Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry

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Dedicated to my late grandparents Thozamile Wilson Lamani† and Welekazi Dorah Lamani†, my mother Nosipho "Spakes" Lamani and most of all, my husband Abraham J Dixon and our precious kids Ratrudis, Jarius and Jethro Dixon

Summary

Over the years, it has been revealed that some aromatic amines (AAs) have a potential to cause bladder cancer in humans, with cigarette smoking as a major source. However, in relation to tobacco-smoking bladder cancer only few of these compounds have been reported. Hence, an interest to study more about the existence of AAs in human body fluids was developed. The use of a comprehensive multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS) enabled the detection of several isomeric compounds of the same molecular mass and it has given a clear picture of the existence of aromatic amines in human urine due to its fingerprint analysis. For the investigation of urine samples, which were of smokers, non-smokers and past-smokers in this study; pre-treatment steps are necessary prior to analysis to make the analytes suitable for GC. The steps include: (i) thermal acidic hydrolysis to cleave aromatic amine adducts, (ii) liquid-liquid extraction (LLE) of the hydrolyzed analytes, (iii) derivatization of the extracted amines through diazotization and iodination and (iv) enrichment of the iodinated derivatives with headspace solid-phase microextraction (SPME), before introducing the analytes into the GC system. The application of the developed in-situ derivatization SPME GC×GC-qMS technique resulted in more than 100 isomeric aromatic amines identified in human urine and with regard to the numbers and peak intensities, the urine of smokers were more burdened compared to non- and past-smokers urine. Also, some alternative developments were investigated in order to improve sample preparation procedures which are mainly the time and solvents consumed during preparation. Microwave-assisted technique showed a possibility of potentially minimizing the time from 12 hours of the conventional hydrolysis to ≤ 1 hour. And for the organic solvents, less amounts were achieved by thin-film microextraction (TFME) including the amount of

sample needed. In conclusion, the work done here offers a better understanding on the prevalence of several aromatic amines in humans, than previously reported. This creates a platform for further investigations probably in the urine samples of the patients who are diagnosed with bladder cancer. This may eventually be useful in finding biomarkers which could be used to determine the level of cancer-causing aromatic amines in urine samples.

Zusammenfassung

Im Laufe der Jahre hat sich gezeigt, dass einige aromatische Amine (AA) das Potenzial haben, beim Menschen zu Blasenkrebs zu führen, wobei das Rauchen von Zigaretten als Hauptquelle gilt. In Bezug auf durch Tabakrauch verursachten Blasenkrebs wurde jedoch nur über wenige dieser Verbindungen berichtet. Daher wurde ein Interesse daran entwickelt, mehr über die Existenz von AA in menschlichen Körperflüssigkeiten zu erfahren. Die Verwendung einer umfassenden mehrdimensionalen Gaschromatographie-Massenspektrometrie (GC×GC-qMS) ermöglichte den Nachweis mehrerer isomerer Verbindungen mit gleicher Molekülmasse und hat aufgrund ihrer Fingerabdruckanalyse ein klares Bild von der Existenz von aromatischen Aminen im menschlichen Urin gegeben. Um die in dieser Studie verwendeten Urinproben von Rauchern, Nichtrauchern und ehemaligen Rauchern für die GC geeignet zu machen, waren vor der Analyse mehrere Vorbehandlungsschritte erforderlich. Die Schritte umfassten: (i) thermische saure Hydrolyse zur Spaltung aromatischer Aminaddukte, (ii) Flüssig-Flüssig-Extraktion (LLE) der hydrolysierten Analyten, (iii) Derivatisierung der extrahierten Amine durch Diazotierung und Iodierung und (iv) Anreicherung der Iodderivate mit Headspace-Festphasen-Mikroextraktion (SPME), bevor die Analyten in das GC-System eingeführt wurden. Die Anwendung der entwickelten In-situ-Derivatisierungs-SPME GC×GC-qMS-Technik führte zu mehr als 100 isomeren aromatischen Aminen, die im menschlichen Urin identifiziert wurden. Im Hinblick auf die Anzahl und die Peakintensitäten war der Urin der Raucher höher belastet im Vergleich zu dem Urin von Nichtrauchern und ehemaligen Rauchern. Es wurden auch einige alternative Entwicklungen untersucht, um die Probenvorbereitungsprozedur hinsichtlich des Zeitaufwands und des Einsatzes vonLösungsmitteln zu verbessern.

Die mikrowellenunterstützte Technik zeigte eine Möglichkeit, die Zeit von 12 Stunden der herkömmlichen Hydrolyse auf ≤ 1 Stunde potentiell zu minimieren. Und für die organischen Lösungsmittel wurden geringere Mengen durch Dünnschicht-Mikroextraktion (TFME) einschließlich der benötigten Probenmenge erreicht. Zusammenfassend lässt sich festhalten, dass diese Arbeit ein besseres Verständnis der Prävalenz von aromatischen Aminen im Menschen ermöglicht als bisher bekannt. Dies schafft wahrscheinlich eine Plattform für weitere Untersuchungen in den Urinproben der Patienten, bei denen Blasenkrebs diagnostiziert wird. Dies kann letztendlich nützlich sein, um Biomarker zu finden, die verwendet werden können, um den Gehalt an krebserregenden aromatischen Aminen in Urinproben zu bestimmen.

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CHAPTER 1

General introduction

1.1 Application and use of aromatic amines

Aromatic amines are known to be a potent group of carcinogens that are present in the environment, mainly as a result of human activity [1]. They can be sub-divided into mono-, poly- and heterocyclic amines, of which some mono-and polycyclic amines are classified as carcinogenic to humans including 2-methylaniline, 4-chloro-2-methylaniline, benzidine, 4-aminobiphenyl and naphthylamine [2, 3]. There are various fields in which aromatic amines are widely used such as pesticides, drugs and other synthetic products, as well as in the production of specific pigments a.k.a azo dyes [4]. Humans can easily get exposed to aromatic amines during everyday-life activities like cigarette smoking, permanent hair dyes, consumption of some drugs and food packaged in plastic material [5, 6]. Some aromatic amines such as benzidine have been determined in finger paints for children [7] and also 2-methylaniline in magenta dyes [8]. The Federal Institute for Risk Assessment (BfR) has recommended consumers to have as minimum contact with these substances as possible, by avoiding long-term storage of food in printed paper packages (e.g. bakery bags) or food wrapped in printed paper napkins. They stated that primary aromatic amines (PAA) should be undetectable in water extracted from the finished products, inconsiderate of whether they come directly from the ago dye or from the recycled fibers. They propose rather the use of colour pigments that do not contain any carcinogenic aromatic amine component [9]. Also, according to EUDirective 2007/19/EC for materials which contact food, the release of PAA such as aniline, methylaniline, methoxyaniline, etc. which are also categorized as carcinogenic or potentially carcinogenic must not be detectable (Detection limit (DL) = 0.01 mg/kg of food or food stimulant and it applies to the sum of the PAA released) [6].

In cigarette smoke aromatic amines have been identified as biologically active substances [10]. Their biological activity is dependent of the substance structure, for e.g., of the three isomers of aminobiphenyl (2-, 3-, and 4-aminobiphenyl), only 4-aminobiphenyl is a carcinogen. Also, in some studies with experimental animals aromatic amines are known of their organ specificity, where for instance p-phenylenediamine initiates skin cancer and its isomer o-phenylenediamine produces cancer in the liver [11].

1.2 Human exposure to aromatic amines and bladder cancer

There are several routes of exposure to aromatic amines which are attributed to the development of urinary bladder cancer in humans. The occurrence of urinary bladder tumors was first reported by the German surgeon Ludwig Wilhelm Carl Rehn, among workers of the local aniline dye-factory in the late nineteenth century (1895) [12, 13]. Rehn put blame on aniline as a causal agent of the bladder tumors in those dye-factory workers. Later, the German scientist August Wilhelm Hofmann demonstrated that the color of the aniline dyes was rather produced from a mixture of aniline and toluidines and not just pure aniline [14]; however at the time no one considered toluidines to be a potential risk for the development of bladder tumors in humans [8]. Since then, several incidences of bladder cancer among workers in aniline factories, textile dyeing, paper printing and leather finishing industries were reported, leading to more studies on aromatic amines as occupational and environmental carcinogens [8, 15-16]. It was only established in 1954 that 2-naphthylamine was the main cause of bladder cancer in dye industry workers [16].

Other reported potential sources of human exposure to aromatic amines include hair coloring, where aromatic amines constituents are deposited to the hair and scalp with some passing through the skin and accumulate or get excreted in the human body [17]. Also, certain vegetables, fresh salads, vegetable oil and grilled meat impose a potential risk of exposure of humans to aromatic amines [18]. The information though about these substantial sources is still very limited. In addition, cigarette smoking is considered a major risk factor for the development of urinary bladder cancer in humans [5, 19-20]. Many cohort- and case-control studies implicated cigarette smoking as being responsible for up to 50 % of bladder cancer risks in males and 25 % in females [21]. In certain areas the risk is about 65 % in males and 20 - 30 % in females [22] and in some cases it is even higher in women than in men who smoked comparable amounts of cigarettes [23]. The changes done in the early 50s in the composition of tobacco and cigarette design, with intent to lower the content of nicotine in cigarettes may have led to increased depth and frequency of inhalation so as to satisfy the need for nicotine and therefore resulting in higher exposure to bladder carcinogens [24]. Vineis presented concentration data of some aromatic amines that are present in the smoke of air-cured (black) and flue-cured (blonde) tobaccos, as shown in table 1.1 below [25]. These data suggest that the risk of exposure depends also on the type of tobacco smoked. Many other factors such as the number of cigarettes smoked per day, the duration etc. are also to be considered for the risk. In countries where smoking of air-cured or black tobacco is common, bladder cancer risks are higher [26]. It is suggested that quitting smoking may reduce the risk, nonetheless there is little evidence on whether the excess risk disappears even after a long time since quitting [20].

Table 1.1 Aromatic amines present in the smoke of air-cured and flue-cured tobaccos

Concentration of aromatic amines (ng/cigarette) in condensates of air-cured (French) and flue-cured (U.S.) tobacco in mainstream smoke [25]		
	U.S	**France**
Aniline	102	364
o-methylaniline	32	162
m-methylaniline	15	30
p-methylaniline	14	34
2-ethylaniline + 2,6-dimethylaniline	15	54
2,5-dimethylaniline	19	87
3-ethylaniline + 2,4-dimethylaniline	14	57
4-ethylaniline + 2,3-dimethylaniline	8	27
1-naphthylamine	4	3
2-naphthylamine	1	2
2-aminobiphenyl	2	3
3-aminobiphenyl	3	5
4-aminobiphenyl	2	5
2-methyl-1-naphthylamine	6	4

Generally, the uptake of aromatic amines takes place through the gastrointestinal tract, the lungs, and, with some exceptions, the skin [11]. In order for aromatic amines to exert their carcinogenic effect and to initiate the carcinogenic response, they require a metabolic activation by host enzymes (CYP1A); where aromatic amines are oxidized to N-hydroxylamines in the liver and transported to the bladder

to undergo O-acetylation by an N-acetyl transferase (NAT1) to form chemically reactive compounds (DNA adduct). The metabolic pathway is depicted in figure 1.1 below.

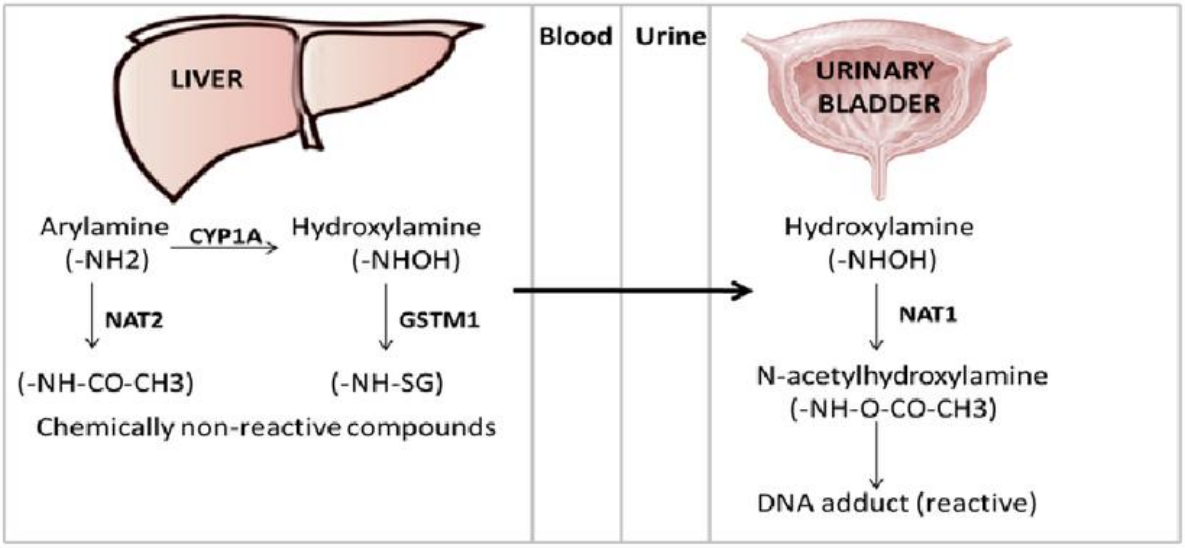

Fig. 1.1 Simplified scheme of arylamine metabolism pathway. Arylamines are N-acetylated by NAT2 in the liver, transforming them to relatively nonreactive species. Alternatively, they may be N-hydroxylated by CYP1A, transported to the bladder, and undergo O-acetylation by NAT1, to form highly reactive species [27]

When the urine is stored in the bladder, aromatic amines get in contact with the cells that line the bladder (urothelium) where they react and may form high-grade cancer cells [28]. For bladder cancer research urine is highly recommended [29] because the urothelium is exposed to and affected by urinary tract carcinogens through urine rather than through blood [30]. Figure 1.2 demonstrates the stages of bladder cancer where the tumor develops in the urothelium (stage 0) to a stage when the cancer spreads from the body to the wall of the abdomen, pelvis and/or other body parts (stage IV).

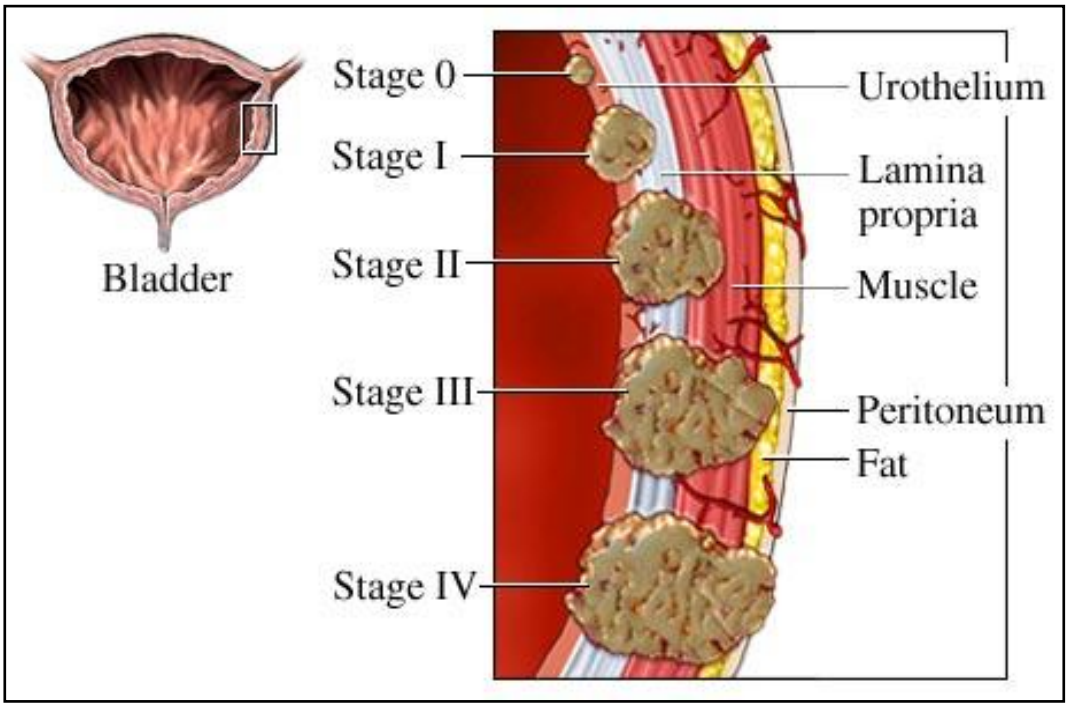

Fig. 1.2 Stages of bladder cancer showing **stage 0**: formation of tumor in the lining of the bladder, **stage I**: cancer spreading under the lining of the bladder, **stage II**: cancer spreading to the inner or outer half of the muscle wall of the bladder, **stage III**: cancer spreading from the bladder to the fatty layer surrounding it and **stage IV**: cancer spreading from the bladder to the wall of abdomen and pelvis and other parts of the body [31].

Jimenez et al. [32] presumed that an increase in water intake can potentially reduce the impact of urinary bladder carcinogen exposure; thereby decrease the urinary DNA adducts levels and mutagenicity. However, more studies are still required to confirm this hypothesis.

1.3 Sample preparation

Sample preparation steps are required for the achievement of accurate and reproducible results, especially in highly complex matrices such as urine, blood and organ tissues. A challenge in monitoring human urine is that arylamines tend to undergo significant metabolism in the human body, resulting in the excretion of only a small amount of parent amines together with a large number of different metabolites [8]. To separate the aromatic amines adducts, urine samples can be hydrolyzed acidic, basic or enzymatic using ß-glucuronisade or arylsulfatase; however the latter is mostly preferred for drug screening and also requires several hours for it to be effective [33-35]. Acidic or basic hydrolyses are often applied for the analysis of aromatic amines in urine [36-39]. Zimmermann [40] compared both processes to evaluate aromatic amines in human urine by using hydrochloric acid (HCl) and sodium hydroxide (NaOH), with samples heated at 110 °C for 12 hours. After extraction, derivatization and detection by gas chromatography-atomic emission detector (GC-AED), more peaks were found after acidic hydrolysis than after basic hydrolysis.

Following the hydrolysis, pre-concentration steps such as liquid-liquid extraction (LLE) and/or solid-phase extraction (SPE) are typically used to extract and concentrate the analytes of interest. Liquid-liquid extraction is probably the most often used method for the isolation of a compound from the matrix [41]. Here the sample to be analyzed is thoroughly mixed with a water-immiscible organic solvent whereby the analyte according to its equilibrium distribution, partitions into the organic layer and the interferences such as salt and proteins remain in the aqueous

layer [42]. The organic layer can be further treated to fit the requirements of the analytical instrument to be used, e.g. liquid or gas chromatography.

In order to enhance sensitivity and/or specificity of detection, further steps such as derivatization are required. A number of derivatization techniques have been employed for gas chromatography, which include acylation, silylation, carbamate formation, sulfonamide formation and phosphonamide formation [43]. To derivatize aromatic amines the most frequently used reagents are acylation reagents, e.g. carboxylic acid anhydrides, silylation reagents, chloroformates, dinitrophenylation reagents, carbonyl compounds forming Schiff bases as well as sulfonyl chlorides and phosphoryl chlorides [44]. Some of these derivatization procedures have potential drawbacks such as the formation of unwanted derivatives, the presence of unchanged derivatization reagents and requirements for non-aqueous reaction conditions [44]. To enhance the gas chromatographic properties, changing the structure of aromatic amines through diazotization of the amino group followed by subsequent substitution of the diazo group by iodine (in a one-pot reaction), does not only enable a decrease in the polarity of the compounds and an increase in the volatility but it also allows the analytes to be detected selectively. This process simply converts anilines to aromatic iodine compounds as demonstrated by the scheme of derivatization shown in the figure below.

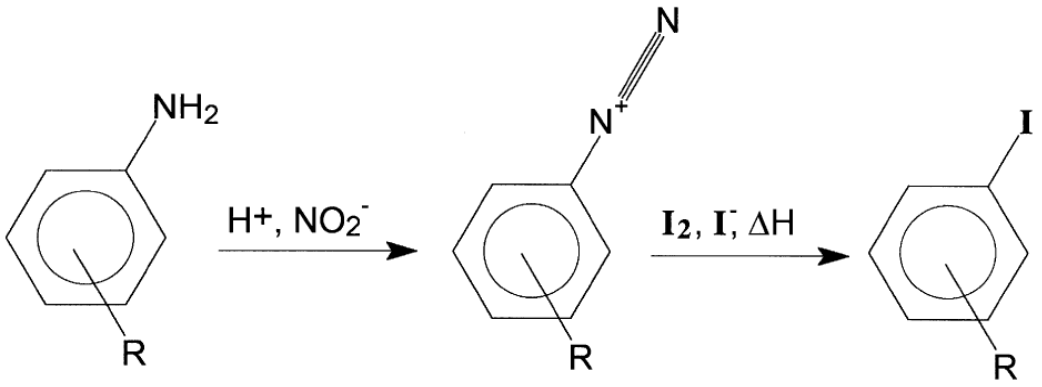

Fig. 1.3 Reaction scheme of derivatization of aromatic amines with iodine [45-46]

The derivatives of aromatic amines can be further enriched by the use of solid phase microextraction (SPME) technique, where compounds are extracted in liquid samples (immersion sampling) or in the headspace area above the liquid or solid samples (headspace sampling) as shown in figure 1.4 below. Generally, the difference between the two methods is that in immersion sampling, the compounds in the solution interact directly with the sorption phase (a), whilst in the headspace sampling the compounds are initially transferred to the headspace (b), where they interact with the sorption phase above the solution (c). This lack of direct contact between the sorption phase and the solution may be of advantage for headspace sampling, since undesirable matrix constituents that may contaminate the sorption phase can be adequately avoided and therefore sustain the life time of the SPME fiber. Furthermore, the SPME fiber can be cleaned and purged in a needle heater after every extraction to prevent cross-contamination.

The headspace sampling technique offers a simpler automated sample extraction procedure for organic compounds and in the case of compounds with poor volatility, direct immersion sampling may be a suitable technique [47].

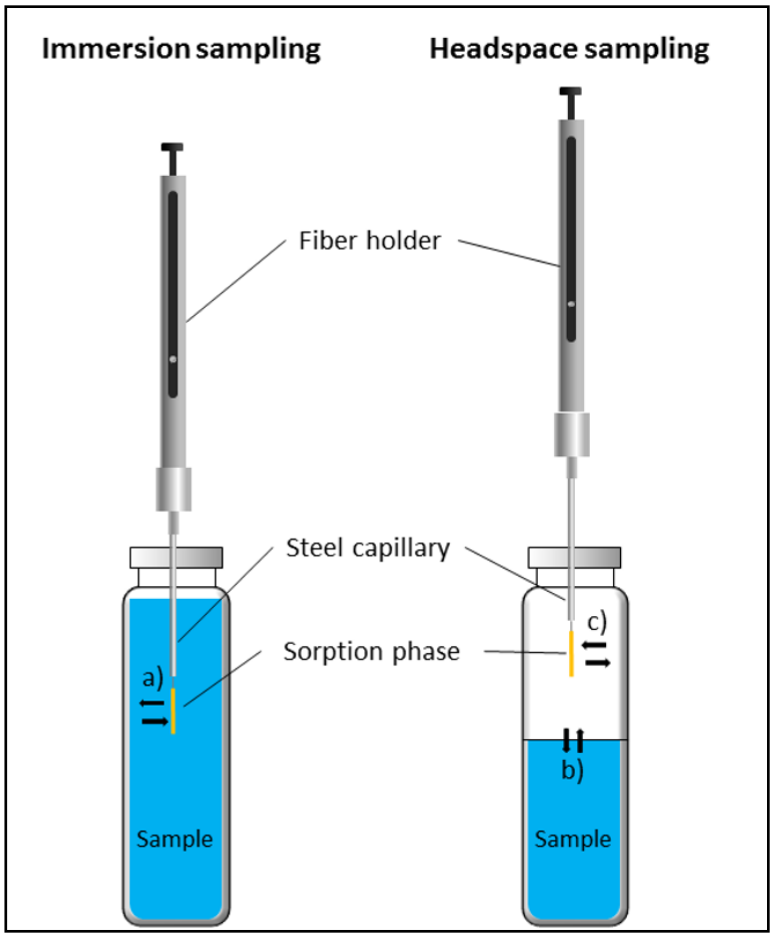

Fig. 1.4 Schematic diagram of SPME showing immersion and headspace sampling of **(a)** analytes interacting with the sorption phase in the solution, **(b)** transfer of analytes to the headspace and **(c)** analytes interacting with the sorption phase in the headspace [48]

1.4 Instrumentation

Chromatography is the technique used to separate, identify and quantify the individual components of a mixture. Among many, gas chromatography (GC) is one of the most commonly used techniques for the analysis of volatile compounds that is widely known for its great separation power, relative simplicity as well as its widespread application including petroleum analysis, environmental analysis, forensics etc. [40]. Since the invention of GC in the early 50s [49], a remarkable progress towards the increase of separation power has been made. Some major milestones include the invention of fused-silica capillary columns [50] and the introduction of the comprehensive two-dimensional GC (2D-GC×GC) [51]. The concept of multi-dimension was revealed in the 80s by Giddings who defined it as a separation technique that will increase the peak capacity and thus the resolution of the components of complex mixtures [52-53]. Hence, the invention and initial development of GC×GC techniques was motivated by the need to significantly increase the peak capacity in the same timeframe as available in one-dimensional (1D) separations [54]. The inventors stated in their patent that GC×GC can have "substantially increased peak capacity per unit time as compared with a single column gas chromatograph" [55].

Peak capacity ($\mathbf{n_c}$), is the major limitation for 1D-GC that hinders the resolution of the entire components especially in highly complex mixtures [56]. Also, samples to be analysed often consist of several groups of compounds from different classes with essentially different physicochemical properties. For example, whilst a non-polar stationary phase can be required for the successful separation of the components of one group, a different stationary phase with different selectivity may be required to

separate the components of another group [57]. In such cases, 2D-GC-GC, also known as “heart-cutting” may be an alternative where only part of the effluent is transferred to the second column. Its advantage over the 1D-GC is that the transferred fractions are subjected to a further separation on a full-length conventional column; resulting in the peak capacity being the sum of the first dimension and that of the second dimension, multiplied by the number (*x)* of the transferred fractions [$\mathbf{n_{c1} + (n_{c2} * x)}$] [58]. The drawback of this technique is that the continuous transfer of the effluent fractions may cause considerable overlapping of compounds previously resolved in the first dimension. Hence, this method is useful if only a limited number of analytes shall be separated from many matrix components. Once the entire sample needs a separation with different dimensions, the 2D-GC×GC becomes a suitable technique.

The 2D-GC×GC is suggested as having peak capacities of two GCs multiplied and result in an enormous resolving power, $\mathbf{(n_{c1} * n_{c2})}$ [58]. This technique can offer a peak capacity of more than an order of magnitude higher than the peak capacity of 1D-GC. Generally, the separation process is carried out on two columns with different selectivity, typically a non-polar normal bore 25-30 m × 0.25 mm ID × 0.25 µm column and a polar micro bore 1-2 m × 0.10 mm ID × 0.10 µm column. A figure illustrating the difference in the peak capacities of conventional one-dimensional GC (1D-GC), conventional multi-dimensional GC (2D-GC-GC) and comprehensive multi-dimensional GC (2D-GC×GC) is shown below.

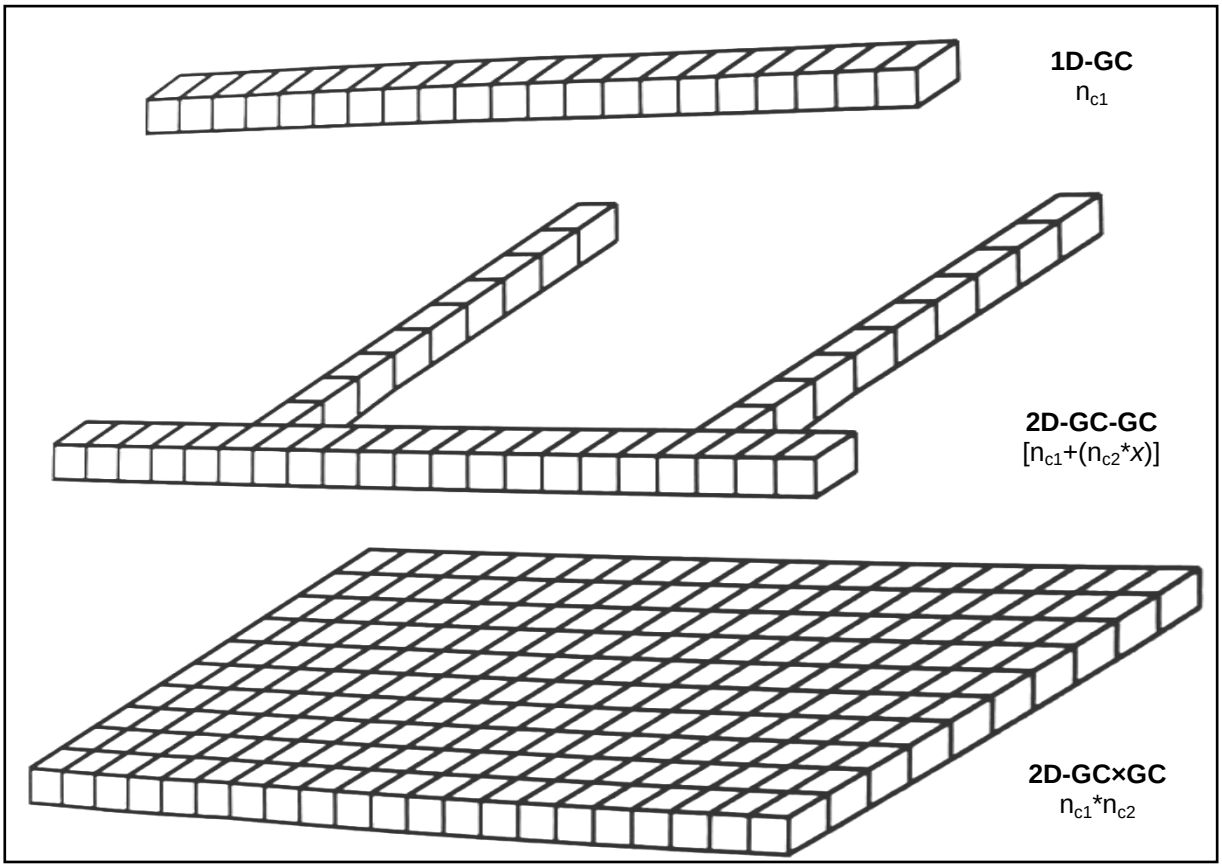

Fig. 1.5 Information capacities of 1D-GC, 2D-GC-GC and 2D-GC×GC [59]

In 2D-GC×GC the two columns are connected together by an interface also known as a modulator, which periodically cuts the effluent from a first GC column and transmits it to the second column, hence acting as a repetitive injector for the second column. The time required to complete this process is called the modulation period, which is typically 4 to 8 seconds. All the components should be eluted from the second column before the modulation period ends, i.e. the modulation time should not be shorter than the longest retention time on the second column; failure to do so may create what is called a “wrap-around”. An example of a peak wrap-around is shown in figure 1.6 below, assuming that the modulation time is 6 sec and the

retention time of one of the compounds (red blob) in 2D is 8 sec. Then in the 2D plot this compound may only appear at the “aliased” or “false” retention position (green blob) of 2 sec in the y-axis (a) and shifted by 6 sec in the x-axis (b).

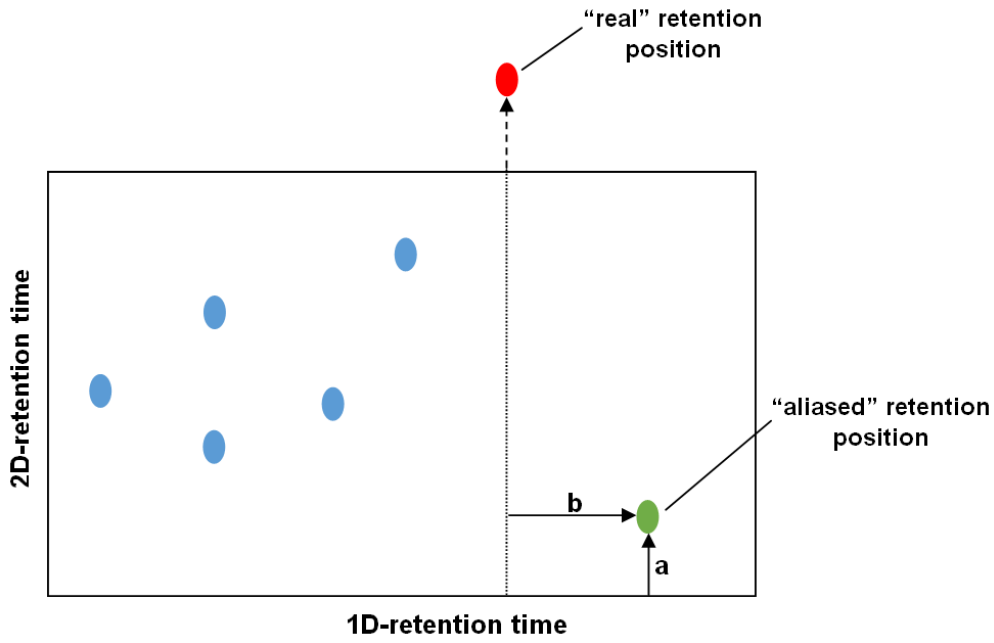

Fig. 1.6 An example of a peak wrap-around effect showing a compound eluted at the “aliased” retention position (green blob) both in 2D (**a**) and 1D (**b**). The vertical line and the red blob illustrates the supposed “real” retention position of the compound

There are different types of developed modulators available, namely (i) heat-based modulators, (ii) cryogenic modulators, and (iii) flow modulators. Cryogenic modulators are mainly used and are known to perform perfectly in many applications, despite their high operation costs due to the cooling gases. One of the most popular cryogenic modulators is called a “loop-modulator” designed by Ledford and co-workers for Zoex Corporation (NE, USA) [60].

It is basically the length of a column looped and placed under the intersecting paths of hot and cold gas (liquid nitrogen) jets. The cold jet is directed vertically downwards onto the looped segment, thus generating two cold spots throughout the GC×GC analysis and the hot jet (nitrogen gas) is placed perpendicular to the cold jet. An example of a loop modulator is shown in figure 1.7 below. The components of the mixture get trapped in the cold spots and every few seconds the hot jet fires, thereby heating the cold spots and releasing trapped analytes which proceed or are “injected” onto the second column. It is important for the modulation period to be kept short, while obtaining the necessary separation in the second dimension.

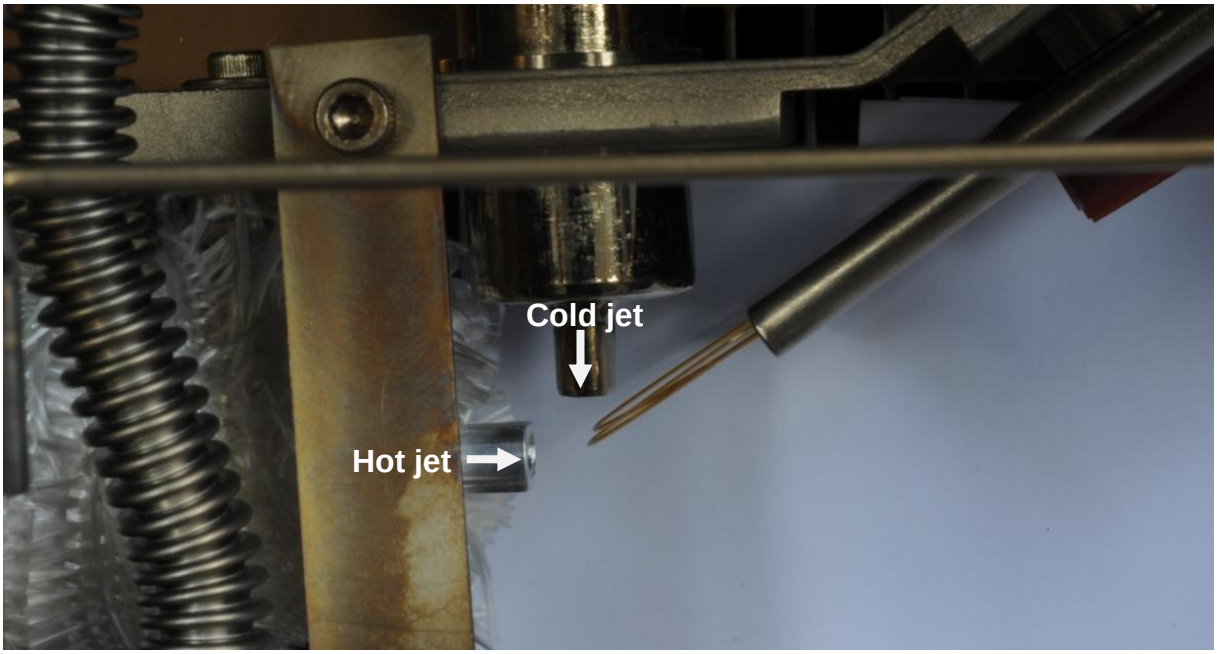

Fig. 1.7 A loop-modulator installed in the GC-oven on the intersecting paths of cold gas jet (vertical tube) and the hot gas jet (horizontal tube)

Considering that each modulation produces short and fast 2D chromatograms as depicted in figure 1.8 below, the separation from 1D once expanded to an orthogonal plane becomes very high (raw 2D chromatogram). Therefore, the transformation process becomes necessary to elaborate and visualize the results. This can be achieved by a software that is capable of recognizing which 2D peak corresponds to which compound, for example, GC Image (Zoex, TX, USA) used with Shimadzu GC×GC system, ChromaToF (Leco Corps MI, USA) which is part of Pegasus ToF-MS instrument and ChromSquare by Chromaleonts.r.l (Messina, Italy) used in data processing of both GC×GC and LC×LC. As highly organized patterns of compounds with the same functional groups are formed, the 2D chromatograms are stacked or piled side by side and with the modulation time considered, the first and second dimension retention times are acquired. These are the so-called “blobs” in the 2D plot.

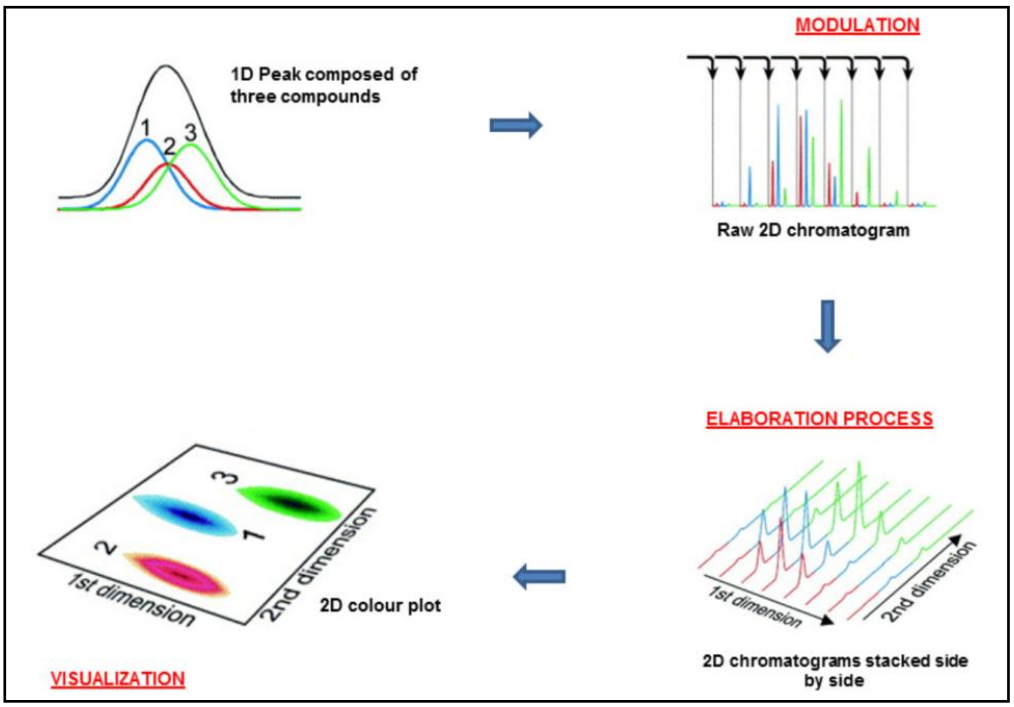

Fig.1.8 Schematic process from the raw data to final 2D contour plot [61]

Finally, apart from column selection and modulation, a choice of a suitable detector is also very important for GC×GC in order to achieve a complete resolution of the compounds of interest. The detectors required should be very fast with the capability to collect data at the rate of atleast 50 Hz, as the peaks eluted from GC×GC systems are very narrow (typically 150 - 400 ms at the base) [62]. Some examples of the detectors used are Flame Ionization Detector (FID), Electron Capture Detector (ECD), Atomic Emission Detector (AED), Time-of-Flight Mass Spectrometer (ToF-MS)and quadrupole Mass Spectrometer (qMS). The latter (GC×GC-qMS) was successfully used in this study.

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CHAPTER 2

Scope of work and aims

This thesis aims to contribute to the tremendous global work done on bladder cancer research, by taking an advantage of powerful techniques such as GC×GC to reveal a broader perception on the existence of aromatic amines in human urine. The development of bladder cancer in humans has frequently been associated with high exposure to aromatic amines [1] and tobacco smoking as an important source [2-3]. However, the information in relation to bladder cancer-causing-aromatic amines is still very limited. **Chapter 1** of this thesis discusses various routes in which humans can get exposed to aromatic amines, the association of aromatic amines with the formation of bladder cancer, as well as the analytical procedures and instrumentation that are used in this study to identify the analytes of interest. Chapters 3 to 5 describe the main content of this thesis and are also shown in figure 2.1 as a graphical visualization to demonstrate the contribution of these chapters to the achievement of the overall goal. Basically, **Chapter 3** focuses mainly on the qualitative analysis of aromatic amines in human urine, which includes pretreatment steps such as thermal acidic hydrolysis, liquid-liquid extraction (LLE) of the hydrolyzed analytes, derivatization of the aniline group through diazotization and iodination in a one-pot reaction and the enrichment of the derivatives using solid phase microextraction (SPME). The extraction efficiency of the SPME fiber was also investigated to ensure a maximum extraction of all analytes of interest. In addition, ionic liquid columns were tested to evaluate whether they could offer a better selectivity for the iodinated derivatives. The high number of isomeric compounds obtained led to the investigation of the quantity of aromatic amines in the urine of smokers, past-smokers and non-smokers **(Chapter 4)**. The analytical procedures, instrumentation and parameters used in this chapter were the same as in Chapter 3.

Due to excess time and solvents needed during sample pretreatment steps, **Chapter 5** dealt with some alternative methods investigated for sample preparation where microwave-assisted hydrolysis (MH) and thin-film microextraction (TFME) were tested to substitute the conventional hydrolysis (CH) and liquid-liquid extraction (LLE) methods, respectively. Finally, in **Chapter 6** the overall conclusions and an outlook on future prospects are discussed.

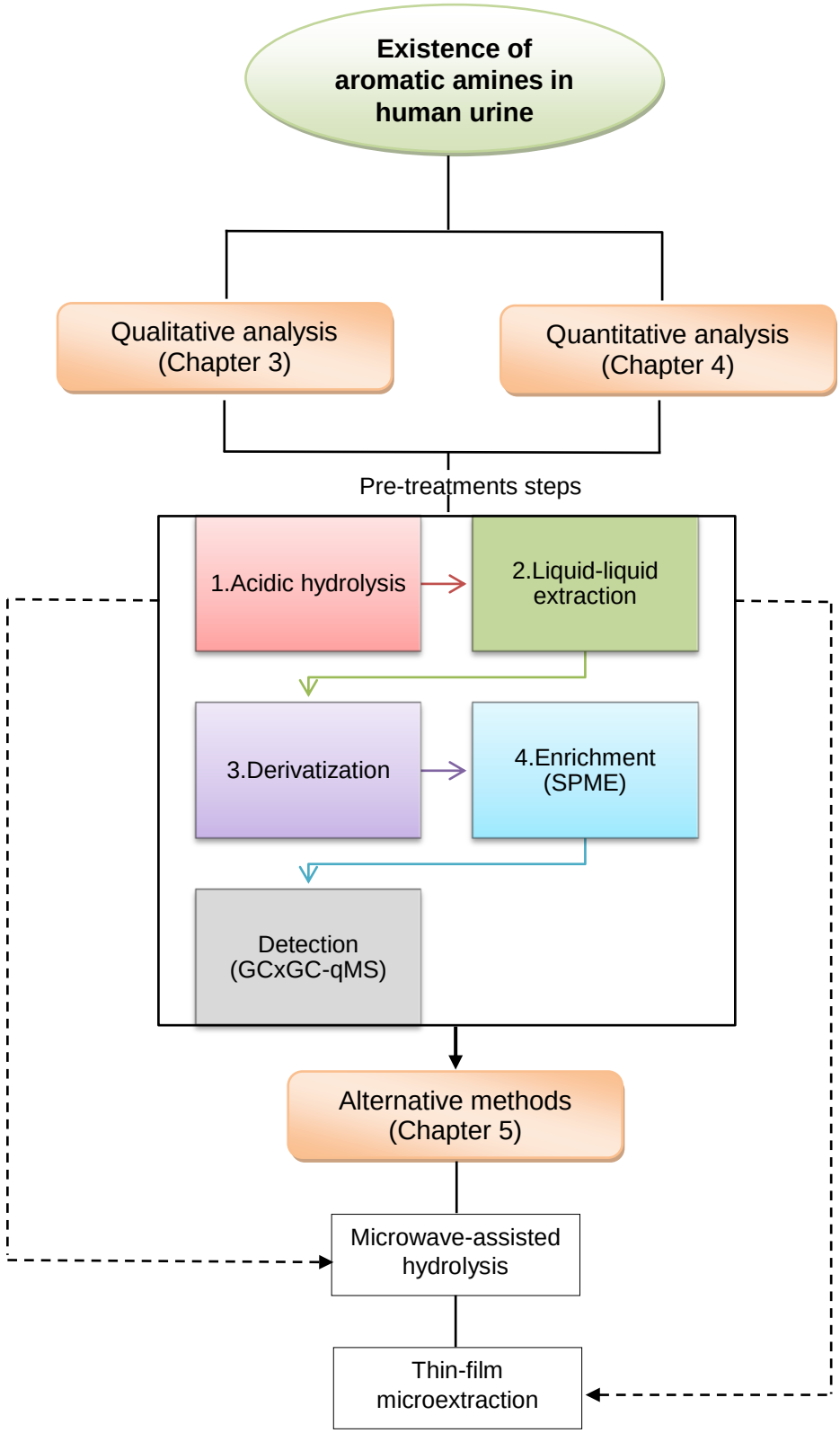

Fig. 2.1 Graphical visualization of various chapters of this thesis

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CHAPTER 3

Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS)

Adapted from a publication by Lamani X, Horst S, Zimmermann T, Schmidt TC, Analytical and Bioanalytical Chemistry (2015), 407 (1): 241-252

3.1 Abstract

Aromatic amines are an important class of harmful components of cigarette smoke. Nevertheless, only few of them have been reported to occur in urine, which raises questions on the fate of these compounds in human body. Here we report on the results of a new analytical method, in-situ derivatization solid phase microextraction (SPME) multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS), that allows for a comprehensive fingerprint analysis of the substance class in complex matrices. Due to the high polarity of amino compounds, the complex urine matrix and prevalence of conjugated anilines, pretreatment steps such as acidic hydrolysis, liquid-liquid extraction (LLE), and derivatization of amines to their corresponding aromatic iodine compounds are necessary. Prior to detection, the derivatives were enriched by headspace SPME with the extraction efficiency of the SPME fiber ranging between 65 and 85 %. The measurements were carried out in full scan mode with conservatively estimated limits of detection (LOD) in the range of several ng/L and relative standard deviation (RSD) less than 20 %. More than 150 aromatic amines have been identified in the urine of a smoking person, including alkylated and halogenated amines as well as substituted naphthylamines. Also in the urine of a non-smoker, a number of aromatic amines have been identified, which suggests that the detection of biomarkers in urine samples using a more comprehensive analysis as detailed in this report may be essential to complement the approach of the use of classical biomarkers.

3.2 Introduction

Aromatic amines are highly polar organic compounds that are used or produced in various fields such as drugs, pesticides, dyes, tobacco smoke and intermediates for the manufacturing of other chemicals [1-2]. Aromatic amines in tobacco smoke are suggested to be important causing agents for the development of bladder cancer in humans [3]. In early studies human bladder cancer was frequently associated with occupational exposure to carcinogenic aromatic amines such as 4-aminobiphenyl, 2-napthylamine, 2-methylaniline and 4,4´-diaminobiphenyl as well as to some polycyclic aromatic hydrocarbons (PAHs), and since it has been known that these chemicals are carcinogenic to humans, their exposure was highly reduced [2, 4]. However, in the 1950s the composition of tobacco and cigarette design changed, resulting in high concentrations of bladder carcinogens such as 2-naphthylamine to occur in cigarette smoke, and hence an increased risk of smoking-related bladder cancer [5-6]. The International Agency for Research on Cancer (IARC) so far classified six aromatic amines as carcinogenic (4-aminobiphenyl, 2-naphthylamine and 4,4´-diaminobiphenyl) or probably carcinogenic (4-chloro-2-methylaniline, 2-methylaniline, 4,4'-methylene bis (2-chloraniline)) to humans [7]. They suggested tobacco smoking to be a major cause of transitional-cell carcinoma (TCC), the most common type of bladder cancer, where the risk increases with the duration of smoking and the number of cigarettes smoked [4, 8-9]. A cigarette is known to contain up to 600 ingredients and, when burned, it produces more than 4000 chemicals, of which some are known to be carcinogenic.

Among these, some aromatic amines are found to be present both in the main stream and in the side stream smoke [3, 10]. During the smoking process, aromatic amines are carried into the bloodstream where they are filtered out of the blood by the kidneys and end up in the urine. When the urine is stored in the bladder, aromatic amines get in contact with the cells that line the bladder where they react and may form high-grade cancer cells [4]. There is still limited information about the amount and/or specific aromatic amines that can cause bladder cancer in humans. There is evidence though, that more anilines have to be considered as several amines were mutagenic in toxicological testing or caused cancer in testing animals [11], but the results are difficult to extrapolate to humans. In the past few years, several analytical methods have been published on the analysis of aromatic amines in human urine [3, 12-14]. However, the studies were somehow limited to analysis with conventional gas chromatography, where many isomeric compounds may not be separated due to their similar retention times and, as a result, a rather small number of aromatic amines are detected in urine. To our knowledge, no results have yet been reported where analysis of aromatic amines in urine was made by comprehensive two-dimensional chromatography. The method described here offers a combination of very sensitive and precise analytical techniques that do not only isolate and extract the analytes from the matrix but also decrease their polarity and introduce iodine to the aromatic ring for the analytes to be detected selectively. Furthermore multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS) in combination with solid phase microextraction (SPME) was applied to enrich, separate and make it possible to distinguish isomers of several aromatic amines of the same molecular mass.

In addition to the method development, ionic liquid columns (ILC) were tested to validate if their use could possibly offer better selectivity for iodinated derivatives. The columns covered polar, highly polar, and extremely polar stationary phases. This study demonstrates exemplarily patterns of aromatic amines found in urine of a smoker and non-smoker, due to their potent carcinogenic effect that can be caused by tobacco smoking and may lead to bladder cancer.

3.3 Experimental

3.3.1 Chemicals and reagents

Aromatic amines and reference substances with a purity of >97 % were purchased from Sigma-Aldrich, Steinheim, Germany and Alfae-Aesar, Karlsruhe, Germany. The reagents used for hydrolysis and derivatization, namely concentrated hydrochloric acid (37 %), hydriodic acid (55 %, unstabilized, A.C.S), sodium nitrite (≥97 %), sodium sulphite (≥98 %), alizarinsulfonic acid (98 %) and amidosulfonic acid (99,3 %) were all products of Sigma-Aldrich. Sodium hydroxide (99 %) was from VWR, Darmstadt, Germany and sodium acetate (≥99 %) from Applichem, Darmstadt, Germany. Analytical grade methanol was purchased from KMF Laborchemie, Lohmar, Germany and the ultra-filtered water used was a product of PureLab Ultra (ELGA LabWater, Celle, Germany).

3.3.2 Stock solution and standard preparation

Stock solutions were prepared by weighing approximately 10 mg of each pure substance in a 10-mL volumetric flask and diluting it with analytical grade methanol to make a final concentration of 1 g/L. From the stock solution, standard solutions with concentrations from 1 ng/L to 500 ng/L were prepared in an aqueous medium by dilution using Hamilton syringes. The stock solution, as long as it was not used, was kept cool in a refrigerator at 4 °C for at most 1 month. The diluted standards were freshly prepared from the stock solution for every analysis.

3.3.3 Urine sample preparation

Urine samples were collected randomly from smoking and nonsmoking donors in 50-mL polypropylene tubes. Into 20 mL urine sample, 10 mL concentrated hydrochloric acid (37 %) was added and the mixture was heated in properly closed vials for 12 h at 110 °C to cleave aromatic amines adducts. After cooling to room temperature, the samples were made alkaline by adding 20 mL of a 10-*M* sodium hydroxide solution so that the amines will be present in their non-charged form. During this process, a black precipitate forms that could disturb extraction and, therefore, the samples had to be filtered using a filter paper. The filtered samples were extracted three times, with 5-mL diethylether while shaken manually for about 1 min and then left to stand for another 1 min to allow the separation of aqueous and organic layers. The aqueous phase was discarded and the organic phase was kept and washed once with 2 mL of 0.1 *M* sodium hydroxide solution.

The amines in the organic phase were extracted back into the aqueous phase with 5 mL water acidified with 100 µL concentrated hydrochloric acid (37 %). The remaining diethylether in the aqueous samples was gently evaporated in a nitrogen stream for 5 min. Urine samples of both smoker and non-smoker were prepared in duplicate.

3.3.4 Derivatization

To decrease the polarity of the extracted amines as well as introducing iodine in the aromatic ring, the samples were derivatized through diazotization and iodination in a one-pot reaction, as partly described by Schmidt et al. [15]. Into each 5 mL extracted sample, 100 µL hydriodic acid (55 %) and 200 µL sodium nitrite (50 g/L) were added and the sample was shaken for 20 min. During the reaction, the aromatic amines are diazotized followed by a subsequent substitution by iodine at the aromatic ring, which results in aromatic iodine compounds. To destroy the surplus of nitrite, 0.5 mL of amidosulfonic acid (50 g/L) was added. The sample was shaken for another 45 min and then heated in a water bath at 95 °C for 5 min to convert the unreacted diazonium ions to phenols and also decompose excess amidosulfonic acid. After cooling to room temperature, 125 µL saturated sodium sulfite was added to reduce the iodine residue. This is demonstrated by immediate discoloration of an initially brownish solution. Finally, a 100 µL of 1 % Alizarinsulfonic acid was added into the sample followed by 0.5 mL of saturated sodium acetate to adjust the pH of the sample to pH 5.

3.3.5 SPME

Headspace SPME was used to enrich the iodinated derivatives before measuring by GC×GC-qMS. Prior to extraction, the headspace vial containing the sample was pre-incubated for 10 s at the incubation temperature of 60 °C, while agitating at the speed of 500 rpm. The SPME fiber was then auto-injected into the headspace vial to extract the solution in the headspace for 25 min, followed by desorbing the extracted analytes into the GC-injection port for 5 min. After every extraction, the SPME fiber was conditioned in the needle heater for 20 min. For the analyses of authentic standards and urine samples, the same extraction conditions with the SPME fiber were used and the solutions were extracted and measured once. To determine the extraction efficiency of the SPME fiber, depletion SPME experiments were done by analyzing four reference substances, namely iodobenzene, methyl-iodobenzene, chloro-iodobenzene and pentafluoro-iodobenzene. These substances are the derivatives of very common aromatic amines except for pentafluoro-iodobenzene, which was used as an internal standard. A 5 µg/L mixture of these four compounds was prepared in a 10-mL volumetric flask and made up to the mark with water; 2 mL of the solution was transferred into a 20-mL headspace vial and diluted with 8 mL water to make a final concentration of 1 µg/L, in triplicate. Each solution was extracted with the SPME fiber and measured five times consecutively.

3.3.6 Instrumentation

All analyses were performed by a Shimadzu GC system consisting of a GC-2010 and GCMS QP2010 Plus (Shimadzu GmbH, Duisburg, Germany) with a built-in loop-type modulator from Zoex Corporation (Houston (Texas), USA). The system is coupled with a Shimadzu AOC-5000 liquid, headspace and SPME GC injection system, which has a control panel for mobilizing samples and injectors. The SPME fiber used for sample enrichment was 65 µm PDMS/DVB (Sigma-Aldrich, Schnelldorf, Germany) with a SPME liner of 0.75 mm x 5.0 mm x 95 mm made for Shimadzu GCs. The GC columns used were, for the first dimension, a DB-5, 30 m, 0.25 mm i.d., 0.25 µm film from Agilent Technologies, Waldbronn, Germany and for the second dimension, a BPX-50, 2.7 m, 0.15 mm i.d., 0.15 µm film from SGE Analytical Science, Griesheim, Germany. Ionic liquid columns namely SLB IL-59 (polar), SLB IL-61 (polar), SLB IL-76 (highly polar), SLB IL-82 (highly polar), and SLB IL-100 (extremely polar) were supplied by Sigma-Aldrich, Taufkirchen, Germany with 2.7 m, 0.1 mm i.d. and 0.08 µm film. The second column was coiled around the Zoex loop-type modulator allowing 1 meter after cryo-focussing to the detector. The modulation time was set at 6 s. Helium was used as carrier gas and the nitrogen cooled by liquid nitrogen for cryogenic effect. In the case where GC/MS was used, the cold jet was set off, hence no modulation and cryogenic effect was needed. The column temperature gradient was set initially at 60 °C and held for 3 min, then increased to 230 °C at 5 °C/min and held for another 3 min. At this start temperature (60 ° C) the column head pressure was 97 kPa. The column flow was set at 1 mL/min with a linear velocity of 29 cm/s.

The temperature of injection port and MS interface were set at 250 °C and the ion source temperature at 230 °C. The instrument was operated in the splitless injection mode with the detector voltage at 1.2 kV and scan rate of 10,000 u/s in full scan mode. The scanned mass-to-charge ratios were between *m/z* 70 and 300 for authentic standards (scan speed=0.02) and *m/z* 70 - 460 for urine samples (scan speed=0.03). The data was processed via GC×GC Software, GC Image (Shimadzu GmbH, Duisburg, Germany).

3.3.7 Safety considerations

Some pure substances of aromatic amines are highly toxic and/or carcinogenic. Therefore, care should be taken during the preparation of stock solutions by strictly using protection wear such as nose masks, hand gloves, laboratory coat etc. as well as working strictly under the fume hood. Some symptoms of contact with the substances are itching skin, irritation in the nose and sudden sharp headaches when inhaled.

3.4 Results and discussions

3.4.1 Determination of extraction ratio by depletion SPME

To test the depletion SPME model as described by Zimmermann et al. [16], a 10 mL sample (n=3) containing four reference substances at a concentration of 1 µg/L was analyzed. A 65 µm PDMS/DVB fiber was used where each sample placed in a 20-mL headspace vial was extracted in the headspace (vial penetration of 22 mm) and analyzed by GC/MS five times consecutively. The conditions for extraction with the SPME fiber are as described previously. The exponential decay of the peak areas for each analyte, in correlation with the number of extraction, was observed. This is demonstrated in Fig. 3.1a where the mean peak areas for each analyte are plotted against the number of extraction (*x*). For methyl-iodobenzene, more than 80 % of the analytes were extracted after the third extraction time and no more analytes could be extracted from the solution. Hence, the number of extractions (*x*) shown is up to three. The same applies for pentafluoro-iodobenzene with the number of extraction (*x*) shown up to four. To determine the SPME fiber extraction efficiency, the following equation was used:

$$n_{f,x} = n_{s,o} E\,(1- E)^{x-1} \qquad (1)$$

where $n_{f,x}$ is the extracted amount of analytes, $n_{s,o}$ the initial amount of analytes in the sample, E the extraction ratio and x the number of the consecutive extraction steps. The results can be compared with the fitting function $f(x) = ab^x$ with $a = n_{s,o}E$ and $b = 1- E$. From this equation, the extraction ratio E could be easily determined from the slope b after linearization:

$$\log n_{f,x} = \log (n_{s,o}E) + (x - 1) \log (1- E) \quad (2)$$

Figure 3.1b shows the log peak areas plotted against the number of extraction, *x*.

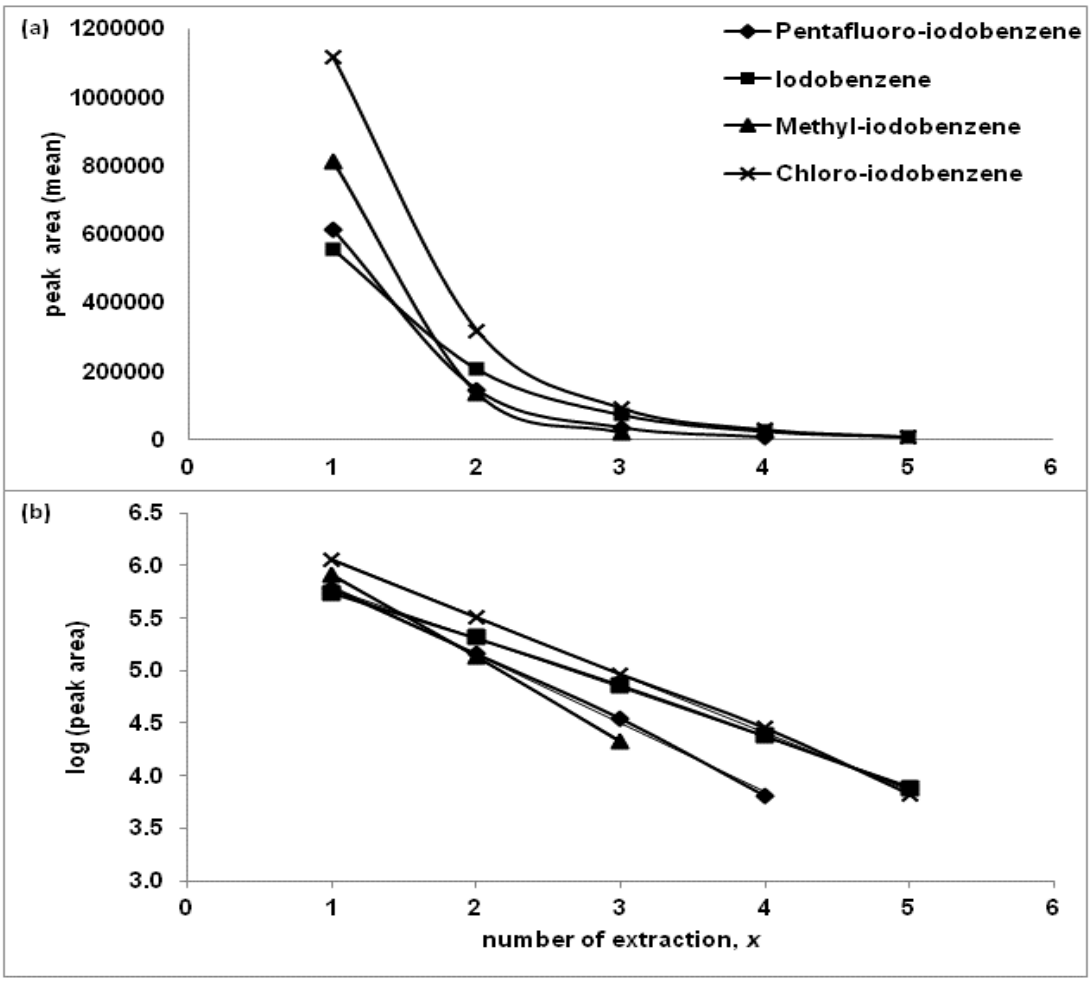

Fig. 3.1 Multiple extraction of a sample containing four iodinated compounds at a concentration of 1 µg/L using a 65 µm PDMS/DVB SPME fiber and the determination of peaks area with GC/MS. In **(a)** mean peak area and in **(b)** log (peak area) are plotted against the number of extraction, *x* where n=3. Relative standard deviations (RSD) were ≤7 % for the first two extractions and ≤20 % for extractions 3 to 5. The RSDs are not shown here to increase legibility.

The slopes obtained from the plots and the calculated extraction ratios of the analytes are shown in Table 3.1 with extraction efficiency of the SPME fiber ranging between 65 % and 85 %, with confidence intervals between 5.8 % and 7.1 % and RSD of 4 % - 7 %. The fiber-sample partition coefficient (K_{fs}) of each compound was calculated from the extraction ratios using the equation below:

$$K_{fs} = V_s E / V_f (1-E) \quad (3)$$

where K_{fs} is the partition coefficient between the fiber coating *f* and the aqueous sample *s* and V_s and V_f are the volumes of the sample (10 mL) and the fiber coating (0.440 µL), respectively. Due to the good precision and the efficient extraction of the sample, the PDMS/DVB fiber was retained for further experiments, and hence no other SPME fibers were tested.

Table 3.1 Calculated extraction ratios, *E* and fiber sample partition coefficients, K*fs* for depletion SPME

Compound	**R^2**	**Slope [log (1-E)]**	**E (%)**	**K_{fs}**
Pentafluoro-iodobenzene	0.9981	-0.6565	78±6.2	80579
Iodobenzene	0.9991	-0.4657	66±5.8	43727
Methyl-iodobenzene	0.9998	-0.7950	84±7.1	119318
Chloro-iodobenzene	0.9989	-0.5489	72±6.0	57581

3.4.2 Method development for GC×GC using pure substances

A standard solution with 16 aromatic amines at 100 ng/L in aqueous solution was used to optimize instrument parameters and to develop a method for the determination of aromatic amines in urine samples. The standards were derivatized as described in the Experimental section and analyzed by GC×GC-qMS. In Fig. 3.2, a 100 ng/L standard is shown as an example and Table 3.2 summarizes the names, the derivative fragments (*m/z*) and estimated LODs of the analyzed substances. All the compounds were eluted and well separated within 30 min in the first-dimension (1D) and within 6 s in the second-dimension (2D), except for the methylaniline derivatives (analytes 2 and 3) which could not be separated. Conversely, dimethylaniline derivatives (analytes 7 and 8) were well separated. The mass spectra of all derivatives showed the typical iodine fragment ion signal with 127 *m/z*, which enables the analytes to be detected selectively, even in complex samples such as urine. The method was used further to detect aromatic amines in urine samples.

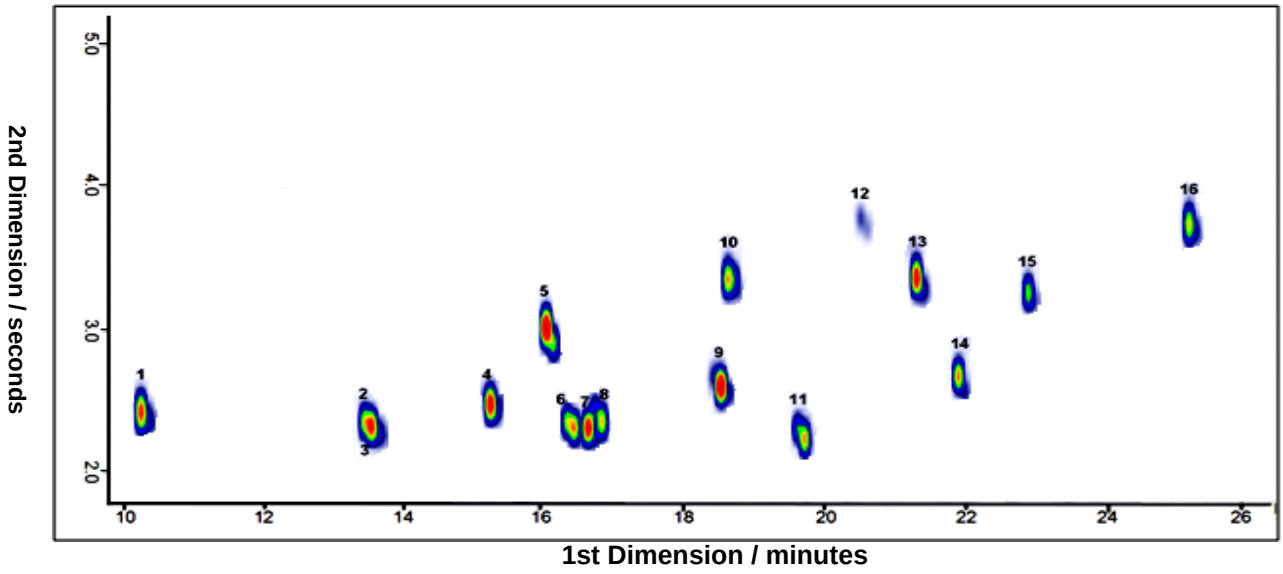

Fig. 3.2 A 100 ng/L standard mixture of 16 derivatized aromatic amines and analysis by GC×GC-qMS for the method development. The analytes are numbered based on their elution time and are listed in Table 3.2

Table 3.2 Names and fragments of the iodinated derivatives analyzed for method development with estimated LODs indicated for aqueous standards

No.	Substance	Derivatives	Fragments of the iodinated derivatives (m/z)	LODs estimated from calibration [17] in ng/L
1	Aniline	Iodobenzene	204, 77	24.4
2	o-methylaniline	1-iodo-2-methylbenzene	218, 91	5.2[a]
3	p-methylaniline	1-iodo-4-methylbenzene	218, 91	5.2[a]
4	3-chloro-4-fluoroaniline	3-chloro-4-fluoro-1-iodobenzene	256, 129	18.8
5	2-chloroaniline	2-chloro-1-iodobenzene	238, 111	3.8
6	4-ethylaniline	4-ethyl-1-iodobenzene	232, 217,105	11.4
7	2,6-dimethylaniline	2,6-dimethyl-1-iodobenzene	232, 105	9.3
8	2,4-dimethylaniline	2,4-dimethyl-1-iodobenzene	232, 105	9.3
9	4-chloro-2-methylaniline	4-chloro-1-iodo-2-methylbenzene	252, 125	4.3
10	2-bromoaniline	2-bromo-1-iodobenzene	282, 155	10.9
11	2,4,6-trimethylaniline	1-iodo-2,4,6-trimethylbenzene	246, 119	22.4
12	2-aminoacetophenone	2-iodoacetophenone	246, 203, 76	n.a.
13	2,6-dichloroaniline	2,6-dichloro-1-iodobenzene	272, 145	6.1
14	3-chloro-2,6-dimethylaniline	3-chloro-2,6-dimethyl-1-iodobenzene	266, 139	13.4
15	3-chloro-4-methoxyaniline	3-chloro-1-iodo-4-methoxybenzene	268, 141	13.1
16	2-naphthylamine	2-iodonaphthalene	254, 127	19.3

n.a.: No calibration performed due to low signal intensity below 100 ng/L

[a]Compounds 2 and 3 could not be separated and therefore the sum of both was integrated

3.4.3 Choice of second dimension column

In order to investigate further the separation of isomers in the second dimension, five ILC were studied and compared with the standard BPX-50 column. The columns, in the order of increasing polarity were: SLB IL - 59, 61, 76, 82 and 100. In the first dimension, the same column was kept throughout the experiments (DB-5 column, non-polar). For these experiments, a standard consisting of a mixture of aromatic amines mentioned in Table 3.2, except for 3-chloro-4-methoxyaniline, was used. The standards were derivatized and enriched by headspace SPME as described before and analyzed by GC×GC-qMS. To compare the column combinations, peak distribution of the analytes in 2D space was plotted for all five ILC as shown in Fig. 3.3 below. The analytes were better spread in one of the polar IL columns, IL 61, and two of the highly polar columns, IL 76 and 82. The results obtained imply that different ionic liquids interact in a comparable way with the iodinated derivatives analyzed here as the retention times in the second dimension are similar. Another possibility could be that as compounds elute from the first dimension they are already near the elution temperature for the second column and are, therefore poorly retained. The results are comparable to the BPX-50 column, a traditional mid-polar GC column used in the experiments mentioned previously in this study (for example Fig. 3.2). As interesting as the ILC features are, BPX-50 was found to be as well suited for our analytes of interest and, therefore, was retained for further analysis.

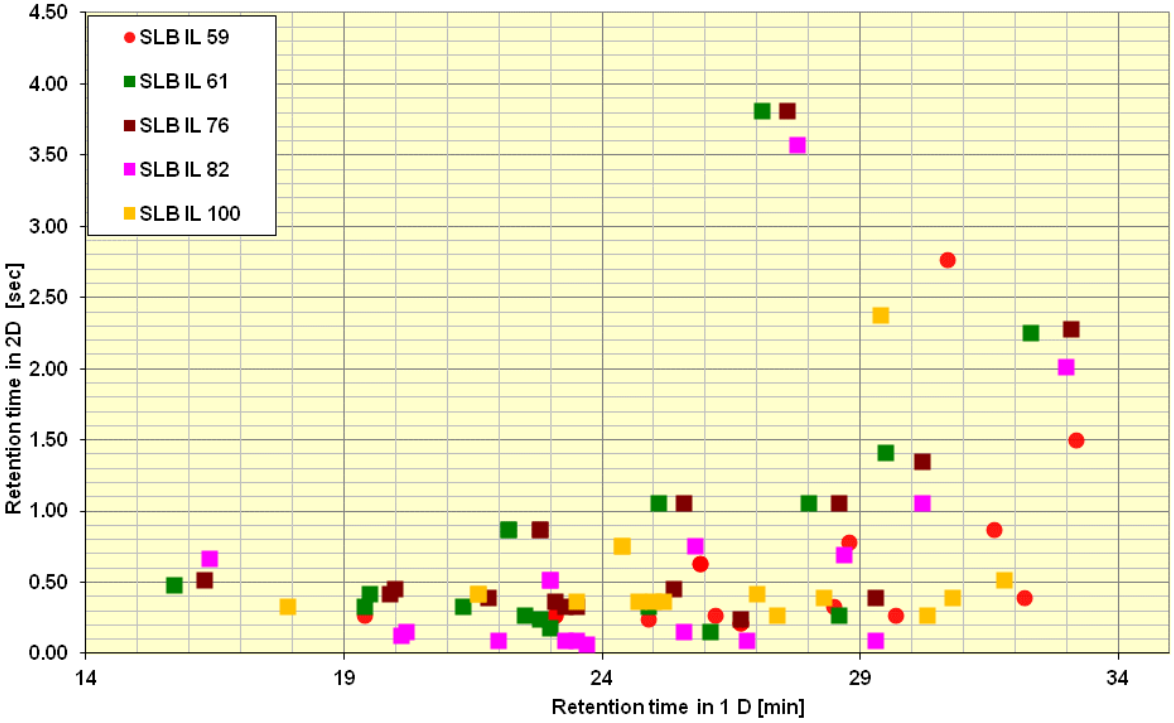

Fig. 3.3 Distribution of the 15 derivatized aromatic amines peaks mentioned in table 3.2 (except for 3-chloro-4-methoxyaniline) on the DB-5/ILC combinations and analysis by GC×GC-qMS

3.4.4 Aromatic amines in urine samples

Urine samples of a tobacco smoker and a non-smoker were hydrolyzed, extracted, derivatized and analyzed by GC×GC-qMS. The analyses were carried out in full-scan mode so that all extractable and GC-suitable compounds were detected. Fig. 3.4a and b show the total ion chromatogram (TIC) of the aromatic iodine compounds that are proposed to be present in the urine of non-smoking and smoking donors, respectively.

In both Fig. 3.4a and b not all the compounds can be visualized because of low intensity, especially in the urine of a non-smoker, but their identification is possible. The mass spectra of all derivatives showed the typical iodine fragment ion signal with 127 *m/z*; as a consequence, aromatic amines present in the samples could be identified by monitoring the iodine fragment ion. Since intensity of the mass fragment 127 is typically small, it would be beneficial to monitor instead the neutral loss of iodine. However, so far there is no opportunity in GC×GC software to do so. For each compound, the isomers (viz. blobs) were counted to obtain the total sum of analytes present in the sample. This is summarized in Table 3.3 below with the names of the anilines and the fragment ions of the iodinated derivatives. In the urine of a donor exposed to cigarette smoke, ca. 150 aromatic amines were tentatively identified. Among the substances found, less than 10 are described in literature in relation with tobacco smoking – bladder cancer [2, 4, 8]. In the urine of a person without known exposure to cigarette smoke, many anilines (ca. 120) were identified; nonetheless, regarding the number and peak intensities of occurring iodinated derivatives, the sample was less burdened. Amongst the detected compounds, alkylated anilines were the largest group (n = 39), as shown in Fig. 3.5a in groups of isomeric homologues (C1- to C4-anilines). Here, the number of isomers rapidly increases with larger or more numerous alkyl substituents and the intensities vary substantially among the isomers. In addition, compounds of the same nominal mass, such as aminoacetophenone (*m/z* 246) and C3-anilines (*m/z* 246), derivatives may elute at similar retention time in the first dimension but as in this case could be well separated in the second dimension.

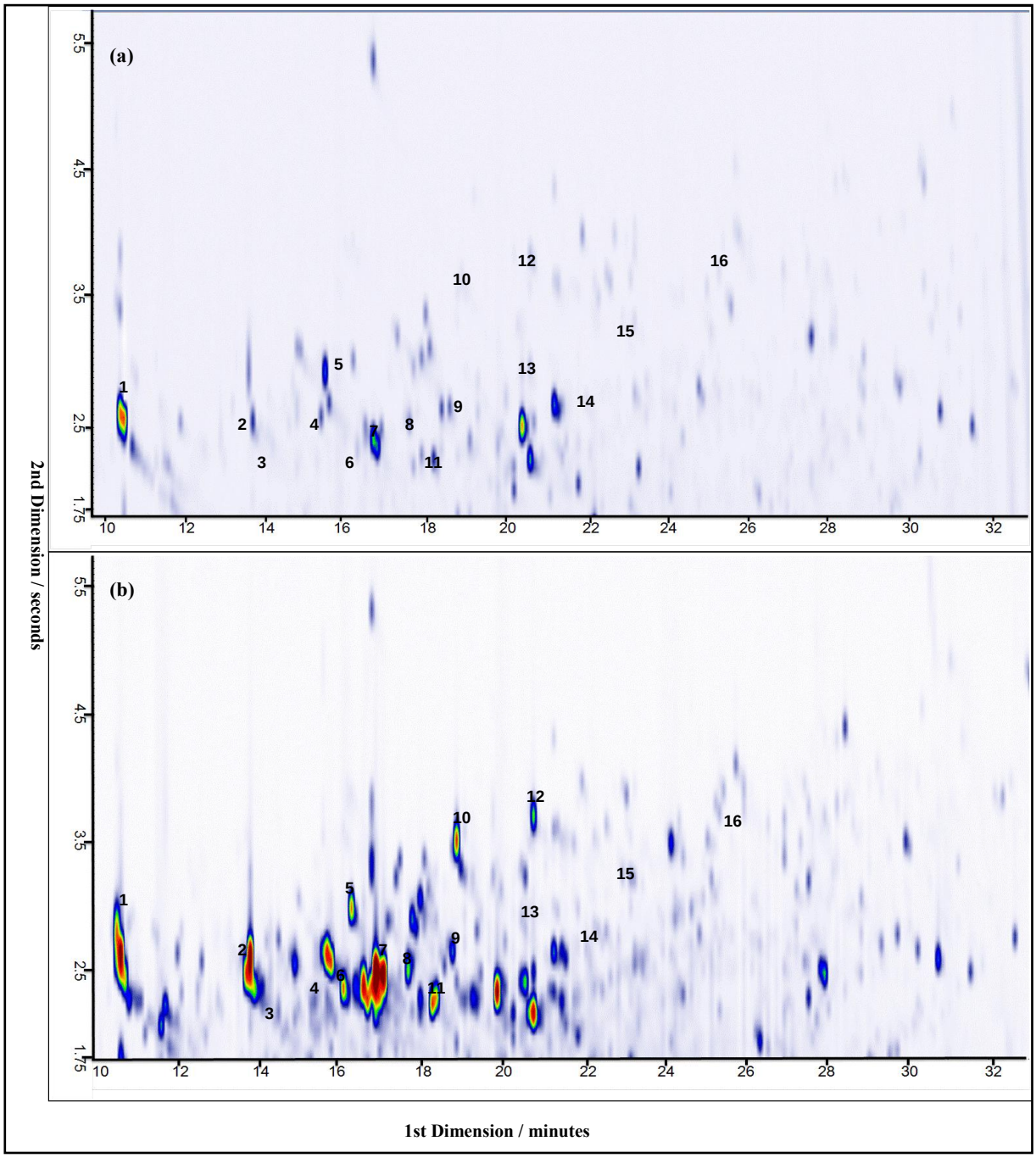

Fig. 3.4 TIC from *m/z* 70-460 showing aromatic iodine compounds proposed to be present in the urine of **(a)** a person without exposure to cigarette smoke and **(b)** a smoking person. The same scale was used on both chromatograms. The red color indicates the high intensity of the analyte followed by yellow, green and blue as the

intensity decreases. The samples were measured by SPME GC×GC-qMS after hydrolysis, liquid-liquid extraction and derivatization. Numbers in the figure refer to compounds listed in Table 3.2 and shown in Fig. 3.2. Note that not all of the shown spots are necessarily caused by aniline derivatives

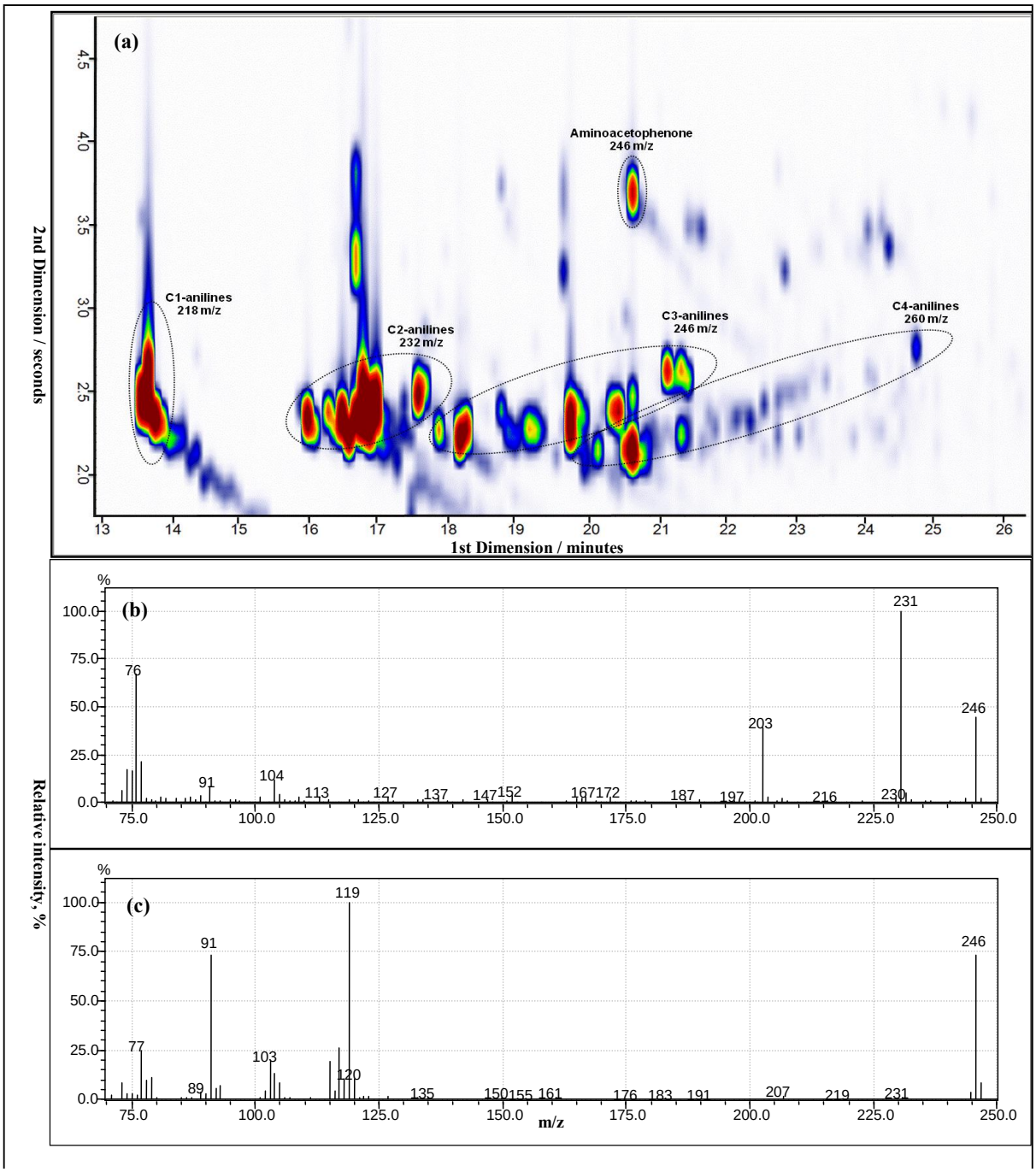

Fig. 3.5 (a) Reconstructed ion chromatogram (RIC) of a urine sample of a smoker showing typical *m/z* associated with homologous alkylated aromatic iodine compounds and a separation of aminoacetophenone and C3–aniline derivatives with the same mass-to-charge ratio, *m/z* 246. The compounds selected are derivatives of

C1-anilines (*m/z* 218), C2-anilines (*m/z* 232), C3-anilines/aminoacetophenone (*m/z* 246), and C4-anilines (*m/z* 260), and the RIC is generated by summing up signals at these four *m/z*; **(b)** and **(c)** show mass spectra of iodinated derivatives of aminoacetophenone and C3-aniline, respectively, with fragmentation patterns and loss of *m/z* 127 (iodine)

Here the aminoacetophenone derivative (viz. iodoacetophenone, *m/z* 246) loses at first a methyl group (fragment ion *m/z* 231) followed by a carbonyl group (fragment ion *m/z* 203) and an iodo group (fragment ion *m/z* 76). In the case of the C3-aniline derivative (*m/z* 246) an iodo group is lost (fragment ion *m/z* 119) followed probably by an ethyl group (fragment ion *m/z* 91) and a methyl group (fragment ion *m/z* 77). Separation of compounds with the same nominal mass such as these is not possible when measuring with a conventional GC-MS with unit mass resolution; hence, this result underlines the importance of GC×GC-MS for the separation of multiple compound mixtures in complex matrices. Due to the large number of compounds and the GC×GC data files, which have much bigger size compared to conventional GC, analysis and handling of data especially for quantitative analysis, is still a great challenge. In that regard, further progress has been described by Tranchida et al. [18]. We, therefore, suggest the use of a fingerprint analysis to easily monitor the exposure to aromatic amines providing a better understanding about their role on the formation of bladder cancer.

Table 3.3 Tentative list of aromatic amines proposed to be present in the urine of a smoker and a non- smoker based on the fragmentation patterns and the loss of iodine fragment ion *m/z* 127

Substance	**m/z of iodinated derivatives [M^+]**	**Main fragments *(m/z)***	**Isomers found in urine of a smoker**	**Isomers found in urine of non-smoker**
Diaminobenzene	330	203, 76	2	2
C1- Diaminobenzene	344	217, 90	3	2
C2- Diaminobenzene	358	231, 104	3	2
C3- Diaminobenzene	372	357, 245	3	3
C4- Diaminobenzene	386	371, 244	1	1
C1- Triaminobenzene	456	329, 202, 75	3	1
Chlorodiaminobenzene	364	237, 110	2	1
C1- Chlorodiaminobenzene	378	343, 251, 124	2	0
Bromodiaminobenzene	408	281, 154, 75	1	1
Tetrahydronaphthylamine	258	131, 102	10	2
C1- Tetrahydronaphthylamine	272	257, 130	7	7
C2- Tetrahydronaphthylamine	286	271, 159	7	10
C3- Tetrahydronaphthylamine	300	258, 173, 157	5	5
Aminoindane	244	117	5	4
C1- Aminoindane	258	244, 131	1	2
Aminoquinoline	255	128	2	2
C1- Aminoquinoline	269	142	4	3
C2- Aminoquinoline	283	156, 141	3	0
Aminopyridine	205	78	2	0
Aminothiophene	210	83	1	1
C1- Aminothiophene	224	97, 81	1	2

Table 3.3 (continued)

Aminoacetophenone	246	231, 203, 76	3	3
Aminophenol	220	93	2	1
C1- Aminophenol	234	107, 89	3	2
C2- Aminophenol	248	220, 93	1	1
C3- Aminophenol	262	247, 135	0	3
Aniline	204	77	1	1
C1- Aniline	218	91	2	2
C2- Aniline	232	217, 105	7	5
C3- Aniline	246	119	12	10
C4- Aniline	260	245, 118	17	16
Chloroaniline	238	111	2	3
C1- Chloroaniline	252	125	2	2
C2- Chloroaniline	266	231, 139	6	4
C3- Chloroaniline	280	265, 138	5	3
C4- Chloroaniline	294	279, 152	2	1
Dichloroaniline	272	145	2	2
Bromoaniline	282	155	3	2
Methoxyaniline	234	219, 92	1	1
C3- Methoxyaniline	276	261, 134	2	2
Chloromethoxyaniline	268	253, 126	1	2
Thioaniline	236	109	1	3
C1- Thioaniline	250	123, 108	1	1
Naphthylamine	254	127	2	2
C1- Naphthylamine	268	141	4	4
C2- Naphthylamine	282	155	2	2
C1- Aminobiphenyl	294	279, 152	1	1
C2- Aminobiphenyl	308	181, 166	3	4
Diaminobiphenyl	406	279, 152	1	0
Methylenedianiline	420	293, 166	1	0
C1- Diaminonaphthalene	394	267, 140	3	1

3.5 Conclusions

An analytical method was developed to reveal a more complete picture of the occurrence of aromatic amines in urine by investigation of smoking and non-smoking persons. This method was at first developed for the detection of aromatic amines and not for quantification. More aromatic amines based on peak intensities at higher concentration were identified in the urine sample of a smoker compared with the urine of a non-smoker. Since many more aromatic amines occur in urine of a person exposed to cigarette smoke than hitherto known, it seems that the health risk potential by these compounds might exceed by far previous expectations. The use of GC×GC-MS to detect aromatic amines in urine samples was important for the identification of many structurally related compounds. The method is very efficient with high sensitivity such that single ion monitoring is not required, allowing recording of full-scan mass spectra for compound identification. In the future, quantification of aromatic amines in urine based on the presented method will be evaluated as well as the analysis of these compounds in the urine of patients who are diagnosed with bladder cancer. This eventually may be beneficial for further investigations of biomarkers, which could be used in the future to diagnose the level of cancer-causing aromatic amines in urine samples.

3.6 References

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CHAPTER 4

Quantitation of aromatic amines in human urine by comprehensive multi-dimensional gas chromatography mass spectrometry(GC×GC-qMS)

4.1 Abstract

A reliable analytical method, in-situ derivatization solid phase microextraction (SPME) multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS), was used for the quantification of selected aromatic amines in human urine. To determine the repeatability and linearity of the method, a standard mixture consisting of 15 aromatic amine pure substances in synthetic urine was used. The relative standard deviations (% RSD, n=8) obtained were between 6 - 20 %, with limits of detection (LOD) and limits of quantification (LOQ) ranging between 10 - 90 ng/L and 10 - 100 ng/L respectively. In the urine of non-smokers (n=8), past-smokers (n=8) and smokers (n=8), the selected aromatic amines (14, except for 3-chloro-4-fluoroaniline) were identified and quantified by means of external calibration curve with the recovery rates (%) of analytes in the standards ranging between 75 - 160 %. The calculated mean concentrations of the selected analytes were ca. 15 - 400 ng/L in the urine of non-smokers (NS), ca. 40 - 650 ng/L in the urine of past-smokers (PS) and ca. 25 - 660 ng/L in the urine of smokers (S). Several other aromatic amine isomers were also identified in the urine samples of non-smokers (ca. 85), past-smokers (ca. 130) and smokers (ca. 160).

4.2 Introduction

Tobacco smoking is known to be the major risk factor for the development of bladder cancer in humans [1-3]. The risk for smokers is approximated to be four-fold in comparison with non-smokers [3], having a potential of escalation with the duration of smoking as well as the number and the type of cigarettes smoked [1, 4-5]. Over the last few decades some aromatic amines (AAs) such as 2-naphthylamine, 4-aminobiphenyl and others, have been studied in order to clarify the underlying mechanism of bladder carcinogenesis [6]. The carcinogenicity of aromatic amines is identified in research studies as having the ability to cause bladder cancer through DNA adduct formation and mutagenicity [1, 7]. This class of chemicals is known to exert its genotoxic effects after being activated by metabolism [8-9]. A simple illustration of biotransformation of aniline is used here as an example and is shown in Fig. 4.1 below; where initially an amino group is oxidized by the cytochrome P450 enzymes (CYP1A2) or to a much lesser extent, N-methylation or peroxidation. The hydroxyarylamine formed from the reaction is then transported from the liver to the blood and by the blood to the bladder, where it undergoes hydrolysis at the acidic pH of urine [10]. A reactive intermediate (electrophilic nitrenium cation) is then formed which can bind directly to the DNA of uroepithelial cells and form covalent adducts. These persistent arylamine-DNA adducts are widely believed to be etiologically involved in the genesis of bladder cancer [11-12].

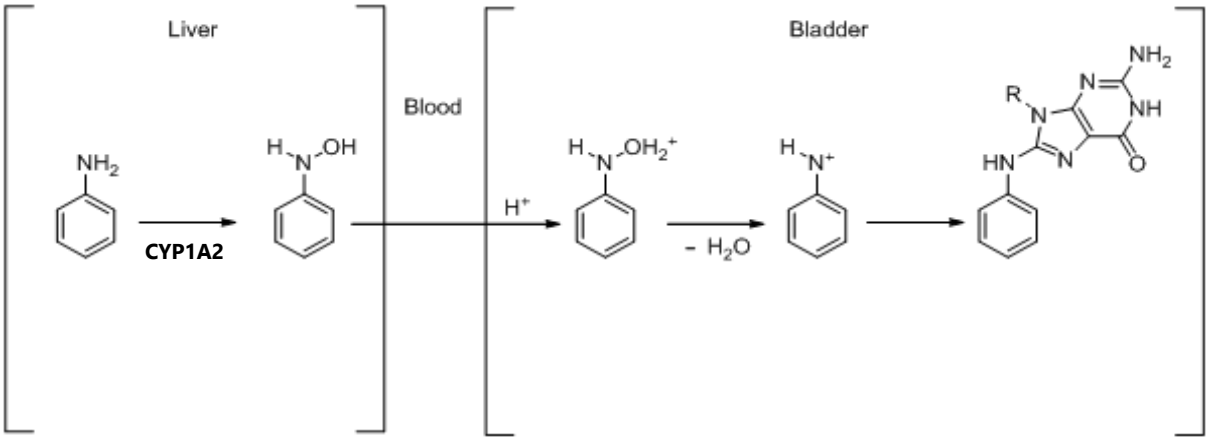


Fig. 4.1 Biotransformation of aniline used as an example, to illustrate the formation of persistent aromatic amines-DNA adducts for the genesis of bladder cancer [6, 13]

Whilst the perspective on the DNA adduction, the mutagenicity and the etiological involvement of AAs in the genesis of bladder cancer have been provided [6, 8, 11]; in this study we focus mainly on the development of an analytical method, which rather reveals a more complete picture of the occurrence (qualitative) and the quantity of some aromatic amines in human urine. The combination of the analytical procedures and techniques used, viz., the liquid-liquid extraction (LLE), in-situ derivatization solid phase microextraction (SPME) and multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS) enable the analytes of interest to be detected selectively even in the complex mixture of urine. In this report the proposed quantity of selected aromatic amines in the urine of smokers, non-smokers and past-smokers is demonstrated.

4.3 Experimental

4.3.1 Chemicals and reagents

Synthetic urine was purchased from Synthetic Urine e.K., Nußdorf, Germany. This product is described as corresponding to the original urine with respect to all relevant chemical properties; however, free from undesirable accompanying substances such as metabolites or bacteria. Pure aromatic amines substances with a purity of >97 %, as well as the reagents used for hydrolysis and derivatization, namely concentrated hydrochloric acid (37 %), hydriodic acid (55 %, unstabilized, A.C.S), sodium nitrite (≥97 %), sodium sulphite (≥98 %), alizarinsulfonic acid (98 %) and amidosulfonic acid (99, 3 %) were all products of Sigma-Aldrich, Steinheim, Germany. Sodium hydroxide (99 %) was from VWR, Darmstadt, Germany and sodium acetate (≥99 %) from Applichem, Darmstadt, Germany. Analytical grade methanol was purchased from KMF Laborchemie, Lohmar, Germany and the ultra-filtered water used was a product of PureLab Ultra (ELGA LabWater, Celle, Germany).

4.3.2 Preparation of stock solution and standards

A stock solution consisting of a mixture of 15 AAs was prepared by weighing approximately 10 mg of each pure substance in a 10-mL volumetric flask and diluted with analytical grade methanol, to make a final concentration of 1 g/L. From the stock solution, standard solutions with concentrations from 1 ng/L to 500 ng/L were prepared in synthetic urine by dilution using Hamilton syringes. To test the precision of measurements, a 100 ng/L standard was prepared in a 100-mL volumetric flask and made up to the mark with synthetic urine; 10 mL of the solution (n= 8) was transferred into 20-mL headspace vials and each solution was derivatized and then

measured by GC×GC-qMS. The stock solution, as long as it was not used, was kept cool in a refrigerator at 4 °C for at most one month.

4.3.3 Preparation of human urine samples

Urine samples were collected from the Epidemiology Institution (Institute für Medizinische Informatik, Biometrie und Epidemiologie, Universitätsklinikum Essen, Germany) and analyzed to identify and quantify the amount of aromatic amines in the samples. The samples varied from smoking, non-smoking and past-smoking persons and both males and females. There was no form of diagnosis, historical interview and/or personal details of the donors taken before sample collection. The urine samples were prepared in duplicates as described in detail by Lamani et al. [14]. The preparation steps included (i) hydrolysis of adducts, (ii) matrix reduction by pH-dependent solvent extraction, (iii) derivatization,(iv) enrichment of analytes by headspace solid-phase microextraction (SPME) and (v) two-dimensional gas chromatography (GC×GC) and mass spectrometric detection (qMS).

4.3.4 Instrumentation

A Shimadzu GC system, GC-2010 and GCMS QP2010 Plus coupled with a Shimadzu AOC-5000 liquid, headspace and SPME GC injection system (Shimadzu GmbH, Duisburg, Germany) with a built-in loop-type modulator from Zoex Corporation (Houston (Texas), USA) was used for all analyses. All the equipments (e.g. GC columns, SPME fiber, SPME liner, etc.) and the parameters used are the same as indicated in the previous chapter (section 3.3.6).

4.4 Results and discussions

4.4.1 Precision of measurements

To evaluate the repeatability of the method, synthetic human urine spiked with a 100 ng/L of 15 AAs mixture (Table 4.1) was analyzed. The spiked samples (n=8), before being measured by GC×GC-qMS were derivatized through diazotization and iodination in a one-pot reaction in order to convert anilines to aromatic iodine compounds and thus decreasing the polarity of the analytes. In fig. 4.2a the peak separation of (i) 4-ethyl-1-iodobenzene (4EA) and (ii) 2,6-/2,4-dimethyl-1-iodobenzene (2,6DMA/2,4DMA) is shown as an example in the form of “slices” in a 1D plot. These are isomers of the same molecular mass (*m/z* 232), eluted from the first dimension (1D) column and re-injected for further separation into the second dimension (2D) column where they are trapped and released every 6 s. The loss of the iodine fragment (*m/z* 127) is clearly demonstrated in their mass spectra (Figures 4.2b and c), hence enabling the analytes to be detected selectively. In fig. 4.3a, the contour plot in 2D is shown where the slices are being constructed. To highlight the reliability of peak identification for isomeric compounds when analyzing with the GC×GC, the same isomers (*m/z* 232) were detected also in a complex matrix of human urine, which is shown in fig. 4.3b. These isomers could be easily identified and compared in both spiked and human urine samples, as a result of the 1D and 2D retention times considered for each analyte, as well as the loss of the iodine fragment (*m/z* 127). In human urine, even more isomers of the same molecular mass were identified. All the analytes (15) were well separated and eluted between 10.5 and 26.5 min in 1D and between 2.0 and 4.0 s in 2D; with relative standard deviations, %RSD of the peak areas obtained from the spiked synthetic urine (n=8) ranging

between 6 - 20 % (Table 4.1). This quantitative method was used for further experiments for the analysis of aromatic amine isomers in human urine.

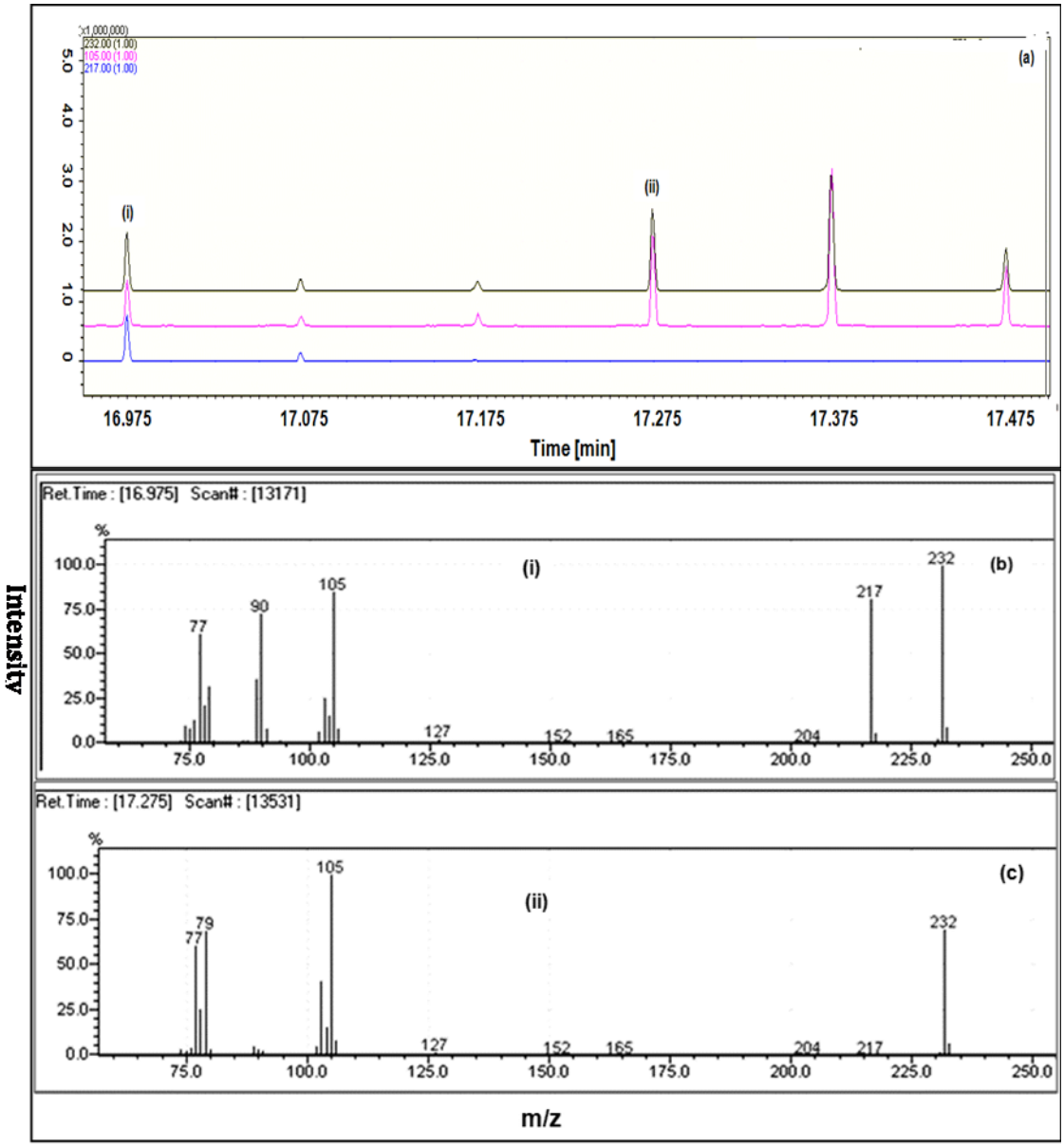


Fig. 4.2 (a) A 1D plot showing a peak separation of the isomers of the same molecular mass (*m/z* 232), **(i)**4-ethyl-1-iodobenzene (4EA) and **(ii)** 2,6-/2,4-dimethyl-1-iodobenzene (2,6DMA/2,4DMA) from a 100 ng/L spiked synthetic urine, after being trapped and released every 0.1 min (6s modulation time)in a second dimension column; **(b)** and **(c)** show mass

spectra of both analytes 4EA and 2,6DMA/2,4DMA respectively, to illustrate the loss of the iodine fragment (*m/z* 127) for the easy identification of the analytes

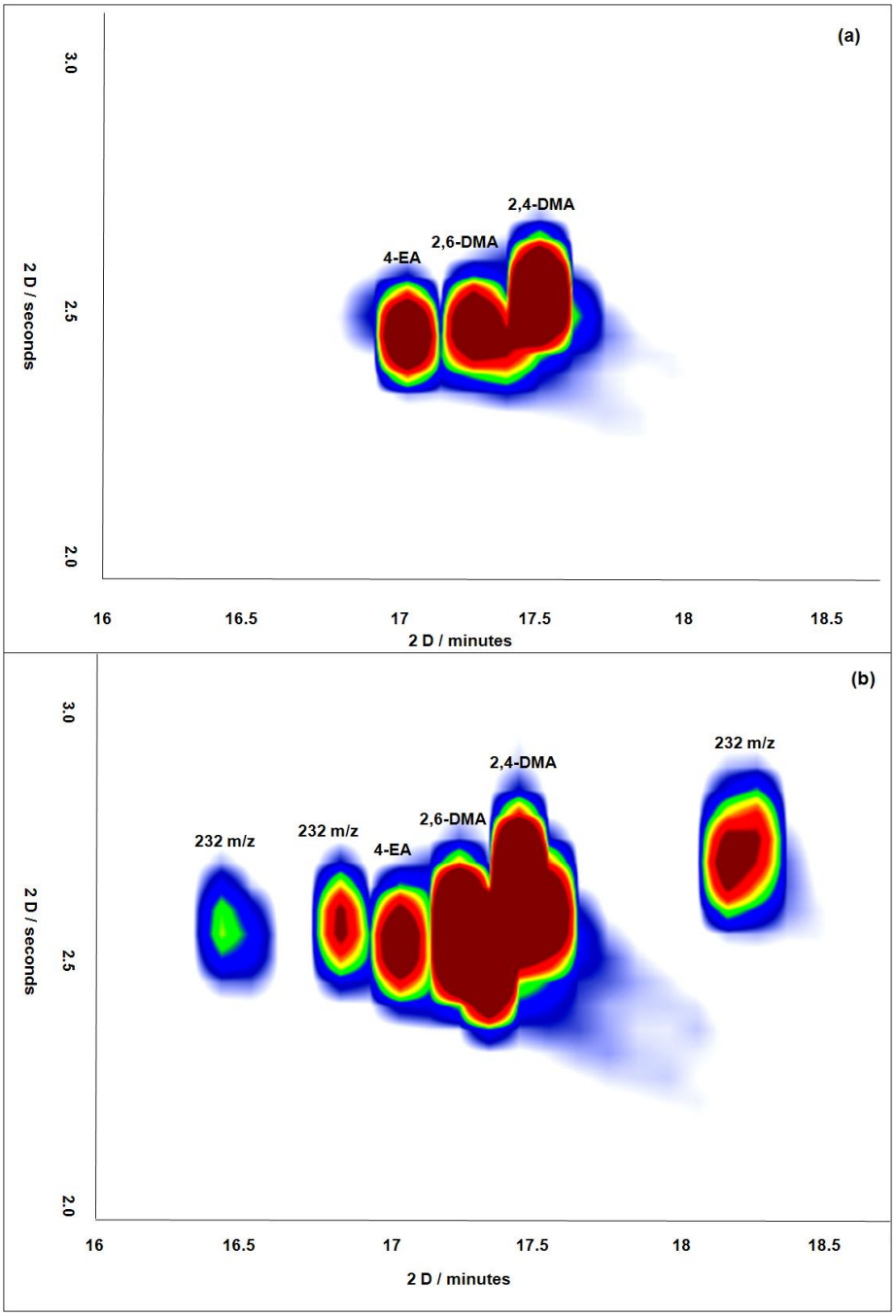


Fig. 4.3 Contour plot in 2D showing the isomers of the same molecular mass [*m/z* 232] viz. 4-ethylaniline derivative (4EA) and 2,6-/2,4-dimethylaniline derivatives (2,6DMA/2,4DMA), in **(a)** 100 ng/L spiked synthetic urine and in **(b)** human urine respectively

Table 4.1 Names, fragments of the iodinated derivatives, retention times and % RSD of peak areas to determine the repeatability of the method

≠	Substance	Fragments of the iodinated derivatives (m/z)	Retention time in 1D and 2D in min / sec respectively[a]		% RSD (n = 8)
1	Aniline (AN)	204, 77	10.8	2.6	13
2	o-methylaniline (o-MA)	218, 91	14.1	2.5	13
3	3-chloro-4-fluoroaniline (3C4FA)	256, 129	15.9	2.6	9
4	2-chloroaniline (2CA)	238, 111	16.7	3.2	7
5	4-ethylaniline (4EA)	232, 217,105	17.1	2.5	15
6	2,6-dimethylaniline (2,6DMA)	232, 105	17.3	2.5	15
7	2,4-dimethylaniline (2,4DMA)	232, 105	17.5	2.6	15
8	4-chloro-2-methylaniline (4C2MA)	252, 125	19.2	2.8	7
9	2-bromoaniline (2BA)	282, 155	19.4	3.6	9
10	2,4,6-trimethylaniline (2,4,6TMA)	246, 119	20.5	2.4	8
11	2-aminoacetophenone (2ACP)	246, 203, 76	21.4	4.0	10
12	2,6-dichloroaniline (2,6DCA)	272, 145	22.1	3.6	6
13	3-chloro-2,6-dimethylaniline (3C2,6DMA)	266, 139	22.7	2.8	12
14	3-chloro-4-methoxyaniline (3C4MOA)	268, 253, 126	23.8	3.5	14
15	2-naphthylamine (2NA)	254, 127	26.1	4.0	20

[a]The retention times are given in minutes (min) for 1D and in seconds (s) for 2D respectively

4.4.2 Linearity validation

To check the linearity of the method, calibration standards with the concentrations of 1 - 5 - 10 - 25 - 50 - 75 - 100 - 250 and 500 ng/L were prepared in triplicates in synthetic human urine solution, derivatized and then measured by GC×GC-qMS. The 15 analytes contained in the standards are as mentioned in Table 4.1 above. A calibration standard point of 1 ng/L was omitted from the calibration curve due to high deviations; hence 8 calibration points were used to plot the calibration curves. These are shown in the Appendix, Fig. A4.1(a – o). The names of the iodinated derivatives, the regression coefficient of the calibration curve, LOD and LOQ obtained from DIN 32645 [15] are shown in Table 4.2. The derivatives of AN, 2,6DMA, 2BA and 2ACP had higher LOD > 50 ng/L and LOQ > 60 ng/L. These results differ from our former study [14] where the LOD for the same analytes ranged between 4 - 24 ng/L. This may be a result of the dilution solution used to prepare the standards, which was previously ultra-filtered water and now synthetic human urine; as well as the narrower concentration range of the prepared standards which was 1 - 5 - 10 - 50 - 100 - and 500 ng/L, with 1ng/L omitted from the calibration curves. The percentage (%) recovery rates obtained for all the analytes in spiked samples and as shown in table 4.3, ranged between 75 - 160 % except for AN, o-MA, 2,4DMA and 4C2MA which had the average recovery starting from 50 %.

Table 4.2 Names, regression coefficients, LOD and LOQ of the iodinated derivatives analyzed to evaluate linearity, n = 3

≠	Substance	Derivatives	R^2	LOD (ng/L)	LOQ (ng/L)
1	AN	Iodobenzene	0.969	70	84
2	o-MA	1-iodo-2-methylbenzene	0.998	13	16
3	3C4FA	3-chloro-4-fluoro-1-iodobenzene	0.998	11	14
4	2CA	2-chloro-1-iodobenzene	0.993	40	50
5	4EA	4-ethyl-1-iodobenzene	0.989	31	38
6	2,6DMA	2,6-dimethyl-1-iodobenzene	0.975	60	71
7	2,4DMA	2,4-dimethyl-1-iodobenzene	0.985	47	57
8	4C2MA	4-chloro-1-iodo-2-methylbenzene	0.991	29	35
9	2BA	2-bromo-1-iodobenzene	0.916	89	103
10	2,4,6TMA	1-iodo-2,4,6-trimethylbenzene	0.996	23	29
11	2ACP	2-iodoacetophenone	0.979	81	99
12	2,6DCA	2,6-dichloro-1-iodobenzene	0.998	13	17
13	3C2,6DMA	3-chloro-2,6-dimethyl-1-iodobenzene	0.992	24	29
14	3C4MOA	3-chloro-1-iodo-4-methoxybenzene	0.993	25	30
15	2NA	2-iodonaphthalene	0.993	22	27

Table 4.3 Percentage (%) recovery rates of the analytes in spiked samples(n=3)

Analytes / Spiked conc. [ng/L][a]	5	10	25	50	75	100	250	500	%Rec (Average)
AN	0	56±5	≥200	115±9	97±15	115±10	120±16	94±10	**55– 120**
o-MA	103±14	57±6	87±4	103±2	94±5	115±3	98±24	100±2	**55 – 115**
3C4FA	91±21	82±9	77±8	105±1	94±4	105±2	104±7	99±6	**75 – 105**
2CA	80±8	137±2	140±1	88±5	64±13	113±1	105±5	99±3	**65– 140**
4EA	0	132±8	156±7	92±9	76±11	97±8	114±5	97±2	**75 – 155**
2,6DMA	≥200	0	125±10	75±6	86±7	110±1	122±9	95±11	**75 – 125**
2,4DMA	0	72±19	87±9	153±19	49±5	105±1	108±17	98±6	**50 – 150**
4C2MA	158±11	49±7	85±3	90±5	90±6	99±2	115±11	97±2	**50– 160**
2BA	0	-≥200	≥200	0	7±6	90±1	109±11	100±5	**90 – 110**
2,4,6TMA	0	75±2	167±1	103±4	91±10	109±1	129±5	100±7	**90 – 165**
2ACP	0	≥200	150±16	87±11	113±13	124±12	140±8	99±6	**85 – 150**
2,6DCA	112±48	81±8	99±6	110±19	83±2	111±14	99±16	100±1	**80 – 110**
3C2,6DMA	0	0	167±12	104±18	95±18	122±9	102±4	99±6	**95 – 165**
3C4MOA	0	≥200	114±4	103±2	78±15	85±4	108±15	99±6	**75 – 115**
2NA	0	≥200	113±27	118±11	100±14	88±3	108±12	98±12	**85 – 120**

The highest values of ≥200 were excluded from the average calculations

[a]- The exact weighted concentration of each analyte is shown in Table A5.1 (Appendix)

4.4.3 Quantification of the selected aromatic amines in urine samples

The urine samples of non-smokers, past-smokers and smokers (n=8 each) were prepared and measured by GC×GC-qMS. For each urine sample, the concentration of the selected 14 aromatic amines (Tables 4.1 and 4.2, except for 3C4FA which was not detected in the urine samples), was calculated. All the analytes of interest could be easily identified in urine samples by monitoring the loss of iodine fragment (*m/z* 127) and also by comparing the 1D and 2D retention times of each analyte to the standards. However, not all the selected analytes could be reliably quantified, as some had very high peak area values which resulted in high calculated concentrations and exceeded the working range of the prepared calibration standards (5 - 975 ng/L, the exact concentrations are shown in the Appendix, Table A4.1). These include the derivatives of AN, o-MA, 2,4DMA, 2BA and 2ACP in non-smokers' (NS) samples and AN, o-MA, 2,6DMA, 2BA and 2ACP in smokers' (S)samples. In past-smokers' (PS) samples, some analytes namely 2CA, 2BA, 2,6DCA, 3C2,6DMA (also in S and NS) and 3C4MOA had values lower than the limit of quantitation (<LOQ). In figure 4.4 the calculated concentrations of the selected analytes that fit in the calibration working range and also proposed to be present in the urine samples of NS, PS and S are shown. In some cases, the analytes' concentration values could be found only in 1 out of 8 samples and they are indicated in the graph as a minus (-) sign. Those that were above the quantification range are indicated by the blue arrows.

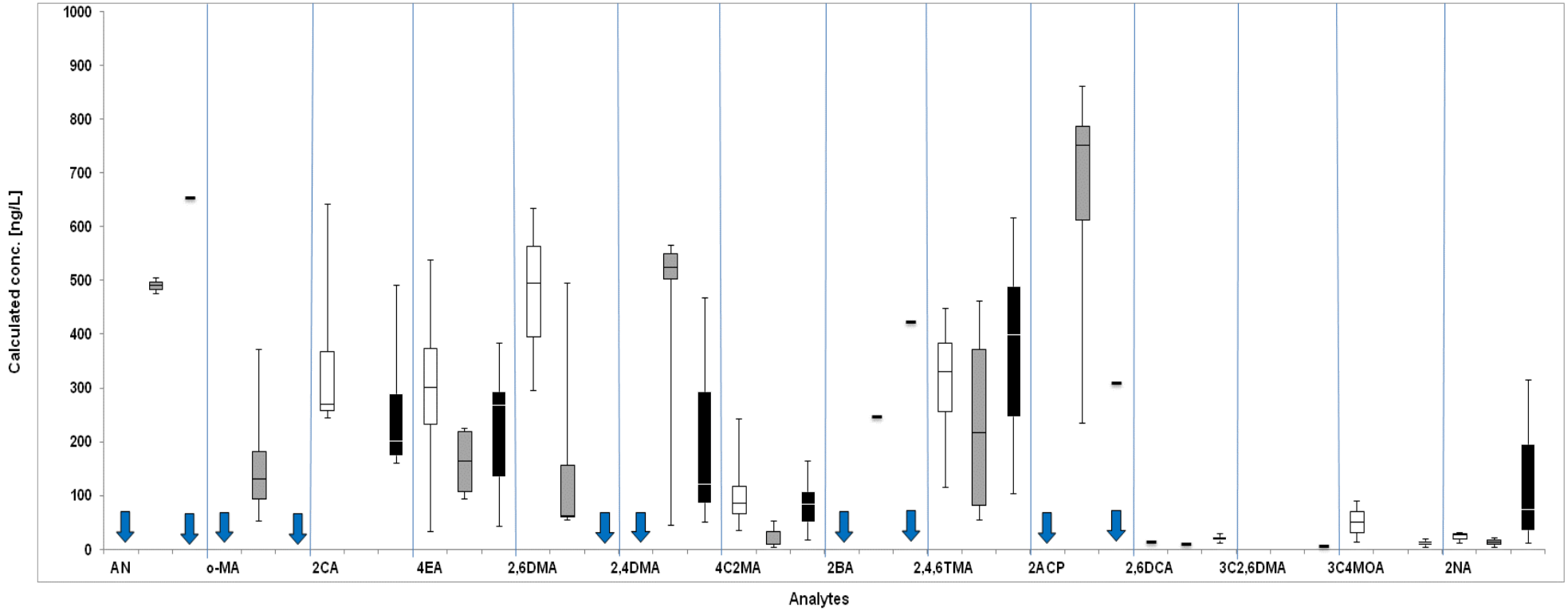


Fig. 4.4 Box-whisker-plot of concentrations of the selected analytes (14) in urine samples of non-smokers (white bars), past-smokers (grey bars) and smokers (black bars). The minus sign (-) indicates the calculated concentration values that fit within the working range which were found only in 1 out of 8 samples in the studied group and the blue arrows indicate the compounds that were above the quantification range

Here, the minimum, maximum and median concentrations of every analyte per studied group (NS - PS - S) are shown. Since some analytes could not be quantified due to higher values that exceeded the calibration work range, comparing the analytes' concentrations within the groups was quite complex. Hence, to simplify the results the mean concentrations of the analytes for every group were considered. In NS, PS and S urine the concentrations ranged between ca. 15 – 400 ng/L, ca. 40 – 650 ng/L and ca. 25 – 660 ng/L respectively. The results expected were a lower concentration for NS, in-between for PS and higher for S; however, here the values for PS were rather similar to that of S samples. The reason for the insignificant difference observed between PS and S concentrations is unknown but some factors such as the background of the individuals' smoking habits might be a major influence on the results and these may include the duration (years) of smoking, how often they smoked, the number of cigarettes they smoked, the time since quitting (for PS), and etc. According to the studies by Welty et al., the risk of bladder cancer in PS remains elevated more than 32 years after quitting, even among those with moderate smoking histories [16]. Finally, the results obtained here for the analytes of interest were compared to other studies as shown in Table 4.4.

Riedel et al. [17], detected o-MA and 2NA in urine samples of smokers and non-smokers. Since in this study the analyte o-MA could only be quantified in PS urine, no comparison was done. But, in the case of 2NA, our concentrations were about ten- and five folds higher in S and NS urine respectively. On the other hand, Grimmer et al. [2] obtained higher concentrations of 2NA in NS samples than in S samples and comparing their results to our study; their concentrations were almost two times lower in the S samples and about five times higher in NS samples.

Also, Riffelman et al. [18] had the highest concentrations of 2NA by more than one order of magnitude higher than in this study (3000ng/L in S and 500 ng/L in NS). This was the same for 4C2MA in both S and NS samples. The comparison for AN and o-MA with Riffelman et al. was not done, since in our study AN could be quantified in only 1 of the smokers' sample and in PS samples; as well as o-MA. The results were also compared with the work done by Lorenzo Parodi [19], where the same experimental and instrumental procedures were applied in both studies. The results were similar for o-MA (in PS) and 4C2MA (in S, NS, PS) derivatives; but for the derivatives of 4EA (in S, NS, PS) and 2,4,6TMA (in S, NS, PS) the results reported here were ca. five- to ten times fold higher. Concerning other analytes such as 2CA, 2BA, 2ACP and 2,6DCA, no publications were found that targeted these analytes and therefore, no comparison was done. In general, it can be noted in all the studies mentioned that, most often the analytes in smokers' urine had higher concentrations compared to non-smokers, even though the differences were not so significant (max. two fold higher), except in Riffelman et al. studies. In cases where analytes in NS had higher concentrations than S, the differences were also minimal, by ca. < one or two folds higher. For the PS (only this study and Lorenzo Parodi), the concentrations were sometimes similar to S or NS samples.

Table 4.4 Concentrations of aromatic amines in urine samples, identified in other studies in the urine of smokers (S), non-smokers (NS) and past-smokers (PS)

Author(s)	Results [ng/L]
Riedel et al., 2006 [17]	**o-MA:** S(10) = 117, NS(9) = 55
	2NA: S(10) = 12, NS(10) = 5
Grimmer et al., 2000 [2]	**2NA:** S(12) = 90, NS(14) = 128
Riffelmann et al.,1995 [18]	**AN:** S(8) = 1400, NS(8) = 200
	o-MA: S(8) = 1700, NS(8) = 0
	4C2MA: S(8) = 3000, NS(8) = 2200
	2NA: S(8) = 3100, NS(8) = 500
Lorenzo Parodi N., 2015 [19]	**o-MA:** S(1) = 96, NS(10) = 102, PS(9) = 107
	4EA: S(5) = 29, NS(3) = 23, PS(4) = 30
	DMA: S(10) = 98, NS(20) = 56, PS(19) = 57
	4C2MA: S(3) = 95, NS(14) = 59, PS(13) = 71
	2,4,6TMA: S(7) = 94, NS(14) = 67, PS(12) = 57
	2NA: S(3) = 44, PS(2) = 82

This study	**AN:** S(1) = 656, PS(2) = 491
	o-MA: PS(7) = 159
	2CA: S(4) = 264, NS(4) = 356
	4EA: S(5) = 227, NS(7) = 299, PS(4) = 163
	2,6DMA: NS(2) = 396, PS(2) = 326
	2,4DMA: S(2) = 294, PS(4) = 536
	4C2MA: S(6) = 96, NS(8) = 104, PS(2) = 44
	2BA: S(1) = 423, PS(1) = 247
	2,4,6TMA: S(7) = 371, NS(8) = 316, PS(4) = 238
	2ACP: S(1) = 310, PS(4) = 650
	2,6DCA: S(2) = 25, NS(1) = 17
	2NA: S(5) = 167, NS(2) = 30

The bracketed numbers () stand for the number of participants

Another interesting finding was reported by Chamssuddin et al. where they evaluated the relationship on the bladder cancer tumor grades, stages and aggressiveness among smoking and non-smoking diagnosed patients [20]. Their patients (n= 300) were categorized to (1) non-smokers, as patients with no smoking history, (2) low dose-smokers, as patients smoking 10 - 29 cigarettes per day, (3) moderate-smokers, as patients smoking 30 - 59 cigarettes per day and (4) high dose-smokers, as patients smoking ≥60 cigarettes per day and for the last 3 years.

The high dose-smokers demonstrated significantly higher grades, stages and aggressiveness of the cancer; however, between low dose- to moderate smokers and non-smokers, there were no significant differences observed in the grades, stages and the aggressiveness of the disease. With these findings, it can be suggested that the smokers'samples evaluated in our studies may fall in the category of low to moderate-dose smokers, where the concentrations of aromatic amines obtained may be similar to and/or moderately higher than PS and NS. Finally, several other aromatic amines derivatives were identified in all the samples but were not quantified. In the urine of non-smokers (n=8) ca. 85 isomers, in past-smokers' urine (n=8) ca. 130 isomers and in the urine of smokers (n=8) ca. 160 isomers were identified. A table with a tentative list of the identified isomers is shown in the Appendix A5. In comparison with the results obtained from the previous chapter, ca. 120 isomers were detected in non-smoker's urine and ca. 150 isomers in the smoker's urine. In both cases the urine of smokers had relatively more isomers than the non-smokers.

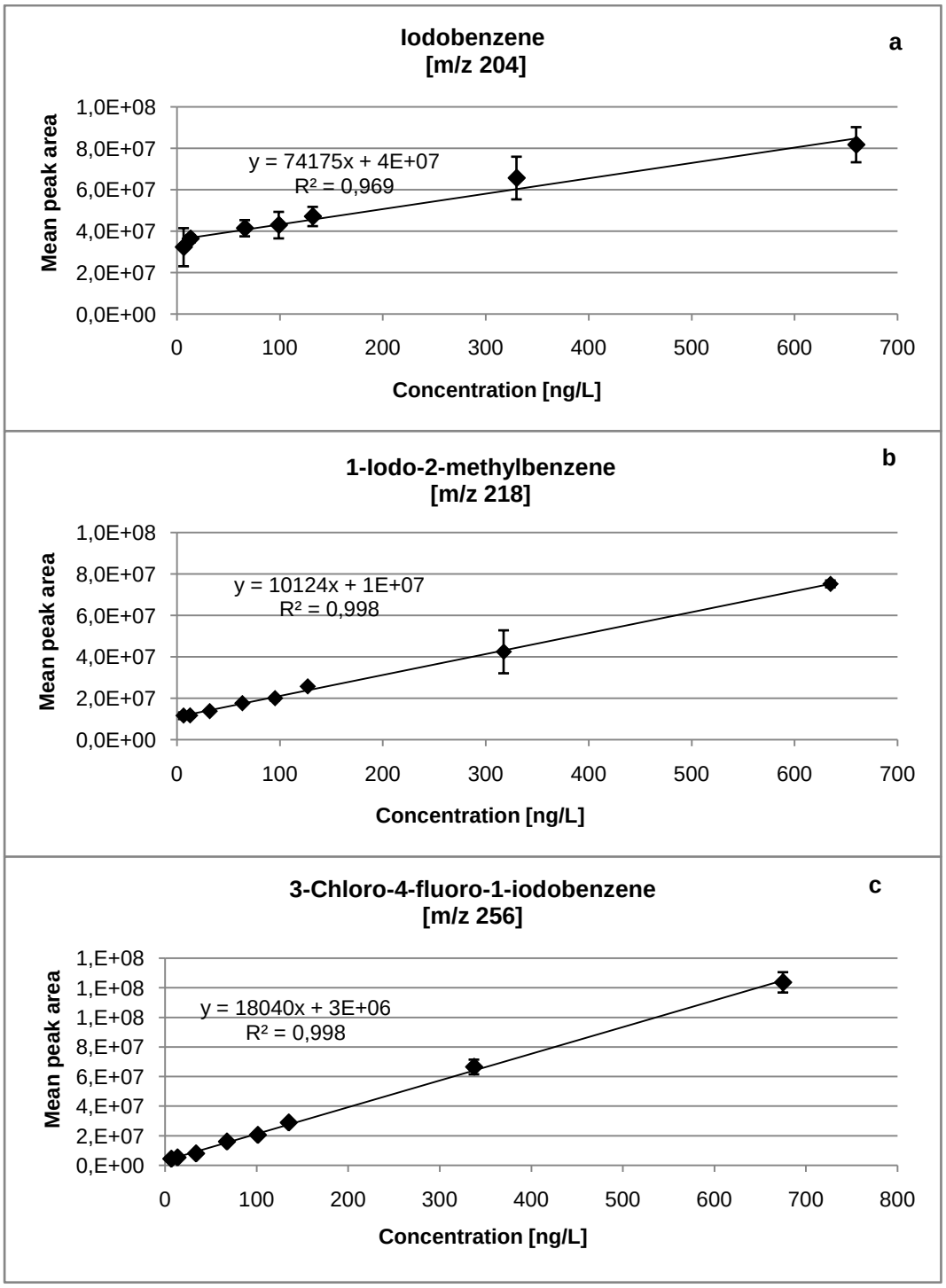
4.5 Conclusions

A number of aromatic amines have been identified and some quantified in the urine of non-smokers, past-smokers and smokers. Selected analytes were obtained at lower concentrations in the urine of non-smokers and at higher or similar concentrations in the urine of smokers and past-smokers. Among the selected analytes, AN, o-MA, 2,6DMA, 2,4DMA, 2BA and 2ACP had higher concentrations in S and NS samples, which exceeded the limit of the calibration curve points. To correct this, the urine samples can be diluted before preparation and/or the calibration points can be extended to a higher concentration. However, both approaches were not successful in this present study because the quantity of each urine sample was limited (10 - 50 mL, where 20 mL in duplicates was required for every analysis) and therefore the samples could not be re-measured. Also, when samples are diluted some analytes in the urine samples such as 2,6DCA which are found at the concentration close to their LOQ and/or even lower than the estimated LOQ (e.g. 3C2,6DMA) may be affected.

In future, this may be avoided by measuring the concentration of urinary creatinine, a substance produced in the body and excreted in the urine, in order to provide information about the dilution of urine samples. This procedure is usually done when measuring drugs of abuse, as excessive or reduced fluid intake may greatly alter the concentration of substances in urine [21]. Based on the European guidelines for workplace drug testing in urine, the samples having a concentration of creatinine within the range of 56- 226 mg/L should be considered as dilute [22].

In addition, substantial historical background information of the sample donors may be necessary for better interpretation of the results. These may include for smokers: the number of cigarettes smoked and the period; for non-smokers: if they are perhaps second-hand smokers (i.e. staying in smokers' environment) and for the past-smokers: the time since quitting. Other factors may include the working environment since environmental exposure to aromatic amines may also be an important source.

4.6 Appendix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100
200
300
400
500
600
700
Concentration [ng/L]
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[m/z 218]
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600
700
Concentration [ng/L]
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[m/z 256]
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Mean peak area
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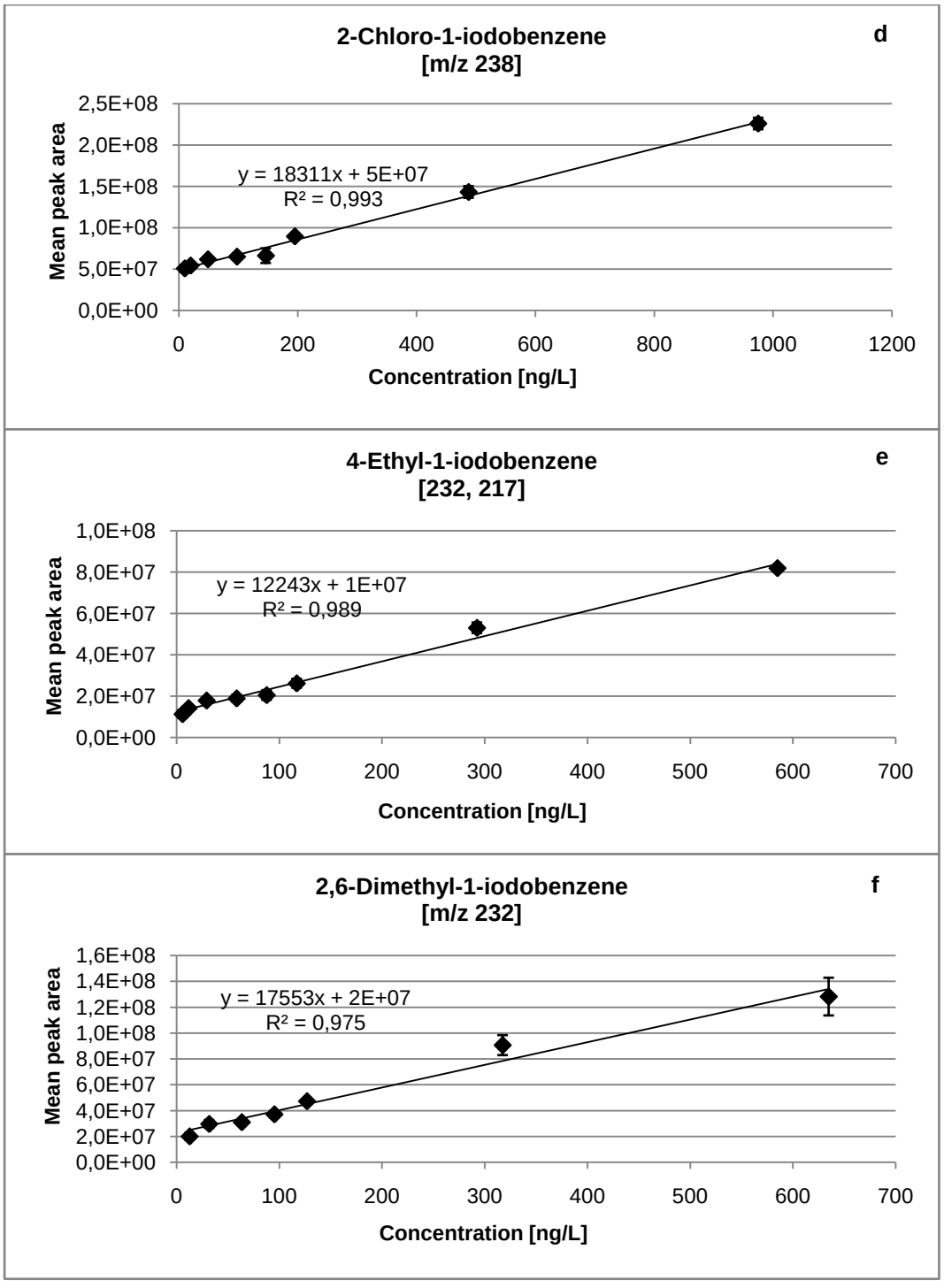
600
700
800
Concentration [ng/L]


2-Chloro-1-iodobenzene
[m/z 238]
d
Mean peak area
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R² = 0,993
0
200
400
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800
1000
1200
Concentration [ng/L]
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[232, 217]
e
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4,0E+07
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0,0E+00
y = 12243x + 1E+07
R² = 0,989
0
100
200
300
400
500
600
700
Concentration [ng/L]
2,6-Dimethyl-1-iodobenzene
[m/z 232]
f
Mean peak area
1,6E+08
1,4E+08
1,2E+08
1,0E+08
8,0E+07
6,0E+07
4,0E+07
2,0E+07
0,0E+00
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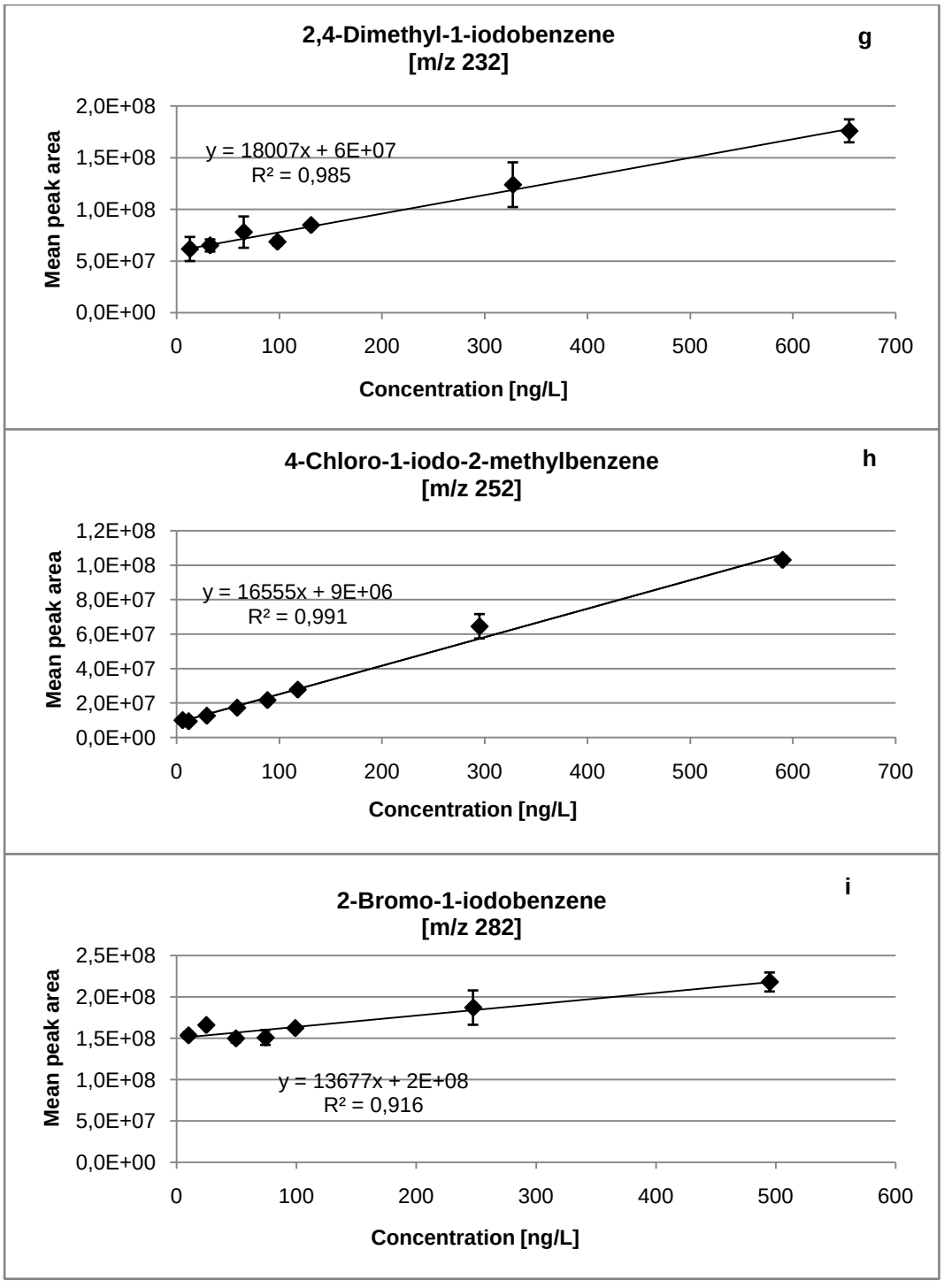
R² = 0,975
0
100
200
300
400
500
600
700
Concentration [ng/L]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Mean peak area
Concentration [ng/L]
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[m/z 252]
h
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R² = 0,991
Mean peak area
Concentration [ng/L]
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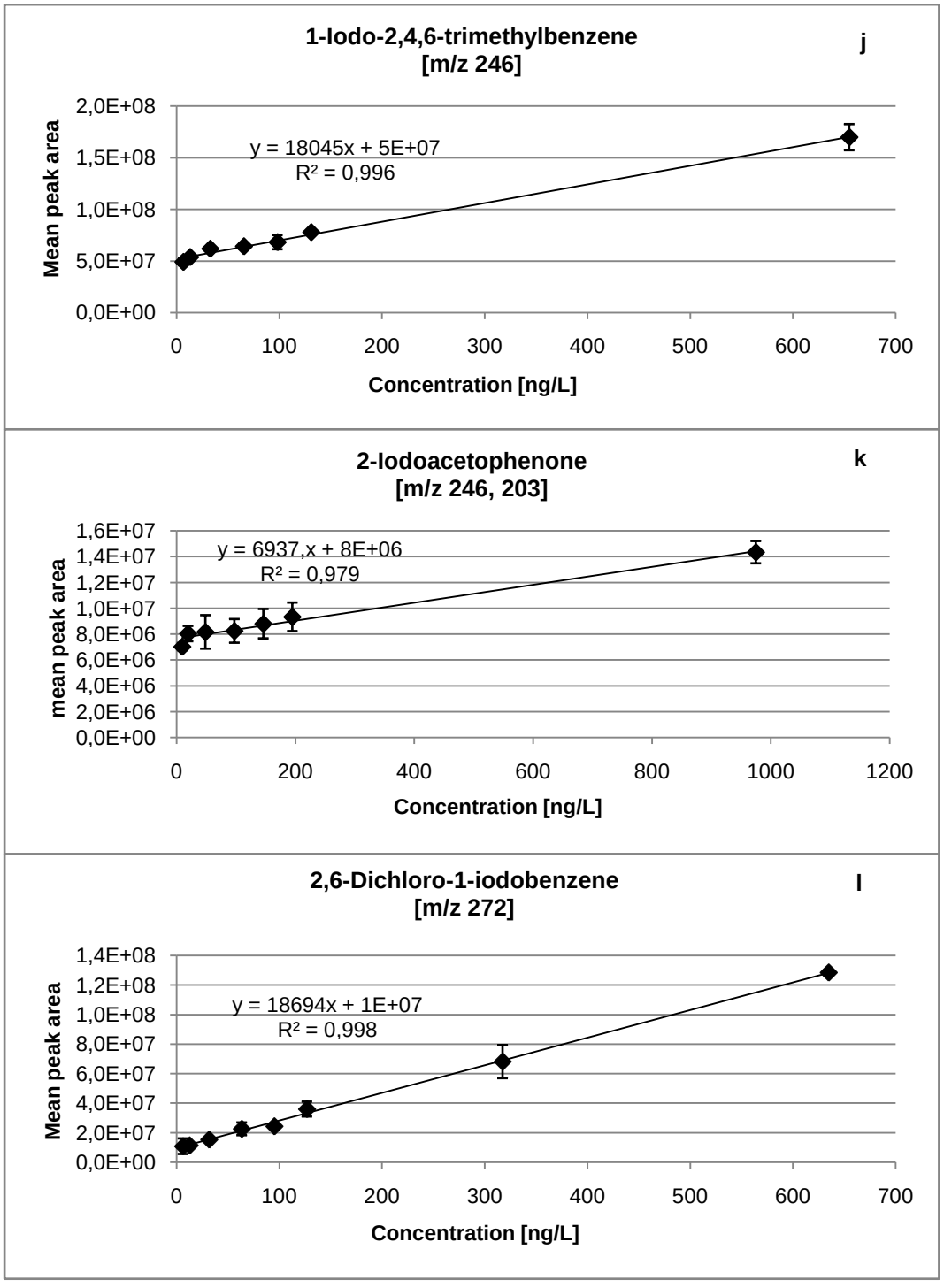
R² = 0,916
Mean peak area
Concentration [ng/L]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[m/z 246]
j
y = 18045x + 5E+07
R² = 0,996
Mean peak area
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1,0E+08
5,0E+07
0,0E+00
0
100
200
300
400
500
600
700
Concentration [ng/L]
2-Iodoacetophenone
[m/z 246, 203]
k
y = 6937,x + 8E+06
R² = 0,979
mean peak area
1,6E+07
1,4E+07
1,2E+07
1,0E+07
8,0E+06
6,0E+06
4,0E+06
2,0E+06
0,0E+00
0
200
400
600
800
1000
1200
Concentration [ng/L]
2,6-Dichloro-1-iodobenzene
[m/z 272]
l
y = 18694x + 1E+07
R² = 0,998
Mean peak area
1,4E+08
1,2E+08
1,0E+08
8,0E+07
6,0E+07
4,0E+07
2,0E+07
0,0E+00
0
100
200
300
400
500
600
700
Concentration [ng/L]

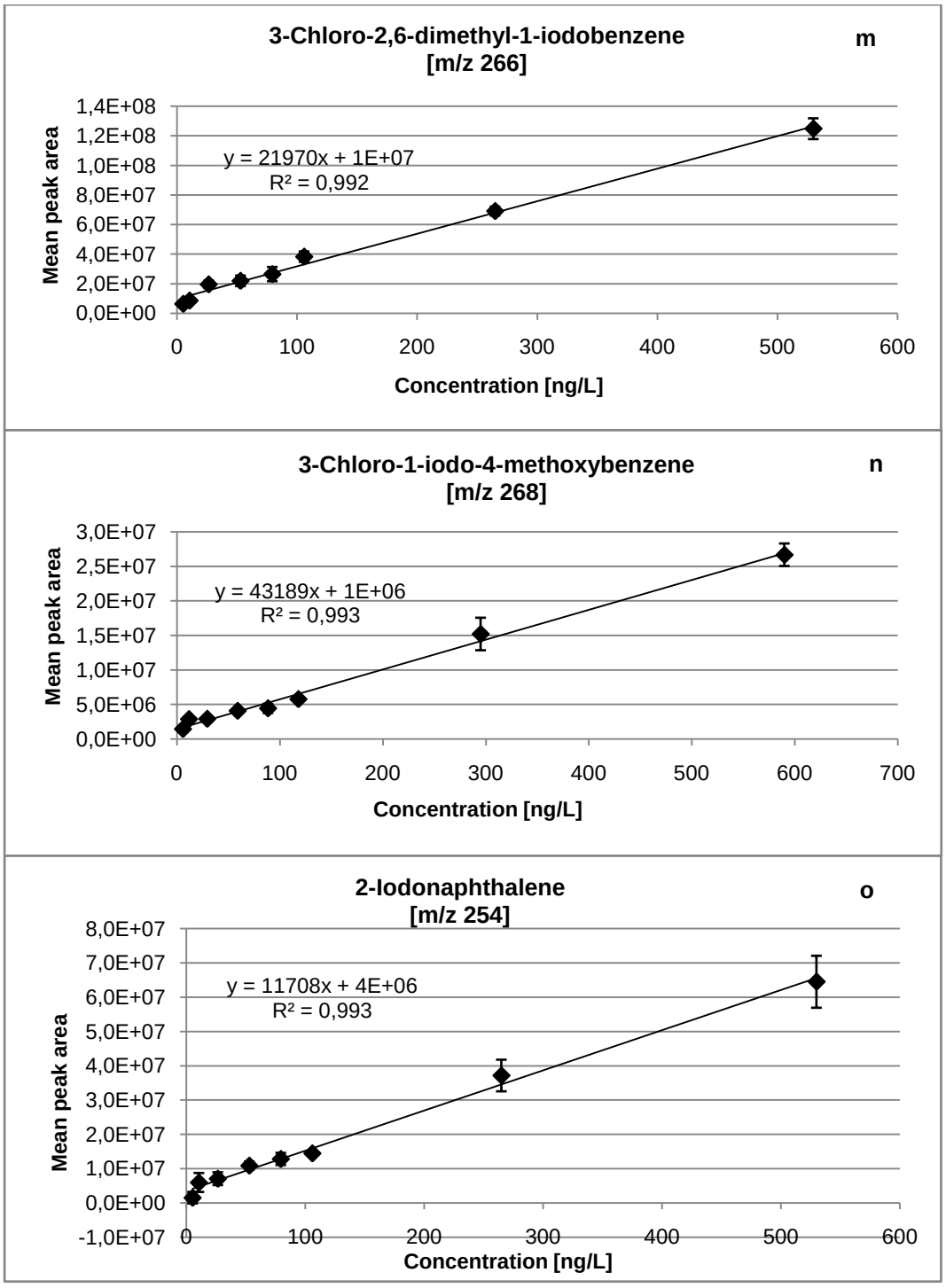

Fig. A4.1(a – o) Calibration curves of 15 selected aromatic amines in synthetic human urine (n = 3)

Table A4.1 Exact concentrations weighed for the preparation of calibration standards, 10 mg of each substance diluted in 10 mL solution to make a final concentration of 1 g/L.

≠	Substance	Amount of substance weighed (mg)	Final concentration per 500 ng/L standard
1	AN	13.2	660
2	o-MA	12.7	635
3	3C4FA	13.5	675
4	2CA	19.5	975
5	4EA	11.7	585
6	2,6DMA	12.7	635
7	2,4DMA	13.1	655
8	4C2MA	11.8	590
9	2BA	9.90	495
10	2,4,6TMA	13.1	655
11	2ACP	19.5	975
12	2,6DCA	12.7	635
13	3C2,6DMA	10.6	530
14	3C4MOA	11.8	590
15	2NA	10.6	530

Table A4.2 Tentative list of the aromatic amine derivatives proposed to be present in the urine of smokers, past-smokers and non-smokers

Substance	*m/z* of iodinated derivatives	Main fragments (*m/z*)	PS (n=8)	NS (n=8)	S (n=8)
Aniline	204	77	1	1	1
C1- Aniline	218	91	1	1	1
C2- Aniline	232	217, 105	6	4	5
C3- Aniline	246	119	10	8	10
C4- Aniline	260	245, 118	14	12	15
Chloroaniline	238	111	2	2	2
C1- Chloroaniline	252	125	2	2	2
C2- Chloroaniline	266	231, 139	7	6	5
C3- Chloroaniline	280	265, 138	5	3	n/a
C4- Chloroaniline	294	279, 152	2	2	1
C5- Chloroaniline	308	181, 145, 109	1	1	n/a
Dichloroaniline	272	145	2	2	2
Bromoaniline	282	155	2	2	2
Methoxyaniline	234	219, 92	1	1	2
C3- Methoxyaniline	276	261, 134	1	2	1
Chloromethoxyaniline	268	253, 126	4	n/a	1
Thioaniline	236	109	3	1	3
C1- Thioaniline	250	123, 108	2	1	1
Naphthylamine	254	127	1	1	2
C1- Naphthylamine	268	141	2	2	4
C2- Naphthylamine	282	155	1	3	5
C1- Aminobiphenyl	294	279, 152	3	n/a	6
C2- Aminobiphenyl	308	181, 166	3	n/a	n/a
Diaminobiphenyl	406	279, 152	n/a	n/a	n/a
Methylenedianiline	420	293, 166	1	n/a	n/a
C1- Diaminonaphthalene	394	267, 140	n/a	n/a	n/a

Table A4.2 Tentative list of the aromatic amine derivatives proposed to be present in the urine of smokers, past-smokers and non-smokers (continues)

Substance	*m/z* of iodinated derivatives	Main fragments (*m/z*)	PS (n=8)	NS (n=8)	S (n=8)
Diaminobenzene	330	203, 76	2	2	2
C1- Diaminobenzene	344	217, 90, 74	2	2	5
C2- Diaminobenzene	358	231, 104	2	3	6
C3- Diaminobenzene	372	357, 245	1	1	2
C4- Diaminobenzene	386	371, 244	1	1	1
C1- Triaminobenzene	456	329, 202, 75	2	2	2
Chlorodiaminobenzene	364	237, 110, 74	4	4	3
C1- Chlorodiaminobenzene	378	343, 251, 124	n/a	n/a	n/a
Bromodiaminobenzene	408	281, 154, 75	n/a	n/a	n/a
Tetrahydronaphthylamine	258	131, 102	5	4	5
C1- Tetrahydronaphthylamine	272	257, 130	7	n/a	14
C2- Tetrahydronaphthylamine	286	271, 159	12	1	16
C3- Tetrahydronaphthylamine	300	258, 173, 157	4	n/a	5
Aminoindane	244	117	5	3	6
C1- Aminoindane	258	244, 131	4	n/a	7
Aminoquinoline	255	128	n/a	n/a	n/a
C1- Aminoquinoline	269	142	n/a	n/a	n/a
C2- Aminoquinoline	283	156, 141	n/a	n/a	n/a
Aminopyridine	205	78	n/a	n/a	n/a
Aminothiophene	210	83	n/a	n/a	1
C1- Aminothiophene	224	97, 81	n/a	1	n/a
Aminoacetophenone	246	231, 203, 76	1	1	2
Aminophenol	220	93	1	1	1
C1- Aminophenol	234	107, 89	2	2	2
C2- Aminophenol	248	220, 93	n/a	n/a	3
C3- Aminophenol	262	247, 135	n/a	1	3

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CHAPTER 5

Alternative developments in sample preparation

5.1 Abstract

Urine is very important in bladder cancer studies and due to its highly complex matrix, pre-treatment steps such as hydrolysis, extraction techniques, etc. are necessary to enable the detection of trace elements. For the determination of aromatic amines, hydrolysis is required in order to separate the amine adducts from the matrix. In this study the urine samples were hydrolyzed by a laboratory microwave set at 600 Watts / 110 °C at the irradiation time of 15, 45 and 65 min consecutively. For comparison, the samples were also hydrolyzed conventionally by using a laboratory hot plate heated at 110°C for 12 hours. After hydrolysis, the samples were liquid-liquid extracted, derivatized and measured by SPME-GC×GC-qMS; where about 74 aromatic amine isomers were identified from the conventionally hydrolyzed samples and about 51, 55 and 38 isomers at 15, 45 and 65 min respectively were identified from the samples hydrolyzed in a microwave. Also, a Thin Film Micro-extraction (TFME) method was developed for the extraction of aromatic amines using different coated blades namely Bondesil-PBA/PAN, Chromabond-Easy/PAN, LC-Diol/PAN and C_{18}-SCX/PAN. A mixture of aromatic amines in water was used for method development. The Bondesil-PBA/PAN (phenylboronic acid/polyacrylonitrile) blade demonstrated better extraction efficiency when compared to other blades, at 50 min adsorption- and 5 min desorption times.

5.2 Introduction

Sample preparation time is one of the most vital elements in analytical laboratories, thus optimization of developed methods in order to minimize the time required for sample pre-treatment is necessary. This is especially true for analytical procedures, where sample pre-treatment steps such as hydrolysis of the sample, extraction of the analytes, derivatization, etc. are likely required; to make it possible to detect trace elements from highly complex samples such as urine. Aromatic amines (AAs) tend to undergo significant metabolism in the human body, resulting in the excretion of small amount of the parents' amines in urine together with a large number of different metabolites [1]. In the studies by Zimmermann [2],acidic and basic hydrolyses were compared in order to cleave the conjugates of anilines in the human urine; by using hydrochloric acid (HCl) and sodium hydroxide (NaOH) and with the samples heated at 110 °C for 12 hours. After extraction, derivatization and detection by gas chromatography-atomic emission detector (GC-AED), more peaks were found after acidic hydrolysis than after basic hydrolysis.

To reduce the time consumed during conventional hydrolysis (CH) process, microwave-assisted hydrolysis (MH) has been widely applied in various fields as a promising alternative [3-5]. This technique was first introduced by Abu-Samra et al. [6] in the preparation of biological samples for trace analysis of metals. Sanchez et al. achieved highest hydrolysis efficiency of some anilines and chloroanilines in urine from 45 minutes with the conventional hydrolysis to 2 minutes using an on-line household microwave-assisted hydrolysis-SPE method [7]. Similar results were obtained by Afiuni-Zadeh et al. for the hydrolysis of proteins, where the time was minimized from 18 - 24 hours (CH) to 2 - 10 minutes (MH) [8].

After hydrolysis, the analytes must be extracted and this can be achieved through techniques such as liquid-liquid extraction (LLE), solid phase extraction (SPE) and solid phase microextraction (SPME)/thin film microextraction (TFME). The LLE technique is a simple, traditional method that is effective for a wide range of analytes [9-11]; however large volumes of solvents are required to extract the analytes from the sample matrix [12]. Also, SPE technique has been widely used especially on automated sample treatment procedures [9], but in both techniques the issues of low sample throughput, large volume of solvents and the costs to dispose the solvents are major draw-backs [12-15]. Alternatively, the SPME/TFME (an equilibrium sample preparation technique) is known to require less organic solvent amounts and sample volumes (< 100 µL) and hence an environmentally friendly method [12, 16]. However, the SPME/TFME high reproducibility and precision rely much on proper handling and/or controlling of factors such as sample temperature, pH, ionic strength, extraction timing etc. and a lack thereof may produce poor results [16-17]. With adequate method development and proper use, the precision of the SPME/TFME can be as good as that of traditional methods [12]. In this study, our objective was to evaluate these innovative methods in order to minimize the time and solvents required for the pre-treatment of urine samples.

5.3 Experimental

5.3.1 Chemicals and reagents

The reagents used for the experiments namely sodium acetate (98 %), sodium nitrite (99 %), sodium sulfite (98 %), potassium dihydrogen phosphate (≥99 %), alizarinsulfonic acid (98 %), amidosulfonic acid (>99 %), concentrated hydrochloric acid (37%), hydriodic acid (55 %, unstabilized, A.C.S), nitric acid (≥90 %) and hydrogen peroxide solution (≥30 %, unstabilized, A.C.S) were all purchased from Sigma-Aldrich, Steinheim, Germany. Methanol (>99 %) was a product of KMF Laborchemie, Lohmar, Germany and the ultra-filtered water used was from PureLab Ultra (ELGA LabWater, Celle, Germany). The aromatic amines used for method validation are listed in table 5.1.

5.3.2 Preparation of stock solutions and standards

The stock solution consisting of a mixture of 6 aromatic amines was prepared by weighing about 0.1 g of each compound in a 100-mL volumetric flask and dilution with methanol to the mark. From the stock solution working standards were prepared for every measurement and the solutions were kept in a refrigerator at 4°C until needed.

5.3.3 Preparation of reagents

The following reagents, namely sodium nitrite (50 g/L), sodium sulfite (saturated), potassium dihydrogen phosphate (0.1 *M*), alizarinsulfonic acid (1 %), amidosulfonic acid (50 g/L), hydrochloric acid (0.1*M*) and sodium acetate (saturated) were prepared in 100-mL volumetric flasks and made up to the mark with ultra-filtered water. The pH of the sodium acetate solution was adjusted to pH 9 by adding 2 drops of concentrated hydrochloric acid. To prepare the phosphate buffer (pH 9), an amount of 95.5 mL of 0.1 *M* potassium dihydrogen phosphate (KH_2PO_4) and 4.5 mL of 0.1 *M* hydrochloric acid (HCl) solutions were mixed in a 100-mL volumetric flask.

5.3.4 Instrumentation

The Digestion Microwave System, model Mars 5 is a product of CEM Corporation, North Carolina, USA that is designed for laboratory use in digesting, hydrolyzing or drying a wide range of materials. The GC×GC and MS parameters used to analyze the urine samples for the comparison of conventional and microwave-assisted hydrolyses are the same as mentioned in the previous chapter (section 3.3.6). For the TFME experiments, the parameters for GC and MS are shown in table 5.2.

Table 5.1 Aromatic amines used for method validation

Substance	Purity	CAS Number	Company, City & Country	Structural formula and molecular weight	m/z of iodinated derivatives [M+]
Aniline (AN)	99.5 %	62-53-3	Sigma-Aldrich, Steinheim in Germany	93.13 g/mol	204
4-methylaniline (4MA)	99.0 %	106-49-0	Sigma-Aldrich, Steinheim in Germany	107.07 g/mol	218
2,6-dimethylaniline (2,6DMA)	99.0 %	87-62-7	Sigma-Aldrich, Steinheim in Germany	121.09 g/mol	232
2-chloroaniline (2CA)	99.7 %	95-51-4	Sigma-Aldrich, Steinheim in Germany	127.57 g/mol	238
3-chloro-4-fluoroaniline (3C4FA) as an IS	98.0 %	367-21-5	Sigma-Aldrich, Steinheim in Germany	145.01 g/mol	256
4-chloro-2-methylaniline (4C2MA)	98.0 %	95-69-2	Sigma-Aldrich, Steinheim in Germany	141.03 g/mol	252

Table 5.2 GC-MS parameters used for method validation by SPME/TFME blades

Material	**GC-MS parameters**
GC-system	GC-2010 and GCMS QP2010 Plus coupled with AOC-5000 liquid, headspace and SPME GC injection system (Shimadzu GmbH, Duisburg, Germany)
SPME fiber	65 µm PDMS/DVB (Sigma-Aldrich, Schnelldorf, Germany)
SPME liner	0.75 mm x 5.0 mm x 95 mm (made for Shimadzu GCs)
GC columns	DB-5, 30 m, 0.25 mm ID, 0.25 µm film (Agilent Technologies, Waldbronn, Germany)
Colum temperature gradient	40 °C held for 3 minutes and 200 °C at 5 °C /min held for 1 minute
Injection temperature	250 °C
Injection mode	Split / Splitless
Pressure	193.6 kPa
Total flow	32.8 mL / min
Column flow	2.44 mL/min
MS mode	SIM
Ion source temp	200 °C
Interface temp	250 °C
Scanned mass-to-charge ratio (m/z)	40 - 400
Software	GCMSsolution

5.3.5 Microwave-assisted hydrolysis (MH)and conventional (CH) hydrolysis processes

Into a 4 mL urine sample in a microwave vessel, 3 mL nitric acid (HNO_3) and 2 mL hydrogen peroxide (H_2O_2) were added and the vessels were placed in the laboratory microwave, with an extra vessel filled with 50 mL water as a blank. The procedure followed for the microwave hydrolysis was prescribed in the installed microwave programs, where the total amount of a sample required for MARS 5 cavity was up to 10 mL with acid and/or 50 mL with water. The suggested solvents to be used were HNO_3 and H_2O_2 with the following wattages; 300 Watts (1 - 2 vessels), 600 Watts (3 - 5 vessels) and 1200 Watts (6 or more vessels). In our case, 3 vessels (2 for samples and 1 for water) were used for the tests and therefore the microwave power and temperature were set using the Marslink program at 600 Watts and 110 °C for 15, 45 and 65 min irradiation time consecutively. Each experiment started automatically with a fixed ramp time (5 min) until the desired temperature was reached. For the conventional hydrolysis, 10 mL concentrated hydrochloric acid (37 %) was added into a 20 mL urine sample and heated by the laboratory hot plate at 110 °C for 12 hours. The procedures for both conventional and microwave-assisted hydrolyses are illustrated in figure 5.1 below. The urine samples were provided by the same donor for all the experiments and were prepared in duplicates. After hydrolyses (CH and MH), the samples were extracted (LLE), derivatized and measured by GC×GC-qMS. Finally, the microwave vessels were cleaned in the microwave twice with 8 mL HNO_3 after every hydrolysis.

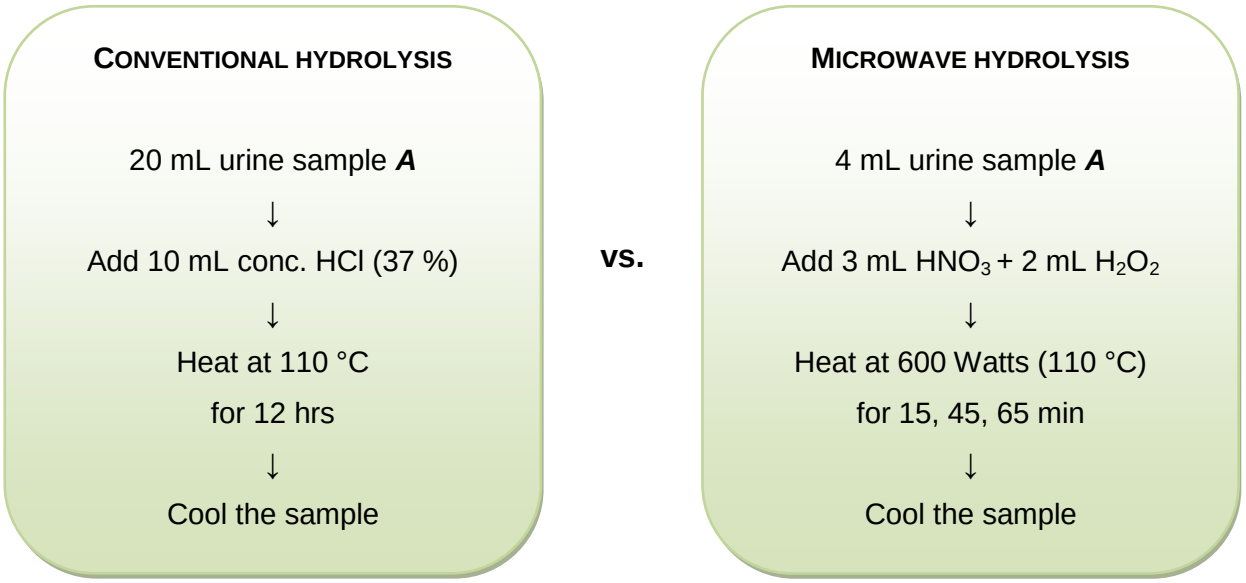


Fig. 5.1 Diagram illustrating the procedure for the conventional and microwave-assisted hydrolysis of urine samples. The symbol ***A*** stands for the same donor

5.3.6 Extraction of aromatic amines by Thin Film Microextraction (TFME)

The SPME/TFME blades coated with Bondesil-PBA/PAN, Chromabond-Easy/PAN, LC-Diol/PAN and C_{18}-SCX/PAN were supplied by Professional Analytical System (PAS) Technology, Magdala, Germany. The coated blades and the SPME/TFME device are shown in figure 5.2. Prior to extraction, the blades were conditioned overnight by placing them in a well plate filled with 2 mL methanol in each well. After conditioning, the methanol was replaced with 1mL water and shaken with the blades for 15 minutes in the middle position (850 rpm) and then discarded. To extract the analytes, 900 µL of phosphate buffer (pH9) was placed in the plate wells and spiked with 100 µL of 1 mg/L aromatic amine mixture.

The blades were immersed in the solution and shaken for desired extraction times of 10 - 20 - 30 - 40 - 50 - 60 min separately. To desorb the analytes from the coated blades, the solution was discarded and replaced by 1 mL of water spiked with 10 µL hydriodic acid (HI) and then shaken all for another 20 min. Finally, the solution was transferred into 20-mL vials containing 9 mL water where the extracts were further derivatized as described by Lamani et al. [18] and measured by GC-MS. After obtaining the suitable extraction time, the desorption experiments were carried out by testing the time that will be suitable to desorb the analytes from the coated blades efficiently, which were 5 - 10 - 20 - 30 - 40 - 50 - 60 min consecutively. The validated parameters (i.e. the SPME/TFME blade, extraction- and desorption times) were then used further to evaluate the concentration of aromatic amines that can be extracted by the SPME/TFME blade from the prepared standards.

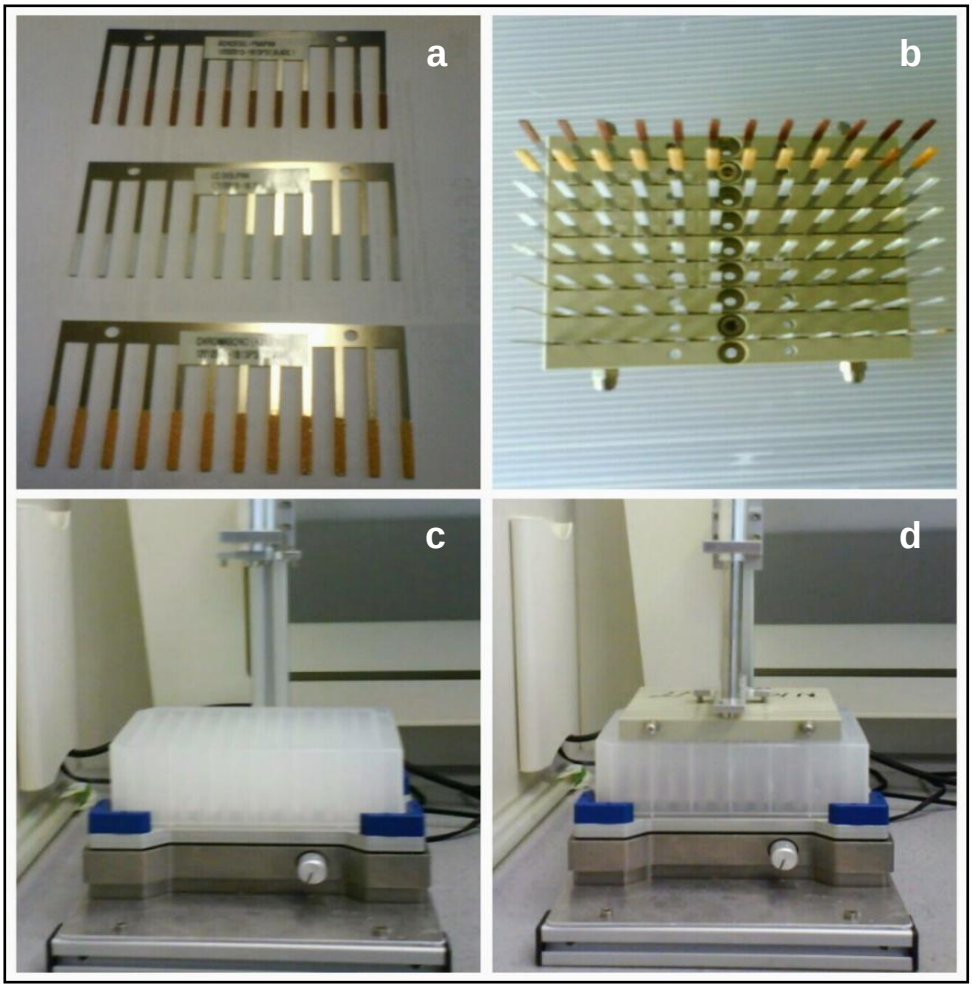


Fig. 5.2 SPME/TFME device showing **(a)** and **(b)** the SPME/TFME blades coated with different materials, **(c)** the 96-SPME well plate attached to the SPME/TFME device and filled with 2 mL methanol to condition the blades overnight and **(d)** the SPME/TFME blades placed on the well plate in the middle position and immersed in 2 mL methanol for conditioning

5.4 Results and discussions

5.4.1 Microwave-assisted hydrolysis (MH) and conventional hydrolysis (CH)

Urine samples taken from the same donor were used to evaluate the hydrolysis of aromatic amine conjugates by using a laboratory microwave and a hot plate. As shown in figure 5.3 below, aromatic amines were found in both microwave assisted- and conventionally hydrolyzed samples (n=2); though the number of analytes obtained was higher in the CH (ca. 74 analytes) compared to the MH. At 600 W / 110 °C about 51, 55 and 38 analytes were obtained at the irradiation times of 15, 45 and 65 min, respectively. When the power and the temperature of the microwave were increased to 800 W / 130 °C, to further evaluate the behavior of the analytes; the number of analytes obtained was ca. 51, 43 and 38 at 15, 45 and 65 min, respectively. The adjustment in the parameters from 600 W to 800 W did not show any significant difference in the number of analytes obtained, except for a loss of some analytes in the samples hydrolyzed for 45 min. Also, it was obvious that keeping the samples in the microwave for a longer time (up to 60 min) had a negative effect on the analytes as more than 10 analytes were lost when compared to 15 min irradiation time, in both 600 and 800 W.

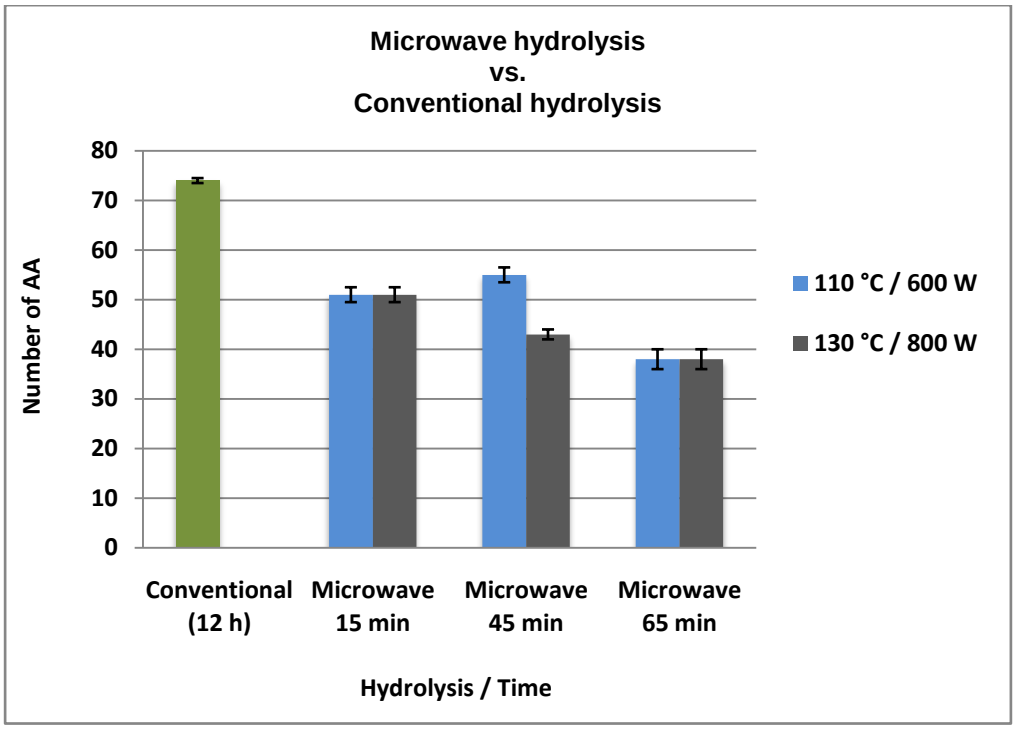


Fig. 5.3 A diagram showing the number of aromatic amines obtained after hydrolysis by conventional (CH) method at 110 °C (green bar) and microwave-assisted (MH) method at 600 watts (blue bars) and 800 watts (grey bars); followed by liquid-liquid extraction, derivatization of the hydrolyzed analytes and analysis with GC×GC-qMS

The results obtained from CH and MH processes at 600 W / 110 °C are also shown as an example in the GC×GC-qMS contour plot in figure 5.4 below. Here, it can be noticed that the blobs which represent analytes peaks, were much more intense in the MH for 45 min (fig. 5.4c) than the blobs in the MH for 15 min (fig. 5.4b); even though the number of analytes identified at these two irradiation times were almost equal (51 isomers at 15 min and 55 isomers at 45 min). To confirm this, the peak areas of some of the analytes which seem to be more dominant in figures 5.4a – d, numbers 1 to 5 were compared.

These include **(1)** Aniline (AN), **(2)** 2-methylaniline (2MA), **(3)** 2,6-dimethylaniline (2,6DMA), **(4)** 2,4-dimethylaniline (2,4DMA) and **(5)** 2,4,6-trimethylaniline (2,4,6TMA). The comparison of peak areas in figure 5.5 shows the analytes hydrolyzed for 45 min more concentrated than the analytes hydrolyzed for 15 min and as anticipated in figure 5.4b and c below.

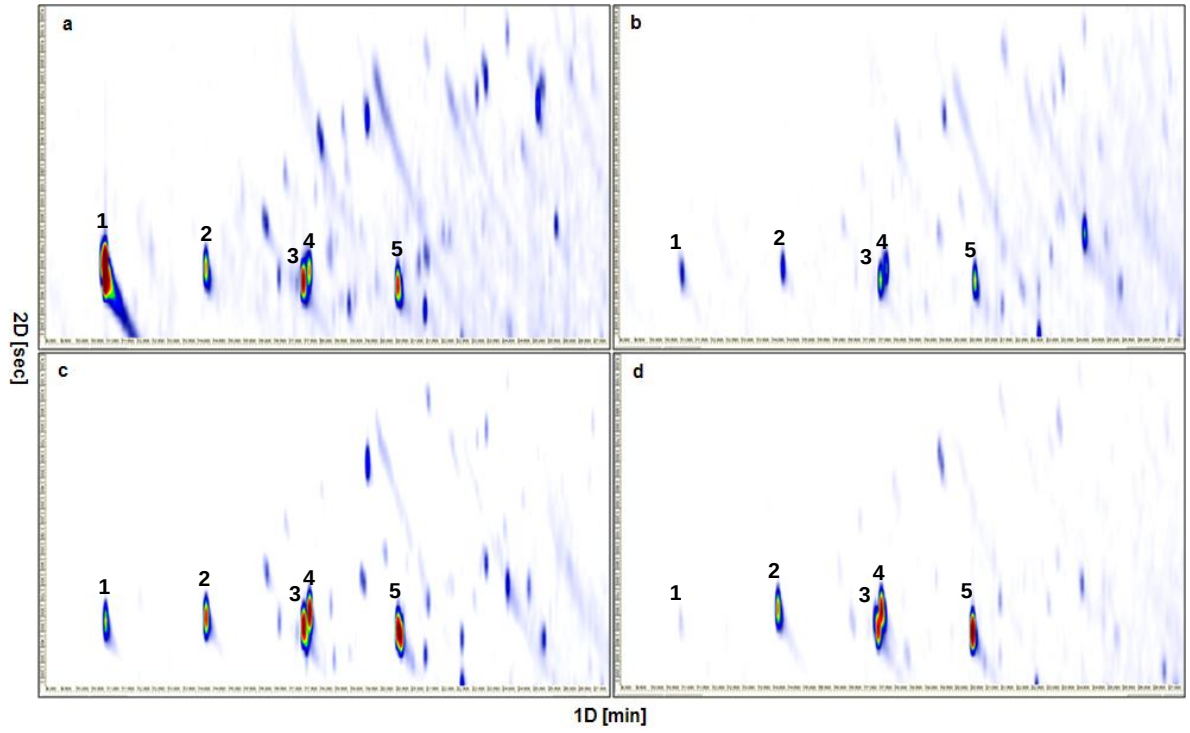


Fig. 5.4 GC×GC-qMS contour plots of urine samples after hydrolysis with the conventional (**CH**) and microwave-assisted (**MH**) methods followed by liquid-liquid extraction, derivatization and measure by GC×GC-qMS. **(a)** CH at 110 °C / 12 hrs, **(b)** MH at 600 W / 15 min, **(c)** MH at 600 W / 45 min, **(d)** MH at 600 W / 65 min. The numbers 1 – 5 are the analytes selected for the comparison of peak areas

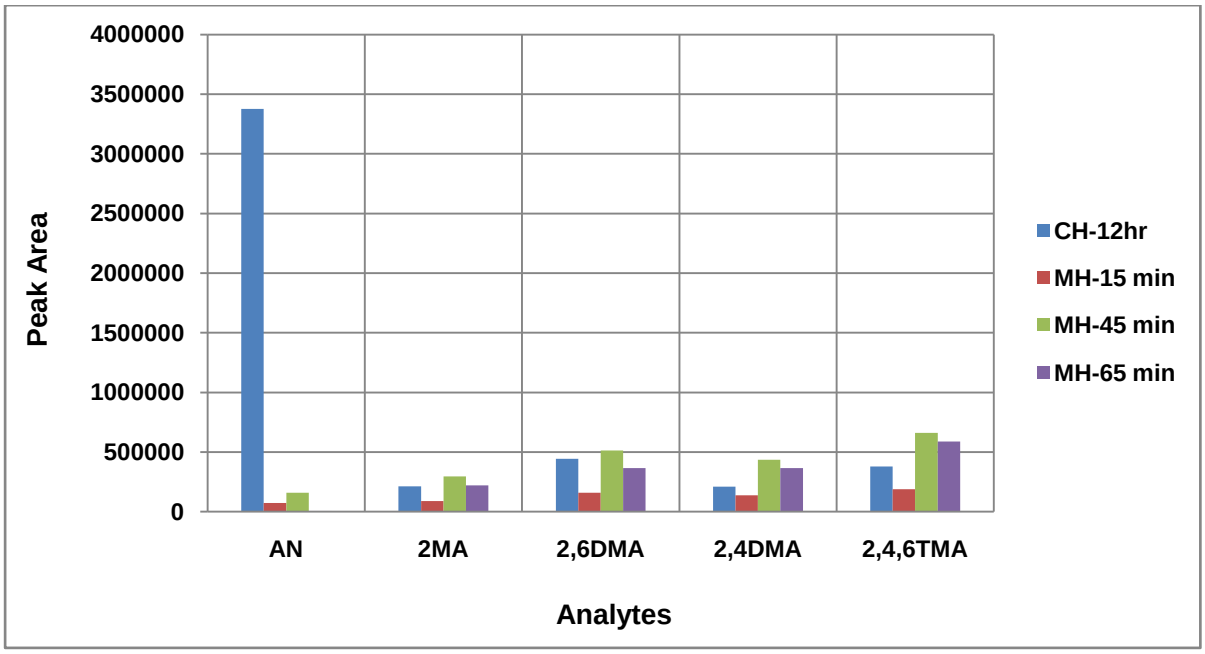


Fig. 5.5 Comparison of peak areas of the selected analytes identified in urine samples of the same donor and hydrolyzed by **CH** at 12 hr and **MH** at 15, 45 and 65 min irradiation time. The analytes are as indicated by numbers in figures 5.4a – d above

Further, the suggested list of all other analytes detected in both hydrolysis processes is shown in table 5.3 below. Here, Aniline and C1- to C4-anilines were identified in all the experiments (CH and MH) and also in similar numbers. Other analytes such as Aminobiphenyl, C1-aminobiphenyl, Aminoquinoline, C1- and C2-aminoquinoline, C2- and C3-tetrahydronaphthalene, Aminophenol, Aminothiophene and C1-aminothiophene could only be identified in CH and were not detected in MH. It is only C2-chloroaniline that could not be detected in CH but present in all MH experiments done and at all tested irradiation times.

Table 5.3 List of proposed analytes obtained from CH and MH methods followed by the extraction of the hydrolyzed analytes, derivatization and analysis by GC×GC-qMS

Substance	**m/z of iodinated derivatives [M+]**	**Main fragment (*m/z*)**	**Isomers in CH at 110°C**	**Isomers in MH at 600W**			**Isomers in MH at 800W**		
			12 hr	**15min**	**45min**	**60min**	**15min**	**45min**	**60min**
Aniline	204	77	1	1	1	1	1	1	1
C1-Aniline	218	91	1	1	1	1	1	1	1
C2-Aniline	232	217,105	5	3	4	3	4	3	3
C3-Aniline	246	119	9	6	8	6	7	8	7
C4-Aniline	260	245,118	6	3	4	3	3	4	4
Chloroaniline	238	111	2	2	2	2	3	2	2
C1-Chloroaniline	252	125	2	2	2	2	2	2	3
C2-Chloroaniline	266	139	**n.d**	3	4	1	5	3	2
C3-Choroaniline	280	265,138	3	5	5	n.d	4	4	3
C4-Chloroaniline	294	279,152	1	1	1	1	1	1	1
Chlor-methoxyaniline	268	253,126	4	2	n.d	n.d	n.d	n.d	n.d
Dichloroanline	272	145	2	2	n.d	2	2	2	2
Bromoaniline	282	155	2	1	1	1	2	1	1
C1-Bromoaniline	296	169	n.d	1	n.d	1	1	1	1
Methoxyaniline	234	107,92	1	1	1	n.d	n.d	n.d	n.d
C3-Methoxyaniline	276	261,134	1	2	2	2	2	1	1
Naphthylamine	254	127	2	1	1	n.d	1	n.d	n.d
C2-Naphthylamine	282	155	n.d	1	1	n.d	n.d	1	n.d

Table 5.3 List of proposed analytes obtained from CH and MH methods (continues)

Substance	m/z of iodinated derivatives [M+]	Main fragment (*m/z*)	Isomers in CH at 110°C	Isomers in MH at 600W			Isomers in MH at 800W		
			12 hr	15min	45min	60min	15min	45min	60min
Amino-biphenyl	280	153	3	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
C1-Amino-biphenyl	294	279,152	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
Diamino-benzene	330	203,76	2	2	2	2	2	2	2
C1-Diamino-benzene	344	217,90	1	1	2	1	1	2	n.d
C2-Diamino-benzene	358	231,104	2	1	1	2	n.d	n.d	n.d
C3-Diamino-benzene	372	245,118	1	n.d	1	1	1	1	1
C4-Diamino-benzene	386	371,244	1	1	n.d	n.d	n.d	n.d	n.d
Chlordiamino-benzene	364	237,110	2	n.d	2	1	n.d	n.d	n.d
C1-Diamino-naphthalene	394	267,140	n.d	n.d	2	n.d	n.d	n.d	n.d
Triamino-toluene	456	329,202,75	2	3	2	2	3	n.d	2
Amino-quinoline	255	128	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
C1-Amino-quinoline	269	142	2	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
C2-Amino-quinoline	283	156,128	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**

Table 5.3 List of proposed analytes obtained from CH and MH methods (continues)

Substance	m/z of iodinated derivatives [M+]	Main fragment (*m/z*)	Isomers in CH at 110°C	Isomers in MH at 600W			Isomers in MH at 800W		
			12 hr	15min	45min	60min	15min	45min	60min
Aminoindane	244	117,91	2	n.d	1	n.d	2	1	n.d
Tetrahydro-naphthylamine	258	131	3	2	n.d	n.d	1	n.d	n.d
C1-Tetrahydro-naphthylamine	272	145	2	2	3	2	2	2	1
C2-Tetrahydro-naphthylamine	286	271,144	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
C3-Tetrahydro-naphthylamine	300	285,158	2	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
Aminophenol	220	93	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
C2-Aminophenol	248	121	n.d	1	1	1	n.d	n.d	n.d
Aminothiophene	210	83	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
C1-Aminothiopene	224	97	1	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
TOTAL			**74**	**51**	**55**	**38**	**51**	**43**	**38**

n.d - not detected

Due to some restrictions in the MH use such as microwave malfunctioning and/or unavailability, further tests could not be done. However, in future a number of potential parameters can be optimized to achieve better results. These may include testing (i) irradiation time below 15 minutes and/or between 15 and 45 min, (ii) microwave power below 600 watts and/or up to 1000 watts and (iii) other strong acids such as hydrochloric acid.

5.4.2 Method validation by Thin Film Microextraction (TFME)

Four blades coated with different materials (Chromabond-Easy/PAN, C_{18}-SCX/PAN, LC-Diol/PAN and Bondesil-PBA/PAN) were used to develop a less time- and less solvent-consuming method for the extraction of aromatic amines in human urine. To validate the method a mixture of six aromatic amines in water, including 3C4FA as an IS, was used (table 5.1). The spiked water samples were extracted for 10 - 20 - 30 - 40 - 50 - 60 min each time in duplicates with every blade, at a selected desorption time of 20 min. Thereafter, the samples were derivatized and measured by GC-MS. Figure 5.6 shows the extraction efficiency of (a) C_{18}-SCX/PAN (b) LC-Diol/PAN and (c) Bondesil-PBA/PAN coated blades over time. The results obtained from Chromabond-Easy/PAN coated blade are not reported due to very high standard deviations obtained. In figure 5.6a, the results from the extraction with C_{18}-SCX/PAN blade demonstrated similarity in the sorption behavior of the analytes with slightly higher values at 30 minutes; except for 2CA (*m/z* 238) where the extraction efficiency of the coated blade increased with time.

In the case of LC-Diol/PAN and Bondesil-PBA/PAN coated blades (figures 5.6b and c), the extraction efficiency was higher at 50 min for all the analytes. However, AN (*m/z* 204) and 4-MA (*m/z* 218) had very low intensities from the extraction with LC-Diol/PAN blade and therefore could not be integrated and/or plotted in the graphs.

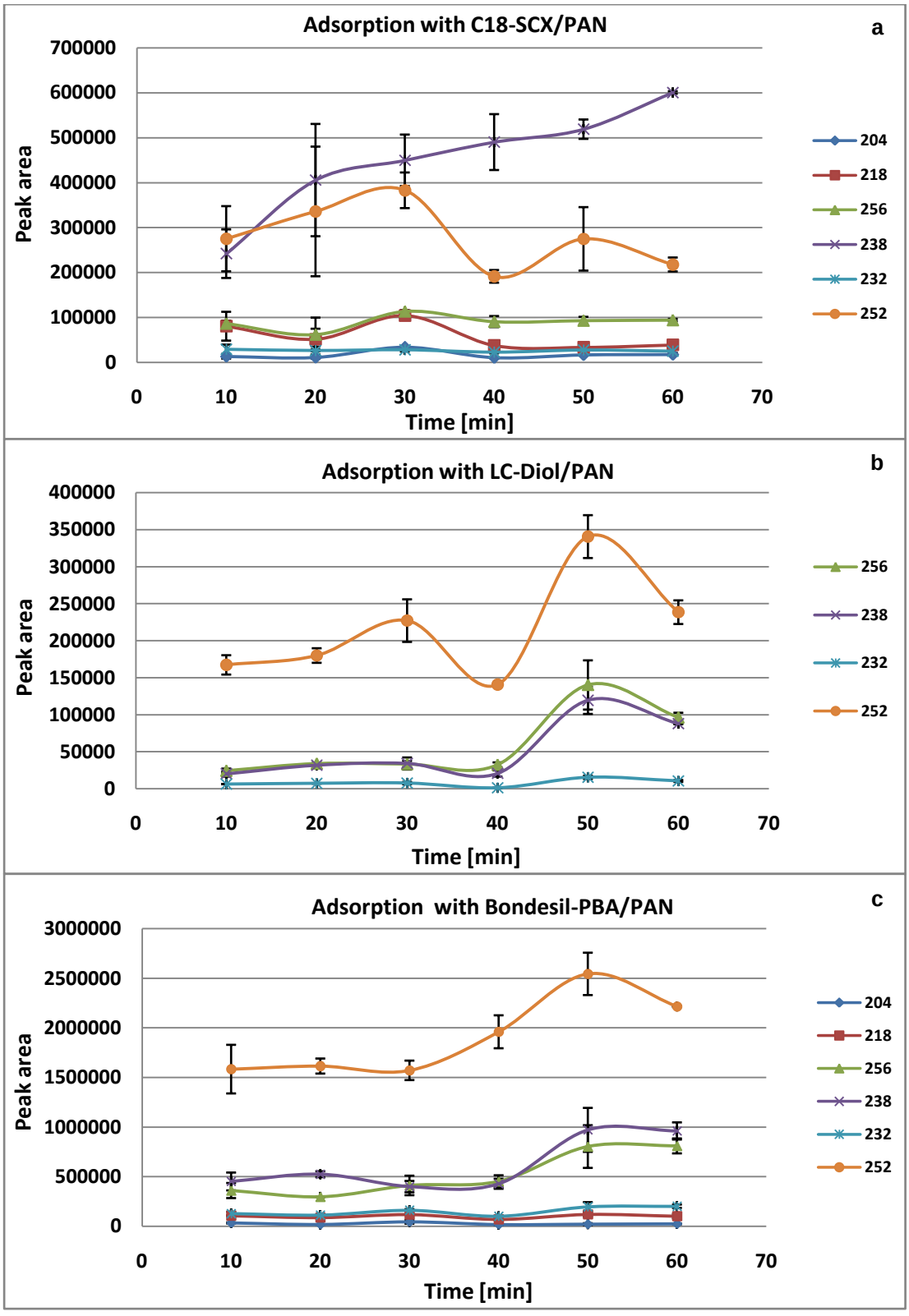


Fig. 5.6 Extraction efficiency of SPME/TFME coated blades for the extraction of selected AAs with **(a)** C_{18}-SCX/PAN, **(b)** LC-Diol/PAN, **(c)** Bondesil-PBA/PAN blades

at the adsorption times of 10 - 20 - 30 - 40 - 50 - 60 min consecutively and 20 min selected desorption time that was kept constant for all the tests

To continue with the method validation, desorption experiments were done using the Bondesil-PBA/PAN coated blade, where the spiked water samples were extracted for 50 min using phosphate buffer (pH 9)and desorbed with acidified water for 5 - 10 - 20 - 30 - 40 - 50 - 60 min successively. The samples were then derivatized and measured by GC-MS. The results are shown in figure 5.7a below where the desorption of the analytes from 5 to 60 min was found to be almost the same or constant for some analytes (*m/z* 204, 218 and 232) and/or gradually increased after 30 min with the ascending time for others, even though in such cases the difference was minimal. This may indicate that desorption of the analytes from the coating material to the solvent was already sufficient within the first 5 min of the process. The chromatograms of adsorption/desorption at 50/5 min and 50/50 min respectively using Bondesil-PBA/PAN coated blade are shown in figures 5.7b and c. For further experiments 50 min adsorption and 5 min desorption times were selected for the extraction of the analytes.

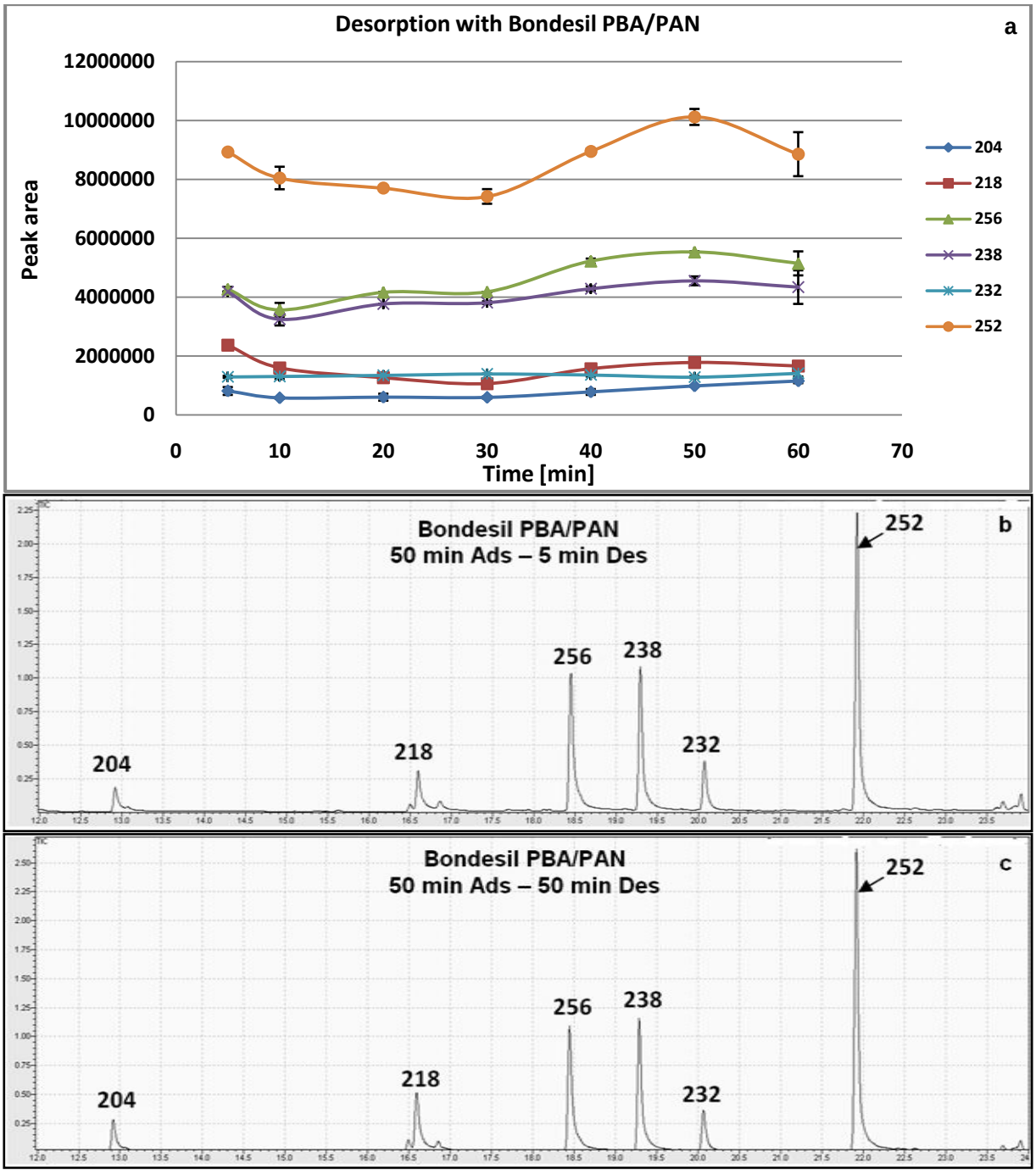
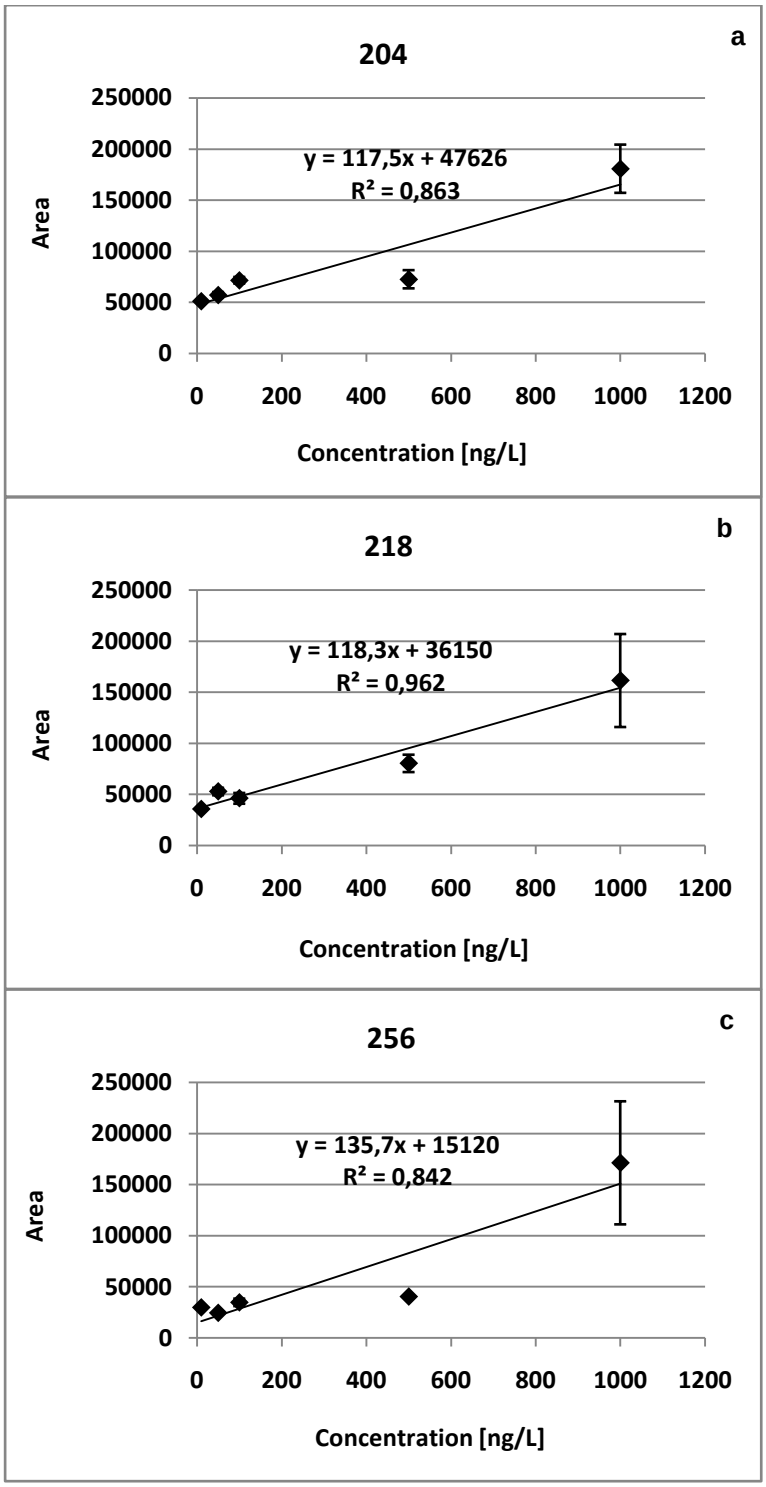


Fig. 5.7 (a) A diagram showing a mixture of six AAs spiked in water, extracted by Bondesil-PBA/PAN coated blade and allowed to adsorb for 50 min and desorb for 5 to 60 min consecutively. In **(b)** and **(c)**, the chromatograms of six AAs extracted with Bondesil-PBA/PAN blade at the adsorption/desorption times of 50 min / 5 min and 50 min / 50 min respectively are shown

In order to evaluate the concentration of the analytes that can be extracted by the Bondesil-PBA/PAN coated blade, a mixture of six AAs spiked in water and having the concentrations of 10 - 50 - 100 - 500 - 1000 ng/L in duplicates were extracted as described in section 5.3.6, at 50 min adsorption- and 5 min desorption time. Figures 5.8a - f show the calibration curves of each analyte with the peak areas plotted against the spiked concentrations. Here, it can be noted that the change or an increase in the peak areas over the concentrations is minimal and as indicated by the smaller regression slopes obtained; which may suggest that the extraction blades used are no longer useful for the quantitative analysis. For this reason, the results obtained from these particular experiments were not found reliable enough and therefore, it was not found meaningful to continue this TFME study on the analysis of human urine due to foreseen challenges of recovering the analytes from the complex urine samples.


a
204
y = 117,5x + 47626
R² = 0,863
Area
Concentration [ng/L]
b
218
y = 118,3x + 36150
R² = 0,962
Area
Concentration [ng/L]
c
256
y = 135,7x + 15120
R² = 0,842
Area
Concentration [ng/L]

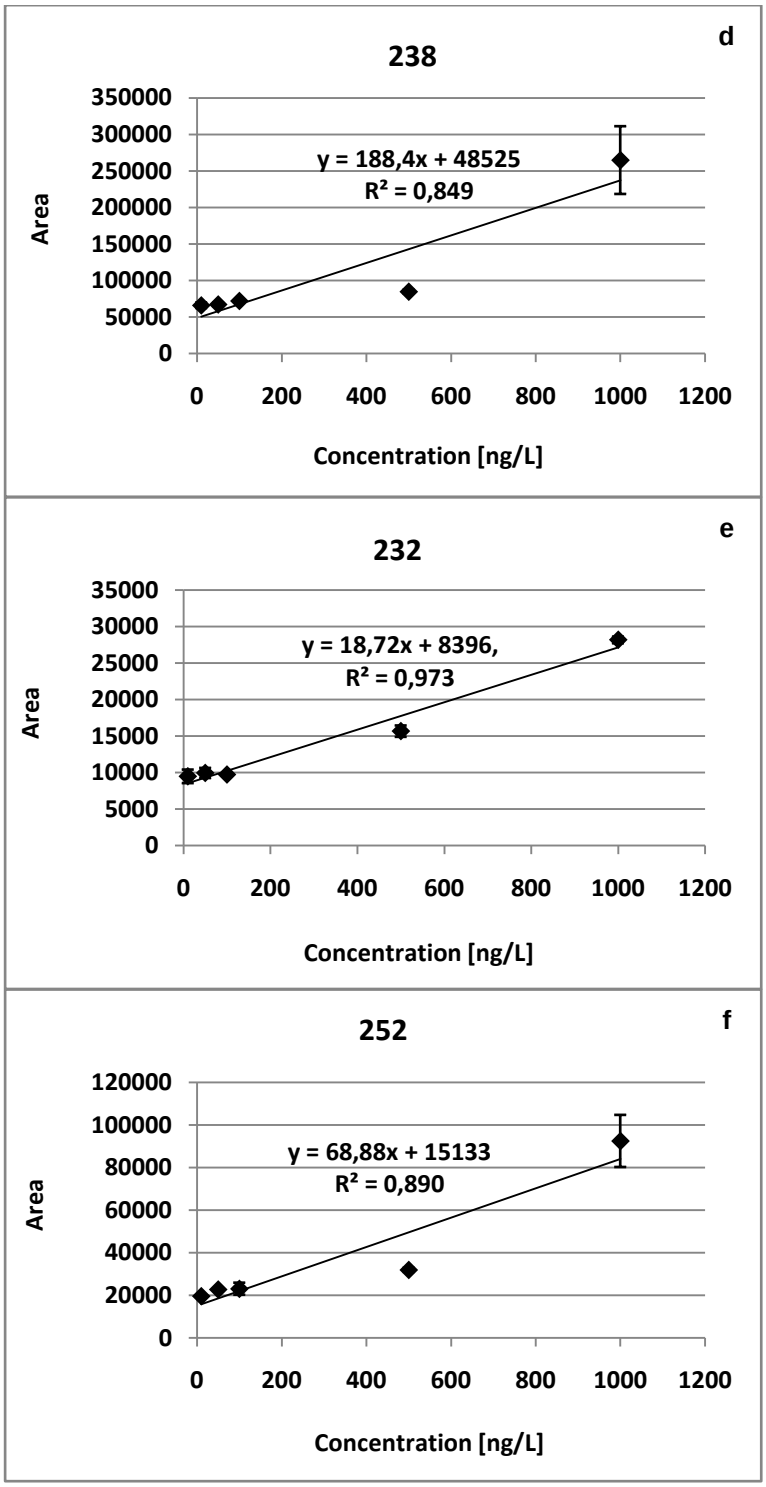


Fig. 5.8 The extraction efficiency of the Bondesil-PBA/PAN coated blade from a mixture of six AAs (a – f) spiked in water at different concentrations of 10 - 50 - 100 - 500 – 1000 ng/L, (n=2)

5.5 Conclusions

This study aimed to investigate alternative methods that can be used to minimize the time required for sample treatment for the analysis of aromatic amines in human urine. Hence, microwave-assisted hydrolysis (MH) and thin film microextraction / solid phase microextraction (TFME/SPME) methods were investigated to potentially replace the methods used in the previous chapters, namely conventional thermal-acid hydrolysis (CH) and liquid-liquid extraction (LLE) methods respectively. The preliminary results attained from the few experiments done with MH suggested that this method can be a promising alternative to the CH method; since a reasonable number of aromatic amines in urine samples were identified. Further experiments in the future may bring better results. In the case of TFME/SPME method, the adsorption and desorption times for the analytes with a suitable coated blade (Bondesil-PBA/PAN) were achieved. However, the results obtained were not satisfactory and hence not reliable. It is suggested that the results might have been adversely affected by different factors such as the improper handling or control of the SPME/TFME device and/or the excess use of the extraction blades which might have degraded the quality of the coating materials and resulted in poor reproducibility and precision of the technique. Hence, the urine samples were not investigated as it was estimated that the possibility to recover the analytes of interest from the complex matrix may be minimal. Finally, the conventional hydrolysis and liquid-liquid extraction methods used in previous chapters are considered for this study as reliable and simple methods for the analysis of aromatic amines in human urine and in future further investigations on the microwave-assisted hydrolysis and TFME/SPME as alternative methods may be essential.

5.6 References

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CHAPTER 6

General conclusions and outlook

In this study, a deeper insight on the occurrence of aromatic amines in human urine has been provided for a better understanding of their role in the formation of bladder cancer (BC) in humans. The use of sample pre-treatment steps such as acidic hydrolysis, liquid-liquid extraction, derivatization and enrichment of the analytes was beneficial for the selective and efficient detection of the target compounds using comprehensive multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS). With this developed method it was possible to identify the isomers of several aromatic amines of the same molecular mass. This finding is very important here because the biological activity of aromatic amines is known to be dependent of the substance structure, for example, of the three isomers of aminobiphenyl (2-, 3- and 4-aminobiphenyl) only 4-aminobiphenyl is known as a carcinogen [1]. Also, the use of ionic liquid columns (ILC) to further investigate the separation of isomers in the second dimension has shown similar results with the traditional mid-polar column, BPX-50 in combination with the non-polar column, DB-5 in the first dimension. Hence, the ILC (polar-highly polar-extremely polar) were not further explored.

The number of target compounds (>100) identified in most urine samples analyzed here may suggest a greater risk of aromatic amines exposure to humans than previously known, especially to the smokers (S) where most of the target compounds had higher concentrations when compared to the non-smokers (NS). This may confirm the hypothesis that cigarette smoking does increase the risk of exposure to aromatic amines. In the case of past-smokers (PS), the concentrations were similar to the S and higher when compared to the NS. This insignificant difference in the concentrations of PS and S may imply that the high risk of bladder cancer for a past-smoking person might not necessarily decrease immediately or drastically just after

quitting. This suggestion may be partly supported by a study of Pira et al. [2], where a number of dyestuff workers who were heavily exposed to aromatic amines such as o-methylaniline, naphthylamines and benzidine between the years 1922 and 1972; were still found to be at high risk of bladder cancer even more than 30 years after the last exposure.

Another aspect of this study was to develop alternative methods to reduce high consumption of time and solvents during sample preparation. In the case of microwave-assisted hydrolysis (MH), the hydrolysis time could be potentially reduced from 12 hours when using conventional hydrolysis (CH) to ≤ 1 hour. However, more tests are still necessary to further validate some parameters such as irradiation time, microwave power, etc. to achieve better results. Also, for the liquid-liquid extraction (LLE) versus thin-film microextraction (TFME) the amount of solvents and sample required as well as the preparation time could be minimized. Nonetheless, the cost of TFME coated blades may probably be a drawback for this technique when compared to LLE method.

Finally, the association of aromatic amines with the development of bladder cancer has been widely known. However, to our knowledge there are no studies where the existence of AAs in human urine has been widely revealed, as done in this study. Also, the use of AAs as urine biomarkers to investigate BC in humans is not among the known and currently available urine biomarkers; which include urine dipstick analysis for hematuria, urine cytology, nuclear matrix protein 22 (NMP22), bladder tumour antigen, fluorescence in situ hybridization (FISH), cytokeratin fragment test, multigene RNA test, epigentic profile test and morpho-histochemical staining test[3-

14]. These urine biomarkers are typically used to detect blood, tumour cells, genes and proteins in the urine. However, they are reported as not being reliable enough for routine screening, primary detection or inspection of BC; due to their insufficient sensitivity and specificity (<100%), which restricts the tests from correctly identifying all patients with or without bladder cancer. Rather, they are recommended as the source of information for cystoscopy, a further BC examination used to inspect bladder tumors inside the bladder to confirm or rule out bladder cancer [15-18].

In view of that, it is suggested that the analytical method developed in this study maybe useful to the progressing global BC studies. With this method, aromatic amines can be investigated probably in the urine of patients diagnosed with bladder cancer, both qualitatively and quantitatively. The results can be further used to test exclusively some aromatic amines such as 2-naphthylamine (2NA), 4-chloro-2-methylaniline (4C2MA), 2-methylaniline (2MA) and etc. as urine biomarkers to be able to detect the level of BC risk in humans. The substances mentioned above as an example, were identified in almost all urine samples analyzed in this study and moreover they are classified by the IARC [19] as carcinogenic or probably carcinogenic to humans. Hence, they are anticipated to be potential future urine biomarkers for the detection and inspection of bladder cancer in humans.

In addition, the high risk of bladder cancer to humans as a result of exposure to AAs has received much less attention both in research and the public than other harmful smoke components such as polycyclic aromatic hydrocarbons and therefore, more research studies building upon the results presented in this thesis are necessary in order to create awareness and/or provide prevention measurements to save lives.

These may include more collaboration with the urology or bladder cancer institutions in order to have access to a larger number of urine samples from people with different backgrounds. Secondly, further developments on some pre-treatments steps mentioned earlier here such as hydrolysis and extraction procedures, to relatively reduce the time used during sample preparation. Last but not least, analysis with comprehensive gas chromatography results in large amounts of data that can make the integration of chromatograms challenging. Therefore, there is still a need for data software that can provide easier analysis of the information gathered from GC×GC analysis.

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CHAPTER 7

Appendix

7.1 List of abbreviations (in numeric and alphabetic order)

%	:	Percent
°C	:	Degrees Celsius
µg	:	Microgram (10^{-6})
1D	:	First dimension
2,4,6TMA	:	2,4,6-trimethylaniline
2,4DMA	:	2,4-dimethylaniline
2,6DCA	:	2,6-dichloroaniline
2,6DMA	:	2,6-dimethylaniline
2ACP	:	2-aminoacetophenone
2BA	:	2-bromoaniline
2CA	:	2-chloroaniline
2D	:	Second dimension
2NA	:	2-naphthylamine
3C2,6DMA	:	3-chloro-2,6-dimethylaniline
3C4FA	:	3-chloro-4-fluoroaniline

3C4MOA	:	3-chloro-4-methoxyaniline
4C2MA	:	4-chloro-2-methylaniline
4EA	:	4-ethylaniline
A.C.S	:	Analytical Chemical Society
AAs	:	Aromatic amines
AN	:	Analine
BC	:	Bladder cancer
ca.	:	Circa
cm/s	:	Centimeter per second
Conc	:	Concentration
DNA	:	Deoxyribonucleic acid
E	:	Extraction ratio
g/L	:	Gram per liter
GC-AED	:	Gas Chromatography Atomic Emission Detector
GC×GC-qMS	:	Multi-dimensional gas chromatography mass spectrometry
IARC	:	International Agency for Research and Cancer

ILC	:	Ionic liquid columns
i.d.	:	Inner diameter
K_{fs}	:	Fiber-sample partition coefficient
kPa	:	Kilopascal
kV	:	Kilovolt
LLE	:	Liquid-liquid extraction
LOD	:	Limit of detection
LOQ	:	Limit of quantitation
M	:	Molar
m/z	:	Mass-to-charge ratio
mL	:	Milliliter
n	:	Number of samples
n.d	:	Not detected
$n_{f,x}$	:	Extracted amount of analytes
ng	:	Nanogram (10^{-9})
NS	:	Non-smoker

$n_{s,o}$	:	Initial amount of analytes in the sample
o-MA	:	o-methylaniline
PAH	:	Polycyclic aromatic hydrocarbons
PS	:	Past-smoker
r^2	:	Regression coefficient
RIC	:	Reconstructed ion chromatogram
Rpm	:	Revolutions per minute
RSD	:	Relative standard deviation
s	:	Seconds
S	:	Smoker
SPME	:	Solid phase microextraction
TCC	:	Transitional-cell carcinoma
TIC	:	Total ion chromatogram
V_f	:	Volume of the sample
viz.	:	Namely
V_s	:	Volume of the fiber coating

7.2 List of tables

7.3 List of figures

7.4 List of publications

Publications in peer-reviewed Journals

Lamani X.; Horst S.; Zimmermann T.; Schmidt T.C. Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GC×GC-qMS). *Analytical and Bioanalytical Chemistry 2015, 40 (1): 241-252*

Abadi D.R.; Dobaradaran S.; Nabipour I.; **Lamani X**.; Ravanipour M.; Tahmasebi R.; Nazmara S. Comparative investigation of heavy metal, trace and macro element contents in commercially valuable fish species harvested off from the Persian Gulf. *Environmental Science and Pollution Research 2015, 22 (9): 6670-6678*

Burkhardt M.; Zuleeg S.; Vonbank R.; Schmid P.; Hean S.; **Lamani X**.; Bester K.; Boller M. Leaching of additives from construction materials to urban storm water runoff. *Water Science and Technology 2011, 63 (9): 1974-1982*

Bester K.; **Lamani X**. Determination of biocides as well as some biocide metabolites from facade run-off waters by solid phase extraction and high performance liquid chromatographic separation and tandem mass spectrometry detection. *Journal of Chromatography A 2010, 1217: 5204-5214*

Burkhardt M.; Junghans M.; Zuleeg S.; Schoknecht U.; **Lamani X**.; Bester K.; Vonbank R.; Simmler H.; Boller M. Biozide in Gebäudefessaden – ökotoxikologische Effekte, Auswaschung und Belastungsabschätzung für Gewässer. *UmweltwissSchadstForsch 2009, 21: 36-47*

Poster presentations

Lamani X.; Horst S.; Zimmermann T.; Schmidt T.C. Determination of aromatic amines in the urine of smokers using comprehensive two-dimensional GCxGC-qMS. *ANAKON 2013, 04.-07.03.2013, Essen, Germany*

Lamani X.; Zimmermann T.; Schmidt T.C. Determination of aromatic amines in the urine of smokers using comprehensive two-dimensional GC×GC as a method of analysis. *36th International Symposium on Capillary Column and 9th GC×GC Symposium, 27.05 – 01.06.2012, Riva del Garda, Italy*

7.5 Curriculum vitae

This CV was removed from online publishing for the protection of personal data.

7.6 Declaration (Erklärung)

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel:

Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry

selbst verfasst und keine außer den angegebenen Hilfsmittel und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, 03.05.2018

Xolelwa Lamani-Dixon

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