estrogenic chemicals found in the discharges of sewage-treatment plants (STP) [11].

STP effluents have been shown to be estrogenic to fish [12]. Therefore, there has been growing concern over the release of various endocrine disrupting chemicals into the aquatic environment. The estrogenic component of domestic STP effluents has been shown to consist mainly of E_1 , 17 β - E_2 and the synthetic contraceptive steroid 17 α -ethinylestradiol (EE) [13, 14]. Under normal circumstances, non-pregnant premenopausal women excrete approximately 7.4 μ g of urinary E_1 and 3.1 μ g of urinary 17 β - E_2 per day [15]. These values

are considerably lower than the quantities excreted by most postmenopausal women who receive various formulations of HRT [16]. It has been shown that serum and urine estrogen levels in postmenopausal women who receive estradiol supplementation for example, are far greater than those obtained for their premenopausal counterparts [16, 17]. Standard HRT doses and/or dose guidelines may be too high for a lot of women, therefore frequent monitoring of estrogen levels during HRT has been recommended [10, 17].

In the clinical setting, urinary estrogen levels are frequently determined by means of biological assay, including enzyme immunoassay (EIA) and radioimmunoassay (RIA). These methods are often selected because of their affordability, ease of implementation, and high throughput which make them amenable to large scale investigations [18]. However, a number of limitations such as cross reactivity owing to a wide range of structurally similar compounds, and poor inter-laboratory reproducibility caused by batch-to-batch variation of the antibodies, may complicate the interpretation of results [20, 21].

Gas chromatography in combination with mass spectrometry (GC/MS) has addressed many of the shortcomings associated with immunoassays. The technique is highly specific and accurate, and has been used as a reference method to validate EIA [19, 21] or RIA methods [20, 21]. Unfortunately, the routine application of GC/MS for the detection of the estrogens has been hampered by the need to perform extensive sample preparation, which in turn has led to rather slow turnaround times. One of the ways to improve sample throughput, is to combine the extraction, concentration and purification of the compounds in a single step. Several investigators have already reported a significant reduction in analysis time by using solventless sample preparation techniques such as stir bar sorptive extraction (SBSE) [22, 24] and solid phase microextraction (SPME) [23, 24].

One of the main advantages of using sorptive extraction methods is the ability to analyze organic compounds, such as the estrogens, directly from an aqueous sample. The compounds are enriched by a polymeric phase that is coated onto a solid support, such as a glass stir bar (i.e. SBSE) [25] or an optical silica fiber (i.e. SPME) [26]. Affinity of the estrogens for the polymer layer can be enhanced by optimizing the stirring speed, temperature, pH and ionic strength of the sample solution. Furthermore, the phenolic

hydroxyl groups of the estrogens can be derivatized *in situ* with acetic acid anhydride to increase the affinity of the compounds for the polymeric phase coating [27, 28]. The aliphatic hydroxyl groups on the other hand, are more difficult to derivatize and this step can only be accomplished after the compounds have been extracted from the sample. Two headspace derivatization methods for SBSE have recently been described to improve the gas chromatographic properties of hydroxyl containing compounds that cannot be derivatized in aqueous solution. The first approach involves the in-tube silylation of the extracted compounds by BSTFA [29], whereas the second approach is based on the formation of the acetate derivatives of the extracted compounds by exposing the stir bars to acetic acid anhydride vapors in modified headspace vials [30].

In this investigation, the development of a new method for the analysis of E₁ and 17 β -E₂ in human urine samples is described. The method is based on the *in situ* derivatization of the estrogens with acetic acid anhydride; extraction of the derivatives by aqueous SBSE; and final exposure of the stir bars to acetic acid anhydride vapors before thermal desorption and GC/MS. The method has been applied to determine the effect of conjugated equine estrogens intake (i.e. Premarin) on the excretion of E₁ and 17 β -E₂ in human urine samples.

9.2 Experimental

9.2.1 Materials, standard solutions and urine samples

Estrone (E₁), 17 β -estradiol (17 β -E₂) and equilin (Eq) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Equilenin (Eqn), used as internal standard, was supplied as a 200 $\mu\text{g}\cdot\text{mL}^{-1}$ standard solution in acetonitrile by Riedel-de Haën (Sigma-Aldrich, Johannesburg, South Africa). 17 α -dihydroequilin (17 α -Eq) and 17 α -dihydroequilenin (17 α -Eqn) were obtained from Steraloids (Newport, RI, USA). The chemical structures of the compounds are shown in Figure 9.1. Concentrated solutions of the individual estrogens (80 $\mu\text{g}\cdot\text{mL}^{-1}$) and Eqn (20 $\mu\text{g}\cdot\text{mL}^{-1}$) were prepared in methanol. From these solutions, a combined working solution was prepared for E₁ and 17 β -E₂ at a concentration of 1 $\mu\text{g}\cdot\text{mL}^{-1}$. A separate solution was prepared for the internal standard (Eqn) at the same concentration. All solutions were stored at -20°C until used.

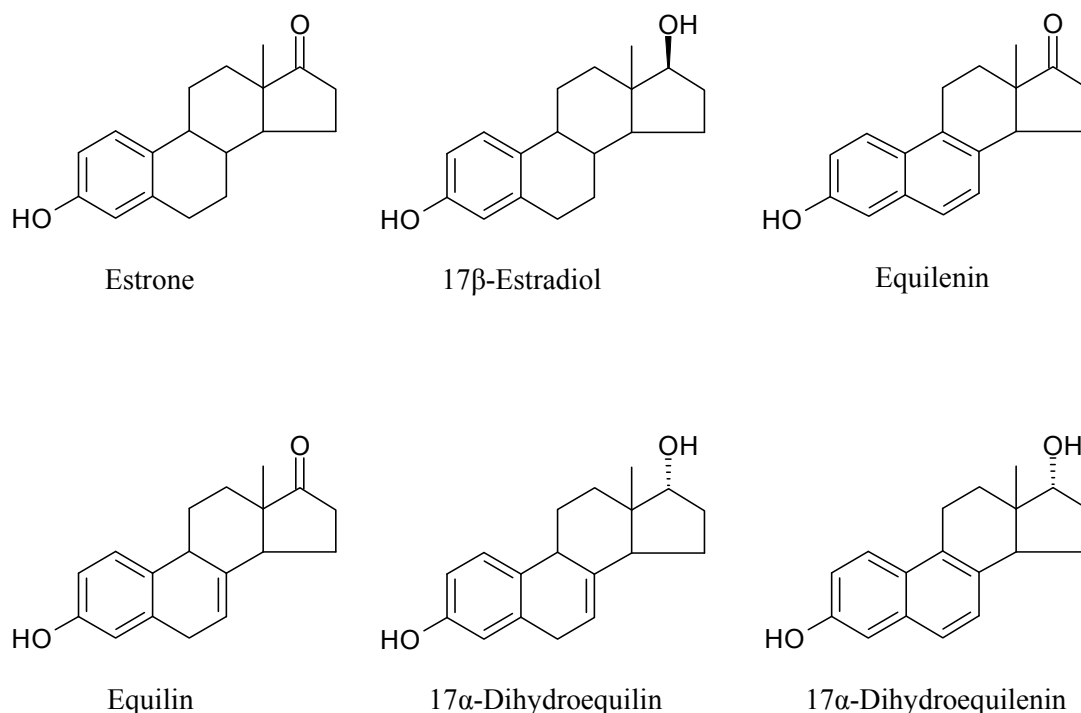


Figure 9.1 Chemical structures of the human and equine estrogens analyzed by SBSE-HD-TD-GC/MS.

Premarin tablets (0.625 mg; Wyeth, Johannesburg, South Africa) were purchased from a local pharmacy. A solution of the conjugated equine estrogens (62.5 $\mu\text{g}\cdot\text{mL}^{-1}$) in methanol was prepared according to a modification of the procedure described by Seibert et al. [31]. The outer coating of one tablet was removed using a piece of damp, lint-free tissue paper until the shellac layer was exposed. The tablet was crushed to a powder using a mortar and pestle. The conjugated equine estrogens were extracted by vortexing with two 6 mL portions of methanol. The fractions were combined, evaporated under nitrogen, and reconstituted in 10 mL methanol.

Sodium hydroxide pellets (NaOH), β -glucuronidase/sulfatase from *Helix Pomatia* (Type H-2), anhydrous sodium carbonate (Na_2CO_3) and pyridine were obtained from Sigma-Aldrich (Johannesburg, South Africa). Acetic acid anhydride and dichloromethane were supplied by Merck (Darmstadt, Germany). Ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) and vitamin C were obtained from Fluka (Sigma-Aldrich, Johannesburg, South Africa). The 15 mL screw cap vials were from Supelco (Sigma-Aldrich, Johannesburg, South Africa) and the headspace

derivatization vials [30] were prepared from 2 mL autosampler vials that were obtained from Agilent Technologies (Chemetrix, Johannesburg, South Africa). A 10 position magnetic stirrer combined with a convection oven was designed and built by J. Blom and colleagues (Department of Mechanical Engineering, University of Stellenbosch). Twister stir bars (10 mm \times 0.5 mm d_f PDMS) were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany). The stir bars were pre-conditioned by sonication in a 1:1 mixture of dichloromethane:methanol for 5 min after which they were heated at 280°C for 10 min under a nitrogen flow of 50 mL.min⁻¹.

First morning urine samples were obtained from two postmenopausal women aged 54 and 57 years, respectively. At the time of the study, the 57 year old woman received a formulation of conjugated equine estrogens as a hormone replacement (i.e. 0.625 mg Premarin per day). Control urine samples were obtained from 3 non-pregnant, premenopausal women and two male volunteers that were collected at random between 9h00 and 12h00. All urine samples were stored at -25°C prior to analysis. Urine creatinine levels were determined by Pathcare (Cape Town, South Africa).

9.2.2 Enzymatic hydrolysis, SBSE and derivatization procedure

After allowing the urine samples to thaw to room temperature, 1 mL aliquots were transferred to 15 mL screw cap vials containing 2 mL of a buffer solution (pH 4.6) that consisted of 1 M (NH₄)₂SO₄, 5.7 mM vitamin C and 2 mM NaOH. Twenty μ L of a crude solution of *Helix Pomatia* was added to the sample mixtures and the vials were incubated at 40°C for 16 hours [32]. The samples were spiked with 2 ng.mL⁻¹ of the internal standard (Eqn), and 500 mg Na₂CO₃ and 50 μ L pyridine were added. The vials were vortexed gently until the salt was dissolved. Using a stop-watch, the *in situ* derivatization step was performed in a fume hood as follows. After adding 500 μ L of acetic acid anhydride, 6 s elapsed before the open vials were vortexed for 5 s; another 9 s elapsed until the samples were vortexed for a further 20 s. Three mL of the derivatized samples were transferred to clean 15 mL vials prior to performing SBSE.

A conditioned stir bar was placed in each vial and the samples were stirred at 1100 rpm for 60 min using a home-built multiposition magnetic stirrer/oven that was heated to 40°C. After SBSE extraction, the stir bars were removed from the sample vials, washed with distilled water and dried with tissue paper. A second derivatization step was performed by placing the stir bars inside 2 mL headspace vials as previously described [30] and 20 μ L acetic acid anhydride and 20 μ L pyridine were added. The vials were incubated at 80°C for 30 min to form the acetate derivatives of the extracted compounds. Urine samples that were obtained from two postmenopausal women and water samples spiked with different concentrations of the Premarin solution (Section 9.2.1) were prepared as described above, except that the internal standard Eqn was not added.

9.2.3 Thermal desorption – gas chromatography/mass spectrometry (TD-GC/MS)

TD-GC/MS analyses of the derivatized estrogens were performed with an Agilent 6890 gas chromatograph that was equipped with a TDS 2 thermodesorption system and a TDS A autosampler (Gerstel). Thermal desorption of the compounds was accomplished in the solvent-venting mode using helium at a flow rate of 100 mL.min⁻¹. The temperature of the TDS 2 was ramped from 50°C to 150°C (held for 1 min), and from 150°C to 300°C (held for 10 min) at a rate of 60°C.min⁻¹. The split-valve was closed after 2 min. The desorbed compounds were transferred through a heated transfer line (320°C) to a programmable vaporization (PTV) inlet (CIS 4, Gerstel) that was cooled to 10°C using liquid nitrogen. The sample was injected by increasing the PTV inlet temperature to 300°C (held for 5 min) at a rate of 12°C.s⁻¹. The splitless time was 2.5 min. Chromatographic separation of the derivatives was performed on an HP5MS capillary column (30 m L \times 0.25 mm ID \times 0.25 μ m d_f; Agilent) using helium at a flow rate of 1.25 mL.min⁻¹. The oven temperature was programmed from 70°C (held for 2 min) to 220°C at 10° C.min⁻¹, and from 220°C to 300°C (held for 2 min) at 3.2°C.min⁻¹. The total run time was 44 min.

The gas chromatograph was interfaced with a 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA) that was operated in the full scan and selected ion monitoring (SIM) modes. The GC/MS interface, ion source and quadrupole temperatures were maintained at 280°C, 230°C and 150°C, respectively. Mass spectra of the acetate

derivatives were recorded in the electron impact mode by scanning over a mass range of 50 – 550 amu (ionization voltage 70 eV). For SIM, two to three ions were selected from each spectrum to detect trace amounts of the compounds in human urine. The monitored ions included the base peak and one or two other target ions of each derivative, i.e.: E₁ (*m/z* 270; 312), 17 β -E₂ (*m/z* 225; 314), Eq (*m/z* 268; 310), Eqn (*m/z* 266; 308), 17 α -Eq (*m/z* 237; 252; 294) and 17 α -Eqn (*m/z* 235; 250; 277). The underlined values are the base peaks of the acetate derivatives.

9.3 Results and discussion

9.3.1 Mass spectrometry

The mass spectra of the derivatized estrogens are shown in Figure 9.2. Pure water samples, spiked at a concentration of 0.48 $\mu\text{g}\cdot\text{mL}^{-1}$ of each estrogen were analyzed as described in the experimental section. The most intense fragment ions for the acetate derivatives of E₁ and 17 β -E₂ (Figure 9.2-A and 9.2-B) were formed by the loss of [CH₂=C=O] from the C₃-acetyl groups of the compounds. The loss of [CH₃COOH] from the base peak of 17 β -E₂ (*m/z* 314) and subsequent fragmentation of the D rings in both compounds, resulted in complex spectra containing several low intensity fragment-ions [33]. Similar fragmentation patterns to that of E₁ acetate were observed for the equine estrogens, namely Eq and Eqn (Figure 9.2-C and 9.2-D). The ions at *m/z* 268 and *m/z* 266 were formed by the loss of [CH₂=C=O] from the molecular ions of Eq acetate (*m/z* 310) and Eqn acetate (*m/z* 308). Further decompositions corresponded to that of E₁ acetate as reported previously [34].

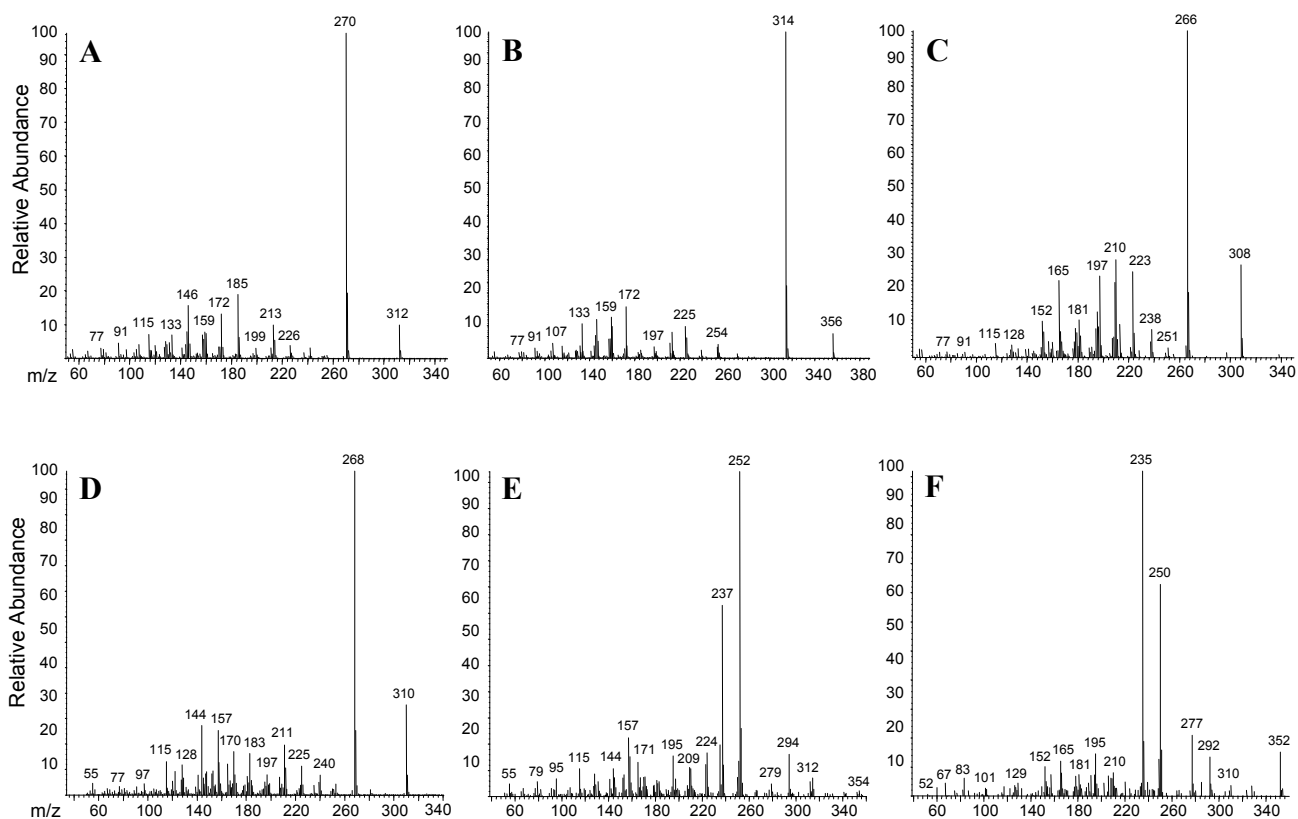


Figure 9.2 Electron impact mass spectra of the acetate derivatives of A) Estrone (E_1); B) 17 β -Estradiol (17 β - E_2); C) Equilenin (Eqn); D) Equilin (Eq); E) 17 α -Dihydroequilin (17 α -Eq); F) 17 α -Dihydroequilenin (17 α -Eqn).

The mass spectra of the acetate derivatives of 17 α -Eq and 17 α -Eqn differed significantly from those obtained for the simple estrogens (Figure 9.2-E and 9.2-F). Ions corresponding to the loss of $[CH_2=C=O]$ and $[CH_3COOH]$ from the C_3 - and C_{17} -acetyl substituents were present in the spectra of both compounds, namely m/z 312 and 294 for 17 α -Eq and m/z 310 and 292 for 17 α -Eqn. The base peak (m/z 252) of the diacetate derivative of 17 α -Eq was formed by the loss of 42 mass units from the ion at m/z 294. An additional loss of 57 mass units from this ion (i.e. m/z 294) resulted in the formation of the ion at m/z 237. The fragmentation of 17 α -Eqn appeared to follow the same pattern, except that a complete reversal in the intensity of the ions at m/z 235 and m/z 250 was demonstrated. A summary of the monitored ions is presented in Table 9.1.

Table 9.1 Summary of the ions monitored by GC/MS in SIM mode.

Compound (Abbreviation)	M ^a	Monitored ions
1. Estrone (E ₁)	312	<i>m/z</i> <u>270</u> ^b , 312
2. 17 β -Estradiol (17 β -E ₂)	356	<i>m/z</i> 225, <u>314</u>
3. Equilin (Eq)	310	<i>m/z</i> <u>268</u> , 310
4. Equilenin (Eqn)	308	<i>m/z</i> <u>266</u> , 308
5. 17 α -Dihydroequilin (17 α -Eq)	354	<i>m/z</i> 237, <u>252</u> , 294
6. 17 α -Dihydroequilenin (17 α -Eqn)	352	<i>m/z</i> <u>235</u> , 250, 277

^aMolecular mass of the acetate derivatives.

^bUnderlined values are the base peaks of each derivative.

9.3.2 Method optimization

A number of experimental conditions that affect the sensitivity of the SBSE – headspace derivatization (HD) – TD –GC/MS procedure were optimized to improve the recovery of the estrogens from the urine samples. Prior to performing SBSE, the deconjugated estrogens were derivatized in the aqueous sample to enhance the extraction of the compounds by the stir bar coating. The aqueous derivatization step is performed with acetic acid anhydride in the presence of Na₂CO₃ and pyridine within a few seconds. During the reaction, the polar phenolic hydroxyl groups of the estrogens are replaced with less polar acetate groups, thus increasing the affinity of the compounds for the non-polar, polydimethylsiloxane coating of the stir bar. The efficiency of the reaction was optimized by adding different amounts of acetic acid anhydride and Na₂CO₃ to the samples.

Urine samples that were obtained from two male volunteers were spiked with 2 ng.mL⁻¹ of E₁, 17 β -E₂ and Eqn. The samples were analyzed as described in the experimental section. Firstly, the amount of Na₂CO₃ used was optimized by adding different quantities of the carbonate (i.e. 200 mg – 600 mg) and a fixed volume of the reagent to the samples (i.e. 0.5 mL acetic acid anhydride). Thereafter, the amount of reagent used was optimized by adding different volumes of acetic acid anhydride to the samples (i.e. 0.2 mL – 0.6 mL), while keeping the amount of Na₂CO₃ constant (i.e. 500 mg). In both experiments the estrogen derivatives were extracted at 50°C during 60 min. Figure 9.3 shows the results of the mean values for $n = 3$. The R.S.D.s were less than 5% for all points.

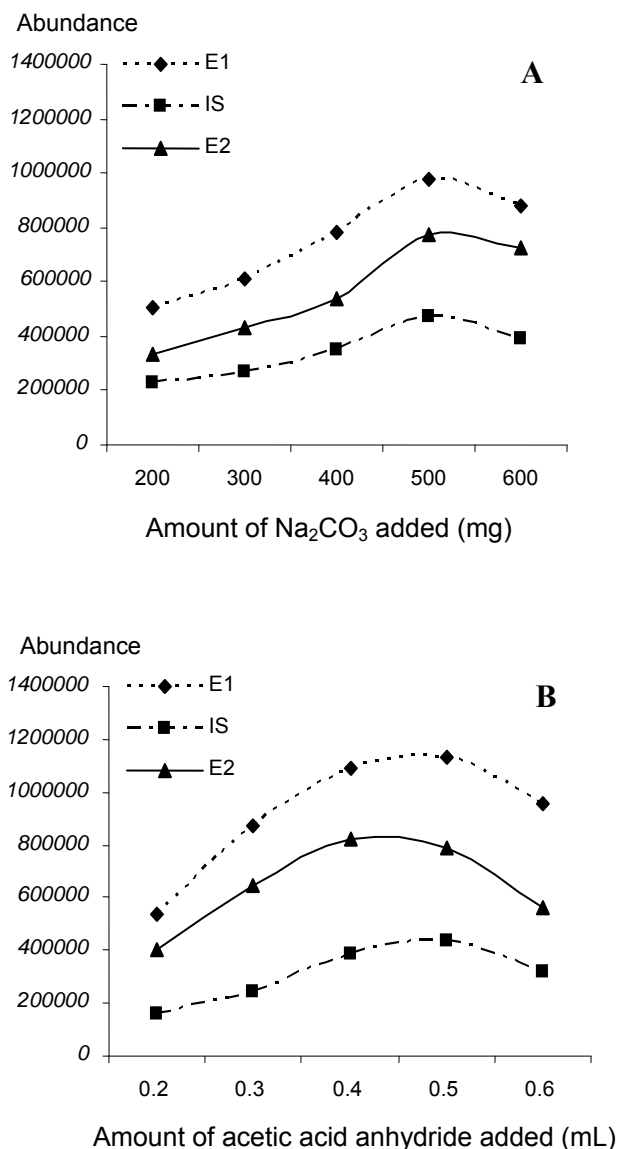


Figure 9.3 The amount of Na₂CO₃ (A) and acetic acid anhydride (B) added to prepare the monoacetate derivatives of E₁, 17 β -E₂ and the IS. One mL urine samples were spiked with 2 ng.mL⁻¹ of each compound and were analyzed by SBSE-TD-GC/MS. Conditions are given in the experimental section.

The peak areas of the derivatives increased gradually and reached a maximum after 500 mg of Na₂CO₃ and 0.5 mL of acetic acid anhydride (0.4 mL for 17 β -E₂) were added to the samples. Further increases in the volume of the reagent and the quantity of the catalyst resulted in decreased amounts of the compounds being extracted. As well as improving the efficiency of the derivatization reaction, the amount of acetic acid anhydride and Na₂CO₃

added to the samples also influenced the pH and ionic strength of the solutions. It is known that to achieve optimal extractions by SBSE, the compounds should be present in their neutral form [35]. Since the estrogens are ionizable compounds, the pH and ionic strength of the sample will affect the overall amount of estrogens extracted by the stir bar. Under the optimized derivatization conditions, a final pH of ca. 5.5 was obtained prior to the extraction of the estrogen derivatives. The efficiency of the extraction at different pH levels was not investigated because of the buffering effect produced by the reagent and carbonate. However, Figure 9.3-A demonstrates that by increasing the ionic strength of the sample solution, increased amounts of the estrogens are recovered by the stir bar. Therefore, an amount of 500 mg Na₂CO₃ and a volume of 0.5 mL acetic acid anhydride were selected as optimal to prepare the monoacetate derivatives of the estrogens prior to extraction by SBSE.

Further improvements in the recovery of the estrogens were accomplished by optimizing the temperature and the time of extraction. By increasing the temperature of the sample solution, the diffusion coefficients of the analytes will increase, but at the same time their partition coefficients may decrease [35]. Therefore, the optimal extraction temperature for E₁, 17 β -E₂ and Eqn was determined by performing a series of experiments at 40, 50, 60, 70 and 80°C, while the extraction time was held constant at 60 min. The recovery of the estrogens decreased slightly at extraction temperatures of 60°C and higher. The initial peak areas of E₁ and Eqn remained unchanged between the temperature interval 40°C to 50°C, whereas the peak areas of 17 β -E₂ increased slightly up to 50°C and then leveled off between 50°C and 60°C. To select the most appropriate extraction temperature for the compounds, the repeatability of the method was evaluated at 40°C and 50°C, respectively. The repeatability ($n = 4$) for E₁ and 17 β -E₂ improved when a lower extraction temperature was used (i.e. 2.3% for E₁ and 3.3% for 17 β -E₂ at 40°C versus 3.9% for E₁ and 6.2% for 17 β -E₂ at 50°C). Therefore, 40°C was chosen as the optimal extraction temperature for the compounds. Thereafter, the extraction-time profiles of E₁, 17 β -E₂ and Eqn were obtained by stirring the samples for various lengths of time which ranged from 30 – 120 min. Sixty minutes was sufficient to achieve equilibrium extraction for the studied estrogens.

Lastly, the headspace acylation of 17 β -E₂ was optimized by exposing the stir bars to acetic acid anhydride and pyridine vapors for 30 min at temperatures ranging from 60°C to 90°C. The peak areas obtained for 17 β -E₂ remained relatively unchanged within the selected temperature range. To determine the most efficient headspace derivatization temperature, the repeatability of the method was evaluated at 70°C and 80°C, respectively. Repeatability ($n = 4$) for 17 β -E₂ improved significantly when a higher headspace derivatization temperature was used (i.e. 1.3% for E₁ and 6.3% for 17 β -E₂ at 70°C versus 1.3% for E₁ and 3.5% for 17 β -E₂ at 80°C). Therefore, 80°C was selected as the optimal temperature to derivatize the extracted compounds. The optimum time required to form the diacetate derivative of 17 β -E₂ was investigated by exposing the stir bars to acetic acid anhydride and pyridine vapors for various lengths of time i.e. 15-60 min. An optimal yield was obtained after 30 min.

9.3.3 Quantification of E₁ and 17 β -E₂ in human urine samples

The efficiency of the optimized SBSE-TD-HD-GC/MS method was demonstrated by measuring trace levels of E₁ and 17 β -E₂ in urine samples that were obtained from 3 healthy, non-pregnant, premenopausal women. The levels were determined by the standard addition method in samples that were spiked at two concentration levels, namely 2 ng.mL⁻¹ and 4 ng.mL⁻¹ of each compound. A fixed amount of the internal standard (Eqn) was added (i.e. 2 ng.mL⁻¹) prior to analyzing the spiked and non-spiked samples. The slopes of the curves were obtained by plotting the peak area ratios of E₁ (m/z 270) and 17 β -E₂ (m/z 314) corrected for the IS (m/z 266) against the concentration of the analytes. The correlation coefficients (r^2) of both compounds ranged between 0.995 – 0.999. To determine the original steroid concentrations, the linear curves were extrapolated to the negative axis, whereby levels of 3.6, 4.4 and 2.9 ng.mL⁻¹ were obtained for E₁ and 1.1, 1.4 and 0.96 ng.mL⁻¹ were obtained for 17 β -E₂. The levels were corrected for the amount of creatinine measured in each sample as shown in Table 9.2. The limits of detection for the method were calculated at a signal to noise (S/N) level of 3 and were 0.02 ng.mL⁻¹ for E₁ and 0.03 ng.mL⁻¹ for 17 β -E₂. The limits of quantitation were calculated at a S/N of 10 and were 0.05 ng.mL⁻¹ and 0.1 ng.mL⁻¹ for E₁ and 17 β -E₂, respectively. The precision of the SBSE method was estimated from the relative standard deviation of 12 replicate analyses (i.e. 4

replicates that were analyzed on 3 different days). The intra-day repeatability was 1.8% for E₁ and 4.2% for 17 β -E₂, whereas the inter-day repeatability was 1.6%, 1.8% and 1.9% for E₁ and 2.7%, 3.9% and 4.5% for 17 β -E₂.

Table 9.2 Levels of urinary E₁ and 17 β -E₂ obtained for 3 healthy, premenopausal women. One mL urine samples spiked with 2 ng.mL⁻¹ of the IS (Eqn) were analyzed by SBSE-TD-GC/MS. Quantification was performed by the standard addition method.

No.	Age	r ^{2*}	E ₁ (ng.mL ⁻¹)	E ₁ [#]	r ^{2*}	17 β -E ₂ (ng.mL ⁻¹)	17 β -E ₂ [#]
1	20	0.995	3.6	2.2	0.999	1.1	0.68
2	23	0.999	4.4	2.6	0.998	1.4	0.84
3	20	0.998	2.9	2.2	0.999	0.96	0.72

*Correlation coefficients

[#]Levels corrected for creatinine content (ng.mg creatinine⁻¹)

9.3.4 Increased urinary excretion of E₁ and 17 β -E₂ following the oral administration of Premarin.

The estrogenic components of Premarin were identified by analyzing a pure water sample spiked at a concentration of 0.19 μ g.mL⁻¹ of the conjugated equine estrogens (Section 9.2.1). Accurate identification of the steroids was made by comparing the GC/MS scan results of each compound with the retention times and mass spectra of the corresponding standards. All the samples were analyzed by SBSE-TD-GC/MS as described in the experimental section. SIM chromatograms of the target compounds, namely E₁, Eq, Eqn, 17 β -E₂, 17 α -Eq and 17 α -Eqn were obtained by analyzing a pure water sample spiked at a concentration of 31.3 ng.mL⁻¹ of the Premarin solution. Figure 9.4A-i illustrates that a number of the estrogens co-eluted under the present gas chromatographic conditions, i.e. E₁ (*m/z* 270) co-eluted with Eq (*m/z* 268), and 17 β -E₂ (*m/z* 314) co-eluted with 17 α -Eq (*m/z* 252). However, it was possible to detect the individual compounds by using the extracted-ion SIM chromatograms of each estrogen as shown in Figure 9.4A-ii and Figure 9.4A-iii.

Significant differences were observed between the GC/MS profiles of Premarin and those obtained for the postmenopausal urine samples. Figure 9.4B-i shows that the peaks identified in sample A (i.e. peak 1 – 4) were also detected in a urine sample obtained from a 57 year old postmenopausal woman who received a preparation containing conjugated

equine estrogens (i.e. 0.625 mg Premarin per day). A significant amount of E₁ was excreted by this volunteer, whereas only trace amounts of Eq and 17 α -Eq were detected in the sample (Figure 9.4B-ii and -iii). The main components of Premarin have been identified as the sulfate conjugates of E₁ (50-60%), Eq (20-30%) and 17 α -Eq (14-20%) [36]. Possible metabolic pathways for the equine estrogens have previously been reported by Bhavnani et al [37]. These authors demonstrated that the equine estrogens, including Eq and 17 α -Eq are extensively metabolized *in vivo* [38, 39]. Given that low quantities of Eq and 17 α -Eq are excreted in human urine, it is expected that these estrogens will have a negligible impact on the quantification of E₁ and 17 β -E₂ during conjugated equine estrogen supplementation.

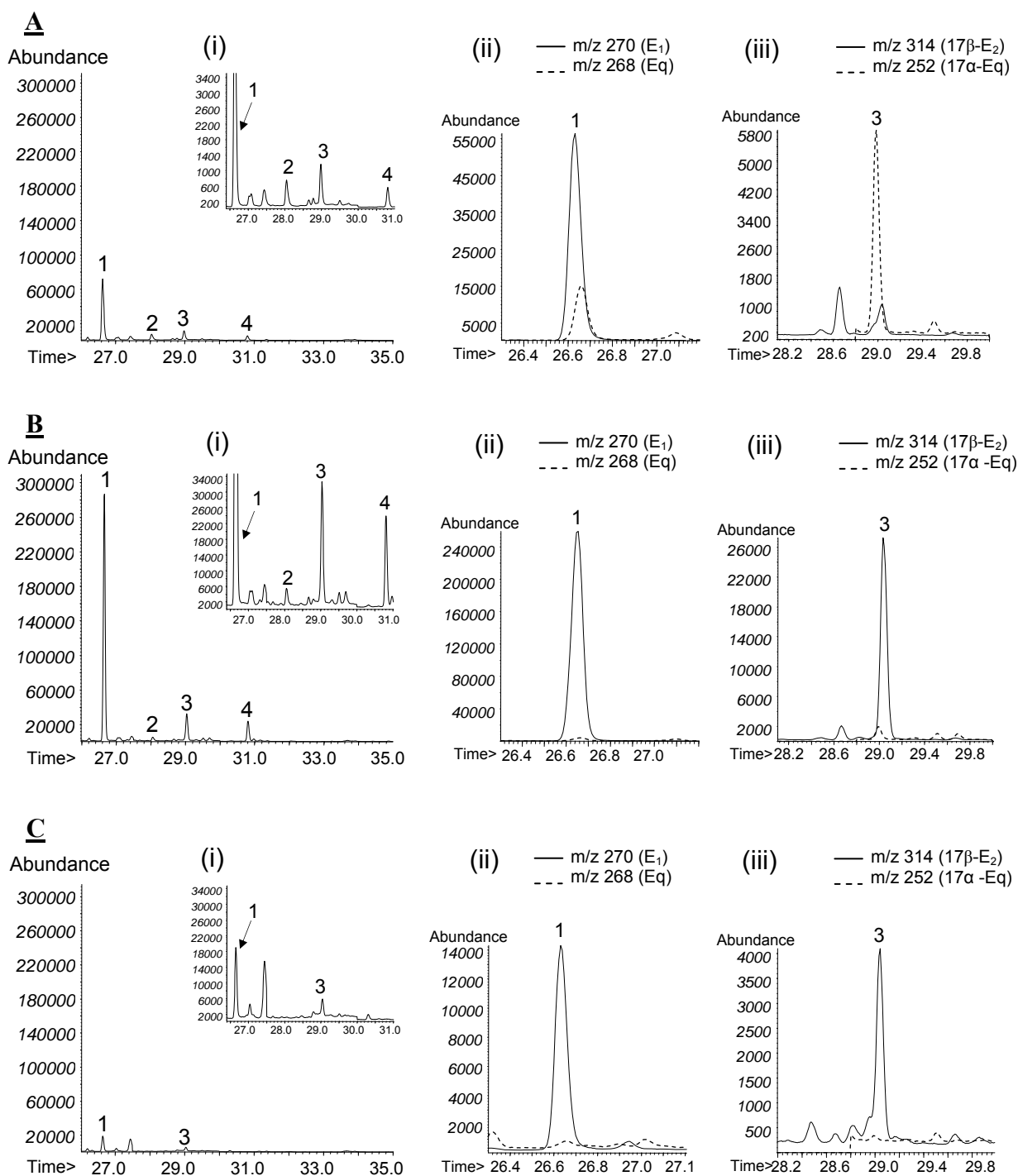


Figure 9.4 SIM chromatograms of A) a water sample spiked with 31.3 ng.mL⁻¹ of the Premarin solution; B) the non-spiked urine sample of a postmenopausal woman who received Premarin; and C) a postmenopausal urine sample without Premarin. The peak identities are 1) E_1 and Eq; 2) Eqn; 3) 17β - E_2 and 17α -Eq; and 4) 17α -Eqn. Additional chromatograms represent (i) magnified sections of the corresponding chromatograms; (ii) extracted-ion SIM chromatograms of peak 1; and (iii) extracted-ion SIM chromatograms of peak 3. Conditions are given in the experimental section.

Reference levels for E₁ and 17 β -E₂ were obtained by analyzing a urine sample that was donated by a 54 year old postmenopausal woman who did not receive any form of HRT (Figure 9.4C-i). This volunteer excreted much lower levels of the endogenous estrogens as compared to the postmenopausal woman who received daily estrogen supplementation (Figure 9.4B-i). To investigate this finding further, the results were compared against normal levels of the estrogens found in healthy premenopausal women. Figure 9.5 demonstrates that the excretion of E₁ and 17 β -E₂ in a urine sample of a 23 year old premenopausal woman (A; E₁: 2.6 ng.mg creatinine⁻¹, 17 β -E₂: 0.84 ng.mg creatinine⁻¹) were substantially lower than the levels detected in a 57 year old postmenopausal woman who received a formulation containing conjugated equine estrogens (B; E₁: 56.9 ng.mg creatinine⁻¹, 17 β -E₂: 11.6 ng.mg creatinine⁻¹).

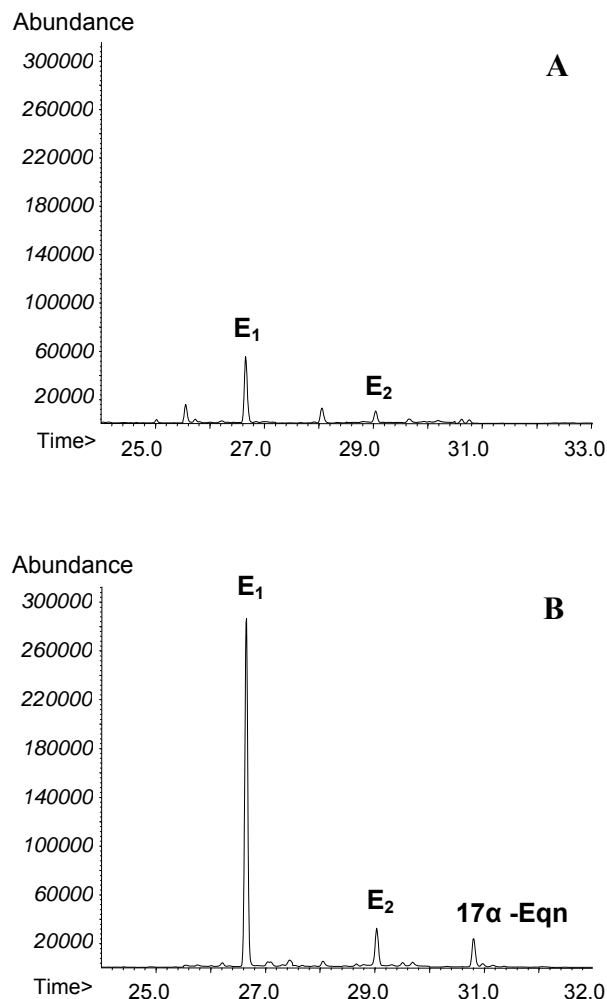


Figure 9.5 SIM chromatograms of A) a 1 mL urine sample of a 23 year old premenopausal woman (E_1 : 2.6 ng.mg creatinine⁻¹; 17 β - E_2 : 0.84 ng.mg creatinine⁻¹); and B) a 1 mL urine sample of a 57 year old postmenopausal woman who received Premarin (E_1 : 56.9 ng.mg creatinine⁻¹; 17 β - E_2 : 11.6 ng.mg creatinine⁻¹). The extraction, derivatization and chromatographic conditions are described in the experimental section.

Similar findings to those observed in this study have been reported by other investigators. Tepper et al. demonstrated that approximately 57% of postmenopausal women who received 2 mg of an oral estradiol formulation, experienced serum estrogen levels 5 times in excess of what was required to manage their menopausal symptoms [17]. They concluded that it may be inappropriate to treat all women with the same steroid dosage. In a similar study, Friel et al. reported that after studying hundreds of women who received various formulations of HRT, they noticed that some women excreted higher quantities of E_1 and 17 β - E_2 as compared to those seen in healthy premenopausal women [16]. Both studies

concluded that currently marketed pharmaceutical preparations contain doses of the estrogens in excess of what are required to manage the symptoms of a large number of postmenopausal women. Doses containing as little as 0.3 mg.day⁻¹ of the conjugated equine estrogens have been shown to be as effective in controlling menopausal symptoms as the standard doses that are currently prescribed (i.e. 0.625 mg.day⁻¹) [10,40]. Estrogen replacement therapy is based on fixed-dose regimens, which are infrequently monitored during long periods of treatment [17]. The short-term risks of exposure to high estrogen levels are still unknown [41], although recent findings from a large scale clinical trial provided convincing evidence that the long-term use of HRT is associated with an increased risk of breast and endometrial cancer [42].

Another important consideration is the fact that surplus estrogens will be excreted into domestic waste removal systems. It is currently estimated that 20 – 50% of women in the western world use some form of HRT (i.e. those aged 45 to 70 years) [43]. In view of the frequent use of these products and the fact that standard HRT doses may be too high for many women, it is likely that the use of HRT formulations will make a significant contribution to the levels of estrogens found in STP effluents. The presence of these chemicals in wastewater discharges have been attributed to the widespread intersexuality detected in fish [44], although the overall threat to fish populations from this source is still unknown [45]. In light of the potential health risks to women who use various formulations of HRT, as well as the potential harmful effects of these compounds in the environment, it seems reasonable to recommend that urinary estrogen levels be frequently monitored during HRT. Future work should aim to establish the usefulness of these measurements in determining the dose-response relationships of HRT preparations.

9.4 Conclusion

Regulatory authorities have become increasingly concerned about the presence of estrogenic chemicals in the aquatic environment. Possible sources for these compounds in wastewater discharges have been attributed to the frequent use of pharmaceutical products that may contain endogenous, chemically modified or conjugated equine estrogens. Estrogen replacement therapy is currently based on fixed-dose regimens that are seldom

tailored to meet individual requirements. Therefore, excessive amounts of the estrogens may be excreted by postmenopausal women who receive conventional doses of various HRT preparations. In the present study it was demonstrated that increased amounts of E₁ and 17 β -E₂ were excreted by a postmenopausal volunteer who received a standard oral dose of the conjugated equine estrogens (i.e. 0.625 mg.day⁻¹). Urine levels of the estrogens were determined by aqueous SBSE that was combined with two derivatization steps to form the mono- and diacetate derivatives of the extracted compounds. The derivatives were thermally desorbed and analyzed on-line by GC/MS. The sensitivity of the method was improved by optimizing a number of experimental conditions which enhanced the recovery of the compounds by the stir bar coating. Detection limits of 0.02 ng.mL⁻¹ and 0.03 ng.mL⁻¹ were obtained for E₁ and 17 β -E₂, respectively. The developed SBSE procedure is highly sensitive and easy to perform, which are important considerations for the routine clinical monitoring of urinary estrogen levels by GC/MS.

References

- [1] M.N. Weitzman, R. Pacifici, *J. Clin. Invest.* 116 (2006) 1186.
- [2] E.P. Smith, J. Boyd, G.R. Frank, H. Takahashi, R.M. Cohen, B. Specker, T.C. Williams, D.B. Lubahn, K.S. Korach, *N. Eng. J. Med.* 331 (1994) 1056.
- [3] C.M. dos Reis, N.R. de Melo, E.S. Meirelles, D.P. Vezozzo, A. Halpern, *Maturitas* 46 (2003) 59.
- [4] M. Fontoura, R. Brauner, C. Prevot, R. Rappaport, *Arch. Dis. Child.* 64 (1989) 1170.
- [5] E. Hogervorst, J. Williams, M. Combrinck, A.D. Smith, *Eur. J. Endocrinol.* 148 (2003) 67.
- [6] S.R. Cummings, T. Duong, E. Kengon, J.A. Cauley, M. Whitehead, K.A. Krueger; *JAMA.* 287 (2002) 216.
- [7] T. Key, P. Appelby, I. Barnes, G. Reeves, *J. Natl. Cancer Inst.* 94 (2002) 606.
- [8] M.C. Pike, D.V. Spicer, L. Dahmouh, M.F. Press, *Epidemiol. Rev.* 15 (2003) 17.
- [9] D. Farquhar, *CMAJ.* 167 (2002) 377.
- [10] J.S. Cohen, *Arch. Intern. Med.* 161 (2001) 957.
- [11] I.R. Falconer, H.F. Chapman, M.R. Moore, G. Ranmuthuqala, *Environ. Toxicol.* 21 (2006) 181.
- [12] C.E. Purdom, P.A. Hardiman, V.J. Bye, N.C. Eno, C.R. Tyler, J.P. Sumpter, *Chem. Ecol.* 8 (1994) 275.
- [13] C. Desbrow, E.J. Routledge, G.C. Brighty, J.P. Sumpter, M. Waldock, *Environ. Sci. Technol.* 32 (1998) 1549.
- [14] R. Gibson, M.D. Smith, C.J. Spary, C.R. Tyler, E.M. Hill, *Environ. Sci. Technol.* 39 (2005) 2461.
- [15] H. Adlercreutz, T. Fotsis, C. Bannwart, E. Hämäläinen, S. Bloigu, A. Ollus, *J. Steroid Biochem.* 24 (1986) 289.
- [16] P.N. Friel, C. Hinchcliffe, J.V. Wright, *Altern. Med. Rev.* 10 (2005) 36.
- [17] R. Tepper, S. Goldberger, I. Cohen, J. Segal, S. Yarkoni, M. Fejgin, Y. Beyth, *Gynecol. Obstet. Invest.* 38 (1994) 113.
- [18] K.A. O'Connor, E. Brindle, D.J. Holman, N.A. Klein, M.R. Soules, K.L. Campbell, F. Kohen, C.J. Munro, J.B. Shofer, B.L. Lasley, J.W. Wood, *Clin. Chem.* 49 (2003) 1139.
- [19] R.T. Falk, S.C. Rossi, T.R. Fears, D.W. Sepkovic, A. Migella, H. Adlercreutz, J. Donaldson, H.L. Bradlow, R.G. Ziegler, *Cancer Epidemiol. Biomarkers Prev.* 9 (2000) 81.

- [20] R.T. Falk, M.H. Gail, T.R. Fears, S.C. Rossi, F. Stanczyk, H. Adlercreutz, P. Kiura, K. Wahala, J.L. Donaldson, J.B. Vaught, C.M. Fillmore, R.N. Hoover, R.G. Ziegler, *Cancer Epidemiol. Biomarkers Prev.* 8 (1999) 567.
- [21] R.W. Giese, *J. Chromatogr. A* 1000 (2003) 401.
- [22] M. Kawaguchi, Y. Ishii, N. Sakui, N. Okanouchi, R. Ito, K. Saito, H. Nakazawa, *Anal. Chim. Acta* 533 (2005) 57.
- [23] L. Yang, T. Luan, C. Lan, *J. Chromatogr. A* 1104 (2006) 23.
- [24] C. Blaso, M. Fernandez, Y. Picó, G. Font, *J. Chromatogr. A* 1030 (2004) 77.
- [25] E. Baltussen, P. Sandra, F. David, C.A. Cramers, *J. Microcolumn Sep.* 11 (1999) 737.
- [26] C.L. Arthur, J. Pawliszyn, *Anal. Chem.* 62 (1990) 2145.
- [27] K.D. Buchholz, J. Pawliszyn, *Anal. Chem.* 66 (1994) 160.
- [28] B. Tienpont, F. David, K. Desmet, P. Sandra, *Anal. Bioanal. Chem.* 373 (2002) 46.
- [29] M. Kawaguchi, R. Ito, N. Sakui, N. Okanouchi, K. Saito, H. Nakazawa, *J. Chromatogr. A* 1105 (2006) 140.
- [30] A. Stopforth, B.V. Burger, A.M. Crouch, P. Sandra, *J. Chromatogr. B* 834 (2006) 134.
- [31] D.S. Seibert, C.F. Poole, *J. High Resol. Chromatogr.* 21 (1998) 481.
- [32] C.H. Shackleton, *J. Chromatogr.* 379 (1986) 91.
- [33] D.H. Smith, B.G. Buchanan, W.C. White, E.A. Feigenbaum, J. Lederberg, C. Djerassi, *Tetrahedron* 29 (1973) 3117.
- [34] C. Djerassi, J.M. Wilson, H. Budzikiewicz, J.W. Chamberlin, *J. Am. Chem. Soc.* 84 (1962) 4544.
- [35] H. Lord, J. Pawliszyn, *J. Chromatogr. A* 902 (2000) 17.
- [36] A.O. Mueck, H. Seeger, D. Wallwiener, *Climacteric* 6 (2003) 221.
- [37] B. Bhavnani, C.A. Woolever, D. Wallace, C.C. Pan, *J. Clin. Endocrinol. Metab.* 68 (1989) 757.
- [38] B.R. Bhavnani, C.A. Woolever, *J. Steroid Biochem.* 17 (1982) 217.
- [39] B.R. Bhavnani, *Endocr. Rev.* 9 (1988) 396.
- [40] D.F. Archer, *Clin. Obstet. Gynecol.* 46 (2003) 317.
- [41] N. Mikhail, *Mayo Clin. Proc.* 78 (2003) 379.
- [42] V. Beral, *Lancet* 362 (2003) 419.
- [43] T. Largo-Janssen, W.W. Rosser, C. van Weel, *Lancet* 362 (2003) 414.

- [44] S. Jobling, M. Nolan, C.R. Tyler, G. Brighty, J.P. Sumpter, *Environ. Sci. Technol.* 32 (1998) 2498.
- [45] A.C. Johnson, J.P. Sumpter, *Environ. Sci. Technol.* 35 (2001) 4697.

Chapter 10

Concluding comments

The main part of this study focuses on the development of simplified analytical methods for the analysis of target compounds in biological samples by stir bar sorptive extraction and gas chromatography/mass spectrometry. GC/MS is often used to analyze complex matrices because of its ability to separate different sample constituents as well as providing accurate structural information for the resolved compounds. Due to the complexity of most biological samples, direct analysis by GC/MS is rarely performed. Very often, highly laborious and time consuming sample preparation steps are required to isolate the compounds of interest. Current trends in analytical chemistry are aimed at simplifying standard sample preparation procedures, as well as minimizing the use of organic solvents that are potentially harmful to humans and the environment. In sorptive extraction, the use of organic solvents is avoided because the analytes partition between the aqueous sample and a polymeric extraction phase based on their octanol-water distribution constants. Stir bar sorptive extraction (SBSE) and solid phase microextraction are the two most frequently used sorptive extraction methods. Both techniques utilize polydimethylsiloxane (PDMS) as the stationary phase to extract volatile and semi-volatile components from aqueous and gaseous samples. In SBSE, the PDMS layer is attached to a glass stir bar. Several analyses can be performed using a single stir bar due to the remarkable hydrophobic recovery of the polymer surface (Chapter 2). A wide range of analytes can be extracted by the PDMS layer; therefore the specificity of the analysis should be verified before proceeding with the actual quantification of the compounds. In Chapter 5, the versatility of the SBSE technique is highlighted by demonstrating the detection of a wide range of drugs of abuse in different biological fluids. The SBSE-TD-GC/MS technique is highly sensitive, which allows the mass spectrometer to be used in the scan mode. Further improvements in the specificity and sensitivity of the analysis are accomplished by using the MS in the selected ion monitoring (SIM) mode. Both specificity and sensitivity are essential to achieving the trace detection of biological compounds in complex matrices. Specificity is optimized by monitoring a few ions that are characteristic for the compounds being analyzed, whereas sensitivity is improved by controlling several external and instrumental parameters. Apart from ensuring the optimal operation of the analytical instrument, sensitivity is mainly determined by the affinity of the analytes for the stir bar coating. Several parameters may be optimized to improve the extraction of various compounds by the polymer layer, namely stirring speed, temperature, pH, salt addition, as well as the derivatization of polar functional groups.

These parameters should be held constant at all times to ensure the reproducibility of the extraction. Once the compounds can be detected with a certain level of confidence (i.e. as measured by precision, sensitivity and detection limit), quantification is performed using the internal standard or standard addition technique. In this study, standard addition proved to be quite valuable for the quantification of compounds analyzed by SBSE, because it corrects for matrix effects, controls for changes taking place on the stir bar surface, and minimizes alterations occurring in the operating conditions of the GC/MS system. Furthermore, it is probably one of the most efficient ways of validating the selection of ions used for SIM analysis. To enable the extraction of polar analytes by the stir bar coating, a derivatization step will be required that may be performed directly in the aqueous sample prior to performing SBSE, or in the headspace of a vial containing vapors of the reagent after the compounds have been extracted from the sample. This study describes the analysis of four distinct biomarkers in human bodily fluids by SBSE-TD-GC/MS, using different derivatization techniques. Until recently, one of the limitations in the analysis of compounds containing polar functional groups by aqueous SBSE has been the lack of a suitable derivatization method for these analytes. This limitation has recently been overcome by the development of two new headspace derivatization procedures, one of which has been presented in this study. Chapter 6 describes the direct measurement of a chemical marker of tuberculosis, namely tuberculostearic acid (TBSA) in sputum samples. The clinical samples were decontaminated and concentrated before being analyzed by SBSE-TD-GC/MS. Prior to performing SBSE, the mycobacterial lipids were hydrolyzed and then derivatized with ethyl chloroformate to increase the sorption of the compounds by the stir bar coating. The method is sufficiently sensitive to detect TBSA directly in sputum samples without the need to culture the organisms. Future work should focus on establishing the diagnostic accuracy of the method, as well as measuring other markers for *M. Tuberculosis* that will increase the specificity of the TBSA method. In Chapter 7, the detection of 4-hydroxynonenal (4HNE) by SBSE and GC/MS has been demonstrated. 4HNE is a highly toxic end-product of lipid peroxidation that may be used as a marker of oxidant injury. The measurement of 4HNE is based on the formation of an oxime derivative using *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine, followed by acylation of the hydroxyl group by means of headspace derivatization prior to thermal desorption. The high sensitivity of the method allows the detection of physiological quantities of 4HNE excreted

daily. Future measurements of 4HNE, particularly in larger clinical trials, will enable clinicians to clarify the role of free radicals in the pathophysiology of human diseases, as well as providing certain end-points for various therapeutic interventions. Chapter 8 describes the measurement of the T/ET ratio in a group of healthy volunteers and HIV+ patients. The extraction of T and ET was accomplished by aqueous SBSE, followed by headspace acylation and TD-GC/MS. Decreased levels of T were detected in the HIV+ group, whereas the excretion of ET was comparable to levels obtained for the control group. This finding has not previously been reported in the literature. Therefore, further clinical research will be required to elucidate the clinical significance of the T/ET ratio in HIV infection. Lastly, Chapter 9 demonstrates the increased urinary excretion of E₁ and 17β-E₂ following the administration of a standard dose of Premarin to a female volunteer. Current estrogen replacement therapies are based on fixed-dose regimens that are seldom tailored to meet individual requirements. Therefore, surplus estrogens may be released into the aquatic environment due to inadequate removal by sewage treatment plants. The routine monitoring of estrogen levels is recommended to avoid the high urinary excretion of E₁ and 17β-E₂ during hormone replacement therapy. Analysis of the estrogens is accomplished by *in situ* acylation and aqueous SBSE, followed by headspace acylation and TD-GC/MS. The method is simple to perform, which is an important consideration for the routine clinical monitoring of urinary estrogen levels by GC/MS.

In conclusion, the work presented in this study illustrates the versatility of SBSE to enrich various classes of biological compounds directly from aqueous samples. The successful application of SBSE in combination with GC/MS for the analysis of complex biological matrices has also been demonstrated. Furthermore, new derivatization possibilities create the exciting possibility of developing additional SBSE methods for application in the clinical and biomedical fields.