Aromatic amines in human urine: optimization and automation of the analytical method for their analysis as iodinated derivatives

Dissertation

zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

– Dr. rer. nat. –

vorgelegt von

Nerea Lorenzo Parodi

geboren in Madrid, Spanien

Instrumentelle Analytische Chemie

der

Universität Duisburg-Essen

2024

Die vorliegende Arbeit wurde im Zeitraum von Februar 2016 bis August 2019 im Arbeitskreis von Prof. Dr. Torsten C. Schmidt in der Fakultät für Chemie im Bereich Instrumentelle Analytische Chemie der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 22.04.2024

Gutachter: Prof. Dr. Torsten C. Schmidt

Prof. Dr. Astrid Gjelstad

Vorsitzender: Prof. Dr. Stephan Barcikowski

DOI: 10.17185/duepublico/82115
URN: urn:nbn:de:hbz:465-20240628-101233-8

Acknowledgements

First of all, I would like to express my deepest gratitude to Prof. Dr. Torsten C. Schmidt, for the wonderful supervision and mentoring that extended beyond the thesis period. Especially for the availability for meetings and discussions, the very insightful and very helpful feedback, and the constant support, encouragement, and patience.

I am also deeply grateful to Prof. Dr. Astrid Gjelstad for her encouragement and support, especially but not limited to the liquid phase microextraction step. For the willingness to help no matter the request, for giving a talk at our group, hosting me during a short stay and taking the role of second reviewer, my deepest and sincerest thank you!

I would like to sincerely thank all my co-authors for the excellent cooperation and professional exchange. Especially Prof. Dr. Erich Leitner for the very valuable research stay opportunity and the interesting and motivating exchanges ever since, and Prof. Dr. Susanne Moebus, for the access to the samples from the Heinz Nixdorf Recall Study and the very insightful revision of our joint publication.

I am also grateful to Evonik Industries AG for the funding of my thesis, including conferences and research stays, which allowed me to share my results at a national and international level. And to Shimadzu Europa GmbH for allowing me work part-time several months in order to focus on the thesis, and all the colleagues there for their support.

Thank you to all the students I had the honor to supervise and who provided valuable preliminary work for the chapters of this thesis: Hyerin, Irene, Xenia, Leonard, Wiebke, Milad, Pradeep and Mona. And special thanks to Hyerin and Wiebke, for the support inside and outside of the laboratory during their HiWi times.

I would like to sincerely thank all the staff of the Department of Instrumental Analytical Chemistry at the University of Duisburg-Essen for cultivating a wonderful working environment. I would like to thank Nenad, Sajjad, Sasho, Daniel, Xochitli, Oliver, Marcel, Christian, and everyone else in the IAC group, for the many stimulating discussions, and especial thanks go to Lydia, Robert, Uschi, Claudia, Klaus, Robert, Maik, Holger and Mr. Molt.

Last but not least, I would like to thank from the bottom of my heart my life partner Erik, my parents Alejandra and Juan Carlos, my brother Jordi, and all of my family and friends, for their unwavering and constant support. I love you all!

Summary

Several Aromatic amines (AA) have been classified as human carcinogens, and tobacco smoke is one of the most important sources of human exposure. They are metabolized in the body and can ultimately be excreted in urine as metabolites or free AA. They can be analyzed with gas chromatography-mass spectrometry (GC-MS), typically after a complex and labor-intensive sample preparation procedure, involving hydrolysis, extraction, and derivatization steps. The objective of this thesis was therefore to optimize and automate the sample preparation procedure for the analysis of aromatic amines in urine.

As a proof-of-concept, an existing procedure was evaluated and it´s suitability for such analysis studied. Therefore, the relationship between the smoking status of the urine donors and the amount of AA present was studied in 68 samples from 10 smokers (S), 28 past smokers (PS) and 30 never smokers (NS). Furthermore, three different data evaluation approaches were presented: a qualitative analysis, a quantitative analysis, and a quantitative screening. Due to the high variability in concentrations typically observed in biological samples, the quantitative screening was proven a very promising alternative to the quantitative analysis. And a relationship between the smoking status of the donors and the AA present could be established. The method was therefore deemed suitable, and it was further optimized and automated.

The Liquid-Liquid Extraction (LLE) step was the first step studied because of the large volumes of sample and toxic organic solvents needed, and the fact that it is a very time-consuming and labor-intensive step. Two alternatives were evaluated, namely Hollow Fiber - Liquid Phase Microextraction (HF-LPME) and Parallel Artificial Liquid Membrane Extraction (PALME) and relevant extraction parameters were optimized. Because significantly higher recoveries could be observed with PALME when comparing the optimized methods, PALME was further validated. PALME was proven a very promising alternative to LLE, with limits of detection (LOD) of 45-75 ng/L, and repeatability and peak area relative standard deviations (RSD) below 20 %.

The next step was to evaluate different detectors. To this end, GC-MS in single-ion monitoring (SIM) mode with (1) electron ionization (GC-EI-MS) and (2) negative chemical ionization (GC-NCI-MS), and (3) GC-EI-MS/MS in multiple reaction monitoring (MRM)

mode using electron ionization were studied. Most analytes showed excellent LOD (50, 3.0-7.3, and 0.9-3.9 pg/L for (1), (2), and (3) respectively), good precision (intra and inter-day repeatability < 20 %) and excellent recoveries (between 80 and 104 %). From the three techniques studied, the most promising one was GC-EI-MS/MS. GC-NCI-MS was proven an interesting alternative for qualitative/non-target analysis, since all the derivatized iodinated amines could be easily identified and the significant loss in sensitivity observed over time would not be as detrimental as in quantitative analysis.

Finally, in order to minimize the need for human intervention and the opportunities for errors, and improve the overall greenness of the analytical procedure, the sample preparation was automated. Different problems encountered, like volume limitations or needle penetration depth adjustments, are discussed in detail. And thanks to the less labor-intensive and time-consuming sample preparation procedure, several steps, such as reaction/extraction times or some of the reagents which were not optimal for the automated set-up, could be further optimized. The sample preparation procedure for the analysis of aromatic amines in human urine, could therefore be successfully optimized and automated.

Having an automated and optimized sample preparation procedure would enable the analysis of enough real samples so that the relationship between AA, smoking status and smoking-related diseases could be determined. Furthermore, the analytical method could be used to analyze collective samples, for example, from workers at risk of AA exposure and the surrounding population and could enable the real time monitoring of occupational exposure. Moreover, this method could also be used for wastewater-based epidemiology and could help monitor a population´s exposure to tobacco smoke and its health status.

Zusammenfassung

Mehrere aromatische Amine (AA) wurden als Karzinogene für den Menschen eingestuft, und Tabakrauch ist eine der wichtigsten Expositionsquellen für den Menschen. Sie werden im Körper verstoffwechselt und können schließlich als Metaboliten oder freie AA im Urin ausgeschieden werden. Sie können mit Gaschromatographie-Massenspektrometrie (GC-MS) analysiert werden, in der Regel nach einer komplexen und arbeitsintensiven Probenvorbereitung, die Hydrolyse-, Extraktions- und Derivatisierungsschritte umfasst. Ziel dieser Arbeit war es daher, die Probenvorbereitung für die Analyse von aromatischen Aminen im Urin zu optimieren und zu automatisieren.

Ein bestehendes Verfahren wurde evaluiert und seine Eignung für eine solche Analyse untersucht. Dazu wurde in 68 Proben von 10 Rauchern, 28 ehemaligen Rauchern und 30 Nie-Rauchern der Zusammenhang zwischen dem Raucherstatus der Urinspender und der Menge der vorhandenen AA untersucht. Außerdem wurden drei verschiedene Ansätze zur Datenauswertung vorgestellt: eine qualitative Analyse, eine quantitative Analyse und ein quantitatives Screening. Aufgrund der hohen Variabilität der Konzentrationen, die typischerweise in biologischen Proben beobachtet werden, erwies sich das quantitative Screening als vielversprechende Alternative zur quantitativen Analyse. Ein Zusammenhang zwischen dem Raucherstatus der Spender und den vorhandenen AA konnte festgestellt werden. Die Methode wurde daher als geeignet erachtet und weiter optimiert und automatisiert.

Die Flüssig-Flüssig-Extraktion (LLE) war der erste Schritt, der untersucht wurde, da große Probenmengen und toxische organische Lösungsmittel benötigt werden und es sich um einen sehr zeit- und arbeitsintensiven Schritt handelt. Es wurden zwei Alternativen bewertet, nämlich die Hohlfaser-Flüssigphasen Mikroextraktion (HF-LPME) und die Parallele Künstliche Flüssigmembranextraktion (PALME), und die entsprechenden Extraktionsparameter wurden optimiert. Da mit PALME bei Vergleich der optimierten Methoden deutlich höhere Wiederfindungsraten erzielt werden konnten, wurde PALME weiter validiert. PALME erwies sich als vielversprechende Alternative zu LLE, mit Nachweisgrenzen (LOD) von 45-75 ng/L und Wiederholbarkeit und relativen Standardabweichungen der Peakflächen (RSD) unter 20 %.

Der nächste Schritt bestand darin, verschiedene Detektoren zu bewerten. Zu diesem Zweck wurden GC-MS im Einzel-Ionen-Überwachung (SIM) Modus mit (1)

Elektronenstoßionisierung (GC-EI-MS) und (2) negativer chemischer Ionisierung (GC-NCI-MS) sowie (3) GC-EI-MS/MS im „Multiple Reaction Monitoring“ (MRM) Modus mit Elektronenstoßionisierung untersucht. Die meisten Analyten zeigten ausgezeichnete LOD (50, 3,0-7,3 und 0,9-3,9 pg/L für (1), (2) bzw. (3)), gute Präzision (Wiederholbarkeit innerhalb und zwischen den Tagen < 20 %) und ausgezeichnete Wiederfindungen (zwischen 80 und 104 %). Von den drei untersuchten Techniken war die GC-EI-MS/MS die vielversprechendste. GC-NCI-MS erwies sich als interessante Alternative für die qualitative/nicht zielgerichtete Analyse, da alle derivatisierten iodierten Amine leicht identifiziert werden konnten, und der im Laufe der Zeit beobachtete erhebliche Empfindlichkeitsverlust nicht so nachteilig ist wie bei der quantitativen Analyse. Die GC-EI-MS wies die schlechteste Empfindlichkeit und Selektivität auf, ist jedoch eine der am weitesten verbreiteten und kostengünstigsten Techniken unter den untersuchten, und könnte für Studien geeignet sein, bei denen ihre relativ schlechtere Leistung kein Problem darstellt.

Schließlich wurde die Probenvorbereitung automatisiert, um die Notwendigkeit menschlicher Intervention und die Fehleranfälligkeit zu minimieren, und das Analyseverfahren insgesamt umweltfreundlicher zu gestalten. Verschiedene aufgetretene Probleme, wie Volumenbegrenzungen oder Anpassungen der Nadeleinstichtiefe, werden im Detail erörtert. Dank des weniger arbeits- und zeitintensiven Probenvorbereitungsverfahrens konnten mehrere Schritte, wie z. B. die Reaktions-/Extraktionszeiten oder einige der Reagenzien, die für den automatisierten Aufbau nicht optimal waren, weiter optimiert werden. Die Probenvorbereitung für die Analyse von aromatischen Aminen in menschlichem Urin konnte daher erfolgreich optimiert und automatisiert werden.

Ein automatisiertes und optimiertes Probenvorbereitungsverfahren würde die Analyse einer ausreichenden Anzahl von realen Proben ermöglichen, so dass die Beziehung zwischen AA, Raucherstatus und rauchbedingten Krankheiten bestimmt werden könnte. Darüber hinaus könnte die Analysemethode zur Analyse von Sammelproben, z. B. von Arbeitnehmern mit AA-Expositionsrisiko und der umgebenden Bevölkerung, eingesetzt werden und eine Echtzeitüberwachung der beruflichen Exposition ermöglichen. Darüber hinaus könnte diese Methode auch in der abwasserbasierten Epidemiologie eingesetzt werden und dazu beitragen, die Exposition einer Bevölkerung gegenüber Tabakrauch und ihren Gesundheitszustand zu überwachen.

Table of Contents

Chapter 1. Introduction

1.1 Aromatic amines

Aromatic amines (AA) are N-substituted aryl compounds that are used in several industries, such as those that manufacture or use dyes, pharmaceuticals, pesticides, polymers and explosives [1]. Some sources of exposure are hair dyes [2, 3], tattoo ink and permanent make-up [4], cooking utensils [5] and cooking oil emissions [6], finger paints [7], dyed toys [8] or occupational exposure [9-12]. However, the main source of exposure to some AA is tobacco smoke [13, 14].

Several AA, such as 4-aminobiphenyl, 2-methylaniline and 2-naphthylamine, have been classified as carcinogens to humans [15]. Epidemiologic studies indicate that they may cause excess risk of bladder cancer in smokers [16, 17] as well as in exposed workers [2, 11, 18-20]. Furthermore, the International Agency for Research on Cancer (IARC) stated that there is sufficient evidence that tobacco smoking causes transitional-cell carcinomas of the bladder, ureter and renal pelvis in humans [21].

In the human body, carcinogens can undergo different metabolic pathways, for example, they can be excreted, or, if not directly reactive, they can become activated. These reactive metabolites can bind to proteins and DNA and form what is known as adducts (see Fig. 1.1) [22]. These adducts can be responsible for mutations that can be fixed in the genome and contribute to tumor formation [23].

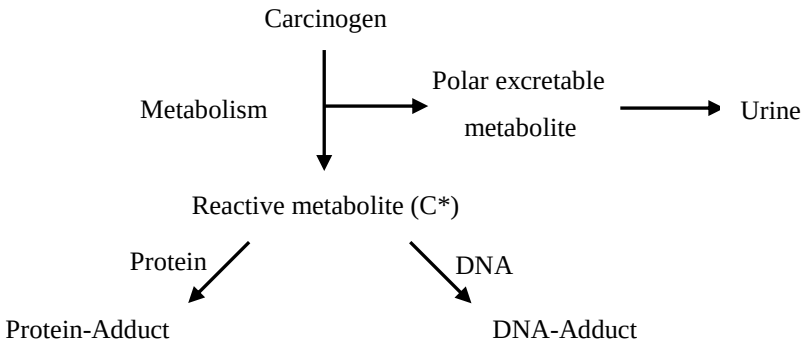


Fig. 1.1. Metabolic pathways of carcinogens, leading to their excretion or to the formation of protein or DNA adducts, after Skipper and Tannenbaum [22].

A metabolic activation seems essential for AA to become carcinogenic, which can vary depending on the tissue- and the cell-specific conditions [1, 24, 25]. The resulting electrophilic species can bind covalently to DNA, forming DNA-adducts [16]. These adducts can lead to mutations during the DNA replication, which, if they occur in key cancer-related genes can lead to tumorigenesis [16]. Several publications, such as [16, 24-32], deal with the possible mechanisms for the metabolic activation of AA in the bladder, including peroxidation, N-glucuronidation, N-oxidation or acetylation. The resulting activated metabolite is an electrophilic nitrenium ion that can interact with proteins, RNA and DNA. The reaction of this ion with the C8 of guanine (Fig. 1.2) is of special importance, since it forms an adduct that has been connected with point mutations [16, 25].

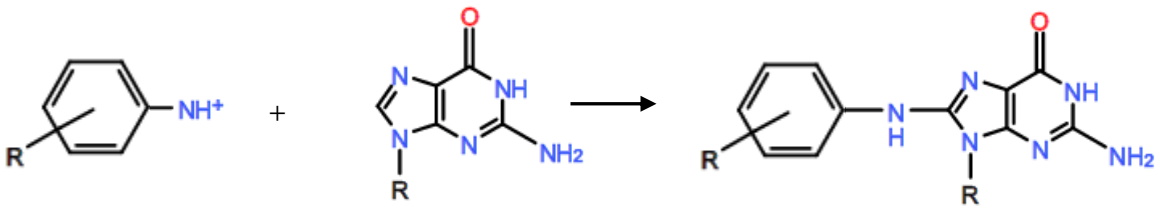


Fig. 1.2. Reaction of the aryl-nitrenium ion of an aromatic amine and a guanine base in the DNA to form a dG-C8-aromatic amine adduct, after Frederick et al. [33].

Some of these activated metabolites can be detoxified, for example, through a process called N-acetylation [16]. This step is particularly interesting considering that 50 % of Caucasians are "slow acetylators", meaning they have a reduced activity of the N-acetyltransferase (NAT) enzyme, and an increased risk of bladder cancer [24, 30].

Another possible fate for the AA is the formation of O-glucuronides, produced by the reaction of C-oxidized or N-oxidized, N-acetylated AA with glucuronic acid, which are considered inactive and are excreted in the urine [23]. It is important to notice how complex the metabolism of AA is, and how the different competing steps can be influenced by the circumstantial situation, the dwelling time of the metabolites in the bladder, the individual susceptibility, etc. [25].

Different approaches have been taken in order to identify the relation between smoking and the intake of AA, such as the direct determination of AA (like aniline, toluidines, or dimethylanilines) in smoke [34-41], the study of DNA [42-46] and protein [45, 47-50] adducts (such as 4-aminobiphenyl), or the analysis of urinary compounds, i.e. the parent arylamines and the metabolic conjugates (for example, naphthylamines, or chloroanilines) [37-39, 51-62]. Because the intake of potential carcinogens during smoking varies depending on the individual smoking topography [21, 63] and the amount of DNA and protein adducts is typically extremely small [64, 65], this thesis focuses on urine samples. Free AA and their metabolites, such as N-acetylaryl-amine, N-glucuronide arylamine, and hemoglobin and DNA adducts, which can be hydrolyzed and converted back to the free AA can thereby be analyzed [47, 66].

The direct analysis of AA is possible with high-performance liquid chromatography (HPLC) [37, 52, 55, 56, 58], however its relative low peak capacity is a critical drawback with such complex samples [67, 68]. Gas chromatography (GC) is a suitable alternative thanks to its high sensitivity, short analysis time, high resolving power and low cost [69]. However, AA tend to be adsorbed and decompose in the GC columns [70], which leads to tailing [71], ghost peaks and low sensitivities [72], and low molecular mass amines are also difficult to extract from water due to their polarity [69]. Therefore, a derivatization step is recommended.

1.2 Sample Preparation

The preparation of the samples is traditionally done manually, significantly contributing to the total analysis time, with approximately two-thirds needed for the sample preparation, and increasing the potential for errors, with 50 % attributed to manual sample preparation [65, 73]. Automation not only minimizes human intervention and errors, but is also considered a key step towards green chemistry [74], especially because less sample and solvent volumes are usually required, and the miniaturization of different applications is facilitated [75]. While the overall time needed for the analysis is typically not reduced through automation, the sample throughput can be significantly improved due to the fact that the autosamplers can work non-stop. Furthermore, and especially interesting for unstable samples, the sample preparation and the measurements can be better timed, so that no time is wasted, and the samples can be measured immediately after preparation. Finally, not only random –human- errors are eliminated, but the overall reproducibility is increased, as the variability of the autosampler is neglectable. The

results are therefore no longer user-dependent, and users with very little to no previous experience can start and run the analysis. To the best of our knowledge, only two other publications discuss the automatic or semi-automatic sample preparation of AA in urine [38, 60], however, they do not report a fully automated method.

1.2.1 Hydrolysis

Human urine samples contain only small amounts of the parent AAs, since, as previously described, AAs are significantly metabolized in the human body [76]. In order to cleave the more abundant metabolized AAs, and obtain the corresponding parent AAs, a hydrolysis step is needed. The hydrolysis step can involve heating, enzymatic treatment, or heating under acidic/basic conditions. The concentration of the AAs found in urine increases from untreated urine < heating < enzymatic treatment < heating under acidic/basic conditions [77]. Furthermore, acidic hydrolysis was reported to be the most common approach for the analysis of AAs in urine samples [38]. Because more AAs were found when using acidic hydrolysis than basic hydrolysis (HCl vs NaOH, at 110 °C for 12 h) [78], this approach was therefore used for this thesis.

An alternative approach, namely, microwave assisted hydrolysis (MWAH) was also considered. Jurado-Sánchez et al. [60] reported a continuous semiautomatic flow-based method using MWAH, solid phase extraction (SPE) and GC-MS for the analysis of amines in urine. The method presented is fast, needing approximately 15 min per 25 mL sample, and provides low limits of detection between 2 and 26 ng/L. They also compared the MWAH-based method to a more conventional method, and while the conventional method needed 45 min to see a maximum in the AAs analytical signals, the MWAH required only 2 min. Unfortunately, there was no comparison of the overall intensity of the AAs obtained, nor real samples were compared with the two methods. Lamani-Dixon [79] tested another MWAH-based method, and found that more AA could be found in urine samples with the conventional approach. The intensity of 5 AAs was also compared, and while in most cases 45 min of MWAH seemed to be slightly better, aniline showed approximately 15 times higher signal with the conventional hydrolysis. Due to the contrasting results, the use of MWAH as an alternative to conventional hydrolysis for the analysis of AA in urine has not yet been sufficiently validated.

1.2.2 Extraction

Liquid-Liquid Extraction

Oftentimes, matrix compounds are present in the samples that can interfere with the analysis. In those cases, an extraction step to remove the matrix compounds is necessary. The most commonly used technique used to extract compounds from aqueous samples is liquid-liquid extraction (LLE) [80]. Usually, two immiscible solvents are mixed (typically water and an organic solvent), and the pH is adjusted so that the analytes of interest are extracted into one solvent, and the matrix compounds stay in the other one.

For the analysis of AA, LLE usually involves at least two to three extraction steps with an acidic solution, a cleaning step with a basic solution, and in some applications, it can be directly combined with a back-extraction step. These steps are usually done manually, and can be prone to human errors, extremely time consuming, and labor intensive. Furthermore, relatively high amounts of toxic organic solvents are used [80]. In the context of this thesis, two alternative microextraction techniques were of particular importance: Hollow Fiber-Liquid-Phase Microextraction (HF-LPME) and Parallel Artificial Liquid Membrane Extraction (PALME).

Hollow Fiber-Liquid Phase Microextraction (HF-LPME)

Hollow Fiber-LPME (HF-LPME) was developed in 1999 by Pedersen-Bjergaard and Rasmussen. The set-up typically consists of a hollow fiber with an end closed with mechanical pressure, to create a lumen where the acceptor can be placed, and the other end glued to a pipette tip, in order to facilitate access to the lumen inside, as seen in Fig. 1.3 and [51].

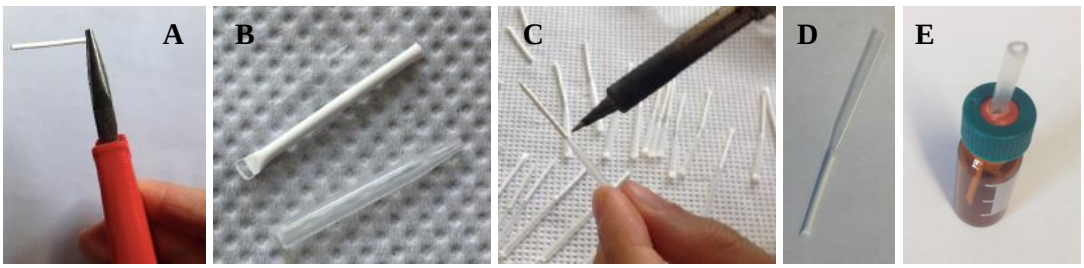


Fig. 1.3. Pictures taken during the preparation process of a hollow fiber, based on [51]. A: Sealing one end of the fiber with mechanical pressure using clamps. B: Clamped fiber and pipette tip piece. C: Soldering the fiber to the pipette tip. D: Final hollow fiber assembly with pipette tip as a needle guide. E: Hollow fiber assembled into a 2-mL vial.

First, the pH of the donor phase, or sample, is adjusted so that the analytes are neutral. Then, a supported liquid membrane (SLM) is created by immersing the fiber in the organic solvent, in that way, the organic solvent is immobilized in the pores of the fiber by capillary forces. Then, the acceptor solution is dispensed into the lumen of the hollow fiber using a syringe, and the fiber is placed in the sample. During this time, the analytes are extracted from the sample, through the SLM into the acceptor solution. The acceptor solution can be the same organic solvent used for the SLM (two-phase extraction) or an aqueous solution (three-phase extraction). Matrix components that could interfere with the analysis, like salts, macromolecules, and polar organic substances are trapped in the donor phase and do not reach the SLM [81-84]. The three-phase systems offer an extra clean-up step, as neutral analytes from the SLM that cannot be ionized in the acceptor phase will also not be extracted [81, 82]. This makes HF-LPME suitable for a wide variety of complex samples, like slurry, blood, saliva, milk, and urine [83]. Furthermore, it enables the measurements of several samples simultaneously, and, due to the disposable nature of the fibers, avoids cross-contamination and the need for regeneration and/or extensive cleaning [85]. The enrichment potential can be easily influenced thanks to the high flexibility regarding the donor and acceptor volumes [86]. On the other hand, unfortunately, there are no commercially available fibers for HF-LPME yet [86]. Nonetheless, there has been several successful automation attempts, as summarized by [87].

Parallel Artificial Liquid Membrane Extraction (PALME)

Parallel Artificial Liquid Membrane Extraction (PALME) was first introduced in 2013 by Gjelstad et al. as an alternative to HF-LPME. It is based on the same principle as HF-LPME, but instead of using a hollow-fiber to support the SLM, it uses the membrane of a commercially available device: 96-well plates, allowing to extract up to 96 samples simultaneously with one plate [88].

The preparation procedure is very similar to that of HF-LPME. First, the donor solution is filled in the wells of a 96-well sample plate, then, the SLM is created by adding 3 to 5 µL of organic solvent to the membranes of a 96-well filter plate, and the acceptor solution is pipetted into the wells above the SLM. Finally, both plates are clamped together, covered with a lid or a sealing foil (Fig. 1.4). This sandwich-like system is placed onto the agitator for a defined amount of time, after which the extraction is stopped by separating the acceptor and donor plate from each other [80].

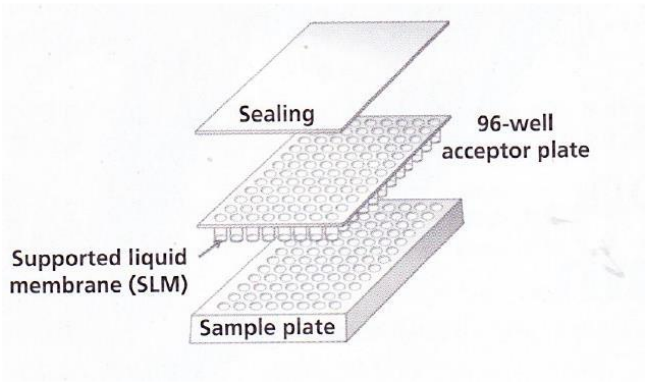


Fig. 1.4. Overview of the sandwich-like set-up of PALME, printed with permission from [80].

This technique offers several advantages over HF-LPME: it is more user friendly, provides a simpler workflow and higher throughput, and it is commercially available, which facilitates a semi or fully automatic extraction [88]. However, the commercially available equipment is very limited in terms of dimensions and membrane material, which not only restricts the range of sample volumes that can be used, but also leads to non-specific binding and non-linear calibration curves for some analytes [80].

1.2.3 Derivatization

While AA can be directly analyzed using liquid chromatography (LC), an extra derivatization step is recommended for their analysis with gas chromatography (GC). This is because due to their physicochemical characteristics, especially their high polarity and water solubility, several problems can arise during GC analysis, such as peak tailing due to adsorption and decomposition on the columns, ghost peaks and overall low sensitivity [89-92]. The derivatization step not only helps increase sensitivity, but also selectivity [89, 90].

There are many different derivatization procedures possible, but the three most common are acylation, silylation and the formation of carbamate derivatives. However, their disadvantages are needing to remove the excess reagent and acidic byproducts, be performed in anhydrous conditions, and having a low yield, respectively [67, 69, 89-92]. The derivatization method developed by Schmidt et al. [67], namely a iodination via a Sandmeyer-like reaction, is not restricted to an anhydrous media, there is no need to remove excess reagents, and the formation of side products -and the subsequent loss of efficiency- is avoided. It consists of two steps, see Fig. 1.5, by which the amino group is diazotized first, and a substitution of the diazo group with iodine takes place afterwards.

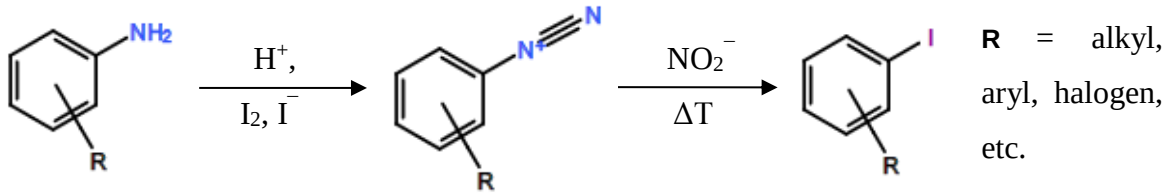


Fig. 1.5. Reaction scheme for the iodination of aromatic amines, after Schmidt et al. [67].

The described derivatization changes the physicochemical characteristics of the AA, minimizing and/or completely eliminating the problems described above, and it allows for a sensitive and selective GC analysis. Nonetheless, because of the very low concentrations of AA expected in the real samples, a further enrichment step is recommended.

1.2.4 Solid-phase microextraction (SPME)

SPME was first used in 1992 [93], and it is based on the partitioning of the analytes between the sample and the extraction phase, and their subsequent desorption into the heated gas chromatography (GC) injector [94, 95]. It therefore combines sampling, extraction, enrichment, and sample introduction in a two-step process (Fig. 1.6).

Some of the biggest advantages in comparison with traditional extraction methods like LLE or SPE, is that it is an automated solvent-less micro extraction technique. These are key features of green analytical chemistry, where the automation and reduction of toxic solvents are especially relevant [94, 95]. Furthermore, SPME offers increased sensitivity, and reduced carry-over and sample losses [96, 97] and it is significantly less laborious [94, 95].

One of the biggest drawbacks of SPME is the relatively short lifetime of the fibers due to bending of fibers, and breaking and stripping of coatings [97]. The latter is less critical when doing headspace (HS) extraction in comparison to direct immersion (DI), since the fibers are not in direct contact with the sample.

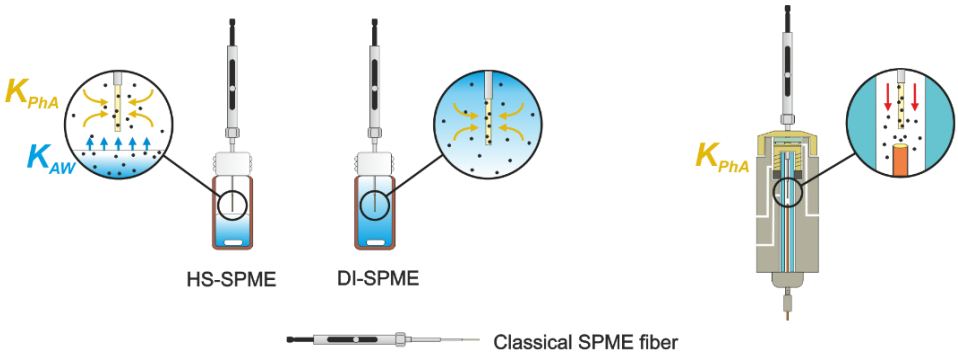


Fig. 1.6. Schematic overview of solid-phase microextraction (SPME). Sorption depends on the partitioning constants of the analytes between the sorbent phase and water, K_{PhW}, for direct immersion (DI), and between air and the water sample, K_{AW}, and the sorbent phase and air, K_{PhA}, for headspace (HS) sampling, based on [98].

1.3 References

1. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Chemical Agents and Related Occupations. Lyon, France: International Agency for Research on Cancer; 2012.

2. Hosseini B, Zendehdel K, Bouaoun L, Hall AL, Rashidian H, Hadji M, Gholipour M, Haghdoost AA, Schüz J, Olsson A. Bladder cancer risk in relation to occupations held in a nationwide case-control study in Iran. Int J Cancer. 2023;153:765-74. https://doi.org/10.1002/ijc.34560.

3. Akyüz M, Ata Ş. Determination of aromatic amines in hair dye and henna samples by ion-pair extraction and gas chromatography–mass spectrometry. J Pharm Biomed Anal. 2008;47:68-80. https://doi.org/10.1016/j.jpba.2007.12.011.

4. Negi S, Bala L, Shukla S, Chopra D. Tattoo inks are toxicological risks to human health: A systematic review of their ingredients, fate inside skin, toxicity due to polycyclic aromatic hydrocarbons, primary aromatic amines, metals, and overview of regulatory frameworks. Toxicol Ind Health. 2022;38:417-34. https://doi.org/10.1177/07482337221100870.

5. Perez MÂF, Daniel D, Padula M, do Lago CL, Bottoli CBG. Determination of primary aromatic amines from cooking utensils by capillary electrophoresis-tandem mass spectrometry. Food Chem. 2021;362:129902. https://doi.org/10.1016/j.foodchem.2021.129902.

6. Chiang TA, Pei-Fen W, Ying LS, Wang LF, Ko YC. Mutagenicity and aromatic amine content of fumes from heated cooking oils produced in Taiwan. Food Chem Toxicol. 1999;37:125-34. https://doi.org/10.1016/S0278-6915(98)00081-7.

7. Garrigos MC, Reche F, Pernias K, Jimenez A. Optimization of parameters for the analysis of aromatic amines in finger-paints. J Chromatogr A. 2000;896:291-8. https://doi.org/10.1016/S0021-9673(00)00742-1.

8. Garrigos MC, Reche F, Marin ML, Jimenez A. Determination of aromatic amines formed from azo colorants in toy products. J Chromatogr A. 2002;976:309-17. https://doi.org/10.1016/S0021-9673(02)01162-7.

9. Alston F, Okorie O. Occupational exposures: chemical carcinogens and mutagens. 1st ed. Boca Raton: CRC Press; 2023.

10. Chamot S, Delzard M, Leroy L, Marhic G, Ganry O. Occupational exposures in the rubber tire industry and risk of cancer: a systematic review. Int Arch Occup Environ Health. 2023. https://doi.org/10.1007/s00420-023-01972-x.

11. Ciocan C, Godono A, Franco N, La Vecchia C, Negri E, Boffetta P, Pira E. Mortality from bladder cancer in dyestuff workers exposed to aromatic amines: A 73-year follow-up. La Medicina del lavoro. 2022;113:e2022017. https://doi.org/10.23749/mdl.v113i2.12893.

12. Labat L, Thomas J, Dehon B, Humbert L, Leleu B, Nisse C, Lhermitte M. [Assessment of occupational exposure to ortho-toluidine using gas chromatography-mass spectrometry]. Acta Clin Belg Suppl. 2006:63-7.

13. Seyler T, Mazumder S, Ahamed R, Zhu W, Blount BC, Apelberg BJ, Wang L. Tobacco Smoke Is a Major Source of Aromatic Amine Exposure in U.S. Adults: 2013–2014 National Health and Nutrition Examination Survey (NHANES). Cancer Epidemiol Biomarkers Prev. 2023:OF1-OF9. https://doi.org/10.1158/1055-9965.epi-23-0071.

14. Pereira L, Mondal PK, Alves M. Aromatic amines sources, environmental impact and remediation. In: Lichtfouse E, Schwarzbauer J, Robert D, editors. Pollutants in Buildings, Water and Living Organisms. Environmental Chemistry for a Sustainable World. Cham: Springer International Publishing; 2015. pp. 297-346. https://doi.org/10.1007/978-3-319-19276-5_7.

15. Tobacco smoke and involuntary smoking: World Health Organization, International Agency for Research on Cancer; 2004

16. Besaratinia A, Tommasi S. Genotoxicity of tobacco smoke-derived aromatic amines and bladder cancer: Current state of knowledge and future research directions. FASEB J. 2013;27:2090-100. https://doi.org/10.1096/fj.12-227074.

17. Skipper PL, Tannenbaum SR, Ross RK, Yu MC. Nonsmoking-related arylamine exposure and bladder cancer risk. Cancer Epidemiol Biomarkers Prev. 2003;12:503-7.

18. Nakano M, Shinagawa T, Eitaki Y, Omae K, Takeuchi A, Iwasawa S, Fukai K, Yoshioka N, Tanaka S, Koda S, Sobue T, Takebayashi T. Risk of bladder cancer in male Japanese workers exposed to ortho-toluidine and other aromatic amines. Int Arch Occup Environ Health. 2021;94:1427-39. https://doi.org/10.1007/s00420-021-01658-2.

19. Carreón T, Hein MJ, Viet SM, Hanley KW, Ruder AM, Ward EM. Increased bladder cancer risk among workers exposed to o-toluidine and aniline: a reanalysis. Occup Environ Med. 2010;67:348-50. https://doi.org/10.1136/oem.2009.051136.

20. de Vocht F, Sobala W, Wilczynska U, Kromhout H, Szeszenia-Dabrowska N, Peplonska B. Cancer mortality and occupational exposure to aromatic amines and inhalable aerosols in rubber tire manufacturing in Poland. Cancer Epidemiol. 2009;33:94-102. https://doi.org/10.1016/j.canep.2009.06.013.

21. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Tobacco smoke and involuntary smoking. Lyon, France: International Agency for Research on Cancer; 2004.

22. Skipper PL, Tannenbaum SR. Protein adducts in the molecular dosimetry of chemical carcinogens. Carcinogenesis. 1990;11:507-18. https://doi.org/10.1093/carcin/11.4.507.

23. Ciotti M, Lakshmi VM, Basu N, Davis BB, Owens IS, Zenser TV. Glucuronidation of benzidine and its metabolites by cDNA-expressed human UDP-glucuronosyltransferases and

pH stability of glucuronides. Carcinogenesis. 1999;20:1963-9. https://doi.org/10.1093/carcin/20.10.1963.

24. Negri E, La Vecchia C. Epidemiology and prevention of bladder cancer. In: Bassi P, Pagano F, editors. Invasive Bladder Cancer. London: Springer London; 2007. pp. 1-14. https://doi.org/10.1007/978-1-84628-377-2_1.

25. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Some Aromatic Amines, Organic Dyes, and Related Exposures. Lyon, France: International Agency for Research on Cancer; 2008.

26. Kobayashi T, Kishimoto S, Watanabe S, Yoshioka Y, Toyoda T, Ogawa K, Watanabe K, Totsuka Y, Wakabayashi K, Miyoshi N. Cytotoxic Homo- and Hetero-Dimers of o-toluidine, o-anisidine, and Aniline Formed by In Vitro Metabolism. Chem Res Toxicol. 2022;35:1625-30. https://doi.org/10.1021/acs.chemrestox.2c00226.

27. IARC Working Group on the Identification of Carcinogenic Hazards to Humans. Some Aromatic Amines and Related Compounds. Lyon, France: International Agency for Research on Cancer; 2021.

28. Wang S, Hanna D, Sugamori KS, Grant DM. Primary aromatic amines and cancer: Novel mechanistic insights using 4-aminobiphenyl as a model carcinogen. Pharmacol Ther. 2019;200:179-89. https://doi.org/10.1016/j.pharmthera.2019.05.004.

29. Blömeke B, Lichter J. Expression and Activity of Arylamine N-Acetyltransferases in Organs: Implications on Aromatic Amine Toxicity. In: Laurieri N, Sim E, editors. Arylamine N-Acetyltransferases in Health and Disease: From Pharmacogenetics to Drug Discovery and Diagnostics. Oxford: World Scientific Publishing Company; 2018. pp. 133-64. https://doi.org/10.1142/9789813232013_0006.

30. Pelucchi C, Bosetti C, Negri E, Malvezzi M, La Vecchia C. Mechanisms of Disease: the epidemiology of bladder cancer. Nat Clin Pract Urol. 2006;3:327-40. https://doi.org/10.1038/ncpuro0510.

31. Talaska G, Al-Zoughool M. Aromatic Amines and Biomarkers of Human Exposure. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2003;21:133-64. https://doi.org/10.1081/GNC-120026234.

32. Hein DW. Molecular genetics and function of NAT1 and NAT2: role in aromatic amine metabolism and carcinogenesis. Mutat Res-Fundam Mol Mech Mutag. 2002;506-507:65-77. https://doi.org/10.1016/S0027-5107(02)00153-7.

33. Frederick CB, Weis CC, Flammang TJ, Martin CN, Kadlubar FF. Hepatic N-oxidation, acetyl-transfer and DNA-binding of the acetylated metabolites of the carcinogen, benzidine. Carcinogenesis. 1985;6:959-65. https://doi.org/10.1093/carcin/6.7.959.

34. Zhang J, Bai R, Zhou Z, Liu X, Zhou J. Simultaneous analysis of nine aromatic amines in mainstream cigarette smoke using online solid-phase extraction combined with liquid

chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2017;409:2993-3005. https://doi.org/10.1007/s00216-017-0245-6.

35. Deng H, Yang F, Li Z, Bian Z, Fan Z, Wang Y, Liu S, Tang G. Rapid determination of 9 aromatic amines in mainstream cigarette smoke by modified dispersive liquid liquid microextraction and ultraperformance convergence chromatography tandem mass spectrometry. J Chromatogr A. 2017;1507:37-44. https://doi.org/10.1016/j.chroma.2017.05.056.

36. Bie Z, Lu W, Zhu Y, Chen Y, Ren H, Ji L. Rapid determination of six carcinogenic primary aromatic amines in mainstream cigarette smoke by two-dimensional online solid phase extraction combined with liquid chromatography tandem mass spectrometry. J Chromatogr A. 2017;1482:39-47. https://doi.org/10.1016/j.chroma.2016.12.060.

37. Chinthakindi S, Kannan K. A liquid chromatography–tandem mass spectrometry method for the analysis of primary aromatic amines in human urine. J Chromatogr B. 2021;1180:122888-90. https://doi.org/10.1016/j.jchromb.2021.122888.

38. Mazumder S, Ahamed RA, McGahee E, Wang L, Seyler TH. A new automated method for the analysis of aromatic amines in human urine by GC-MS/MS. J Anal Toxicol. 2019;43:25-35. https://doi.org/10.1093/jat/bky045.

39. Niu J, Zhao X, Jin Y, Yang G, Li Z, Wang J, Zhao R, Li Z. Determination of aromatic amines in the urine of smokers using a porous organic framework (JUC-Z2)-coated solid-phase microextraction fiber. J Chromatogr A. 2018;1555:37-44. https://doi.org/10.1016/j.chroma.2018.04.059.

40. Smith CJ, Dooly GL, Moldoveanu SC. New technique using solid-phase extraction for the analysis of aromatic amines in mainstream cigarette smoke. J Chromatogr A. 2003;991:99-107. https://doi.org/10.1016/S0021-9673(03)00205-X.

41. Stabbert R, Schäfer KH, Biefel C, Rustemeier K. Analysis of aromatic amines in cigarette smoke. Rapid Commun Mass Spectrom. 2003;17:2125-32. https://doi.org/10.1002/rcm.1161.

42. Rojas M, Cascorbi I, Alexandrov K, Kriek E, Auburtin G, Mayer L, Kopp-Schneider A, Roots I, Bartsch H. Modulation of benzo[a]pyrene diolepoxide-DNA adduct levels in human white blood cells by CYP1A1, GSTM1 and GSTT1 polymorphism. Carcinogenesis. 2000;21:35-41. https://doi.org/10.1093/carcin/21.1.35.

43. Guo J, Villalta PW, Weight CJ, Bonala R, Johnson F, Rosenquist TA, Turesky RJ. Targeted and Untargeted Detection of DNA Adducts of Aromatic Amine Carcinogens in Human Bladder by Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry. Chem Res Toxicol. 2018;31:1382-97. https://doi.org/10.1021/acs.chemrestox.8b00268.

44. Yun BH, Bellamri M, Rosenquist TA, Turesky RJ. Method for Biomonitoring DNA Adducts in Exfoliated Urinary Cells by Mass Spectrometry. Anal Chem. 2018;90:9943-50. https://doi.org/10.1021/acs.analchem.8b02170.

45. Cai T, Bellamri M, Ming X, Koh WP, Yu MC, Turesky RJ. Quantification of Hemoglobin and White Blood Cell DNA Adducts of the Tobacco Carcinogens 2-Amino-9H-pyrido[2,3-b]indole and 4-Aminobiphenyl Formed in Humans by Nanoflow Liquid Chromatography/Ion Trap Multistage Mass Spectrometry. Chem Res Toxicol. 2017;30:1333-43. https://doi.org/10.1021/acs.chemrestox.7b00072.

46. Lee HW, Wang HT, Weng MW, Hu Y, Chen WS, Chou D, Liu Y, Donin N, Huang WC, Lepor H, Wu XR, Wang H, Beland FA, Tang MS. Acrolein- and 4-Aminobiphenyl-DNA adducts in human bladder mucosa and tumor tissue and their mutagenicity in human urothelial cells. Oncotarget. 2014;5:3526-40. https://doi.org/10.18632/oncotarget.1954.

47. Sabbioni G. Hemoglobin Adducts and Urinary Metabolites of Arylamines and Nitroarenes. Chem Res Toxicol. 2017;30:1733-66. https://doi.org/10.1021/acs.chemrestox.7b00111.

48. Pathak KV, Chiu TL, Amin EA, Turesky RJ. Methemoglobin Formation and Characterization of Hemoglobin Adducts of Carcinogenic Aromatic Amines and Heterocyclic Aromatic Amines. Chem Res Toxicol. 2016;29:255-69. https://doi.org/10.1021/acs.chemrestox.5b00418.

49. Bernert JT, Jain RB, Pirkle JL, Wang L, Miller BB, Sampson EJ. Urinary tobacco-specific nitrosamines and 4-aminobiphenyl hemoglobin adducts measured in smokers of either regular or light cigarettes. Nicotine Tobacco Res. 2005;7:729-38. https://doi.org/10.1080/14622200500259762.

50. Richter E, Rösler S, Scherer G, Gostomzyk JG, Grübl A, Krämer U, Behrendt H. Haemoglobin adducts from aromatic amines in children in relation to area of residence and exposure to environmental tobacco smoke. Int Arch Occup Environ Health. 2001;74:421-8. https://doi.org/10.1007/s004200100243.

51. Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

52. Fuller TW, Acharya AP, Meyyappan T, Yu M, Bhaskar G, Little SR, Tarin TV. Comparison of Bladder Carcinogens in the Urine of E-cigarette Users Versus Non E-cigarette Using Controls. Sci Rep. 2018;8:1-6. https://doi.org/10.1038/s41598-017-19030-1.

53. Lamani X, Horst S, Zimmermann T, Schmidt TC. Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GCxGC-qMS). Anal Bioanal Chem. 2015;407:241-52. https://doi.org/10.1007/s00216-014-8080-5.

54. Amiri A, Baghayeri M, Nori S. Magnetic solid-phase extraction using poly(para-phenylenediamine) modified with magnetic nanoparticles as adsorbent for analysis of monocyclic aromatic amines in water and urine samples. J Chromatogr A. 2015;1415:20-6. https://doi.org/10.1016/j.chroma.2015.08.046.

55. Yu J, Wang S, Zhao G, Wang B, Ding L, Zhang X, Xie J, Xie F. Determination of urinary aromatic amines in smokers and nonsmokers using a MIPs-SPE coupled with LC-MS/MS method. J Chromatogr B. 2014;958:130-5. https://doi.org/10.1016/j.jchromb.2014.03.023.

56. Jiang C, Sun Y, Yu X, Gao Y, Zhang L, Wang Y, Zhang H, Song D. Application of C18-functional magnetic nanoparticles for extraction of aromatic amines from human urine. J Chromatogr B. 2014;947-948:49-56. https://doi.org/10.1016/j.jchromb.2013.12.008.

57. Sarafraz-Yazdi A, Ardaki MS, Amiri A. Determination of monocyclic aromatic amines using headspace solid-phase microextraction based on sol-gel technique prior to GC. J Sep Sci. 2013;36:1629-35. https://doi.org/10.1002/jssc.201200940.

58. Riedel K, Scherer G, Engl J, Hagedorn H-W, Tricker AR. Determination of three carcinogenic aromatic amines in urine of smokers and nonsmokers. J Anal Toxicol. 2006;30:187-95. https://doi.org/10.1093/jat/30.3.187.

59. Seyler TH, Bernert JT. Analysis of 4-aminobiphenyl in smoker's and nonsmoker's urine by tandem mass spectrometry. Biomarkers. 2011;16:212-21. https://doi.org/10.3109/1354750X.2010.544755.

60. Jurado-Sánchez B, Ballesteros E, Gallego M. Continuous solid-phase extraction method for the determination of amines in human urine following on-line microwave-assisted acid hydrolysis. Anal Bioanal Chem. 2010;396:1929-37. https://doi.org/10.1007/s00216-009-3395-3.

61. Weiss T, Angerer J. Simultaneous determination of various aromatic amines and metabolites of aromatic nitro compounds in urine for low level exposure using gas chromatography-mass spectrometry. J Chromatogr B. 2002;778:179-92. https://doi.org/10.1016/S0378-4347(01)00542-4.

62. Grimmer G, Dettbarn G, Seidel A, Jacob J. Detection of carcinogenic aromatic amines in the urine of non-smokers. Sci Total Environ. 2000;247:81-90. https://doi.org/10.1016/S0048-9697(99)00471-4.

63. Thielen A, Klus H, Muller L. Tobacco smoke: unraveling a controversial subject. Exp Toxicol Pathol. 2008;60:141-56. https://doi.org/10.1016/j.etp.2008.01.014.

64. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer. 2003;3:733-44. https://doi.org/10.1038/nrc1190.

65. Majors RE. Overview of Sample Preparation. LCGC. 2015;33:46-51.

66. Yih Hui B, Mohamad Zain NN, Mohamad S, Mohamed Fauzi H, Alias Y, Chandrasekaram K, Rahim NY, Yahaya N, Raoov M. Determination of Aromatic Amines in Urine using Extraction and Chromatographic Analysis: A Minireview. Anal Lett. 2019;52:2974-92. https://doi.org/10.1080/00032719.2019.1636057.

67. Schmidt TC, Less M, Haas R, von Löw E, Steinbach K, Stork G. Gas chromatographic determination of aromatic amines in water samples after solid-phase extraction and

derivatization with iodine. I. Derivatization. J Chromatogr A. 1998;810:161-72. https://doi.org/10.1016/S0021-9673(98)00233-7.

68. Zimmermann T, Ensinger WJ, Schmidt TC. In Situ Derivatization/Solid-Phase Microextraction: Determination of Polar Aromatic Amines. Anal Chem. 2004;76:1028-38. https://doi.org/10.1021/ac035098p.

69. Kataoka H. Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. J Chromatogr A. 1996;733:19-34. https://doi.org/10.1016/0021-9673(95)00726-1.

70. Pfundstein B, Tricker AR, Preussmann R. Determination of primary and secondary amines in foodstuffs using gas chromatography and chemiluminescence detection with a modified thermal energy analyser. J Chromatogr A. 1991;539:141-8. https://doi.org/10.1016/S0021-9673(01)95367-1.

71. Terashi A, Hanada Y, Kido A, Shinohara R. Determination of primary and secondary aliphatic amines in the environment as sulphonamide derivatives by gas chromatography—mass spectrometry. J Chromatogr A. 1990;503:369-75. https://doi.org/10.1016/S0021-9673(01)81516-8.

72. Hoshika Y. Gas chromatographic determination of the lower aliphatic primary amines as their Schiff bases. J Chromatogr A. 1975;115:596-601. https://doi.org/10.1016/S0021-9673(01)98965-4.

73. Daryanavard SM, Zolfaghari H, Abdel-Rehim A, Abdel-Rehim M. Recent applications of microextraction sample preparation techniques in biological samples analysis. Biomed Chromatogr. 2021;35. https://doi.org/10.1002/bmc.5105.

74. Wojnowski W, Tobiszewski M, Pena-Pereira F, Psillakis E. AGREEprep – Analytical greenness metric for sample preparation. Trends Anal Chem. 2022;149:116553. https://doi.org/10.1016/j.trac.2022.116553.

75. Kaziur-Cegla W. Automated and solvent-free microextraction techniques for the GC-MS analysis of food and environmental samples: Universität Duisburg-Essen; 2020.

76. Richter E. Biomonitoring of human exposure to arylamines. Frontiers in Bioscience - Elite. 2015;7E:222-38. https://doi.org/10.2741/E727.

77. Sabbioni G, Turesky RJ. Biomonitoring human albumin adducts: The past, the present, and the future. Chem Res Toxicol. 2017;30:332-66. https://doi.org/10.1021/acs.chemrestox.6b00366.

78. Zimmermann T. Analytik aromatischer Amine in wässrigen und biologischen Matrices - Neue Verfahren zur Anreicherung und selektiven Detektion. Marburg/Lahn Philipps-Universität Marburg; 2005.

79. Lamani-Dixon X. Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry: Universität Duisburg-Essen; 2018.

80. Gjelstad A, Andresen AT, Dahlgren A, Gundersen TE, Pedersen-Bjergaard S. High-throughput liquid-liquid extraction in 96-well format: Parallel artificial liquid membrane extraction. LC GC Europe. 2017;30:10-7.

81. Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S. Hollow fiber liquid-phase microextraction. In: Pawliszyn J, Lord HL, editors. Comprehensive Sampling and Sample Preparation. 2: Elsevier; 2012. pp. 475-96.

82. Gjelstad A, Pedersen-Bjergaard S. Perspective: Hollow fibre liquid-phase microextraction - principles, performance, applicability, and future directions. Scientia Chromatographica. 2013;5:181-9. https://doi.org/10.4322/sc.2014.003.

83. Rasmussen KE, Pedersen-Bjergaard S. Developments in hollow fibre-based, liquid-phase microextraction. Trends Anal Chem. 2004;23:1-10. https://doi.org/10.1016/S0165-9936(04)00105-0.

84. Rasmussen KE, Pedersen-Bjergaard S, Krogh M, Grefslie Ugland H, Grønhaug T. Development of a simple in-vial liquid-phase microextraction device for drug analysis compatible with capillary gas chromatography, capillary electrophoresis and high-performance liquid chromatography. J Chromatogr A. 2000;873:3-11. https://doi.org/10.1016/S0021-9673(99)01163-2.

85. Pedersen-Bjergaard S, Rasmussen KE. Liquid-Liquid-Liquid Microextraction for Sample Preparation of Biological Fluids Prior to Capillary Electrophoresis. Anal Chem. 1999;71:2650-6. https://doi.org/10.1021/ac990055n.

86. Gjelstad A. Three-phase hollow fiber liquid-phase microextraction and parallel artificial liquid membrane extraction. Trends Anal Chem. 2019;113:25-31. https://doi.org/10.1016/j.trac.2019.01.007.

87. Soares da Silva Burato J, Vargas Medina DA, de Toffoli AL, Vasconcelos Soares Maciel E, Mauro Lanças F. Recent advances and trends in miniaturized sample preparation techniques. J Sep Sci. 2020;43:202-25. https://doi.org/10.1002/jssc.201900776.

88. Gjelstad A, Rasmussen KE, Parmer MP, Pedersen-Bjergaard S. Parallel artificial liquid membrane extraction: micro-scale liquid-liquid-liquid extraction in the 96-well format. Bioanalysis. 2013;5:1377-85. https://doi.org/10.4155/bio.13.59.

89. Płotka-Wasylka JM, Morrison C, Biziuk M, Namieśnik J. Chemical Derivatization Processes Applied to Amine Determination in Samples of Different Matrix Composition. Chem Rev. 2015;115:4693-718. https://doi.org/10.1021/cr4006999.

90. Kataoka H. 2.1.2. Gas chromatography of amines as various derivatives. In: Molnár-Perl I, editor. Quantitation of amino acids and amines by chromatography: methods and protocols. Journal of Chromatography Library. 70: Elsevier; 2005. pp. 364-404. https://doi.org/10.1016/S0301-4770(05)80016-8.

91. Ferreira AMC, Laespada MEF, Pavón JLP, Cordero BM. In situ aqueous derivatization as sample preparation technique for gas chromatographic determinations. J Chromatogr A. 2013;1296:70-83. https://doi.org/10.1016/j.chroma.2013.04.084.

92. Farajzadeh MA, Nouri N, Khorram P. Derivatization and microextraction methods for determination of organic compounds by gas chromatography. Trends Anal Chem. 2014;55:14-23. https://doi.org/10.1016/j.trac.2013.11.006.

93. Arthur CL, Killam LM, Motlagh S, Lim M, Potter DW, Pawliszyn J. Analysis of substituted benzene compounds in groundwater using solid-phase microextraction. Environ Sci Technol. 1992;26:979-83. https://doi.org/10.1021/es00029a017.

94. Płotka-Wasylka J, Szczepańska N, de la Guardia M, Namieśnik J. Miniaturized solid-phase extraction techniques. Trends Anal Chem. 2015;73:19-38. https://doi.org/10.1016/j.trac.2015.04.026.

95. Kędziora-Koch K, Wasiak W. Needle-based extraction techniques with protected sorbent as powerful sample preparation tools to gas chromatographic analysis: Trends in application. J Chromatogr A. 2018;1565:1-18. https://doi.org/10.1016/j.chroma.2018.06.046.

96. Piri-Moghadam H, Ahmadi F, Pawliszyn J. A critical review of solid phase microextraction for analysis of water samples. Trends Anal Chem. 2016;85:133-43. https://doi.org/10.1016/j.trac.2016.05.029.

97. Nerín C, Salafranca J, Aznar M, Batlle R. Critical review on recent developments in solventless techniques for extraction of analytes. Anal Bioanal Chem. 2009;393:809. https://doi.org/10.1007/s00216-008-2437-6.

98. Lorenzo-Parodi N, Kaziur W, Stojanović N, Jochmann MA, Schmidt TC. Solventless microextraction techniques for water analysis. Trends Anal Chem. 2019;113:321-31. https://doi.org/10.1016/j.trac.2018.11.013.

Chapter 2. Aims and Scope

The aim of this thesis was to optimize and automate the analytical method used for the analysis of aromatic amines, as iodinated derivatives, in human urine. A detailed graphical summary of the chapters of this thesis and their relation to each other can be seen in Fig. 2.1.

In Chapter 3 (marked with light blue in Fig. 2.1) an existing analytical method (delimited with a black line in Fig. 2.1) was evaluated in order to asses if it would be suitable for the determination of relationships between the smoking status of the donor, and the amount of aromatic amines present. The method consisted of the complete sample preparation, including an acidic hydrolysis step to cleave metabolic conjugates, a LLE and back-extraction steps to isolate the AA from the complex urine matrix, and a derivatization step to decrease their polarity, so that they are more readily extracted from water with the last step prior to the analysis, SPME. To evaluate the method, 68 urine samples of never smokers, past smokers and smokers from a population-based study were analyzed, and the results were compared using three different data evaluation approaches (i.e. qualitative analysis, quantitative analysis, and quantitative screening).

Once the method was proven suitable, the optimization and automation of the method was studied in detail. LLE is one of the most time-consuming and labor intensive parts of the sample preparation, so two alternative extraction techniques were studied in Chapter 4 (delimited with orange in Fig. 2.1): Hollow Fiber-Liquid-Phase Microextraction (HF-LPME) and Parallel Artificial Liquid Membrane Extraction (PALME). This was the first time that HF-LPME and PALME were directly compared with each other for the analysis of AA in urine.

In the original method, the analysis was done via comprehensive multidimensional chromatography using electron ionization (GCxGC-EI-MS). While this technique offers a great resolution on the chromatographic side, there are several alternative detection techniques that could offer higher sensitivity and/or selectivity. Therefore, in Chapter 5 (marked with green in Fig. 2.1) three detection techniques were evaluated and compared, namely GC-MS in single-ion monitoring (SIM) mode with electron ionization (GC-EI-MS), and negative chemical ionization (GC-NCI-MS); and GC-MS/MS in multiple reaction monitoring (MRM) mode using electron ionization (GC-EI-MS/MS). To facilitate the comparison and minimize the uncertainty

sources, the techniques were validated by analyzing the iodinated derivatives of the AA and using GC instead of GCxGC.

Finally, in Chapter 6 (marked with purple in Fig. 2.1) the complete sample preparation procedure was automated, including the hydrolysis, the LLE, and the derivatization steps. The original LLE procedure was automated, and not the alternative LLE techniques studied in Chapter 4, due to the limitations of the available autosampler. Taking advantage of the automated set-up, several steps of the sample preparation procedure, such as the reaction and extraction times, could be further optimized. To the best of our knowledge, this was the first time a fully automatic method for the analysis of aromatic amines in urine with GC-MS was reported.

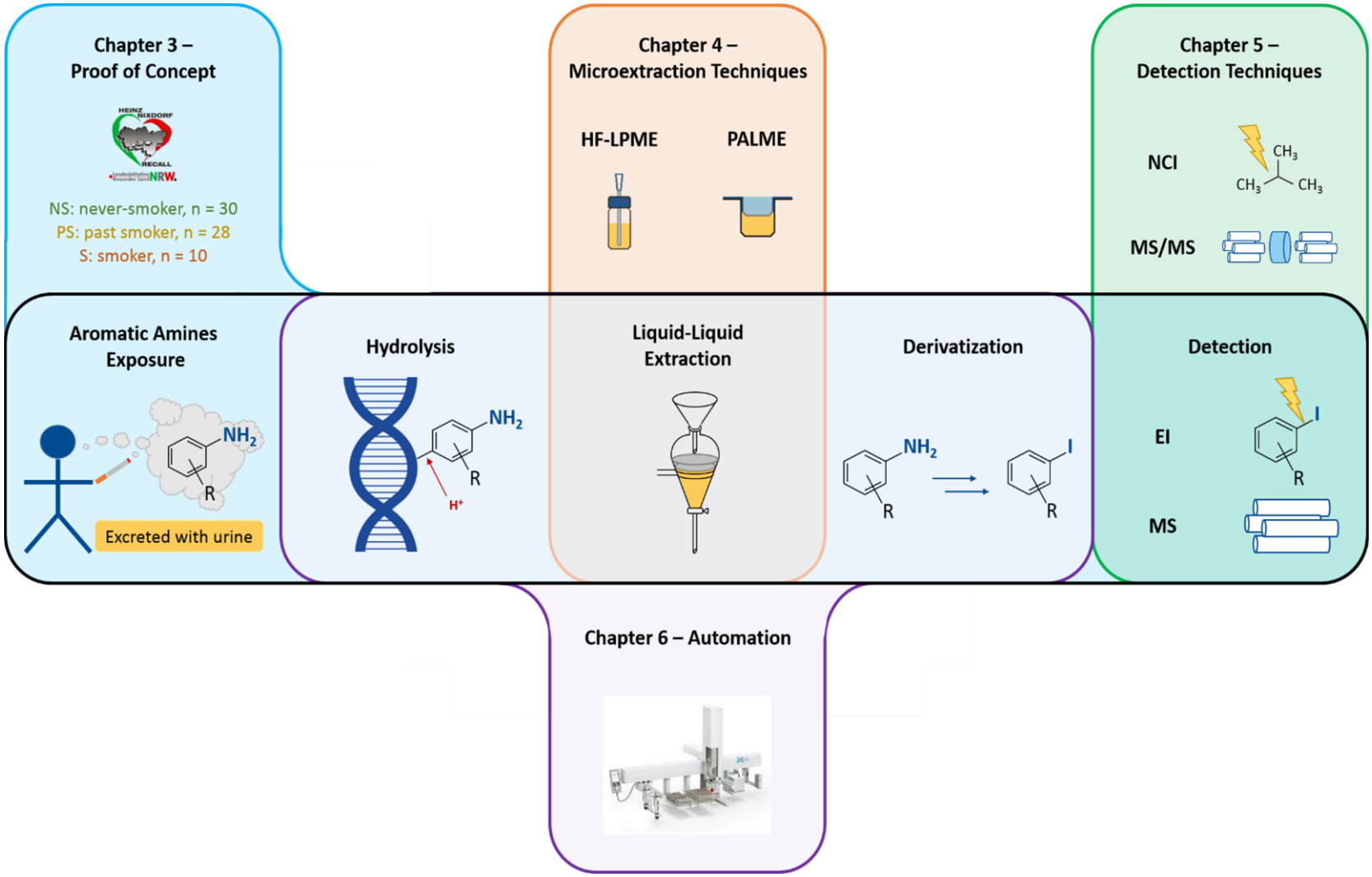


Fig. 2.1. Graphical summary of the thesis showing the relation of the individual chapters.

Chapter 3. Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography-mass spectrometry (GCxGC-MS)

This chapter was adapted from: Lorenzo-Parodi N, Moebus S, Schmidt TC. Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography-mass spectrometry (GCxGC-MS). Int. J. Hyg. Environ. Health. 2024;257:114343. https://doi.org/10.1016/j.ijheh.2024.114343

3.1 Abstract

Several aromatic amines (AA) are classified as human carcinogens, and tobacco smoke is one of the main sources of exposure. Once in the human body, they undergo different metabolic pathways which lead to either their excretion or ultimately to the formation of DNA and protein adducts.

The aim of this study was to investigate AA in 68 urine samples (aged 29-79, 47 % female), including 10 smokers (S), 28 past smokers (PS) and 30 never smokers (NS), and to study if there was a relation between the smoking status and the amount of the AA present. GCxGC-MS was used to analyze AA in complex urine samples due to its high peak capacity and the fact that it provides two sets of retention times and structural information, which facilitates the separation and identification of the target analytes.

First, a qualitative comparison of an example set of a NS, PS and S sample was carried out, in which 38, 45 and 46 AA, respectively, could be tentatively identified. Afterwards, seven AA were successfully quantified in the samples. Of these, 4-ethylaniline (4EA, $p = 0.015$), 2,4,6-trimethylaniline (2,4,6TMA, $p = 0.030$), 2-naphthylamine (2NA, $p = 0.014$) and the sum of 2,4- and 2,6-dimethylaniline (DMA, $p = 0.017$) were found in significantly different ($\alpha = 0.05$) concentrations for the S, 29 ± 14, 87 ± 49, 41 ± 26, and 105 ± 57 ng/L respectively, compared to the NS, 15 ± 6, 42 ± 30, 16 ± 6, and 48 ± 28 ng/L. And 2,4,6TMA (39 ± 26, p =

0.022), 2NA (18 ± 9, p = 0.025) and DMA (53 ± 46, p = 0.030), were also found at significantly higher concentrations in samples from S when compared to PS. However, some samples had AA concentrations outside the calibration curve and could not be taken into account, especially for 2-methylaniline (2MA). Therefore, all the samples were evaluated using a quantitative screening approach, by which the intensities of 4EA (p = 0.019), 2,4,6TMA (p = 0.048), 2NA (p = 0.016), DMA (p = 0.019) and 2MA (p = 0.006) in S were found to be significantly (α = 0.05) higher than in the NS, and 2MA (p = 0.019) and 4EA (p = 0.023) in S were found to be significantly higher than in the PS. An association between the smoking status and the amount of certain AA present could therefore be found. This information could be used to study the relation between the smoking status, the amount of AA present, and smoking related diseases like bladder cancer.

3.2 Introduction

Aromatic amines (AA) are N-substituted aryl compounds with applications in several industries, most importantly those that manufacture or use pharmaceuticals, pesticides, dyes, polymers and explosives [1]. These AA can be discharged into the environment from anthropogenic sources such as the aforementioned industries or through cigarette smoke, which is one of the most important sources of exposure [2]. Other sources by which the population can come into contact with AA are hair dyes [3], cooking oil emissions [4], finger paints [5], dyed toys [6] or due to occupational exposure [7-9]

In spite of their multiple applications, a number of AA, such as 4-aminobiphenyl, 2-methylaniline (also known as o-toluidine) or 2-naphthylamine, are classified as carcinogens to humans [1]. The International Agency for Research on Cancer (IARC) declared that there is sufficient evidence that tobacco smoking causes transitional-cell carcinomas of the bladder, ureter and renal pelvis in humans [10] and epidemiologic studies suggest that AA might be the cause for the excess risk of bladder cancer in smokers [11].

When a carcinogen enters the human body, it can undergo different metabolic pathways, ultimately leading to the formation of reactive metabolites that can bind and form adducts with

proteins and DNA. These adducts can then induce mutations that may become fixed in the genome and contribute to tumor formation [1].

In the case of AA, a metabolic activation seems to be needed in order for them to become carcinogenic, and this can happen in different ways, depending on the tissue- and the cell-specific conditions [1, 12]. Possible mechanisms for the metabolic activation of AA in the bladder are N-hydroxylation, glucuronidation, oxidation and acetylation [13, 14]. These activation reactions lead ultimately to an electrophilic nitrenium ion that can then interact with proteins, RNA and DNA. Other possible fates for the AA is the formation of O-glucuronides, which can be excreted in the urine, or their direct excretion as free non-metabolized AA [14].

The analysis of AA in urine presents two important challenges, namely, the high complexity of the urine matrix and the highly polar character of the AA. With high-performance liquid chromatography (HPLC) a direct analysis of the AA is possible, however, an important drawback of this technique is a considerably low peak capacity [15], which makes it less suitable for the analysis of complex urine samples. In some cases, the use of a triple quadrupole as a detector (LC-MS/MS) can overcome this issue, as exemplified by Chinthakindi and Kannan [16] or Yu et al. [17]. However, in order to take full advantage of the selectivity obtained thanks to the MS/MS, typically multiple-reaction monitoring (MRM) is used, and in those cases non-target screening is no longer possible. Due to its high sensitivity, short analysis time, high resolving power and low cost [18], gas chromatography (GC) was used for this study instead.

Especially with complex samples, the peak capacity of one-dimensional gas chromatography might not be sufficient since it can result in peak overlapping, hindering the proper identification of the analytes. Therefore, a comprehensive two-dimensional gas chromatography mass spectrometry system (GCxGC-MS) is the best tool to improve the separation and subsequent identification of different isomers. With GCxGC, both, a higher peak capacity and two sets of retention times are provided, which allows for a better separation and identification of the target analytes [19]. Furthermore, the use of a mass spectrometer (MS) as the detector, complements the GCxGC system providing structural information of the analytes and facilitating their identification [19], achieving both an enhanced separation power and better sensitivity [20].

Aromatic amines are not easily analyzed in their free form. They tend to be adsorbed and decompose in the chromatographic columns, which translates into tailing, ghost peaks and

low sensitivities, and due to their polarity, low molecular mass amines are difficult to extract from water and are not easily chromatographed [18]. Therefore, a derivatization step is strongly recommended.

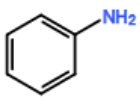
The aim of this work was to investigate fifteen AA in 68 urine samples of never smokers, past smokers, and smokers from a population-based study with the focus on whether there was an association between the smoking status and the amount of AA present. To the best of our knowledge, this is the first time that three different data evaluation approaches (i.e. qualitative analysis, quantitative analysis, and quantitative screening) are compared in the context of AA analysis in urine samples.

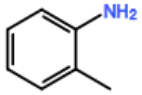
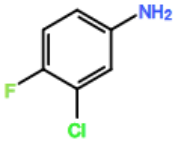
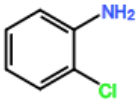
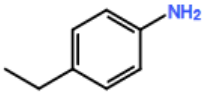
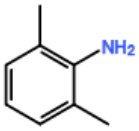
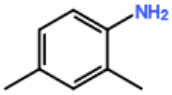
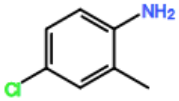
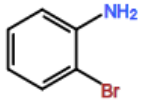
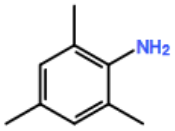
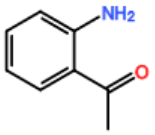
3.3 Materials and methods

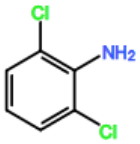
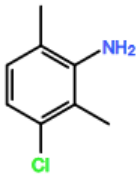
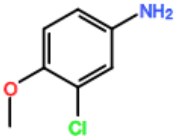
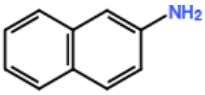
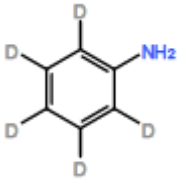
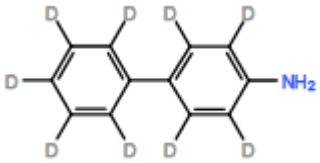
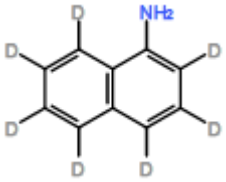
3.3.1 Chemicals and reagents

Reference substances were purchased from various suppliers, namely, Sigma-Aldrich (Steinheim, Germany, DE), Fluka (Buchs, Switzerland), Alfa Aesar (Karlsruhe, DE), Supelco (Steinheim, DE) and Dr. Ehrenstorfer GmbH (Augsburg, DE). The fifteen aromatic amines and three deuterated aromatic amines used for the quantification experiments, including their CAS numbers, structures, suppliers, and purities can be seen in Table 3.1. They were selected as model analytes due to their diverse chemical and physical properties and because most of them have been previously found in smoke, blood, tissue, and/or urine matrixes, as summarized by Lorenzo-Parodi et al. (2023b).

Table 3.1. Aromatic amines and deuterated aromatic amines used, with corresponding CAS numbers, structures, suppliers, and purities. Regulated aromatic amines under the REACH Regulation; No, 1907/2006 (Official Journal of the European Union, 2007) are marked in **bold**, and isomers of regulated aromatic amines are marked in *italics*.

Analyte (Abbreviation)	CAS Nr	Structure	Supplier	Purity
Aniline (A)	62-53-3		Sigma-Aldrich	≥ 99.5 %

Analyte (Abbreviation)	CAS Nr	Structure	Supplier	Purity
2-Methylaniline (2MA)	**95-53-4**		**Aldrich**	**≥ 99.0 %**
3-Chloro-4-fluoroaniline (3C4FA)	367-21-5		Aldrich	98.0 %
2-Chloroaniline (2CA)	*95-51-2*		*Sigma Aldrich*	*≥ 99.5 %*
4-Ethylaniline (4EA)	589-16-2		Aldrich	98.0 %
2,6-Dimethylaniline (2,6DMA)	87-62-7		Aldrich	99.0 %
2,4-Dimethylaniline (2,4DMA)	95-68-1		Aldrich	≥ 99.0 %
4-Chloro-2-methylaniline (4C2MA)	**95-69-2**		**Fluka**	**≥ 98.0 %**
2-Bromoaniline (2BA)	615-36-1		Aldrich	98.0 %
2,4,6-Trimethylaniline (2,4,6TMA)	*88-05-1*		*Aldrich*	*98.0 %*
2-Aminoacetophenone (2AAP)	551-93-9		Aldrich	98.0 %

Analyte (Abbreviation)	CAS Nr	Structure	Supplier	Purity
2,6-Dichloroaniline (2,6DCA)	608-31-1		Aldrich	98.0 %
3-Chloro-2,6-dimethylaniline (3C2,6DMA)	26829-77-6		Alfa Aesar	99.0 %
3-Chloro-4-methoxyaniline (3C4MA)	5345-54-0		Aldrich	97.0 %
2-Naphthylamine (2NA)	**91-59-8**		**Sigma**	**≥ 95.0 %**
Aniline-d_5 (AD5)	4165-61-1		Supelco	99.3 %
4-Aminobiphenyl-d_9 (4ABPD9) -100 ng/µL in Methanol-	344298-96-0		Dr. Ehrenstorfer GmbH	98.5 %
1-Aminonaphthalene-d_7 (1AND7) -100 ng/µL in Methanol-	78832-53-8		Dr. Ehrenstorfer GmbH	97.6 %

Concentrated hydrochloric acid (ACS reagent, 37 %) and sodium hydroxide (98 %) were purchased from Bernd Kraft (Duisburg, DE). Hydriodic acid (ACS reagent, unstabilized,

55 %), sodium nitrite (99 %) and alizarin red S indicator (98 %) were obtained from Sigma-Aldrich. Sodium sulfite (puriss. p.a., ACS reagent, RT, ≥ 98 %) and sulfamic acid (T, ≥ 99 %) were bought from Fluka (Buchs, CH). Sodium acetate (RT, 99 %) was purchased from Applichem (Darmstadt, DE). Diethyl ether and HPLC grade methanol were purchased from Fisher Scientific (Loughborough, UK), and ultrapure water was obtained from a PureLab Ultra water system from ELGA LabWater (Celle, DE). Synthetic human urine imitation was purchased from Synthetic Urine e.K. (Eberdingen-Nußdorf, DE).

All the chemicals and reagents were stored at room temperature except for the three deuterated internal standards (IS), the hydriodic acid and the synthetic urine, that were stored in the refrigerator at 5 °C.

3.3.2 Sample preparation

Preparation of stock and standard solutions

Individual stock solutions of each of the analytes were prepared at 1 g/L in methanol. A standard solution mix, containing all the non-deuterated AA was prepared at 1 mg/L in methanol. A 20 μg/L working solution was prepared in ultrapure water before every use. The IS were prepared separately, first a 10 mg/L AD5 standard solution was prepared in methanol, followed by a 150 μg/L internal standard solution mix in methanol, containing AD5, 4ABPD9 and 1AND7. All the aforementioned solutions were kept refrigerated at 5 °C as long as they were not used. More information can be seen in the supplementary information (SI, "Preparation of stock and standard solutions" section).

Calibration curve preparation

A calibration curve 10, 50, 100, 150 and 200 ng/L was created by spiking 20 mL synthetic urine imitate with the corresponding amount of AAs mix and IS mix to a final concentration of 150 ng/L. More information can be seen in the SI, "Preparation of stock and standard solutions" section.

Urine samples

A total of 68 urine samples (aged 29-79, 47 % female) from the population-based Heinz Nixdorf Recall Study [21] were analyzed. Urine samples from 10 currently smoking (S), 28

past smoking (PS, quit smoking ≥12 months ago) and 30 never smoking (NS) study participants were stored in 50-mL polypropylene tubes at - 5 ºC. After thawing the selected samples, 20 mL of urine was introduced into 30-mL amber bottles, and spiked with the IS mix to a final concentration of 150 ng/L. They were prepared following an adapted procedure from Lamani et al. [22], as described in the following sections.

Hydrolysis

In order to cleave any aromatic amine adducts that might be present in the sample, an acidic hydrolysis step as described by Lamani et al. [22] was followed. Because a water bath was used for heating, and in order to have a stable temperature, the samples were heated at 80 °C instead of 110 °C in a water bath 1086 (Gesellschaft für Labortechnik GmbH, Burgwedel, DE). After hydrolysis, the samples were filtrated into 100 mL beakers using 589^2 white ribbon, ashless, ∅ 150 mm filter papers from Schleicher & Schuell (Dassel, DE).

Since the synthetic urine does not contain any adducts, the acid hydrolysis was not performed for the calibration curve, and only 20 mL of the 10 M sodium hydroxide solution was added before continuing with the LLE step.

Liquid-liquid extraction

The samples were then extracted three times with 5 mL DEE while shaken manually for approximately 1 min. The organic phase was subsequently washed with 2 mL of 0.1 M sodium hydroxide solution, and then the amines in the organic phase were extracted back into the aqueous phase with 10 mL water previously acidified with 200 µL concentrated hydrochloric acid (37 %). The aqueous phase was stored in clean weighted 20 mL vials, and the remaining diethyl ether was gently evaporated in a nitrogen stream for 10-20 min, mixing every 5 min, depending on the DEE content of each individual sample which was checked by the weight of the samples. Two times 4.7 mL of each sample were transferred into 20 mL screw vials, obtaining in this way duplicates.

Derivatization

The samples were derivatized through diazotization followed by iodination as originally described by Schmidt et al. [15], and later adapted by Lamani et al. [22]. The procedure described by the latter was followed, using a KS 260 control shaker (IKA®-Werke GmbH &

Co. KG, Staufen, DE) for shaking and the same water bath used for acidic hydrolysis for incubating the samples at 95 °C. As described in the extraction section, 4.7 mL of sample were used, instead of 5 mL. Furthermore, when the expected discoloration after the addition of sodium sulfite was not complete, an extra 125 µL of saturated sodium sulfite were added.

Blanks were prepared in two different ways, either by adding 4.7 mL ultrapure water into 20 mL screw caps and following the derivatization procedure, or by adding 5 mL instead and directly measuring them. The first type of blank was prepared to check for contaminations in the reactants. Five blanks were prepared per sample batch, and measured between different sample types to control if there was carryover or contamination.

Solid-phase microextraction

To enrich the iodinated derivatives before measuring, headspace SPME was used. This was achieved thanks to the Shimadzu AOC-5000 liquid, headspace, and SPME GC injection system from Shimadzu GmbH (Duisburg, DE), which was controlled with the PAL Cycle Composer program, from CTC Analytics AG (Zwingen, Switzerland). The SPME fiber used for sample enrichment was 65 µm PDMS/DVB, Stableflex, 24 Ga, autosampler from Supelco (Munich, DE) in combination with a SPME liner of 0.75 mm × 5.0 mm × 95 mm made for Shimadzu GCs from Restek (Bad Homburg, DE).

The samples were pre-incubated at 60 °C for 10 min while agitating at 500 rpm. The SPME fiber was then injected into the headspace of the vial (still at 60 ºC and under agitation) at a penetration depth of 22 mm for 25 min. Afterwards, the extracted analytes were desorbed into the GC-injection port for 5 min, followed by the conditioning of the fiber in the conditioning module for 20 min at 250 ºC.

3.3.3 GC-MS analysis

All analyses were performed by a Shimadzu GC-MS system consisting of a GC-2010 gas chromatograph coupled to a QP2010 Plus gas chromatograph mass spectrometer with a built-in ZX1 liquid nitrogen cooled loop modulation system from Zoex Corporation (Texas, USA). The GC columns used were a DB-5 (30 m x 0.32 mm x 0.25 µm) from Agilent Technologies (Waldbronn, DE) for the first dimension and a BPX-50 (2.7 m x 0.15 mm x 0.15 µm) from SGE Analytical Science (Griesheim, DE) for the second dimension, connected

with a deactivated universal press fit connector, suitable for 0.2 to 0.7 mm OD, from BGB Analytik AG (Boeckten, Switzerland). The second column was coiled around the Zoex loop-type modulator allowing 1 meter after cryo-focusing to the detector. Liquid nitrogen was stored in a 150 L Apollo container from Cryotherm (Kirchen/Sieg, DE) and transferred into a SC 20/20 tank from Chart MVE (Ohaio, USA) thanks to the Model 186 Liquid Level Controller from American Magnetics, Inc. (Neuss, DE). The septa used throughout all the experiments were AG3-Schimadzu septa from Macherey-Nagel (Düren, DE).

The temperature of the injection port was set to 250 ºC. Helium (99.999 % from Air Liquide, Krefeld, Germany) was used as the carrier gas with a column flow of 1 mL/min, and a linear velocity of 29.1 cm/s, with the latest parameter being selected as the flow control mode. The modulation time was set to 7.5 s and a split ratio of 20, with a purge flow of 6 mL/min, which translates into a total flow of 26.9 mL/min. The instrument was operated in the splitless injection mode with a sampling time of 5 min. The initial oven temperature of 60 °C was held for 3 min, then increased to 230 °C at a 5 °C/min rate, and subsequently held for another 5 min, adding to a total run time of 42 min. At the starting temperature, and with the parameters aforementioned, the column head pressure was 97.1 kPa. To program the hot jet gradient, the temperature at different times of the run has to be defined individually. To emulate a temperature ramp, the program was set so that every 2.5 min the temperature increased 12.5 ºC up to 265 ºC at minute 33, temperature that was held until the end of the run. The MS interface temperature was set to 250 °C and the ion source temperature to 230 °C. The solvent cut time was 6 min and the detector voltage was 1.3 kV. Full scan mode was used, and in order to achieve a better sensitivity, only the mass-to-charge ratios between m/z 74 and 457 were studied. More information about the method used for the data evaluation can be seen in SI, “Data evaluation” section.

Control of the GCxGC-MS system was done with the GCMS Real Time Analysis software, Shimadzu GmbH. The liquid nitrogen flow was controlled by an E50 E-terminal from Beijer Electronics (Utah, USA). The data was processed via GC Image from GC Image, LLC (Nevada, USA), GCMS Post Run Analysis software from Shimadzu GmbH and using Excel (Microsoft).

3.3.4 Data Evaluation

Three different approaches were followed to evaluate the data. First, a qualitative analysis was carried out to make sure that all the samples were measured successfully. Furthermore, for three samples, the total number of AA was calculated as examples. Afterward, a conventional quantitative analysis was carried out, by which the concentrations of the AA were calculated. With this approach all results outside of the calibration curve cannot be considered for the calculations, which could lead to systematic errors and biases. Therefore, a quantitative screening was also carried out. With this method, the peak intensities of the different samples were compared, independent of whether they were within certain values or not. For both quantitative approaches, outliers determined with Dixon's Q test [23] were excluded from the calculations, and significant differences between the groups were evaluated with Welch´s two-sided t-test [24]. More information, including the equations used, can be found in the SI.

3.4 Results and discussion

3.4.1 Qualitative analysis

Contour plots are useful visual tools that help represent the information obtained with two-dimensional gas chromatography. As example, a set of samples from three female donors (a NS, a PS, and a S) of as similar ages as possible (45, 47 and 39, respectively) were chosen (Fig. 3.1). It can be seen that the S sample presents more analytes and at higher intensities than the NS and PS samples shown in the figure. In Lamani et al. [22], the contour plots of a S and a NS sample were also compared, and a similar observation was reported, i.e., the S sample had more analytes and at higher concentration than the NS sample. A tentative identification of the analytes was also carried out (see SI for detailed information). The results confirm the findings above, the total number of tentatively identified AA, shown in parentheses, is the highest in the S sample (46), followed by the PS (45) and the NS (38). Surprisingly, while the PS plot resembles the NS sample plot, the total number of tentatively identified AA in the PS sample is very similar to the S sample. The number of tentatively identified AA is in general agreement with Lamani et al. [22], who found 46 AA in the urine of a NS donor, and 50 in the urine of a S donor. For all the calculations, different isomers were counted as one AA (see SI for more

information). These results give a general idea of the number of different AA present in the samples but should only be considered examples. A different approach is needed to find statistical differences between the different groups, especially when large sample sizes are considered. The qualitative evaluation of the samples can therefore be complementary to the quantitative analysis or the quantitative screening.

Contour plots are also a very useful tool to identify certain problems with the analysis, namely, modulation problems. In Fig. 3.1, d) such an example is included, where the loop where the hot and cold jets were pointing at was not completely aligned with the jets. This led to an incomplete modulation, which can be easily recognized by the repetitive pattern in the plot.

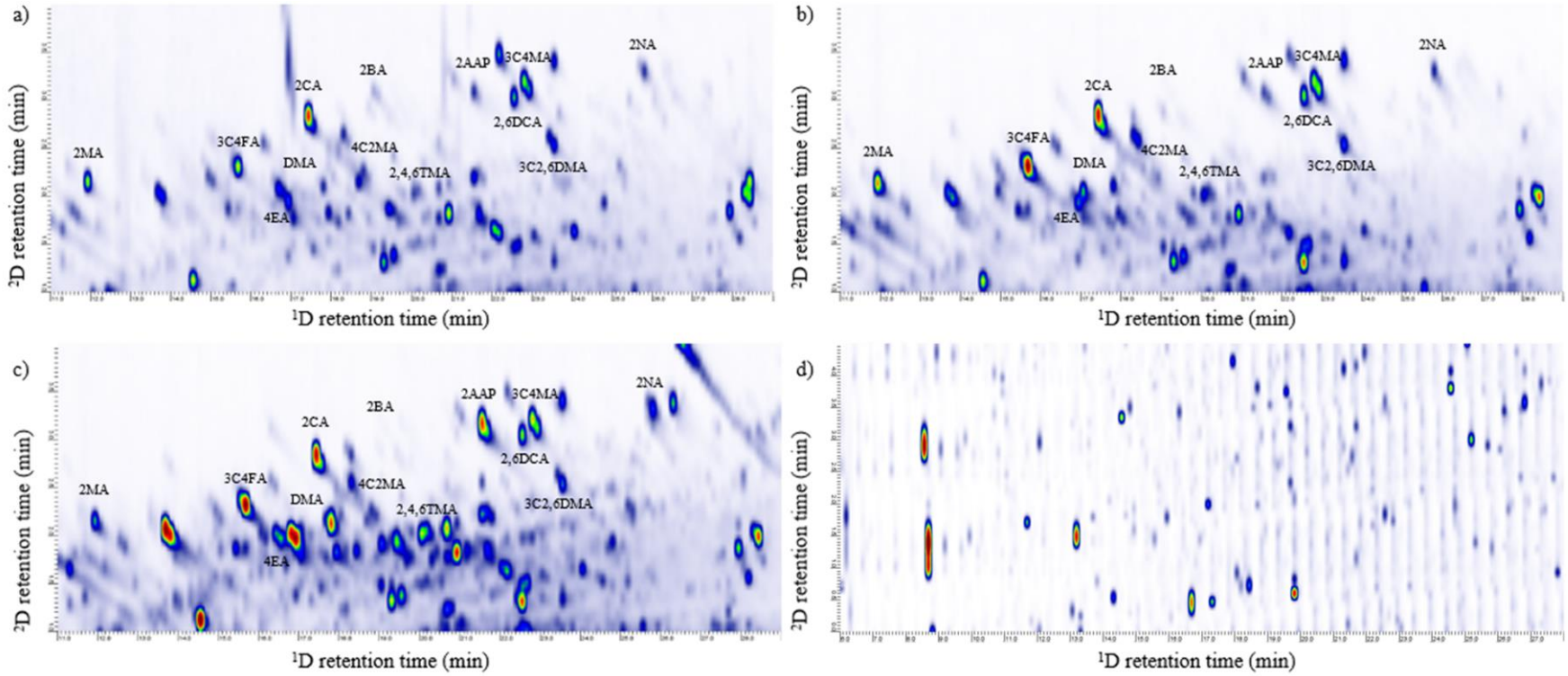


Fig. 3.1. Contour plots of the urine samples from a) a 45 year old never smoker female, b) a 47 year old past-smoker female, c) a 39 year old smoker female and d) an example of a problematic analysis, where the modulation did not work properly. The retention times in the first and second dimension (^{1}D and ^{2}D) are plotted in the x and y axis, respectively, and the color scale indicates different intensities, with red being the most intense (1.51×10^7) followed by yellow, green, and blue (2.09×10^4), and white showing no signal.

3.4.2 Quantitative analysis

The quantitative approaches offer the advantage that the content (or relative content) of the analytes in the samples can be studied. Several calibration possibilities were compared, including using AD5, 1AND7 and 4ABPD9 as internal standards. More information can be found in the SI, "Quantitative analysis". In Table 3.2, the mean concentrations and standard deviations of the different groups (NS, PS and S) for the most promising calibration method, namely considering 4ABPD9 as the IS, can be seen. Since only the samples with concentrations within the calibration curve could be accurately determined, those were the ones included for the calculations of this section, leading to different sample sizes (n) for different analytes and smoking status. In most cases, around 90 % of the measured samples could be taken into account. With two exceptions, 2MA and 2CA, where sometimes less than half the samples could be considered. For all analytes, except 2MA and 2CA, ascending concentrations could be seen: NS<PS<S.

Table 3.2. Mean concentrations (C_i) and the corresponding standard deviations (SD) of the aromatic amines, of never smokers (NS, maximum possible sample number n_{max} = 30), past smokers (PS, n_{max} = 28) and smokers (S, n_{max} = 10), and the total number of samples (n) taken into account for their calculation. Concentrations outside the calibration curve and outliers detected with Dixon's Q test were not considered. Significant differences found with Welch´s t-test (see SI, $\alpha = 0.05$) between NS and S, and PS and S are marked in **bold**. There were no significant differences ($\alpha = 0.05$) found between NS and PS.

	NS		**PS**		**S**	
Analyte	**(n)**	**C_i ± SD (ng/L)**	**(n)**	**C_i ± SD (ng/L)**	**(n)**	**C_i ± SD (ng/L)**
2MA	(17)	119 ± 45	(12)	98 ± 34	> 200 ng/L	
2CA	(22)	127 ± 34	(12)	110 ± 34	(7)	127 ± 52
4EA	**(28)**	**15 ± 6**	(25)	19 ± 12	**(10)**	**29 ± 14**
DMA	**(27)**	**48 ± 28**	**(25)**	**53 ± 46**	**(9)**	**105 ± 57**
4C2MA	(27)	41 ± 22	(24)	52 ± 42	(9)	66 ± 31
2,4,6TMA	**(30)**	**42 ± 30**	**(23)**	**39 ± 26**	**(9)**	**87 ± 49**
2NA	**(26)**	**16 ± 6**	**(24)**	**18 ± 9**	**(10)**	**41 ± 26**

Our results are in reasonable agreement with the publications to date reporting measured concentrations of AA in human urine. In [25], the concentration ranges of AA in urine samples found in different studies are summarized. Here, two of the most commonly studied AA, 2MA and 2NA, were inspected in more detail. To facilitate the comparison of the different publications, results originally presented in ng/24 h or µg/24 h were re-calculated using 1.058 L as the average amount of urine excreted per day [26]. The concentration of 2MA has been found at 130 ng/L [27], 193 ± 56 ng/L [28], and 243 ng/L [29] for S, and 99 ± 24 ng/L [28], 100 ng/L [27], and 150 ± 10 ng/L [16] for NS. El-Bayoumy et al. [30] and Fuller et al. [31] reported concentrations of 2MA one order of magnitude higher: 6000 ± 3500 ng/L [30] and 2330 ± 920 ng/L [31] for S, and 4000 ± 3000 ng/L [30] and 1000 ± 450 ng/L [31] for NS, respectively. Fuller et al. [31] reported 100-fold higher 2NA concentrations than those found here, namely 1460 ± 230 ng/L [31] for S and 1130 ± 360 ng/L [31] for NS. However, other publications found 2NA at similar concentration levels to those reported in this study: 19.7 ± 10.6 ng/L [28], 44.8 ± 47.9 ng/L [17] for S, and 10.1 ± 9.0 ng/L [28], 9.6 ± 6.9 ng/L [17] and 400 ± 50 ng/L [16] for NS. The relatively large differences in concentrations between the studies could be due to differences in population groups in terms of age and sex. However, differences in the amount and duration of smoking, type of cigarette smoked, and other sources of exposure (industrial, traffic, dietary, occupational), are probably the most important factors.

Welch's two-sided t-test [24] was performed comparing the concentration of each analyte in the urine samples (more information in SI, "Quantitative analysis" section). When comparing NS and S, the concentrations of 4EA ($p = 0.015$), DMA ($p = 0.017$), 2,4,6TMA ($p = 0.030$) and 2NA ($p = 0.014$) were found significantly higher ($\alpha = 0.05$) in S than in the NS. DMA ($p = 0.030$), 2,4,6TMA ($p = 0.022$) and 2NA ($p = 0.025$) were also found at significantly higher concentrations ($\alpha = 0.05$) in S when compared to PS. No significant differences were found between NS and PS.

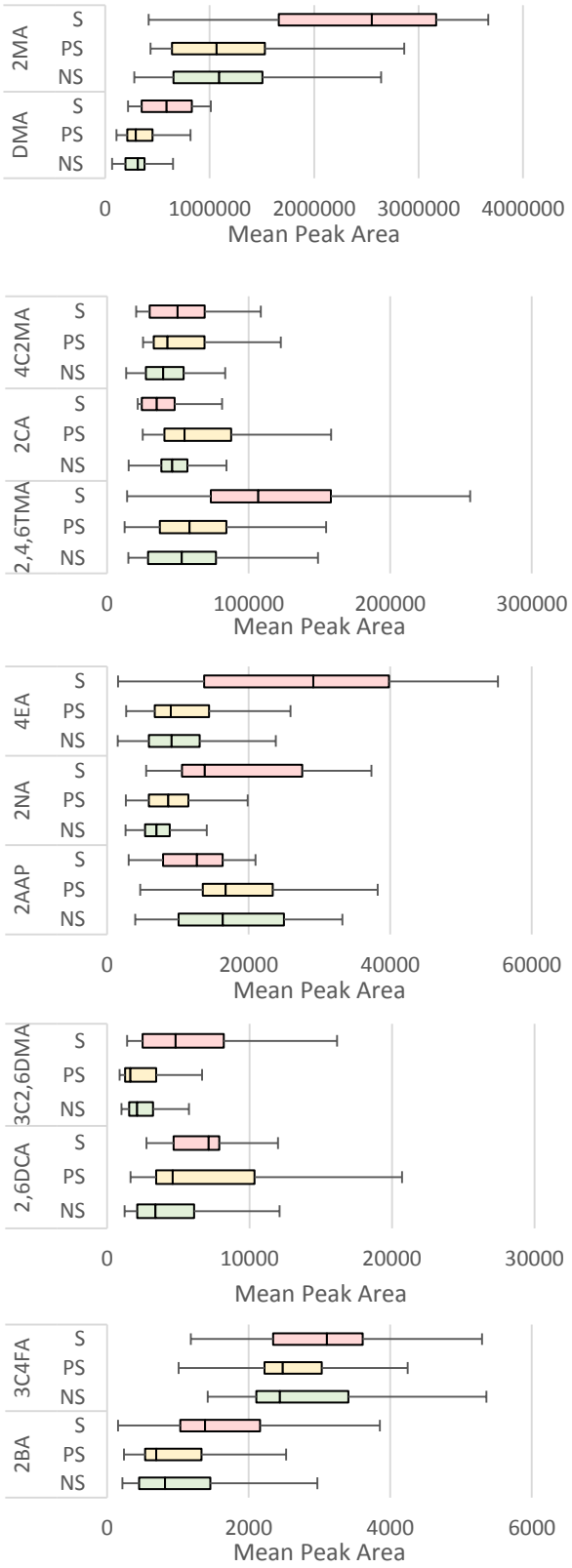
It was not possible to calculate the concentration of 2MA in most samples, as they were above the calibration range measured. To quantify those concentrations above the upper calibration curve limit, different measures could be taken. On one hand, the samples could be diluted and measured again, but then more sample volume would be needed for the second analysis, and the time dedicated to the sample preparation would be doubled. Alternatively, the

calibration curve could be extended, however, because of the high variability of human samples, the linearity of the curves should be carefully studied. Furthermore, there is always a possibility of samples having concentrations outside of the calibration range. Finally, and especially interesting for samples with high variability such as those studied here, the data can be evaluated using a quantitative screening approach.

3.4.3 Quantitative screening

To gain a better insight of the differences between the groups, a quantitative screening was performed: the peak areas of the different analytes were plotted in Tukey box plots [32]. Furthermore, Welch's two-sided t-tests [24] were performed comparing the results from smokers, past smokers, and never smokers.

In the Tukey box plots from Fig. 3.2, a higher median concentration in the urine of smokers for all the analytes except 2CA and 2AAP can be seen. Moreover, after performing Welch's two-sided t-test for unequal variances, it can be stated with a 0.05 significance level, that the amount of 2MA ($p = 0.006$), 2,4,6TMA ($p = 0.048$), DMA ($p = 0.019$), 4EA ($p = 0.019$) and 2NA ($p = 0.016$) present in the urine from S and NS is significantly different. When comparing PS and S, 2MA ($p = 0.019$) and 4EA ($p = 0.023$) were significantly higher ($\alpha = 0.05$) in the samples from S than PS. There were no significant differences ($\alpha = 0.05$) between NS and PS. In contrast to the quantitative analysis approach, with the quantitative screening no samples were excluded from the calculations, which was especially critical for 2MA since a significant portion of the samples had concentrations above the highest measured calibration curve point. Therefore, a significant difference between S and NS for 2MA could now be established. This shows the advantage of this approach, especially for samples with large variabilities.



Analyte	Group	n	M ± SD		
2MA	**NS**	**30**	**$1.2\cdot10^{6}$**	**±**	**$6.3\cdot10^{5}$**
	PS	**28**	**$1.4\cdot10^{6}$**	**±**	**$9.0\cdot10^{5}$**
	S	**10**	**$2.4\cdot10^{6}$**	**±**	**$1.0\cdot10^{6}$**
DMA	**NS**	**29**	**$3.3\cdot10^{5}$**	**±**	**$1.8\cdot10^{5}$**
	PS	27	$3.9\cdot10^{5}$	±	$2.9\cdot10^{5}$
	S	**10**	**$5.9\cdot10^{5}$**	**±**	**$2.9\cdot10^{5}$**
4C 2MA	NS	29	$4.2\cdot10^{4}$	±	$1.9\cdot10^{4}$
	PS	27	$5.3\cdot10^{4}$	±	$3.0\cdot10^{4}$
	S	10	$5.4\cdot10^{4}$	±	$2.8\cdot10^{4}$
2CA	NS	30	$5.5\cdot10^{4}$	±	$3.6\cdot10^{4}$
	PS	27	$1.7\cdot10^{5}$	±	$3.9\cdot10^{5}$
	S	9	$4.2\cdot10^{4}$	±	$2.1\cdot10^{4}$
2,4,6 TMA	**NS**	**30**	**$6.4\cdot10^{4}$**	**±**	**$4.5\cdot10^{4}$**
	PS	27	$7.8\cdot10^{4}$	±	$7.1\cdot10^{4}$
	S	**10**	**$1.2\cdot10^{5}$**	**±**	**$7.7\cdot10^{4}$**
4EA	**NS**	**30**	**$1.2\cdot10^{4}$**	**±**	**$9.4\cdot10^{3}$**
	PS	**27**	**$1.2\cdot10^{4}$**	**±**	**$9.8\cdot10^{3}$**
	S	**10**	**$2.8\cdot10^{4}$**	**±**	**$1.7\cdot10^{4}$**
2NA	**NS**	**30**	**$7.7\cdot10^{3}$**	**±**	**$3.8\cdot10^{3}$**
	PS	28	$1.2\cdot10^{4}$	±	$1.3\cdot10^{4}$
	S	**10**	**$1.9\cdot10^{4}$**	**±**	**$1.2\cdot10^{4}$**
2AAP	NS	29	$1.7\cdot10^{4}$	±	$8.5\cdot10^{3}$
	PS	28	$2.4\cdot10^{4}$	±	$2.2\cdot10^{4}$
	S	10	$1.2\cdot10^{4}$	±	$5.7\cdot10^{3}$
3C2,6 DMA	NS	30	$6.1\cdot10^{3}$	±	$1.4\cdot10^{4}$
	PS	28	$2.6\cdot10^{3}$	±	$1.8\cdot10^{3}$
	S	10	$6.2\cdot10^{3}$	±	$5.0\cdot10^{3}$
2,6 DCA	NS	29	$5.3\cdot10^{3}$	±	$4.5\cdot10^{3}$
	PS	27	$8.8\cdot10^{3}$	±	$1.0\cdot10^{4}$
	S	10	$6.6\cdot10^{3}$	±	$2.8\cdot10^{3}$
3C4FA	NS	30	$2.9\cdot10^{3}$	±	$1.4\cdot10^{3}$
	PS	28	$2.8\cdot10^{3}$	±	$1.2\cdot10^{3}$
	S	9	$3.1\cdot10^{3}$	±	$1.3\cdot10^{3}$
2BA	NS	29	$1.1\cdot10^{3}$	±	$8.6\cdot10^{2}$
	PS	28	$2.0\cdot10^{3}$	±	$3.5\cdot10^{3}$
	S	10	$1.8\cdot10^{3}$	±	$1.3\cdot10^{3}$

Fig. 3.2. Tukey box plots of aromatic amines in urine samples of smokers (S, maximum possible sample number n_{max} = 10), past smokers (PS, n_{max} = 28) and never smokers (NS, n_{max} = 30). Corresponding table with information about the mean peak area (M) of the different groups,

their standard deviation (SD), and the final sample sizes (n), after excluding outliers detected with Dixon's Q test. Significant differences found with Welch´s t-test (see SI, $\alpha = 0.05$) between NS and S, and PS and S are marked in **bold**. There were no significant differences ($\alpha = 0.05$) found between NS and PS.

The very different intensities within a sample class, for example 3C2,6DMA content in the S samples, could be a possible reason why some aromatic compounds show no significant differences. In general, some variation within the samples could be accounted for by correcting the results with the corresponding creatinine concentrations and therefore accounting for different excretion rates. However, S generally have a higher variability than the rest of the groups, which could be due to several reasons such as a different smoking topography or smoking behavior, including the number of puffs per cigarette, or puff volume [10], differences in the amount and type of cigarettes smoked or the time since the last smoking event. The distribution of outliers was relatively consistent across the three different groups, with 5, 6 and 2 outliers for NS, PS and S samples respectively, which represented a 1.4, 1.8 and 1.7 % of the total of each group (360, 336 and 120 respectively). A consistent level of urinary biomarkers could be expected due to the chronic use of tobacco products [33]. However, a study by Modick et al. [34] showed elimination half-times of free aniline of 0.6-1.2 h and of the primary metabolites below 6 h, and Jurado-Sánchez et al. [35] showed biological half-lives of less than 2 h for 2CA, 2,4DCA, 2,6DCA and 2,4,6TCA, without differentiating between free and conjugated AAs. Measuring other biomarkers, such as cotinine or other nicotine metabolites [36] would provide information of other tobacco exposure, such as passive or secondhand exposure, that could also increase the variability of the NS and PS results [37, 38].

Finally, the amount of 2CA and 2AAP present in the S samples is lower than in the other groups, and 2AAP is significantly lower in S compared to PS. This could be due to other types of exposures apart from direct tobacco smoke, such as certain types of honey [39, 40], bread [41] or wine [42]. Furthermore, other sources such as occupational exposure and environmental tobacco exposure cannot be discarded. In order to be able to account for these types of exposure, information about the occupations of the donors and their habits should be assessed and the measurement of other biomarkers, such as cotinine, could be performed.

More information could be gained by including more data about the donors, such as the age of the donor, how many cigarettes they smoke and which type, or if they are likely to be exposed due to their occupation or not. In this study, the gender of the participants was

compared and no significant differences between males and females could be observed, as seen in the SI ("Quantitative screening" section).

3.5 Conclusion

Three different approaches were tested with the aim of establishing a relationship between the content of AA in the urine of 68 donors and their smoking status.

The qualitative evaluation was proven useful as a quality control check, since being able to see the analytes in a contour plot enables the fast identification of problems with the system, and to quickly look at specific samples. Although it can be generally used to compare different samples, it is not the most suitable approach for larger number of samples (n). In order to better evaluate the relationship between the AA and the status of the donor, either the quantitative analysis or the quantitative screening are recommended.

Seven analytes were successfully quantified in the studied samples and were found between 15 and 127 ng/L. Furthermore, the concentrations of 2,4,6TMA, DMA, 4EA and 2NA were significantly higher for smokers than never smokers, and the concentrations of 2,4,6TMA, 2NA and DMA were significantly higher for smokers than past smokers. With this approach, it is important to keep in mind that only those samples within the calibration curve can be used for calculations. This was especially relevant for 2MA, where an underestimation of the total concentrations was suspected, since only the values within the calibration working range could be accurately determined. To prevent this, a broader calibration curve range, encompassing higher concentration points, could be studied.

Finally, a quantitative screening was carried out in order to be able to include all samples. With this approach, 2MA, 2,4,6TMA, DMA, 4EA and 2NA were found at significantly higher intensities in the samples from smokers compared to never smokers, and 2MA and 4EA in samples from smokers compared to past smokers. The quantitative screening takes into account all samples analyzed, and it is therefore the one we would recommend, after a thorough validation, for further studies, especially for samples with high variability like urine samples.

The approach presented here is also suitable for non-target analysis of AA, which could be essential as more AA are expected to be discovered to have negative health effects and

become regulated. More information from the study participants in order to account for the health status, duration and amount of tobacco smoked, occupational exposure, among others, could be considered. The environmental tobacco exposure could also be assessed, for example by measuring nicotine metabolites in urine, such as cotinine. The prevalence of smoking related diseases, like bladder cancer, could also be considered, and the relationship between AA, smoking status and smoking related diseases could be studied. For that, larger sample sizes would be needed, which could become costly. To reduce overall costs, minimize labor time and human errors, an automated sample preparation procedure as described by Lorenzo-Parodi et al. [43] could be used. Furthermore, it would contribute towards a greener analytical chemistry procedure. Miniaturizing the method, i.e. minimizing the sample and reagent volumes needed, would also contribute to a greener chemistry, and would enable the use of samples where the volume is limited, such as archived samples from population studies. This would ultimately help to achieve a better understanding between AA, smoking and smoking related diseases.

3.6 Supplementary information

3.6.1 Materials and methods

Preparation of stock and standard solutions

Stock solutions were prepared by weighing approximately 10 mg of each pure substance in 10 mL volumetric flasks and diluting with pre-cooled methanol to make a final concentration of 1 g/L. The exact weight of each substance, as well as their final concentration can be seen in Table S 3.1. More information can be seen in section 3.3.2.

Table S 3.1. Weighted amounts and final concentrations of the stock solutions of each aromatic amine.

Analyte	Weight (mg ± 0.02 mg)	Concentration (g/L ± 0.02 g/L)	Analyte	Weight (mg ± 0.02 mg)	Concentration (g/L ± 0.02 g/L)
A	9.72	0.97	2BA	11.63	1.16
2MA	12.78	1.28	2,4,6TMA	11.81	1.18
3C4FA	11.47	1.15	2AAP	11.90	1.19
2CA	11.81	1.18	2,6DCA	10.56	1.06
4EA	10.06	1.01	3C2,6DMA	9.86	0.99
2,6DMA	12.36	1.24	3C4MA	12.56	1.26
2,4DMA	9.81	0.98	2NA	10.78	1.08
4C2MA	11.28	1.13			

In Table S 3.2 the preparation of other stock solutions can be seen in detail, as well as their final concentrations. The exact concentration of the 15er - mix, 20 µg/L solution can be seen in Table S 3.3. The uncertainties were calculated doing error propagation [44] and taking the instruments and glassware uncertainties form Table S 3.6 and Table S 3.7 into account.

Table S 3.2. Composition of the intermediate solutions used, prepared by adding a defined volume of stock solutions and filling up with solvent to a defined volume.

Name	Stock Solution	Solvent, Final Volume	Volume (µL)	Concentration (mg/L ± mg/L)
AD5, 10 mg/L	AD5, 1.10 g/L	Methanol, 5 mL	100	11.0 ± 0.4

Name	Stock Solution	Solvent, Final Volume	Volume (µL)	Concentration (mg/L ± mg/L)
15er - mix, 1 mg/L	A, 0.97 g/L	Methanol, 10 mL	10	0.97 ± 0.02
	2MA, 1.28 g/L		10	1.28 ± 0.02
	3C4FA, 1.15 g/L		10	1.15 ± 0.02
	2CA, 1.18 g/L		10	1.18 ± 0.02
	4EA, 1.01 g/L		10	1.01 ± 0.02
	2,6DMA, 1.24 g/L		10	1.24 ± 0.02
	2,4DMA, 0.98 g/L		10	0.98 ± 0.02
	4C2MA, 1.13 g/L		10	1.13 ± 0.02
	2BA, 1.16 g/L		10	1.16 ± 0.02
	2,4,6TMA, 1.18 g/L		10	1.18 ± 0.02
	2AAP, 1.19 g/L		10	1.19 ± 0.02
	2,6DCA, 1.06 g/L		10	1.06 ± 0.02
	3C2,6DMA, 0.99 g/L		10	0.99 ± 0.02
	3C4MA, 1.26 g/L		10	1.26 ± 0.02
	2NA, 1.08 g/L		10	1.08 ± 0.02
15er - mix, 20 µg/L	15er - mix, 1 mg/L	Water, 5 mL	100	Approx. 0.02, see Table S 3.3
3er IS - mix, 150 µg/L	AD5, 11.0 mg/L	Methanol, 10 mL	150	0.164 ± 0.07
	4ABPD9, 100 mg/L		15	0.150 ± 0.02
	1AND7, 100 mg/L		15	0.150 ± 0.02

Table S 3.3. Final concentration (C_F) of the intermediate solution "15er – mix, 1 mg/L" (± 0.02 mg/L) and the diluted standard solution "15er - mix, 20 µg/L" (±1 µg/L).

Analyte	**C_F 15er – mix, 1 mg/L**	**C_F 15er – mix, 20 µg/L**
A	0.97	19
2MA	1.28	26
3C4FA	1.15	23
2CA	1.18	24
4EA	1.01	20
2,6DMA	1.24	25
2,4DMA	0.98	20
4C2MA	1.13	23
2BA	1.16	23
2,4,6TMA	1.18	24
2AAP	1.19	24
2,6DCA	1.06	21
3C2,6DMA	0.99	20
3C4MA	1.26	25
2NA	1.08	22

To prepare the calibration curve, 10, 50, 100, 150 and 200 µL of the 15er - mix, 20 µg/L were added to synthetic urine to have the concentration levels 10, 50, 100, 150 and 200 ng/L, as seen in Table S 3.4. More information can be seen in section 3.3.2.

Table S 3.4. Final concentration and uncertainty at each calibration point, for the 15 aromatic amines used in the quantitation section.

Analyte	Concentration level				
	10 ng/L (ng/L ± ng/L)	**50 ng/L (ng/L ± ng/L)**	**100 ng/L (ng/L ± ng/L)**	**150 ng/L (ng/L ± ng/L)**	**200 ng/L (ng/L ± ng/L)**
A	9.7 ± 0.3	49 ± 1	97 ± 3	146 ± 4	194 ± 6
2MA	12.8 ± 0.3	64 ± 2	128 ± 3	192 ± 5	256 ± 6
3C4FA	11.5 ± 0.3	57 ± 2	115 ± 3	172 ± 5	229 ± 6
2CA	11.8 ± 0.3	59 ± 2	118 ± 3	177 ± 5	236 ± 6
4EA	10.1 ± 0.3	50 ± 1	101 ± 3	151 ± 4	201 ± 6
2,6DMA	12.4 ± 0.3	62 ± 2	124 ± 3	185 ± 5	247 ± 6
2,4DMA	9.8 ± 0.3	49 ± 1	98 ± 3	147 ± 4	196 ± 6
4C2MA	11.3 ± 0.3	56 ± 2	113 ± 3	169 ± 5	226 ± 6
2BA	11.6 ± 0.3	58 ± 2	116 ± 3	174 ± 5	233 ± 6
2,4,6TMA	11.8 ± 0.3	59 ± 2	118 ± 3	177 ± 5	236 ± 6
2AAP	11.9 ± 0.3	60 ± 2	119 ± 3	179 ± 5	238 ± 6
2,6DCA	10.6 ± 0.3	53 ± 1	106 ± 3	158 ± 4	211 ± 6
3C2,6DMA	9.9 ± 0.3	49 ± 1	99 ± 3	148 ± 4	197 ± 6
3C4MA	12.6 ± 0.3	63 ± 2	126 ± 3	188 ± 5	251 ± 6
2NA	10.8 ± 0.3	54 ± 1	108 ± 3	162 ± 4	216 ± 6

To each calibration point and sample, 20 µL of the 3er IS - mix, 150 µg/L were added to have a final concentration of 150 ng/L, Table S 3.5.

Table S 3.5. Final concentrations and associated uncertainties for the internal standards used for the calibration curve and the real samples measurements.

Internal Standard	Final concentration (ng/L ± ng/L)
AD5	164 ± 7
1AND7	150 ± 3
4ABPD9	150 ± 3

Laboratory equipment and glassware used

A list of the general instrumentation and glassware used during the experimental part of the thesis can be seen in Table S 3.6. In Table S 3.7 an overview of the volumetric flasks (hereafter abbreviated VF), syringes and pipettes used can be seen, including their nominal volume, uncertainty, and supplier.

Table S 3.6. General laboratory equipment and glassware used.

Instruments	Model	Supplier
Electronic Semi-microbalance	Sartorius research R 160 P	Sartorius AG (Göttingen, DE)
Precision Balance	M-Power AZ3102	Sartorius AG
Incubation/inactivation bath	1086	GFL, Gesellschaft für Labortechnik GmbH (Burgwedel, DE)
Control shaker	KS 260	IKA®-Werke GmbH & Co. KG (Staufen, DE)
Laboratory glassware washer	G7893	Miele Professional (New Jersey, USA)
Beakers	50, 100, 250, 400, 500	Various suppliers
Filter papers	589[2] White ribbon, ashless, ∅ 150 mm	Schleicher & Schuell (Dassel, DE)

Instruments	Model	Supplier
Funnels	OD 80 mm; Long stem	Various suppliers
Separating funnels	50 mL, Squibb, PTFE plug	Lenz Laborglas GmbH & Co.KG (Wertheim, DE)
Disposable glass Pasteur pipettes	230 mm	VWR International GmbH (Darmstadt, DE)
Micro-pipettes	0.01-0.1 and 0.1-1 mL Eppendorf Research plus	Eppendorf Vertrieb Deutschland GmbH (Wesseling-Berzdorf, DE)
	0.5-5 mL Finnpipette	Thermo Fisher Scientific (Braunschweig, DE)
Pipette tips	EpT.I.P.S. standard: 2-200 µL, 50-1000 µL	Eppendorf AG (Hamburg, DE)
	Plastibrand: 5 mL	BRAND (Wertheim, DE)
Polypropylene tubes	Transparent 50 mL PP centrifuge tubes	VWR International GmbH (DE)
Amber bottles	30 mL, thread, DIN 18	CZT (Kriftel, DE)
Screw caps	DIN 18	CZT (DE)
Schott laboratory bottles	50, 100, 250 mL	DURAN Group GmbH (Wertheim/Main, DE)
Amber vials	20 mL ND18 HS, screw 1.5 mL and 4 mL, screw	BGB Analytik AG (Boeckten, Switzerland (CH))
	12.5 mL crimp top HS vials	Unknown (u/k)
Vial caps	ND18 Magnetic screw caps, Silicone/PFTE ND20 Aluminum crimp cap, Butyl/PFTE ND8-425 and ND13-425 screw craps	BGB Analytik AG (CH)

Table S 3.7. Glassware used, including volume, uncertainty, class/model, and supplier.

Instrument	Vol (mL)	Uncer. (mL)*	Class/Model	Supplier
Glass pipettes	2	± 0.01	AS	BRAND (Wertheim, DE)
	5	± 0.015		
	20	± 0.045		
	10	± 0.02	AS	Hirschmann Laborgeräte (Eberstadt, DE)
	20	± 0.03	B	
Volumetric flask	5	± 0.04	A	VWR International (Darmstadt, DE)
	10	± 0.04	-	Various suppliers
	25	± 0.04	A	Fisher Scientific (Pennsylvania, USA)
	50	± 0.08	AW	Hirschmann Laborgeräte (Eberstadt, DE)
	100	± 0.1	A	BRAND
	250	± 0.15	A	DURAN Group (Wertheim/Main, DE)
Glass syringes	0.025	1 %	Pressure-Lok analytical syringes	VICI AG International (Schenkon, CH)
	0.100	1 %		
	0.250	1 %		
	0.010	1 %	Microliter syringe	Hamilton Laboratory Products (Nevada, USA)
	0.025	1 %		
	0.100	1 %		
	0.250	1 %		

*Unless stated otherwise.

Data evaluation – Peak integration

The peak integration parameters used for the data evaluation in the GCMS Post Run Analysis software can be seen in Table S 3.8. The slope, width and minimum area/height were chosen experimentally, so that as many peaks as possible were properly integrated. The standard retention time (std. tr) was set so that there was enough baseline for the integration, but not too much so that more peaks would be integrated and could interfere with the automation process. The minimum similarity index (S.I.) was low because for smaller concentrations, the background noise was relatively high, interfering with the spectra, and since no background subtraction could be done in an automatic way, it would decrease the similarity index of the peak and it would have to be identified manually.

Especially for higher concentrations, 2,4DMA and 2,6DMA could not be integrated individually in a reliable manner, so it was decided to integrate and evaluate them together, as DMA.

Table S 3.8. Settings selected for the quantitative automatic peak integration with the GCMS Post run Analysis software.

Peak Integration		**Identification**	
Parameter	Value	Parameter	Value
Slope	600 /min	Window for target peak	0.1 %
Width	0.1 s	Window for ref. peak	0.1 %
Min. Area/Height	75	Default band time	0.008 min
Base	Area	Correction of t_r	None
Smoothing	None	Spectrum confirmation	Use mass spect. pattern
Processing time: Std t_r ±	0.01 min	Min. S.I.	30

Abbreviations: min. = minimum, ref. = reference, S.I. = similarity index, spect. = spectra, std = standard, t_r = retention time.

Data evaluation – Dixon's Q test

Before calculating average intensities and concentrations, Dixon's Q test was used to find outliers which were then excluded from further calculations. The tested value was defined as an outlier if $Q_{calc} > Q_{crit}$. Q_{calc} was calculated with Equation 1 to test the smallest value, and Equation 2 to test the largest value, and the Q_{crit} values can be seen in Table S 3.9.

$$Q_{calc} = \frac{|x_2 - x_1|}{|x_n - x_1|}$$ Equation 1 [35]

$$Q_{calc} = \frac{|x_n - x_{n-1}|}{|x_n - x_1|}$$ Equation 2 [35]

Where x_1 is the smallest data point, x_2 the second smallest, x_n the largest and x_{n-1} the second largest.

Table S 3.9. Q_{crit}, or critical Q values of Dixon's Q test for a confidence level of 95 % and different sample sizes (n) [23].

Sample size (n)	Q_{crit} 95 %, α = 0.05	Sample size (n)	Q_{crit} 95 %, α = 0.05	Sample size (n)	Q_{crit} 95 %, α = 0.05
3	0.9411	13	0.3615	23	0.2851
4	0.7651	14	0.3496	24	0.2804
5	0.6423	15	0.3389	25	0.2763
6	0.5624	16	0.3293	26	0.2725
7	0.5077	17	0.3208	27	0.2686
8	0.4673	18	0.3135	28	0.2655
9	0.4363	19	0.3068	29	0.2622
10	0.4122	20	0.3005	30	0.2594
11	0.3922	21	0.2947		
12	0.3755	22	0.2895		

Outliers were excluded when calculating the average concentrations/peak areas of the different groups. It was impossible to know if these outliers were physiologically implausible or not since they could not be measured a second time to confirm. However, some of the outliers were up to 5 times the second highest/lower values, which would indicate that, at least for these cases, the outliers were most likely analytical. Therefore, it was decided not to consider them for further calculations.

Data evaluation – Welch's two-sided t-test

To find significant differences between the groups, Welch's two-sided t-test for unequal variances was chosen because of its more conservative nature in comparison with the student t-test [24]. The calculated t-value (Equation 3)[35], t_{calc}, was compared to the tabulated t-value, t_{crit} (Table S 3.10), and a significant difference could be observed when $t_{calc} > t_{crit}$ [35].

$$t_{calc} = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Equation 3 [35]

Where $\bar{x}_i$ is the average concentration/peak area, s_i the variance, and n_i the number of parallel determinations of the sample set or group i.

Table S 3.10. Critical t value, or t_{crit}, of Welch's two-sided t-test for a confidence level of 95 % and different degrees of freedom, df [24].

df	**t_{crit} 95 %, α = 0.05**	**df**	**t_{crit} 95 %, α = 0.05**	**df**	**t_{crit} 95 %, α = 0.05**
2	4.303	15	2.131	28	2.048
3	3.182	16	2.120	29	2.045
4	2.776	17	2.110	30	2.042
5	2.571	18	2.101	31	2.040
6	2.447	19	2.093	32	2.037
7	2.365	20	2.086	33	2.035

df	t_{crit} 95 %, α = 0.05	df	t_{crit} 95 %, α = 0.05	df	t_{crit} 95 %, α = 0.05
8	2.306	21	2.080	34	2.032
9	2.262	22	2.074	35	2.030
10	2.228	23	2.069	36	2.028
11	2.201	24	2.064	37	2.026
12	2.179	25	2.060	38	2.024
13	2.160	26	2.056	39	2.023
14	2.145	27	2.052	40	2.021

The degrees of freedom, df, used for the determination of t_{crit}, were calculated based on Equation 4.

$$df = \frac{\left(\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}\right)^2}{\frac{\left(s_1^2/n_1\right)^2}{n_1 - 1} + \frac{\left(s_2^2/n_2\right)^2}{n_2 - 1}}$$

Equation 4 [35]

3.6.2 Results and discussion

Qualitative analysis

The four exemplary chromatograms shown in Fig. 3.1 were evaluated using the parameters in Table S 3.11.

Table S 3.11. Settings selected for the qualitative automatic peak integration with the GCMS Post run Analysis software.

Parameter	Value	Parameter	Value
Auto	Area	Peak Top Spectrum (# of averaged points)	3
Width	0.04 s	Calculated From Peak (# of averaged points)	1
Min. Area/Height	0	Min. S.I.	70
Smoothing	None		

The objective was to detect as many peaks as possible, so the number of peaks integrated was the maximum possible: 1000. Because the total number of peaks in the chromatograms was higher than the maximum that could be integrated, the chromatograms had to be integrated several times at different time intervals by selecting "integration off" and "integration on" in the "program" option.

The spectra of the integrated peaks were then searched in a customized library, where only iodinated compounds were included. This library was created by searching in the NIST20 (M1) for compounds with a "Formula" including iodine ("I"), and different compound names ("Cmpd Name"). The compound list used as reference to create the library was based on the AA found in [22]. To cover as many compounds as possible, only the first part of the names was used, for example, by searching for "Phen", different compound types, such as "Phenol" or "Phenyl" could be included. The compound names searched, the corresponding total number of hits found, and how many of those already were included in the library or "repeated", is shown in Table S 3.12. It is important to keep in mind that this identification was fully automatic, and it therefore may happen that isomers of the same compound are identified as one compound. Furthermore, since standards of these compounds were not measured, the identification was only tentative.

Table S 3.12. Compound names (Cmpd Name) searched in the NIST20 (M1) together with the criteria "Formula" includes iodine "I", number of hits for each compound, and how many of them were already included in the previous searches (Repeated). The total number of different compounds included in the library is also shown.

Cmpd Name	Hits	Repeated
Benz	864	-
Phen	732	256
Ani	148	134
Tolu	33	28
Xyl	120	47
Naphth	29	10
Thio	89	73
Pyr	472	141
Quin	57	24
Ind	80	39
Total	2624	752
Total compounds in library		1872

The list of compounds found in each of the samples shown in section 3.4.1 is included in Table S 3.13.

Table S 3.13. Tentatively identified aromatic amines found in the samples from a 45 year old never smoker female (45-F-NS), a 47 year old past smoker female (47-F-PS) and a 39 year old smoker female (39-F-S).

Tentatively identified analyte	45-F-NS	47-F-PS	39-F-S
Benzoyl derivative of m-iodoaniline	x	x	x
Benzyloxyiodomethoxybenzonitrile	x	x	x
Bromoiodobenzene	x	x	x

Tentatively identified analyte	45-F-NS	47-F-PS	39-F-S
Chloroiodobenzene	x	x	x
Chloroiodomethylbenzene	x	x	x
Chloroiodophenylsulfonylethanamine	x	x	x
Dichloroiodobenzene	x	x	x
Dihydrooxopyridinyliodobenzenesulfonamide	x		
Diiodobenzene	x	x	x
Ethyliodobenzene		x	x
Iodoanisole		x	x
Iodobenzaldehyde	x	x	x
Iodobenzene	x	x	x
Iodobenzenemethanol	x	x	
Iodobenzonitrile			x
Iodobenzoyldimethylhydrazine			
Iodobiphenyl	x	x	x
Iodocyclohexane	x	x	x
Iododiethoxytetrahydropyran	x	x	x
Iododihydroindole			x
Iododimethylbenzene			x
Iodoethylbenzene	x	x	x
Iodoindazolamine	x	x	x
Iodoisopropylbenzene	x	x	x
Iodomenthylphenyl phosphine	x	x	x
Iodomethoxybutylbenzene	x	x	x
Iodomethoxymethylbenzyloxybenzaldehyde	x		x

Tentatively identified analyte	45-F-NS	47-F-PS	39-F-S
Iodomethoxypropylbenzene	x	x	x
Iodomethyl hydrochloride phenethylamine		x	
Iodomethylbenzene	x	x	x
Iodomethylcyclohexanecarboxylic acid	x	x	x
Iodomethyldihydrobenzofuran	x	x	x
Iodomethylenzene	x	x	x
Iodomethylphenyadamantanecarboxamide	x	x	
Iodomethylphenylnicotinamide		x	
Iodomethylpropenylbenzene			x
Iodomethylpyridine	x	x	x
Iodomethylpyridinedicarbonitrile		x	x
Iodomethylthiophene			x
Iodopentylbenzene		x	
Iodophenol	x	x	
Iodophenylethanone	x	x	x
Iodophenylformamide		x	x
Iodopicolinol	x		
Iodopropenylmethoxybenzylether		x	x
Iodopyrimidinamine	x	x	x
Iodoquinoline	x	x	x
Iodosobenzene	x	x	x
Iodothioanisole	x	x	x
Iodothiophene		x	
Iodotrimethylisopropenylbicyclononane		x	x

Tentatively identified analyte	45-F-NS	47-F-PS	39-F-S
Iodoundecyloxytetrahydropyran	x	x	x
Succinimidyliodoacetate	x	x	x
t-Butyliodohexahydrobenzodioxinone			x
Trimethyliodobenzene	x	x	x
Tetrahydroiodonaphthalene			x
Total	38	45	46

Quantitative analysis

In order to quantify the aromatic amines present in the urine of smokers, past smokers and never smokers, calibration curves (C.C.) were calculated for each analyte. The use of three internal standard calibration approaches with AD5, 1AND7 and 4ABPD9 as the IS was studied. 4ABPD9 gave the best results and was used for further calculations. More research is needed to understand the underperformance of AD5 and 1AND7. In Table S 3.14 the coefficient of determination (r^2), the slope (b) and the intercept (a) of the calibration curves using 4ABPD9 as IS can be seen. The plotted calibration curves can be seen at the end of the SI, in the "Calibration curves" section.

In general, the linearity of the calibration curves calculated was adequate for the later estimation of the concentrations in real samples, with the exception of A and 2,6DCA. Furthermore, the slopes of 2,6DCA and 2AAP were extremely small, therefore, these AA were excluded from the concentration calculations. In a few cases, and especially for A, a non-zero intercept could be observed. This could be due to the resin used in the purification process, as previously described by Weiss and Angerer [45]. Finally, only 9 of the 68 samples had BA concentrations within the calibration range, and since these results could not be considered representative, this compound was also not included.

Table S 3.14. Determination coefficient (r^2), slope (b) and intercept (a) of the calibration curves (y = bx + a) using 4ABPD9 as IS.

Analyte	Determination coefficient, r^2	Slope, b	Intercept, a
2MA	0.940	60.5	13.985
3C4FA	0.984	24.0	0.073
2CA	0.971	3.49	0.752
4EA	0.972	24.6	-1.423
DMA	0.952	97.3	1.469
4C2MA	0.982	11.57	0.633
2BA	0.951	0.286	0.190
2,4,6TMA	0.966	22.6	-0.452
2AAP	0.981	0.030	0.005
2,6DCA	0.494	0.022	0.094
3C2,6DMA	0.978	4.48	0.410
3C4MA	0.986	0.481	-0.015
2NA	0.991	11.0	-0.388
A	0.761	32	74.2

Once the different calibration curves were calculated, the urine samples from smokers, never smokers and past smokers could be analyzed (see Table 3.2). All the integrated peaks were manually checked to make sure that the right peaks were properly integrated. While doing so, it was noticed that there were a few analytes whose intensity was consistently too low: 3C4FA, 3C4MA and 3C2,6DMA, so they were also not included in the calculations.

To evaluate if there were significant differences between the groups (NS, PS and S), Welch's two-sided t-tests for unequal variances was used to compare their concentrations. The results from the test can be seen Table S 3.15. No t-test could be performed for 2MA when the smokers were compared, because there were not enough samples in the group with concentrations of these analytes within the calibration curve.

Table S 3.15. Degrees of freedom (df), calculated and critical values of the t-distribution (t_{calc} and t_{crit}, respectively) used for the calculation of the Welch's two-sided t-test for unequal variances, and calculated p-values based on the t_{calc} obtained. The test was used to compare the final concentrations of aromatic amines in urine, calculated using 4ABPD9 as internal standard. The significantly different results ($\alpha = 0.05$ or $p < 0.05$) are shown in **bold**. The mean, standard deviation, and sample sizes (n) used for the calculations can be seen in Table 3.2.

	Analyte	**df**	**t_{calc}**	**t_{crit}**	**p-value**
NS vs S	2CA	8	0.004	2.36	0.997
	4EA	**10**	**2.920**	**2.228**	**0.015**
	DMA	**9**	**2.910**	**2.262**	**0.017**
	4C2MA	11	2.185	2.228	0.054
	2,4,6TMA	**10**	**2.584**	**2.262**	**0.030**
	2NA	**9**	**3.062**	**2.262**	**0.014**
PS vs S	2CA	9	0.763	2.262	0.465
	4EA	14	1.946	2.145	0.072
	DMA	**12**	**2.481**	**2.201**	**0.030**
	4C2MA	19	1.037	2.093	0.313
	2,4,6TMA	**10**	**2.777**	**2.262**	**0.022**
	2NA	**10**	**2.695**	**2.262**	**0.025**
NS vs PS	2CA	23	1.361	2.074	0.187
	4EA	34	1.391	2.035	0.173
	DMA	39	0.472	2.024	0.639
	4C2MA	34	1.100	2.035	0.279
	2,4,6TMA	50	0.429	2.009	0.670
	2NA	37	1.244	2.026	0.221
	2MA	27	1.416	2.056	0.169

Quantitative screening

Table S 3.16 shows the results from the Welch's two-sided t-tests for unequal variances used to evaluate if the mean peak areas of the different groups were significantly different (shown in Fig. 3.2).

Table S 3.16. Degrees of freedom (df), calculated and critical values of the t-distribution (t_{calc} and t_{crit}, respectively) used for the calculation of the Welch's two-sided t-test for unequal variances, and calculated p-values based on the t_{calc} obtained. The test was used to compare the mean peak areas of aromatic amines in urine, calculated using 4ABPD9 as internal standard. The significantly different results ($\alpha = 0.05$ or $p < 0.05$) are shown in **bold**. The mean, standard deviation, and sample sizes (n) used for the calculations can be seen in Fig. 3.2.

	Analyte	df	t_{calc}	t_{crit}	p-value
NS vs S	**2MA**	**11**	**3.430**	**2.201**	**0.006**
	DMA	**11**	**2.754**	**2.201**	**0.019**
	4C2MA	12	1.161	2.201	0.270
	2CA	23	1.348	2.069	0.191
	2,4,6TMA	**11**	**2.219**	**2.201**	**0.048**
	4EA	**11**	**2.796**	**2.228**	**0.019**
	2NA	**10**	**2.971**	**2.262**	**0.016**
	2AAP	24	2.023	2.069	0.055
	3C2,6DMA	38	0.051	2.026	0.960
	2,6DCA	26	1.073	2.060	0.294
	3C4FA	14	0.354	2.145	0.729
	2BA	12	1.589	2.201	0.140

	Analyte	df	t_{calc}	t_{crit}	p-value
PS vs S	**2MA**	**14**	**2.641**	**2.145**	**0.019**
	DMA	17	1.907	2.120	0.075
	4C2MA	17	0.079	2.120	0.938
	2CA	26	1.758	2.056	0.090
	2,4,6TMA	15	1.548	2.145	0.144
	4EA	**11**	**2.652**	**2.201**	**0.023**
	2NA	17	1.603	2.120	0.128
	2AAP	**35**	**2.614**	**2.032**	**0.013**
	3C2,6DMA	10	2.240	2.262	0.052
	2,6DCA	33	0.997	2.035	0.326
	3C4FA	13	0.616	2.179	0.549
	2BA	36	0.304	2.030	0.763
NS vs PS	2MA	48	1.065	2.011	0.292
	DMA	42	0.943	2.020	0.351
	4C2MA	44	1.550	2.017	0.129
	2CA	26	1.585	2.056	0.125
	2,4,6TMA	43	0.876	2.017	0.386
	4EA	54	0.266	2.006	0.792
	2NA	32	1.699	2.040	0.099
	2AAP	35	1.568	2.032	0.126
	3C2,6DMA	30	1.361	2.042	0.184
	2,6DCA	35	1.610	2.032	0.117
	3C4FA	56	0.356	2.004	0.724
	2BA	30	1.419	2.042	0.166

In Fig. S 3.1, a comparison between male and female samples can be seen. While the results are more similar than when grouped between NS, PS and S, it can be seen that females usually have a narrower distribution and a lower median. There is limited literature available where such a comparison is made. In Grimmer et al. [46], the individual concentrations for each donor are given, so the means and deviations for males and females can be calculated. Three of the four analytes studied show the same trend as observed here. Interestingly, Chinthakindi and Kannan [16] observed that the median concentrations were higher in females. However, their number of samples was significantly lower, comprising 7 male and 8 female donors.

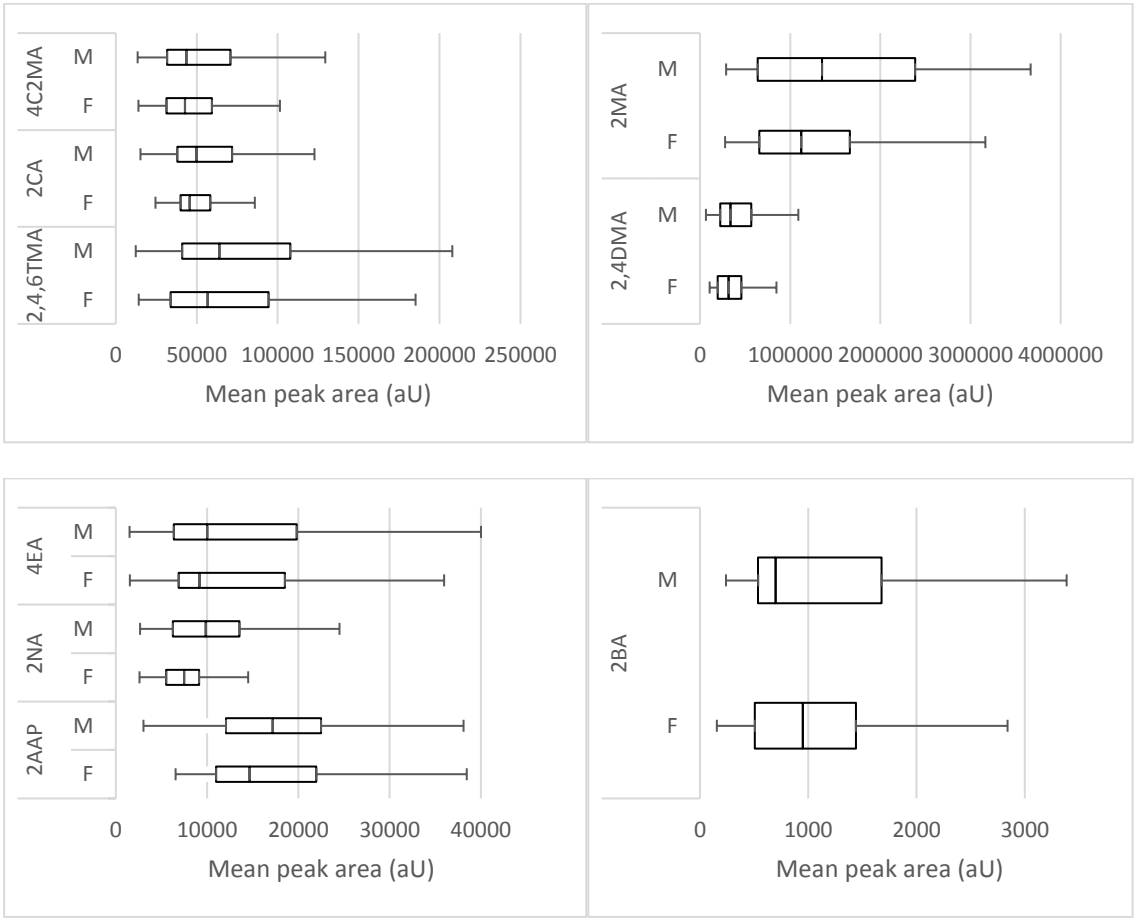
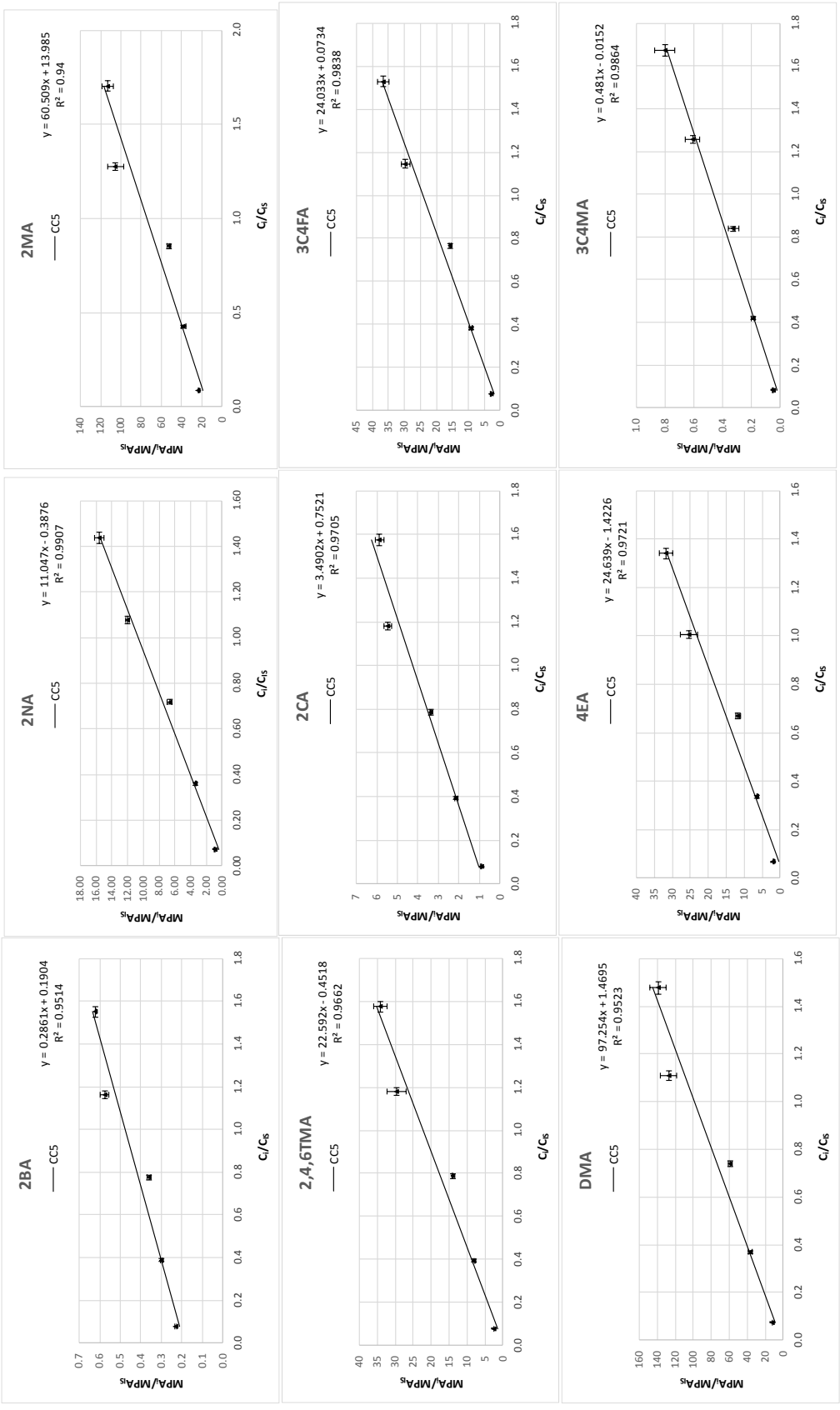
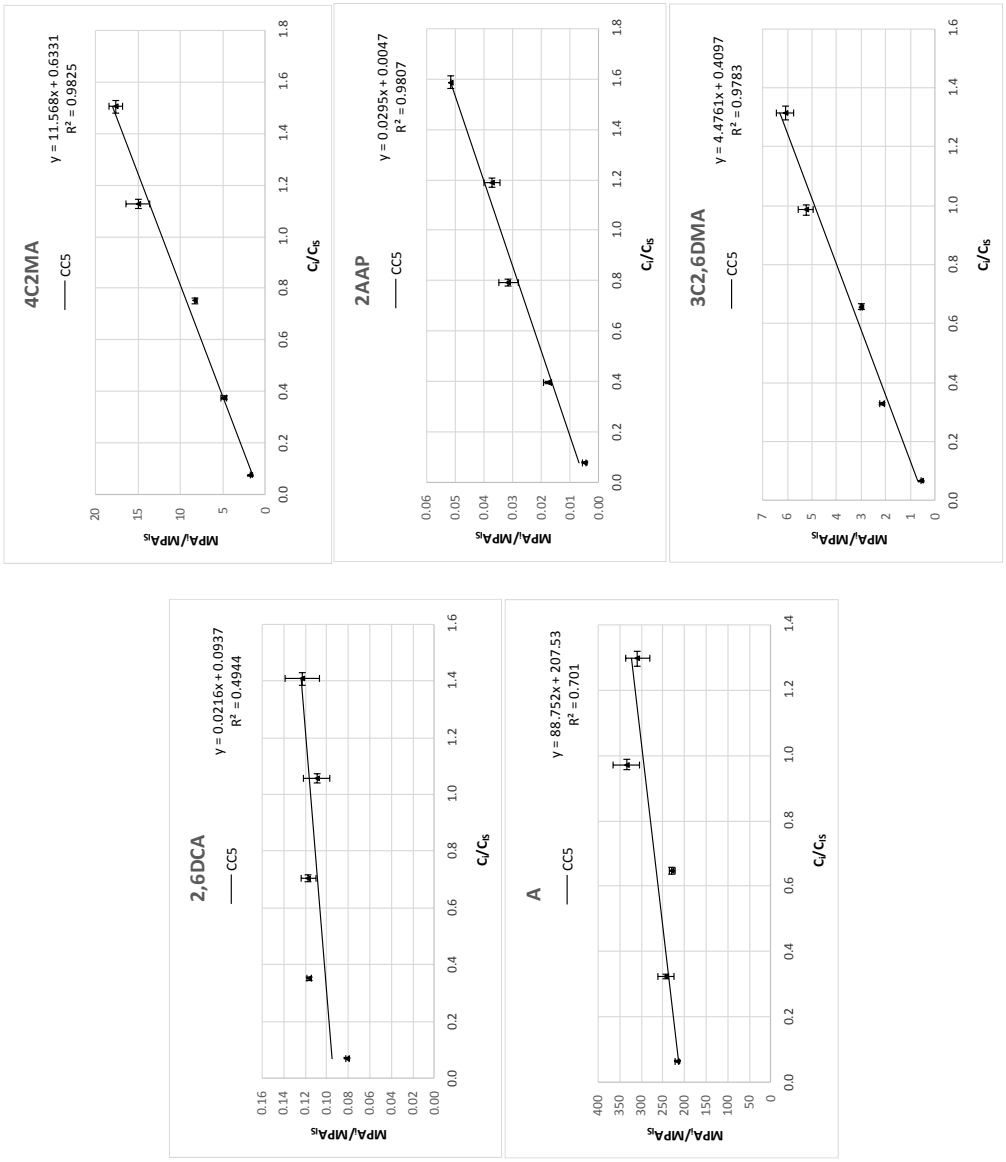


Fig. S 3.1. Tukey box plots for nine aromatic amines in urine samples of females (F, n = 32) and males (M, n = 36).

Calibration Curves





3.7 References

1. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Chemical Agents and Related Occupations. Lyon, France: International Agency for Research on Cancer; 2012.

2. Seyler T, Mazumder S, Ahamed R, Zhu W, Blount BC, Apelberg BJ, Wang L. Tobacco Smoke Is a Major Source of Aromatic Amine Exposure in U.S. Adults: 2013–2014 National Health and Nutrition Examination Survey (NHANES). Cancer Epidemiol Biomarkers Prev. 2023:OF1-OF9. https://doi.org/10.1158/1055-9965.epi-23-0071.

3. Akyüz M, Ata Ş. Determination of aromatic amines in hair dye and henna samples by ion-pair extraction and gas chromatography–mass spectrometry. J Pharm Biomed Anal. 2008;47:68-80. https://doi.org/10.1016/j.jpba.2007.12.011.

4. Chiang TA, Pei-Fen W, Ying LS, Wang LF, Ko YC. Mutagenicity and aromatic amine content of fumes from heated cooking oils produced in Taiwan. Food Chem Toxicol. 1999;37:125-34. https://doi.org/10.1016/S0278-6915(98)00081-7.

5. Garrigos MC, Reche F, Pernias K, Jimenez A. Optimization of parameters for the analysis of aromatic amines in finger-paints. J Chromatogr A. 2000;896:291-8. https://doi.org/10.1016/S0021-9673(00)00742-1.

6. Garrigos MC, Reche F, Marin ML, Jimenez A. Determination of aromatic amines formed from azo colorants in toy products. J Chromatogr A. 2002;976:309-17. https://doi.org/10.1016/S0021-9673(02)01162-7.

7. Alston F, Okorie O. Occupational exposures: chemical carcinogens and mutagens. 1st ed. Boca Raton: CRC Press; 2023.

8. Chamot S, Delzard M, Leroy L, Marhic G, Ganry O. Occupational exposures in the rubber tire industry and risk of cancer: a systematic review. Int Arch Occup Environ Health. 2023. https://doi.org/10.1007/s00420-023-01972-x.

9. Ciocan C, Godono A, Franco N, La Vecchia C, Negri E, Boffetta P, Pira E. Mortality from bladder cancer in dyestuff workers exposed to aromatic amines: A 73-year follow-up. La Medicina del lavoro. 2022;113:e2022017. https://doi.org/10.23749/mdl.v113i2.12893.

10. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Tobacco smoke and involuntary smoking. Lyon, France: International Agency for Research on Cancer; 2004.

11. Besaratinia A, Tommasi S. Genotoxicity of tobacco smoke-derived aromatic amines and bladder cancer: Current state of knowledge and future research directions. FASEB J. 2013;27:2090-100. https://doi.org/10.1096/fj.12-227074.

12. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Some Aromatic Amines, Organic Dyes, and Related Exposures. Lyon, France: International Agency for Research on Cancer; 2008.

13. Wang S, Hanna D, Sugamori KS, Grant DM. Primary aromatic amines and cancer: Novel mechanistic insights using 4-aminobiphenyl as a model carcinogen. Pharmacol Ther. 2019;200:179-89. https://doi.org/10.1016/j.pharmthera.2019.05.004.

14. Talaska G, Al-Zoughool M. Aromatic Amines and Biomarkers of Human Exposure. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2003;21:133-64. https://doi.org/10.1081/GNC-120026234.

15. Schmidt TC, Less M, Haas R, von Löw E, Steinbach K, Stork G. Gas chromatographic determination of aromatic amines in water samples after solid-phase extraction and derivatization with iodine. I. Derivatization. J Chromatogr A. 1998;810:161-72. https://doi.org/10.1016/S0021-9673(98)00233-7.

16. Chinthakindi S, Kannan K. Variability in urinary concentrations of primary aromatic amines. Sci Total Environ. 2022;831:154768. https://doi.org/10.1016/j.scitotenv.2022.154768.

17. Yu J, Wang S, Zhao G, Wang B, Ding L, Zhang X, Xie J, Xie F. Determination of urinary aromatic amines in smokers and nonsmokers using a MIPs-SPE coupled with LC-MS/MS method. J Chromatogr B. 2014;958:130-5. https://doi.org/10.1016/j.jchromb.2014.03.023.

18. Kataoka H. Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. J Chromatogr A. 1996;733:19-34. https://doi.org/10.1016/0021-9673(95)00726-1.

19. Dallüge J, Beens J, Brinkman UAT. Comprehensive two-dimensional gas chromatography: a powerful and versatile analytical tool. J Chromatogr A. 2003;1000:69-108. https://doi.org/10.1016/S0021-9673(03)00242-5.

20. Górecki T, Harynuk J, Panić O. The evolution of comprehensive two-dimensional gas chromatography (GC×GC). J Sep Sci. 2004;27:359-79. https://doi.org/10.1002/jssc.200301650.

21. Hovanec J, Weiß T, Koch HM, Pesch B, Behrens T, Kendzia B, Arendt M, Dragano N, Moebus S, Schmidt B, Brüning T, Jöckel K-H. Smoking intensity and urinary nicotine metabolites by socioeconomic status in the Heinz Nixdorf Recall study. BMC Public Health. 2022;22:302. https://doi.org/10.1186/s12889-022-12609-y.

22. Lamani X, Horst S, Zimmermann T, Schmidt TC. Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GCxGC-qMS). Anal Bioanal Chem. 2015;407:241-52. https://doi.org/10.1007/s00216-014-8080-5.

23. Rorabacher DB. Statistical Treatment for Rejection of Deviant Values: Critical Values of Dixon's "Q" Parameter and Related Subrange Ratios at the 95% Confidence Level. Anal Chem. 1991;63:139-46. https://doi.org/10.1021/ac00002a010.

24. Quinn GP, Keough MJ. Experimental Design and Data Analysis for Biologists. 2nd ed: Cambridge University Press; 2002.

25. Lorenzo-Parodi N, Leitner E, Schmidt TC. Comparison of gas chromatographic techniques for the analysis of iodinated derivatives of aromatic amines. Anal Bioanal Chem. 2023;415:3313-25. https://doi.org/10.1007/s00216-023-04713-8.

26. Addis T, Watanabe C. The volume of urine in young healthy adults on a constant diet. J Biol Chem. 1916; 27:267-72. https://doi.org/10.1016/S0021-9258(18)86912-5.

27. Kütting B, Göen T, Schwegler U, Fromme H, Uter W, Angerer J, Drexler H. Monoarylamines in the general population – A cross-sectional population-based study including 1004 Bavarian subjects. Int J Hyg Environ Health. 2009;212:298-309. https://doi.org/10.1016/j.ijheh.2008.07.004.

28. Riedel K, Scherer G, Engl J, Hagedorn H-W, Tricker AR. Determination of three carcinogenic aromatic amines in urine of smokers and nonsmokers. J Anal Toxicol. 2006;30:187-95. https://doi.org/10.1093/jat/30.3.187.

29. Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

30. El-Bayoumy K, Donahue JM, Hecht SS, Hoffmann D. Identification and quantitative determination of aniline and toluidines in human urine. Cancer Res. 1986;46:6064-7.

31. Fuller TW, Acharya AP, Meyyappan T, Yu M, Bhaskar G, Little SR, Tarin TV. Comparison of Bladder Carcinogens in the Urine of E-cigarette Users Versus Non E-cigarette Using Controls. Sci Rep. 2018;8:1-6. https://doi.org/10.1038/s41598-017-19030-1.

32. Tukey JW. Exploratory data analysis. Massachusetts, USA: Addison-Wesley; 1977. 688 p.

33. Hecht SS. Human urinary carcinogen metabolites: Biomarkers for investigating tobacco and cancer. Carcinogenesis. 2002;23:907-22. https://doi.org/10.1093/carcin/23.6.907.

34. Modick H, Weiss T, Dierkes G, Koslitz S, Käfferlein HU, Brüning T, Koch HM. Human metabolism and excretion kinetics of aniline after a single oral dose. Arch Toxicol. 2016;90:1325-33. https://doi.org/10.1007/s00204-015-1566-x.

35. Otto M. Chemometrics. Statistics and Computer Application in Analytical Chemistry. 3 ed. Weinheim, Germany: Wiley-VCH; 2016. 400 p.

36. Kotandeniya D, Carmella SG, Ming X, Murphy SE, Hecht SS. Combined analysis of the tobacco metabolites cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in human urine. Anal Chem. 2015;87:1514-7. https://doi.org/10.1021/ac504047j.

37. Benowitz NL. Biomarkers of environmental tobacco smoke exposure. Environ Health Perspect. 1999;107 Suppl 2:349-55. https://doi.org/10.1289/ehp.99107s2349.

38. Benowitz NL. Cotinine as a biomarker of environmental tobacco smoke exposure. Epidemiol Rev. 1996;18:188-204. https://doi.org/10.1093/oxfordjournals.epirev.a017925

39. Ozcan-Sinir G, Copur OU, Barringer SA. Botanical and geographical origin of Turkish honeys by selected-ion flow-tube mass spectrometry and chemometrics. J Sci Food Agric. 2020;100:2198-207. https://doi.org/10.1002/jsfa.10244.

40. Bonometti E, Giordana A, Operti L, Rabezzana R, Turco F, Volpi G. Characterization of unifloral Italian (Piedmont region) honeys by headspace solid phase microextraction coupled to gas chromatography–mass spectrometry. JSFA reports. 2022;2:341-50. https://doi.org/10.1002/jsf2.67.

41. Rohleder AR, Scherf KA, Schieberle P, Koehler P. Quantitative Analyses of Key Odorants and Their Precursors Reveal Differences in the Aroma of Gluten-Free Rice Bread and Wheat Bread. J Agric Food Chem. 2019;67:11179-86. https://doi.org/10.1021/acs.jafc.9b04800.

42. Roman T, Nardin T, Trenti G, Barnaba C, Nicolini G, Larcher R. Press Fractioning of Grape Juice: A First Step to Manage Potential Atypical Aging Development during Winemaking. American Journal of Enology and Viticulture. 2020;71:17-25. https://doi.org/10.5344/ajev.2019.19030.

43. Lorenzo-Parodi N, Kaziur-Cegla W, Schmidt TC. Automation and optimization of the sample preparation of aromatic amines for their analysis with GC–MS. Green Anal Chem. 2023;6:100071. https://doi.org/10.1016/j.greeac.2023.100071.

44. Meier PC, Zünd RE. Statistical Methods in Analytical Chemistry. 2nd ed. New York, USA: John Wiley & Sons, Inc.; 2005.

45. Weiss T, Angerer J. Simultaneous determination of various aromatic amines and metabolites of aromatic nitro compounds in urine for low level exposure using gas chromatography-mass spectrometry. J Chromatogr B. 2002;778:179-92. https://doi.org/10.1016/S0378-4347(01)00542-4.

46. Grimmer G, Dettbarn G, Seidel A, Jacob J. Detection of carcinogenic aromatic amines in the urine of non-smokers. Sci Total Environ. 2000;247:81-90. https://doi.org/10.1016/S0048-9697(99)00471-4.

Chapter 4. Liquid phase microextraction of aromatic amines: hollow fiber-liquid phase microextraction and parallel artificial liquid membrane extraction comparison

This chapter was adapted from: Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

4.1 Abstract

Aromatic amines (AA) are carcinogenic compounds that can enter the human body through many sources, one of the most important being tobacco smoke. They are excreted with urine, from which they can be extracted and measured. To that end, Hollow Fiber – Liquid Phase Microextraction (HF-LPME) and Parallel Artificial Liquid Membrane Extraction (PALME) were optimized for the analysis of representative AA, as alternatives to Liquid-Liquid Extraction (LLE). Relevant extraction parameters, namely organic solvent, extraction time, agitation speed and acceptor solution pH, were studied, and the two optimized techniques -HF-LPME: dihexyl ether, 45 min, 250 rpm and pH 1; PALME: undecane, 20 min, 250 rpm and pH 1- were compared.

Comparison of the optimized methods showed that significantly higher recoveries could be obtained with PALME than HF-LPME. Therefore, PALME was further validated. The results were successful for nine different AA, with regression coefficients (R^2) of at least 0.991, limits of detection (LOD) of 45-75 ng/L, and repeatability and peak area relative standard deviations (RSD) below 20 %. Furthermore, two urine samples from smokers were measured as proof of concept, and 2-methylaniline was successfully quantified in one of them.

These results show that PALME is a great green alternative to LLE. Not only does it use much smaller volumes of toxic organic solvents, and sample –enabling the study of samples with limited available volumes- but it is also less time consuming and labor intensive, and it can be automated.

4.2 Introduction

Aromatic amines (AA) are highly toxic compounds, some of which are officially classified as carcinogenic [1]. They are used in several industries, such as during the manufacture of pharmaceuticals, pesticides, dyes, rubber, or resins [2]. Another important source of exposure to humans is tobacco smoke [3]. When the smoke is inhaled, aromatic amines enter the bloodstream and are transported through the body and metabolized until they reach the bladder. There, their metabolites can either be excreted with the urine or form DNA and protein adducts that can induce bladder cancer [2, 4, 5].

Urine samples, like most biological samples, require thorough sample preparation prior to their analysis in order to minimize potential interferences with matrix compounds, such as proteins, peptides or salts, present in the samples [6]. The most commonly used clean-up technique for the extraction of compounds from aqueous samples is Liquid-Liquid Extraction (LLE) [7]. However, LLE presents several disadvantages, such as being time consuming and labor intensive, and therefore, prone to human errors. Furthermore, it typically uses high amounts of organic solvents, which are often highly toxic, and needs relatively large sample volumes, which is especially critical in situations where sample volume is limited, like with archived urine samples. To overcome these drawbacks, two Liquid-Phase Microextraction (LPME) techniques were evaluated as alternatives: Hollow Fiber-LPME (HF-LPME) and Parallel Artificial Liquid Membrane Extraction (PALME).

HF-LPME was developed in 1999 by Pedersen-Bjergaard and Rasmussen [8, 9] and Parallel Artificial Liquid Membrane Extraction (PALME) was first introduced in 2013 by Gjelstad et al. [10] as an alternative to HF-LPME. Both methods are based on creating a supported liquid membrane (SLM) that aids on the extraction process [9]. SLM-based techniques, like HF-LPME and PALME, offer a green alternative to LLE thanks to the much smaller volumes of organic solvent needed, which contributes to reducing the costs and the environmental footprint per sample. They have a simpler workflow than LLE, not only for two-phase extractions, where the acceptor solution is the same organic solvent used for the SLM, but especially for three-phase extractions, where it is an aqueous solution, enabling the extraction into an organic solvent and back extraction into an aqueous solution to be carried out simultaneously. Moreover, they typically extract less matrix interferences thanks to the extra physical barrier, i.e., the solvent-filled porous membrane. Furthermore, the use of disposable

fibers/well plates eliminates the possibility of carry-over and the need for cleaning/regeneration [9]. These characteristics make SLM-based techniques especially suitable for complex biological samples, like blood or urine.

HF-LPME offers a high flexibility regarding the donor and acceptor volumes, and therefore on the enrichment factors observed [9]. In recent years, automation has become one of the trends of current research regarding LPME, leading to several successful automation attempts, as summarized by [11]. Unfortunately, there are no commercially available fibers for HF-LPME yet [9].

There is commercially available equipment suitable for PALME, which facilitates a semi or fully automatic extraction and a successful validation. At the same time, it limits the range of sample volumes that can be used. Because the membranes available are made of polyvinylidene fluoride (PVDF) instead of the more inert polypropylene used for the fibers, non-specific binding for some substances can be observed, leading to non-linear calibration curves [12]. Less time is needed to set-up the extraction and more samples can be processed simultaneously, making it more user-friendly and enabling higher sample throughputs [9].

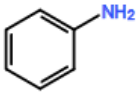
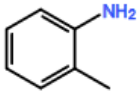
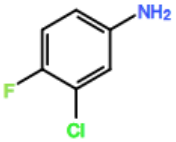
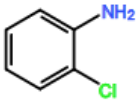
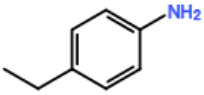
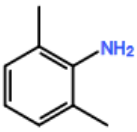
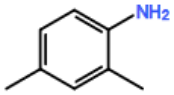
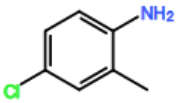
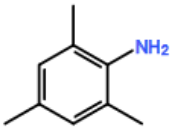
The aim of this chapter was to study the suitability of HF-LPME and PALME for the analysis of aromatic amines in urine. This is, to the best of our knowledge, the first time in which PALME was used for these analytes and the two LPME techniques were compared with each other.

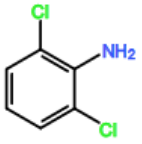
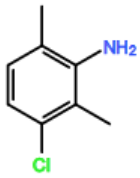
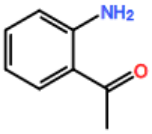
4.3 Materials and methods

4.3.1 Chemicals and reagents

The aromatic amines used (Table 4.1) were purchased from Sigma-Aldrich (Steinheim, Germany), except for 4-chloro-2-methylaniline and 3-chloro-2,6-dimethylaniline which were purchased from Fluka (Darmstadt, Germany) and Alfa Aesar (Karlsruhe, Germany), respectively.

Table 4.1. List of aromatic amines used, with their abbreviation, structure, CAS-number, pKa value of the corresponding anilinium ion, log P-value for the neutral compound, and purity.

Compound	Abbreviation	Structure	CAS-number	pKa [13]	Log P [14]	Purity
Aniline	A		62-53-3	4.63	0.9	≥ 99.5 %
2-Methylaniline	2MA		95-53-4	4.44	1.3	≥ 99.0 %
3-Chloro-4-fluoroaniline	3C4FA		367-21-5	3.60	2.1	98.0 %
2-Chloroaniline	2CA		95-51-2	2.65	1.9	≥ 99.5 %
4-Ethylaniline	4EA		589-16-2	5.00	2.0	98.0 %
2,6-Dimethylaniline	2,6DMA		87-62-7	3.89	1.8	99.0 %
2,4-Dimethylaniline	2,4DMA		95-68-1	4.89	1.7	≥ 99.0 %
4-Chloro-2-methylaniline	4C2MA		95-69-2	3.38	1.9	≥ 98.0 %
2-Bromoaniline	2BA		615-36-1	2.53	2.1	98.0 %
2,4,6-Trimethylaniline	2,4,6TMA		88-05-1	4.38	2.3	98.0 %

Compound	Abbreviation	Structure	CAS-number	pKa [13]	Log P [14]	Purity
2,6-Dichloroaniline	2,6DCA		608-31-1	0.71	2.8	98.0 %
3-Chloro-2,6-dimethylaniline	3C2,6DMA		26829-77-6	3.25	2.6	99.0 %
2-Aminoacetophenone	2AAP		551-93-9	2.3	1.6	98.0 %

For the LPME optimization, the solvents dodecyl acetate (97 %, abbreviated DDA), undecane (≥ 99 %, UD) and dihexylether (97 %, DHE) were purchased from Sigma Aldrich, and 2-octanone (98 %, 2O) was purchased from Alfa Aesar. Concentrated hydrochloric acid (ACS reagent, 37 %, HCl) and sodium hydroxide (98 %, NaOH) were purchased from Bernd Kraft (Duisburg, Germany).

During the derivatization, hydriodic acid (ACS reagent, unstabilized, 55 %), sodium nitrite (99 %) and alizarin red S (98 %) obtained from Sigma-Aldrich, and sodium sulfite (puriss. p.a., ACS reagent, RT, ≥ 98 %) and sulfamic acid (T, ≥ 99 %) from Fluka were used.

Diethyl ether (DEE) and HPLC grade methanol were purchased from Fisher Scientific (Schwerte, Germany), and ultrapure water was obtained from a PureLab Ultra water system from ELGA LabWater (Celle, Germany).

4.3.2 Preparation of stock and standard solutions

Stock solutions were prepared for each aromatic amine studied, by weighing 10 mg of the pure substance in a 10-mL volumetric flask and diluting with methanol to a final concentration of 1 g/L. An intermediate stock containing 50 mg/L of each analyte (standard

mix) was prepared monthly and further diluted in methanol to 2 mg/L and 0.2 mg/L. The solutions were kept refrigerated at 8 °C while not in use.

4.3.3 Sample preparation

In order to achieve the same theoretical acceptor concentration, to minimize the influence of other factors on the results and facilitate the comparison between the techniques, donor concentrations of 250 µg/L and 1 mg/L were used for the HF-LPME and PALME optimization experiments, respectively. The donor solutions were prepared by spiking 10 mM NaOH (pH = 12) with the 50 mg/L standard mix.

Because of the significantly better performance of PALME, only this technique was further used for validation experiments. For the calibration curve experiments, samples with concentrations from 100 to 1200 ng/L in ultrapure water, which were alkalized with NaOH until pH 13.5, were used. As a proof of concept, two real samples from donors (smokers) were measured.

The samples were derivatized and analyzed with solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS), and the performance of the system was checked by measuring control samples on a weekly basis. Samples with a concentration of 2.5 mg/L aromatic amines and pH 2 were prepared, and aliquots of 100 µL were derivatized and analyzed with SPME-GC-MS.

Extraction

The set-ups used and described in the following sections can be seen in Fig. 4.1.

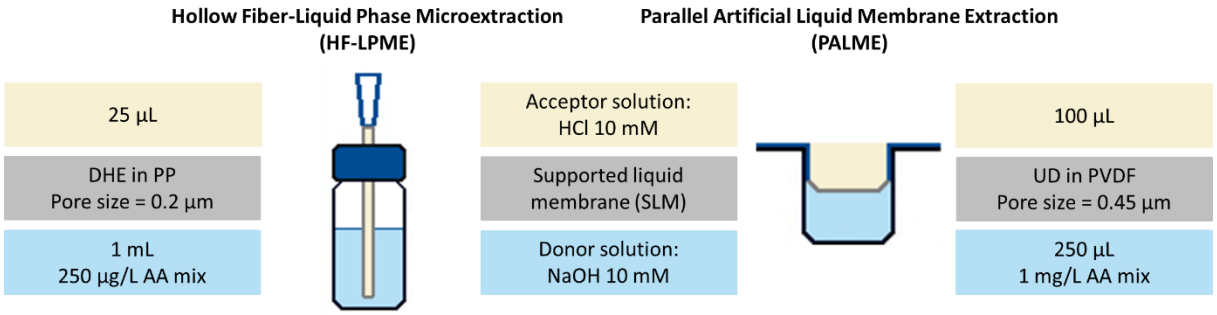


Fig. 4.1. Schematic representation of the HF-LPME and PALME (one well) set-ups, including conditions used in this chapter. Abbreviations: AA: aromatic amines, DHE: dihexylether, HCl: hydrochloric acid, NaOH: sodium hydroxide, PP: polypropylene, PVDF: Polyvinylidene fluoride, UD: undecane.

HF-LPME set-up and procedure

The fiber used was a Q3/2 polypropylene membrane from Membrana (Wuppertal, Germany), with a pore size of 0.2 µm, an internal diameter of 1200 µm and a wall thickness of 200 µm. The HF-LPME setup was prepared following Gjelstad et al. [15]. The fiber was cut into 2 cm long pieces, one end was sealed together with pliers and the other one was fixed to an approximately 2 cm piece of a Finntip 200 Ext pipette tip (Sigma-Aldrich) using a soldering iron (supplementary information, SI, Fig. S 4.1). The HF was then placed, through the lid's septa (Fig. S 4.2), into a 2-mL vial containing the organic solvent for 3-5 s, in order to condition the fiber walls. Afterwards, 25 µL of the acceptor solution was added with a microsyringe (Hamilton Robotics, Bonaduz, Switzerland) into the lumen of the HF, using the pipette tip in the HF as a needle guide. Finally, the HF was placed into a vial containing 1.0 mL of the donor solution.

The vial with the extraction set up was shaken using a KS 260 control shaker (IKA®-Werke GmbH & Co. KG, Staufen, Germany). After a set extraction time, the hollow fiber was directly removed from the donor solution. The acceptor solution was then carefully transferred into a 10-mL amber glass vial with a microsyringe. When multiple samples were extracted, first, all the fibers were taken out of the donor solution and placed into empty vials, and then, the acceptor solutions were collected.

PALME set-up and procedure

96-well plates with 0.5 mL or 1.25 mL wells from Agilent (California, USA) were used as the donor plate and 96-well multiscreen-IP filter plates with polyvinylidene fluoride (PVDF) membranes, a pore size of 0.45 µm and thickness of 100 µm from Merck (Darmstadt, Germany) were used as the acceptor plate.

The membrane was conditioned with 5 µL of organic solvent using a pipette (Eppendorf, Wesseling, Germany, Fig. S 4.3). Then, 250 µL or 1 mL of the donor solution was added into the donor plate and 100 µL of the acceptor solution was pipetted in the well of the membrane-plate and sealed with a multi plate sealing film (HS-300, Axygen Scientific, USA). Both plates were then clamped together and closed with the lid from the acceptor plate. The system was shaken using a KS 260 control shaker for a defined time, after which the plates were separated, and the acceptor solution was collected with a microsyringe and transferred into a 10-mL amber glass vial.

Optimization experiments

A one-factor-at-a-time optimization approach was followed: after each parameter was optimized, the value that provided the best results was used for the subsequent optimizations. First, different organic solvents (DDA, 2O, UD, and DHE) were tested with both techniques. During those experiments the rest of the parameters were kept constant: the samples (or donor solutions) were extracted for 45 min at 250 rpm with a 10 mM HCl (pH = 2) acceptor solution. Agitation speeds of 150, 250 and 350 rpm, extraction times of 15, 30, 45, 60, 75 min and acceptor solutions with pH values of 1, 2, 3 and 4 were studied both with HF-LPME and PALME. A further optimization experiment using PALME was performed by testing 15, 20, 25 and 30 min, and 500 rpm in order to more precisely define the optimal extraction time and speed. HF-LPME was not studied further due to the outstanding capabilities of PALME in comparison. All measurements were done in triplicate.

Derivatization

Prior to derivatization, acceptor solution was added to the extracted sample until a final volume of 100 µL was reached to have constant starting volumes for derivatization and ensure comparability between samples.

The samples were derivatized following a procedure based on a diazotization and subsequent iodination reactions [16]. Into 100 µL of the extracted sample, 100 µL hydriodic acid (55 %) and 200 µL sodium nitrite (50 g/L) were added and the samples were shaken for 20 min at 300 rpm, transforming the amine group of the aromatic amines into diazonium ions. To destroy the surplus of nitrite, 500 µL of sulfamic acid (50 g/L) was added, shaking subsequently for 45 min at 300 rpm. The samples were then heated in a water bath at 95 °C for 5 min to facilitate the substitution of the diazo group by iodine. To reduce the surplus of iodine, 250 µL of sodium sulfite (120 g/L) was added to the cooled down sample, which triggered an immediate discoloration of the initially brownish solution. Finally, 100 µL of alizarin red S (1 % w/v) and 92 µL NaOH (10 M) were added to the samples to adjust the pH of the sample to 5.

The samples used for the optimization tests were derivatized automatically thanks to the PAL RTC from CTC Analytics AG (Zwingen, Switzerland). A few modifications were done to the procedure, such as vortexing the reagents before addition, and the samples after reagent addition. For the method validation experiments, the derivatization was done manually due to the increased throughput needed, since with the PAL RTC only six samples could be derivatized at the same time due to the six positions available in the agitator.

Solid-phase microextraction

To enrich the iodinated derivatives before measuring, a DVB/PDMS SPME fiber with a thickness of 110 µm and a length of 10 mm from BGB Analytik Vertrieb GmbH (Rheinfelden, Germany) was used, in combination with an IP deactivated SPME liner from Restek (Bad Homburg, DE).

The samples were pre-incubated at 60 °C for 10 min under agitation at 500 rpm, while the fiber was being conditioned in the SPME conditioning station for 8 min at 230 ºC. The SPME fiber was then injected into the headspace of the vial (still at 60 ºC and under agitation) for 25 min. Afterwards, the extracted analytes were desorbed into the GC-injection port for 5 min.

4.3.4 Instrumentation

All analyses were performed by a Shimadzu GC-MS system consisting of a GC-2010 Plus gas chromatograph coupled to a GCMS-QP2010 Ultra mass spectrometer from Shimadzu GmbH (Duisburg, Germany). Control of the GC-MS system was done with the GCMS Real Time Analysis software, Shimadzu GmbH. The system was connected to a PAL RTC autosampler, which was controlled with Chronos from Axel-Semrau (Sprockhövel, Germany). Separation of the analytes was achieved with a Rxi-5MS column (30 m, ID: 0.25 mm, film thickness, df: 0.25 μm) from Restek. The septa used throughout all the experiments were AG3-Shimadzu septa from Macherey-Nagel (Düren, DE).

The temperature of the injection port was set to 230 ºC. Helium (99.999 % from Air Liquide, Krefeld, Germany) was used as the carrier gas with a constant column flow of 1 mL/min and a purge flow of 3 mL/min. The linear velocity was selected as flow control mode, and was set to 36.1 cm/s. The instrument was operated in splitless mode with a sampling time of 5 min and using a split ratio of 10:1 afterwards. The initial oven temperature of 40 °C was held for 1 min, then increased to 230 °C with a 10 °C/min rate, and subsequently held for another minute, adding to 21 min total run time. At the starting temperature, and with the parameters aforementioned, the column head pressure was 49.7 kPa.

The MS interface and the ion source temperature were set to 230 °C. The solvent cut time was 5 min and the detector voltage was 1 kV. Full scan mode was used, and in order to achieve a better sensitivity, only the mass-to-charge ratios between m/z 74 and 470 were studied, using a scanning speed of 10000 amu/s. The data were processed with the GCMS Post Run Analysis software from Shimadzu GmbH and evaluated using Excel (Microsoft).

4.3.5 Data evaluation

The m/z ratios used as reference ions and those used for quantitation can be seen in the SI (Table S 4.1). The peaks were automatically integrated with the GCMS Post Run Analysis software (Table S 4.2). All peaks were visually checked for correctness and adjusted if necessary. Outliers were detected using the Dixon test [17]. To check for statistical differences between sample sets Welch's two-sided t-test or the two-variable t-test was used, depending on whether the variances of the two data sets were significantly different or not, which in turn was

determined with Fisher's F-test [17]. Recoveries for HF-LPME and PALME were based on the weekly control samples (2.5 mg/L aromatic amine mix) and calculated according to Gjelstad et al. [18]. More information, including the equations used, can be found in the SI.

The calibration curve and the limits of detection (LODs) and of quantification (LOQs) were calculated according to the DIN 32645 [19]. The repeatability (or intra-day precision) was calculated based on triplicates of the lower calibration point (100 ng/L) and based on the Eurachem Guide [20].

4.4 Results and discussion

Thirteen AA were selected as model analytes due to their diverse chemical and physical properties, such as hydrophobicity (Log P) and pKa values. Furthermore, most of them have either been found in urine samples [3] and/or were successfully extracted by LPME from aqueous samples, such as industrial, environmental and surface/tap water [21-25].

4.4.1 Organic solvent optimization

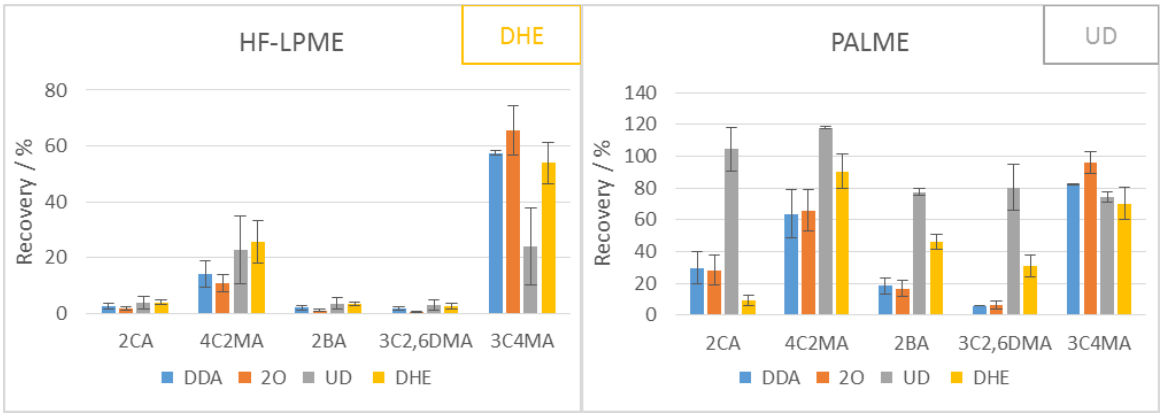
HF-LPME

Four organic solvents (DDA, DHE, 2O and UD) were selected based on literature [15, 21, 25], in order to study the influence of the organic solvent forming the SLM in the extraction process.

There was no solvent that consistently outperformed or underperformed in terms of recovery across the different analytes studied (Fig. 4.2, Fig. S 4.4). For example, 2O showed the best and worst recoveries for five and six of the analytes studied, respectively. DHE showed the best extraction efficiencies for four of the studied analytes and showed good extraction efficiencies for the rest, which translated into a significantly higher geometric mean of the recoveries among the organic solvents tested. Therefore, it was the solvent used for further experiments.

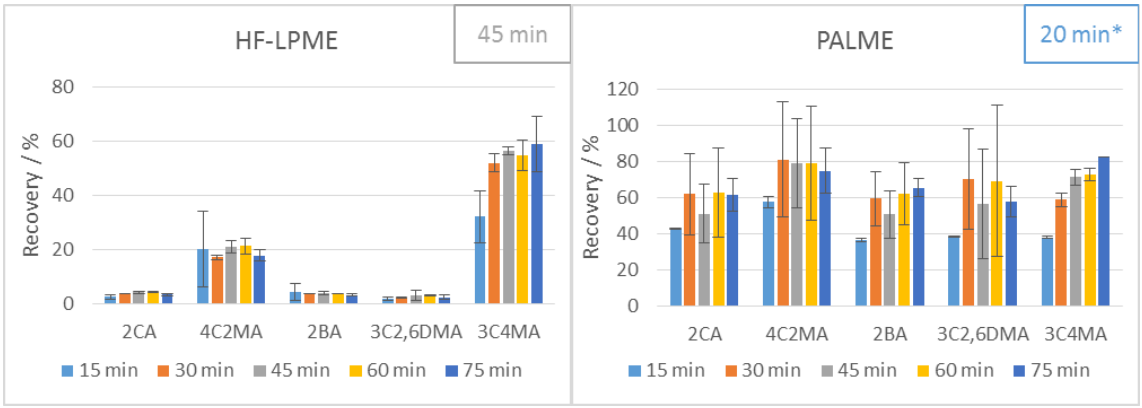
There are a few studies in which aromatic amines were analyzed with different set-ups of HF-LPME (Table 4.2) and different solvents. In agreement with the results shown here, DHE was chosen as optimal solvent in the two papers in which it was tested [21, 25].

Organic solvent optimization



DDA = Dodecyl acetate 2O = 2-Octanol UD = Undecane DHE = Dihexyl ether

Extraction time optimization



*Optimization from 15 min to 30 min in 5 min steps not shown

Acceptor pH optimization

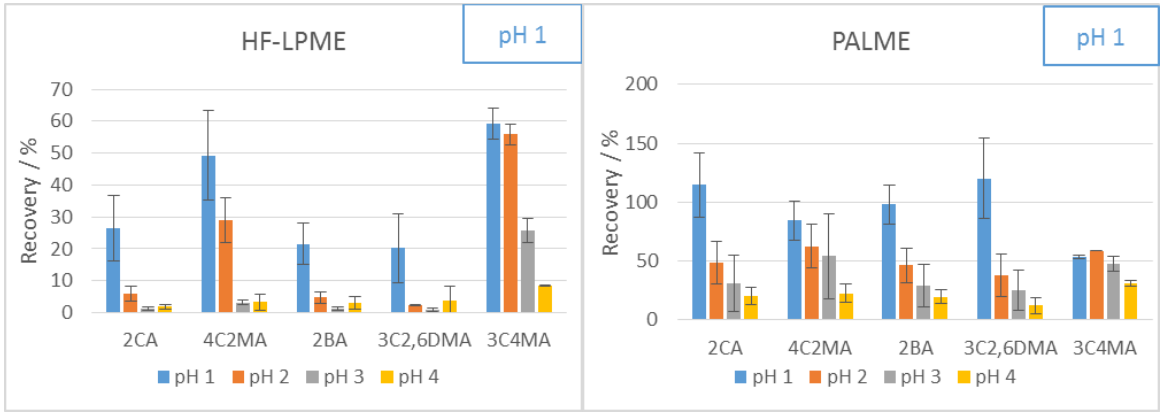


Fig. 4.2. Optimization results of HF-LPME and PALME for a subset of the aromatic amines studied. Optimal values are shown at the top right of the corresponding graph. A one parameter at a time approach was used for optimization, starting with 45 min extraction at 250 rpm, and a pH = 2 acceptor solution.

Table 4.2. Summary of parameters optimized for the analysis of aromatic amines with HF-LPME from literature and from this study. When several values gave optimal results for different amines, the values not used in following experiments are presented in parentheses ( ).

General Information			Organic Solvent		Stirring Speed		Donor pH		Acceptor pH		Extraction time		Ref
Type	Mode	Analytes	Tested	Optimal	Tested (rpm)	Optimal (rpm)	Tested [NaOH] (M)	Optimal [NaOH] (M)	Tested [HCl] (M)	Optimal [HCl] (M)	Tested (min)	Optimal (min)	
Static	HF^2-LPME & HF^3-LPME	A, 4NA, 2,4DNA, 2,6DC4NA	DHE, UD	DHE	-	200**	0.01-0.3	0.1	n.a.	8	10-120	80	[21]
Static	HF^2-LPME	4MA, 3NA, 2,3DMA, 4CA, 3,4DCA, 4ABP	4:6 to 8:2 O:MeOH	6:4 O:MeOH	800-1000	800	-	0.1	-	-	5-50	30 (20, 50)	[22]
Dynamic	HF^3-LPME	3NA, 4BA, 4CA, 3,4DCA	1O, T	1O, T	-	1000	0.01-1	0.1	0.05-0.5	0.5	10-40	20	[23]
Static	HF^3-LPME	3NA, 4BA, 4CA, 3,4DCA	1O, DHE, DAE	DHE	-, 200, 1000	1000	0.001-1	0.1	0.001-0.5	0.5	5-60	30 (50)	[25]
Static	HF^3-LPME*	3CA, 3NA, 4BA	1O, T, B, X, EB, H	T	360-960	800	0.001-1	1	0.001-0.5	0.5	15-90 s / n.a.	75 s / 10	[26]
Static	HF^3-LPME*	See Table 4.1	DDA, 2O DHE, UD	DHE	150-350	250	-	0.01	0.0001-0.1	0.1	15-75	45	This study
	PALME			UD	150-500	500	0.01-0.3	0.3		0.1		20	

* Extraction and back extraction were not performed simultaneously, but in two consecutive steps. ** Agitation speed instead of stirring speed.
Abbreviations: 1O: 1-octanol, 2,3DMA: 2,3-dimethylaniline, 2,4DNA: 2,4-dinitroaniline, 2,6DC4NA: 2,6-dichloro-4-nitroaniline, 3CA: 3-chloroaniline, 3NA: 3-nitroaniline, 3,4DCA: 3,4-dichloroaniline, 4ABP: 4-aminobiphenyl, 4BA: 4-bromoaniline, 4CA: 4-chloroaniline, 4MA: 4-methylaniline, 4NA: 4-nitroaniline, A: aniline, B: benzene, DAE: diamylether, DHE: di-n-hexyl ether, EB: ethylbenzene, H: n-heptane, HCl: hydrochloric acid, HF^n-LPME: n-phase hollow fiber-liquid phase microextraction, MeOH: methanol, n.a.: not available, NaOH: sodium hydroxide, O: octane, PALME: parallel artificial membrane extraction, T: toluene, UD: undecane, X: o-xylene.

PALME

The same solvents were tested with PALME, and UD was either significantly better or similar to the other solvents for all the analytes (Fig. 4.2, Fig. S 4.5). The different optimal solvent found in comparison to HF-LPME could be due to the different thickness of the fibers/membranes (100 µm PALME and 200 µm HF-LPME) or the different material of which they are made of (PVDE PALME and PP HF-LPME).

4.4.2 Extraction time optimization

HF-LPME

Five points were tested at 15 min intervals, from 15 to 75 min. After 60 and 75 min extraction, an intensity loss was observed for most analytes (Fig. 4.2, Fig. S 4.6). A smaller amount of acceptor solution could be recovered from the lumen of the fibers at these extraction times, and it is therefore believed to have leaked through the pores of the HF, as previously reported by Majors et al. [15]. This could explain the recovery decrease observed after 45 min, instead of the expected plateau. Therefore, 45 min was used as extraction time for HF-LPME.

The extraction times used in the literature studied (Table 4.2) ranged from 10 to 80 min, and both in Lin et al. [22] and in Zhao et al. [25], 30 min was used as a compromise between extraction speed and efficiency. The optimal time found in this research, 45 min, would be expected considering that lower agitation speeds would lead to higher extraction times needed (see section 4.4.3).

PALME

Because for most analytes already after 30 min extraction time a plateau was reached, a second experiment with shorter times was performed (Fig. 4.2, Fig. S 4.7). For the majority of the analytes, the maximum recovery was reached after 20 min, and therefore that time was chosen for the following experiments.

A shorter extraction time was needed in comparison to HF-LPME, most likely due to the different geometry of the set-up, e.g. the thinner membrane.

4.4.3 Agitation speed optimization

HF-LPME

Three agitation speeds, namely 150, 250 and 350 rpm were tested, and for most analytes, no significant differences could be observed (Fig. S 4.8). Due to the apparent instability of the setup at higher speeds, and to avoid bubble formation as reported by [27], 250 rpm was used for future experiments.

In most of the literature (Table 4.2), the donor solution was stirred with a magnetic stirrer. In this study, smaller sample volumes were used and placed in 1-mL vials, where standard stirrers would not fit, and therefore the whole set-up was agitated instead. Because of that, smaller agitation speeds were used, comparable to those used in Tao et al. [21], where the complete set-up was also shaken.

PALME

With PALME, the maximum speed of the shaker (500 rpm) was studied in addition to the speeds discussed above. The results with 500 rpm showed an improvement of the extraction efficiencies and were therefore used for the remaining experiments.

4.4.4 Acceptor pH optimization

HF-LPME

Majors et al. recommends using a pH 1 to 3 units below the pKa value of the analytes for the acceptor solution [15]. Because the analytes studied had pKa values between 0.7 and 5.0, the influence of the acceptor solution pH was tested from pH 1 to pH 4.

As expected, and in agreement with literature (Table 4.2), the lower pH showed the best recoveries, with pH 1 and 2 showing significantly better results than pH 3 and 4 for most of the analytes (Fig. 4.2, Fig. S 4.10). Furthermore, pH 1 showed significantly better results than pH 2 for 2CA, and 2BA. This can be explained by the fact that these compounds have the lowest pKa values -after 2,6DCA- among the analytes studied (see Table 4.1), and therefore a lower pH is needed to successfully trap the analytes in the acceptor solution. For 2,6DCA no

significant difference could be observed, probably due to the extremely low pKa of this analyte (0.7), which would indicate even lower pH values are needed.

PALME

A similar trend of increased recoveries with lower acceptor pHs was also observed with PALME, although not as extreme as with HF-LPME (Fig. 4.2, Fig. S 4.11). This could be explained by the higher recoveries already observed with higher pH values. Nonetheless, pH 1 showed the best results and was chosen for the following experiments.

4.4.5 Optimized extraction techniques comparison

HF-LPME presents two major disadvantages. On the one hand, it is a much more labor-intensive setup. As it is not commercially available, it needs to be assembled manually, which not only takes time, but also can introduce small variations in the fibers that could have an influence on the recoveries observed. Furthermore, it is more mistake prone, as steps like insertion or removal of the acceptor solution into/from the lumen of the hollow fiber are much more sensitive: the fiber can easily break, or different size droplets can be left behind in the lumen. Moreover, the recoveries observed were much smaller compared to PALME. The smaller recoveries observed also contribute to small variations having a bigger impact and could explain the fact that the RSDs observed with HF-LPME are generally higher than with PALME.

HF-LPME could be further optimized, for example, by trying different types of carriers and concentrations. However, the results obtained with PALME not only offer the advantage of significantly better recoveries, but also a much less labor-intensive and less error-prone design. Therefore, PALME is recommended over HF-LPME and was used for the validation experiments.

Before the PALME validation, a few further optimization experiments were done (not shown), where a higher donor pH (12 vs 13.5), more donor volume (0.25 vs 1 mL), the addition of an organic modifier (0 vs 25 % methanol) in the acceptor solution, and an increased temperature (40 °C vs room temperature) were studied. Positive effects were observed for a higher donor pH (13.5) and more donor volume (1 mL), so these conditions were used for the validation experiments.

4.4.6 Method validation

The results obtained in this study are generally comparable with literature (Table 4.3, Table 4.4). The selection of the linear range, 100-1200 ng/L, was based on the expected concentrations of AA in urine samples [28-33] and preliminary studies with this set-up, and it includes the lowest calibration point reported for LPME measurements, namely 500 ng/L (Table 4.4). Furthermore, the donor volumes were set to ≤ 1 mL, which is relatively low in comparison to the literature found were LPME is used for the analysis of aromatic amines [21-25]. This value was chosen in order to study if LPME can be suitable for the analysis of valuable archived samples, and it is between 4 times and 100 times smaller than the donor volumes studied in literature. Despite that, the results obtained were satisfactory, with correlation coefficients of 0.991-0.999 for nine AA. Furthermore, the RSD based on peak areas and the repeatability (or intra-day precision) of the lowest calibration point (100 ng/L) were below 20 %. The LODs were calculated based on S/N = 3 for an easier comparison with literature, and based on the calibration curve for a more accurate approach. The LODs obtained in this study based on S/N (100 ng/L, n=3, root mean square calculation method, standard smoothing: 1 time, 1 s width) are 5 to 6500 times smaller than those reported in literature. The S/N approach offers a significant disadvantage, which is that the results can vary significantly based on the concentration point and method used for its calculation, and to which extent peak smoothing was applied, and unfortunately these parameters are usually not reported. Therefore, the LODs based on the calibration curve as described by [34] are also presented. Even when using the more conservative approach based on the calibration curve, the LODs obtained in this study are some of the lowest reported so far for AA with LPME techniques. According to AA concentrations found in literature [28-33], these LODs should generally be sufficient to successfully analyze real samples. Such small LODs and the use of small donor volumes are especially critical when analyzing samples with limited availability where miniaturization is needed, like archived samples. Finally, two real samples were measured: 26DMA could be detected in both samples and 2MA could be quantified in one sample with 243 ng/L and detected in the other. The reason for most target analytes not being detected in these samples might be due to the smoking topography of the donors.

Table 4.3. Figures of merit of the aromatic amines (AA) studied where the regression coefficient (R^2) was > 0.99, and the concentrations observed in two real samples of smokers. Limits of detection and quantification (LOD and LOQ) were calculated based on signal to noise ratios of the lowest calibration point (100 ng/L, n=3, root mean square calculation method, standard smoothing: 1 time, 1 s width), S/N = 3 and 10, respectively (left value) and based on the calibration curves obtained as described by [34] (C.C., right value). The relative standard deviation (RSD) was calculated based on the peak areas observed at the lowest calibration point (100 ng/L, n=3).

AA	**R^2**	**LOD (ng/L)**	**LOQ (ng/L)**	**RSD (%)**	**Sample**	
		S/N / C.C.			**OS1 (ng/L)**	**OS2 (ng/L)**
2MA	0.996	3 / 45	10 / 155	3	< LOQ (C.C.)	243
3C4FA	0.993	7 / 62	24 / 208	7	n.d.	n.d.
2CA	0.994	3 / 55	11 / 186	2	n.d.	n.d.
4EA	0.992	4 / 71	15 / 241	4	n.d.	n.d.
26DMA	0.998	6 / 35	19 / 122	5	< LOQ (C.C.)	< LOQ (C.C.)
2BA	0.993	12 / 60	39 / 203	12	n.d.	n.d.
4C2MA	0.994	3 / 57	9 / 193	2	n.d.	n.d.
246TMA	0.991	3 / 75	11 / 254	11	n.d.	n.d.
3C26DMA	0.996	12 / 45	41 / 156	7	n.d.	n.d.

Table 4.4. Figures of merit of most recent literature regarding the analysis of aromatic amines with LPME. Ranges reported correspond to the minimum and maximum values from different analytes. The relative standard deviation (RSD) was calculated based on the peak areas observed. The limits of detection (LODs) were calculated based on S/N = 3. In this study the LODs were also calculated based on the calibration curves obtained (C.C.) according to [34].

Linear range (µg/L)	**Coefficient of determination (R^2)**	**RSD (%)**	**LODs (µg/L)**	**Reference**
5-200	0.995-0.999	n.a.	0.5-1.5	[21]
5-240	0.992-0.997	4-7	2.1–4.8	[22]
100-10000	0.997-0.999	7-14	8-20	[23]

Linear range (µg/L)	Coefficient of determination (R^2)	RSD (%)	LODs (µg/L)	Reference
0.5-500	0.992-0.999	5-7	0.05-0.10	[25]
0.5-1000	0.998-0.999	4-4	0.05-0.1	[26]
0.1-1.2	0.991-0.999	2-12	0.003-0.01 (S/N) 0.03-0.7 (C.C.)	This study

A few AA could not be successfully analyzed. It is believed that 2,6DCA was mostly trapped in the donor solution and was not successfully extracted into the acceptor solution due to its extremely low pKa value (0.71 [13]). A, AAP and 2,4DMA most likely had too low of a log P value (0.9, 1.6 and 1.7 [14]) and were discriminated by the SLM, as previously reported by [35]. An acceptor solution with a lower pH value could improve the extraction of analytes with low pKa values, and the use of ion-pair reagents could help with polar substances as described by Gjelstad [9].

4.5 Conclusion

The optimized HF-LPME and PALME were compared, and PALME showed significant advantages, not only due to its simpler and less error-prone set-up, but also due to the significantly higher recoveries observed. PALME was proven a very successful extraction technique, providing high enrichment of the AA and LODs in the nanogram per liter range, comparable or lower than those found in literature. Furthermore, compared to LLE it has the extra benefit of being a greener technique, thanks to the significantly lower volumes of organic solvents needed. Moreover, the PALME set-up is disposable, minimizing carry-over and the need for cleaning/regeneration, and thanks to the extra physical barrier, they typically extract less matrix interferences, making it an ideal technique for complex biological matrixes such as urine. Because of the low urine volume needed, this technique would also be suitable for the analysis of archived samples, such as those of completed medical studies, where the available sample volume is limited. And because of the high throughput possible, the method described here could be used in the future for a comprehensive study with different types of donors.

In this study, the PAL RTC was used for derivatization and SPME extraction. Because of the complexity of the automated system, including the autosampler and SPME, a similar set-up may not be available in all routine laboratories, limiting its applicability. However, automation is considered a key step towards green chemistry [36], and offers multiple benefits, including minimized human-intervention and –errors, and increased reproducibility, which in many cases will outweigh the costs. Further automation would be possible thanks to multichannel pipettes or pipetting robots [37]. Although there is no commercially available PAL RTC module for PALME, this autosampler could be used for a more automatic PALME by setting up an external shaker as a new module. The donor plate could be covered with sealing foil instead of the plastic cover so that it could be easily pierced by the autosampler. The donor, organic solvent, and acceptor addition, and the clamping of the plates should still be done manually, since there is no tool available that could perform that task. A main drawback of this semi-automatic approach is that the extraction could not be stopped by separating the plates. With 10 s per sample, it would already take over 15 min to place all 96 acceptor solutions into new vials. Depending on the analytical requirements, this could be accounted for by the use of internal standards, but further research would be needed. An alternative would be to do the extraction separately and put only the acceptor plate into the autosampler. That way the separation is halted for all samples simultaneously. Because of the availability of a pipette tool for the PAL RTC, the risk of contamination when transferring the acceptor solution into vials can still be kept low. If automatic derivatization is needed, an agitator with more positions would be beneficial for higher throughputs.

4.6 Supplementary information

4.6.1 Materials and methods

Hollow fiber assembly

The fiber was cut into approximately 2 cm long pieces (Fig. S 4.1, A and B), from which one end was clamped together (Fig. S 4.1, C and D) before being glued to a piece of a pipette tip using a soldering iron (Fig. S 4.1, E, F and G) to get the final hollow fiber (Fig. S 4.1, H).

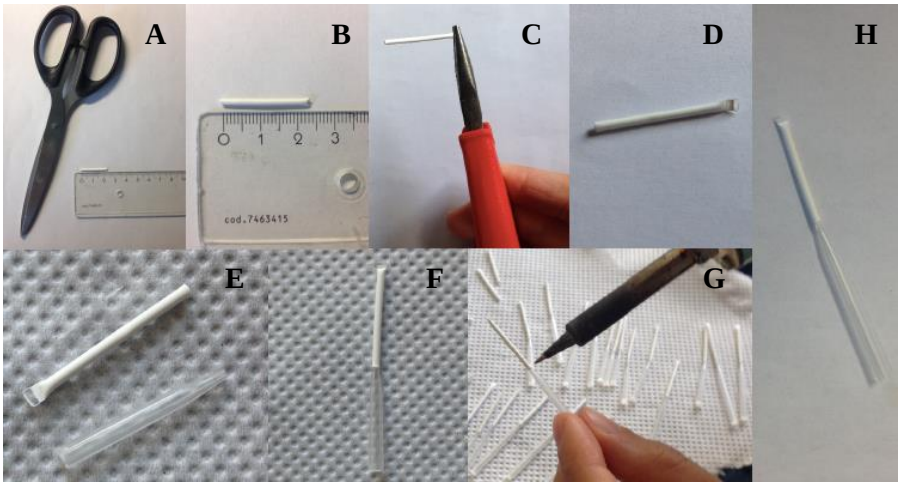


Fig. S 4.1. Pictures taken during the preparation process of a hollow fiber. A: Scissors, ruler and approximately 2 cm long fiber. B: Cut fiber. C: Sealing fiber with pressure at one end using clamps. D: Fiber with one closed end. E: Clamped fiber and pipette tip piece. F: Pipette tip inserted in fiber. G: Soldering the fiber to the pipette tip. H: Final hollow fiber assembly with pipette tip as a needle guide.

The hollow fibers were fixed in the vial caps to avoid risk of losing it into the acceptor solution. First, the septum was perforated with a needle (Fig. S 4.2, B), and the hole was widened with a piece of a pipette tip, called “guiding tip” hereon (Fig. S 4.2, C). The pipette tip piece of the hollow fiber was then connected to the guiding tip (Fig. S 4.2, D), so that the hollow fiber could be pulled through the septum (Fig. S 4.2, E). Then the guiding tip was separated from the hollow fiber (F). The lid was finally screwed on top of a 2 mL amber glass vial (Fig. S 4.2, G).

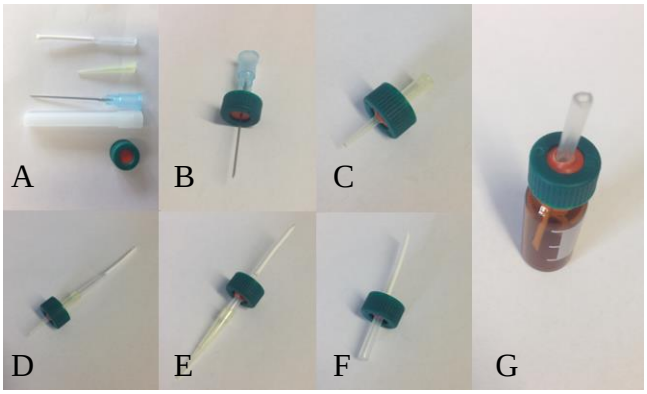


Fig. S 4.2. Fiber assembly into a vial. A: Tools required for the process - from top to bottom: hollow fiber, pipette tip, needle, housing of needle, vial screw cap. B: Septa pierced with needle. C: pipette tip penetrated through the hole in the septa. D: Hollow fiber attached to the pipette

tip. E: Hollow fiber pulled through the septa. F: Hollow fiber in its final position in the septa. G: Hollow fiber assembled into a 2 mL vial.

PALME set-up and procedure

The PALME workflow followed was adapted from [12] (Fig. S 4.3). The 96-well plates used as the donor plate can be seen in Fig. S 4.3 - 1 and 2, and the 96-well filter plates used as the acceptor plate in Fig. S 4.3 - 3, 4 and 6.

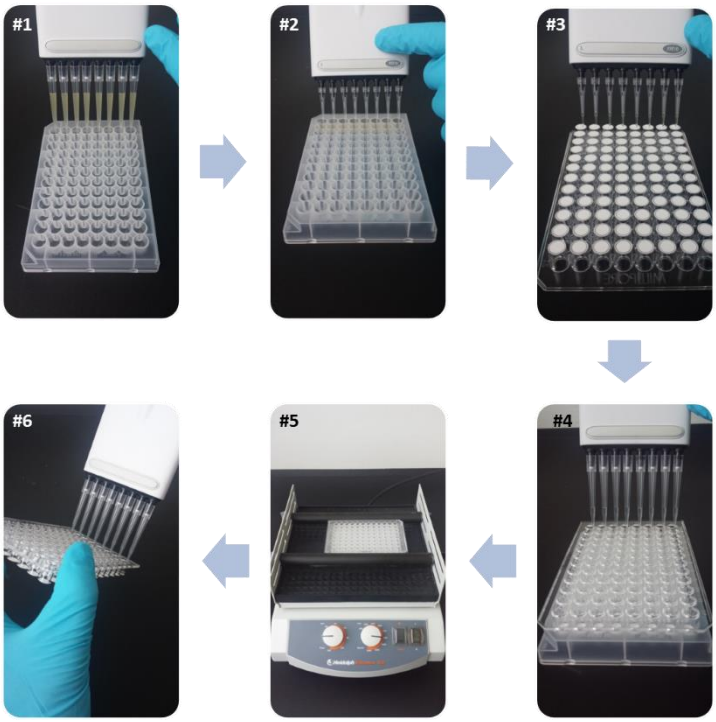


Fig. S 4.3. Typical PALME workflow. Step #1: pipetting samples, Step #2: pipetting internal standards and buffer, Step #3: pipetting SLMs, Step #4: pipetting acceptor solutions, Step #5: clamping the plates together and shaking of the set-up, Step #6: transfer of acceptor solutions. Reproduced from [12] with permission.

Data evaluation – Peak integration parameters

During the sample preparation, all aromatic amines were derivatized into their corresponding iodinated versions. The iodinated compounds were then separated and detected via GC-MS. The quantifier and qualifier ions used for their detection, generally corresponding to the molecular ion and the loss of iodine, can be seen in Table S 4.1.

Table S 4.1. Mass spectrometric parameters used for the detection of the derivatized aromatic amines, including the quantifier and the qualifier ion of each analyte studied.

Analyte	Iodinated aromatic compound	Quantifier ion (m/z)	Qualifier ion (m/z)
A	Iodobenzene	204	77
2MA	1-Iodo-2-methylbenzene	218	91
3C4FA	3-Chloro-4-fluoro-1-iodobenzene	256	129
2CA	2-Chloro-1-iodobenzene	238	111
4EA	4-Ethyl-1-iodobenzene	232	217
2,6DMA	2,6-Dimethyl-1-iodobenzene	232	105
2,4DMA	2,4-Dimethyl-1-iodobenzene	232	105
4C2MA	4-Chloro-1-iodo-2-methylbenzene	252	125
2BA	2-Bromo-1-iodobenzene	282	155
2,4,6TMA	1-Iodo-2,4,6-trimethylbenzene	246	119
2,6DCA	2,6-Dichloro-1-iodobenzene	272	145
3C2,6DMA	3-Chloro-2,6-dimethyl-1-iodobenzene	266	139
2NA	2-Iodonaphthalene	254	127

The chromatograms where automatically integrated using the GCMS Post Run analysis software and the parameters described in Table S 4.2.

Table S 4.2. Settings selected for the automatic peak integration with the GCMS Post run Analysis software.

Peak integration		Peak Identification	
Slope	100 /min	Window for target peak	5 %
Width	3 s	Window for reference peak	5 %
Min. Area	4000	Default Band time	0.8 min
Base	Area	Reference Ion Mode	Relative
Smoothing	None	Ref. Ions based on	Spectrum
Processing time	0.8 min	Correction of Ref. Ions ratio	No Change

Data evaluation – Dixon Q test

Dixon Q test was used to find and exclude outliers from further calculations. Q_{crit} was taken from [38], as shown in Table S 4.3, and Q_{calc} was calculated with Equation 1. If $Q_{calc} > Q_{crit}$, the tested value was an outlier and was neglected during data evaluation. This test was applied to all measured samples.

$$Q_{calc} = \frac{|x_2 - x_1|}{|x_n - x_1|}$$ Equation 1 [17]

Table S 4.3. Critical values of Dixon's test (Q) for a confidence interval of 90 % and sample sizes from three to six [38].

Sample size, n	Q_{crit} 90 %, α=0.1
3	0.941
4	0.765
5	0.642
6	0.560

Data evaluation – Fisher's F-Test, two-variable t-test and Welch's two-sided t-test

The Fisher's F-Test (Equation 2Equation 2) was used to check if the variances of both data groups (s_1^2, s_2^2) were significantly different.

$$F = \frac{s_1^2}{s_2^2}$$ Equation 2 [17]

If the calculated F-value was smaller than the tabulated one (Table S 4.4), the sample variances were not significantly different, and the two-variable t-test could be used.

Table S 4.4. F-quantiles for 95 % confidence interval for the different degrees of freedom f_1 and f_2 [17].

f_2	f_1=1	2	3	4	5
1	161	200	216	225	230
2	18.51	19.00	19.16	19.25	19.30
3	10.13	9.55	9.28	9.12	9.01
4	7.71	6.94	6.59	6.39	6.26
5	6.61	5.79	5.41	5.19	5.05

For the two-variable t-test, the means of each data set were compared using Equation 3, where n_1 and n_2 are the numbers of parallel determinations for the two sets of samples and s_d is the weighted averaged standard deviation, which was calculated using Equation 4.

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{s_d}\sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$ Equation 3 [17]

$$s_d = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$ Equation 4 [17]

If the calculated F-value was bigger than the tabulated one, the general t-test, after Welch, was applied (Equation 5) [17].

$$t = \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$ Equation 5 [17]

The calculated t-value (with either of the methods) was then compared to the tabulated t-value for 95 % significance. If the calculated t-value was smaller than the tabulated one (Table S 4.5), the sample sets were not significantly different [17].

Table S 4.5. Quantile of the t-distribution for the significance level of 95 % and the different degrees of freedom, f [17].

f	**t 95 %**
2	2.920
3	2.353
4	2.132
5	2.015

Data evaluation – Recovery

The recovery calculations were based on the peak intensities as described in Equation 6.

$$R = \frac{Intensity_{LPME\ sample}}{Intensity_{Control\ sample}} \times 100\ \%$$ Equation 6

4.6.2 Results and discussion

Organic solvent optimization

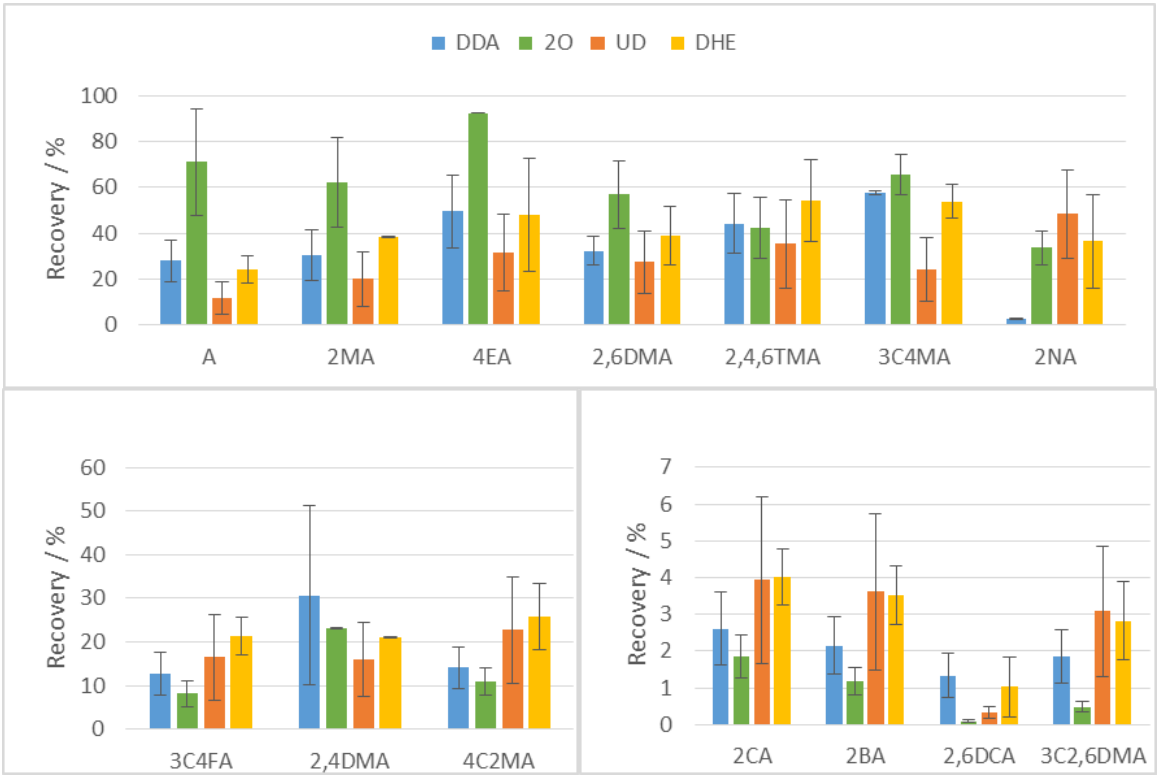


Fig. S 4.4. Influence of organic solvents during HF-LPME on the recovery of the aromatic amines studied. With DDA = dodecyl acetate, UD = undecane, 2O = 2-octanone, and DHE = dihexylether. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered.

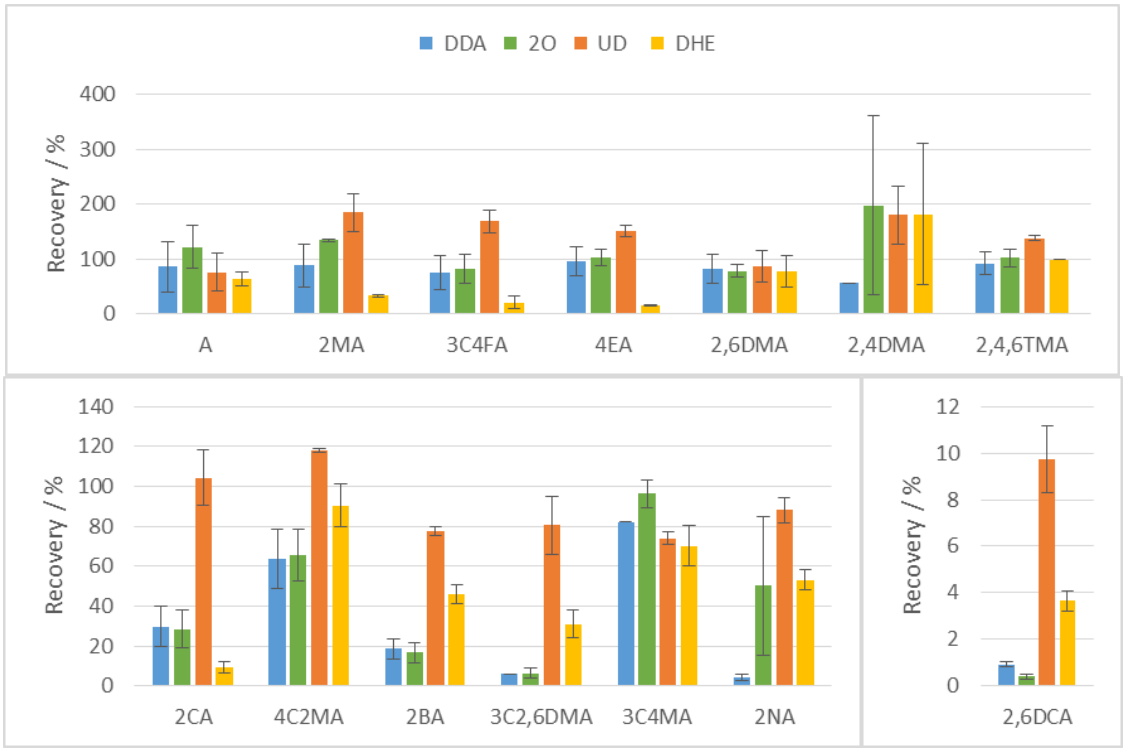


Fig. S 4.5. Influence of organic solvents during PALME on the recovery of the aromatic amines studied. With DDA = dodecyl acetate, UD = undecane, 2O = 2-octanone, and DHE = dihexylether. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered.

Extraction time optimization

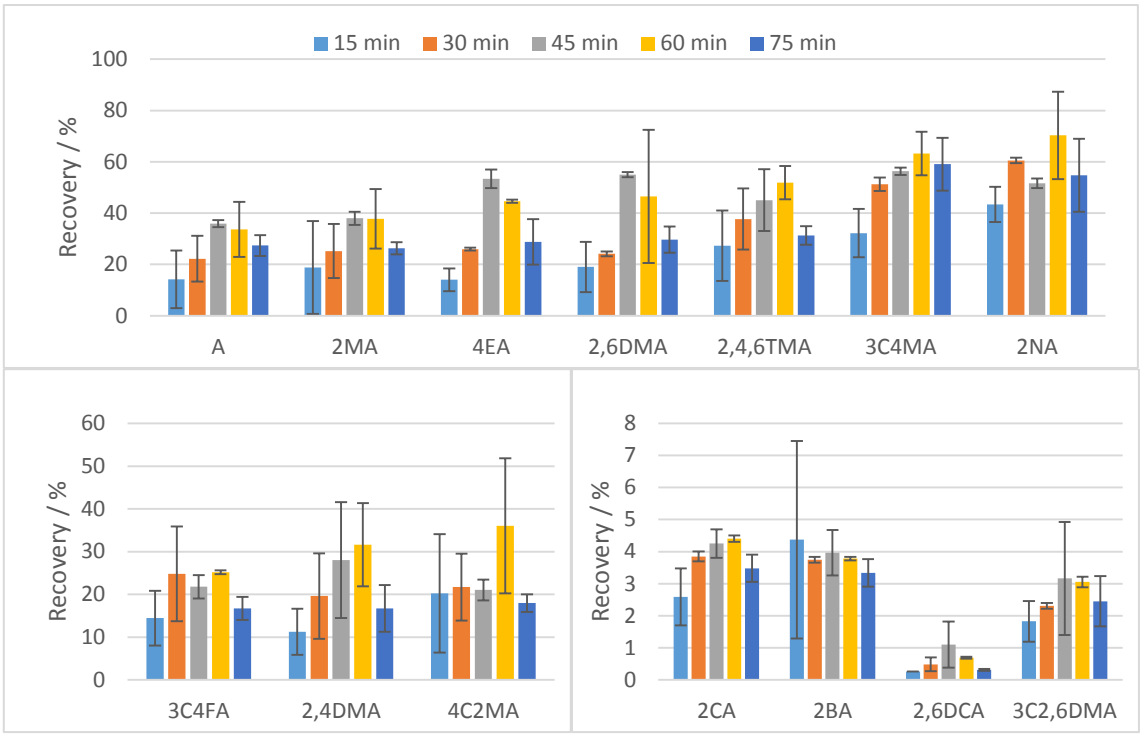


Fig. S 4.6. Influence of extraction times during HF-LPME on the recovery of the aromatic amines studied. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered. Duplicates are presented for 45 min, as the hollow fiber detached from the needle guide, and no acceptor solution could be collected.

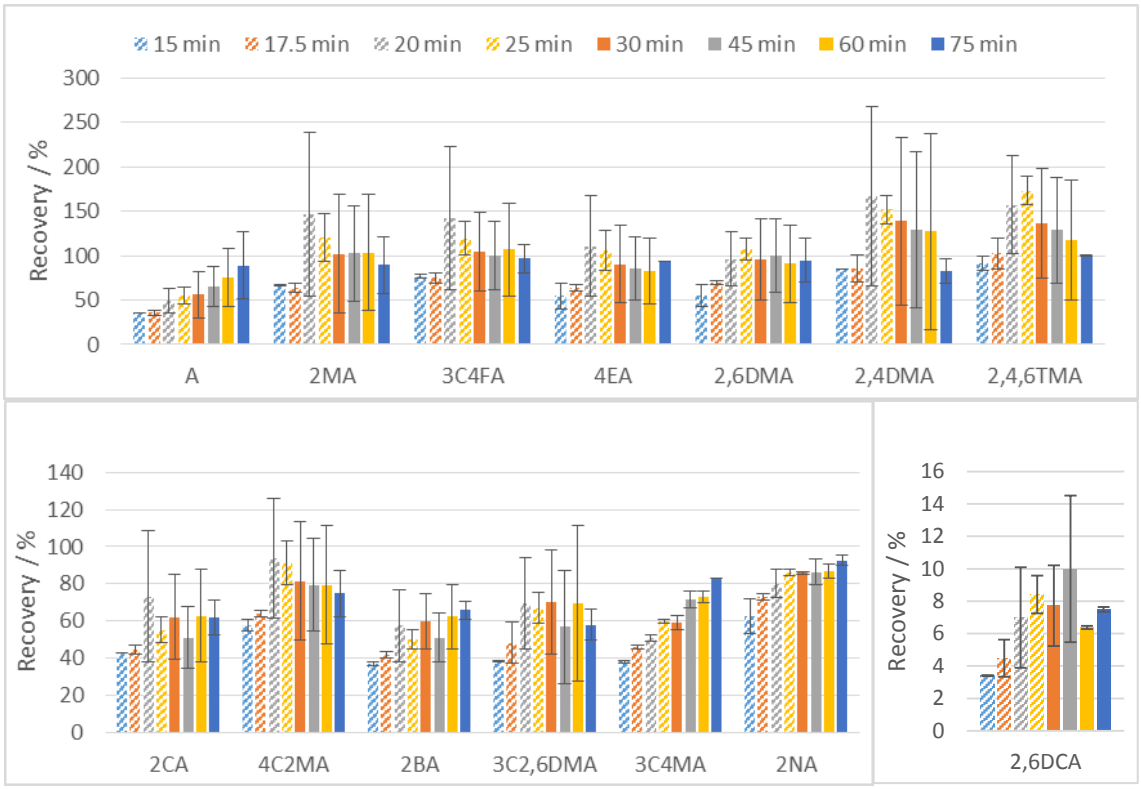


Fig. S 4.7. Influence of extraction times during PALME on the recovery of the aromatic amines studied. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered. The patterned columns were measured in a separate experiment.

Agitation speed optimization

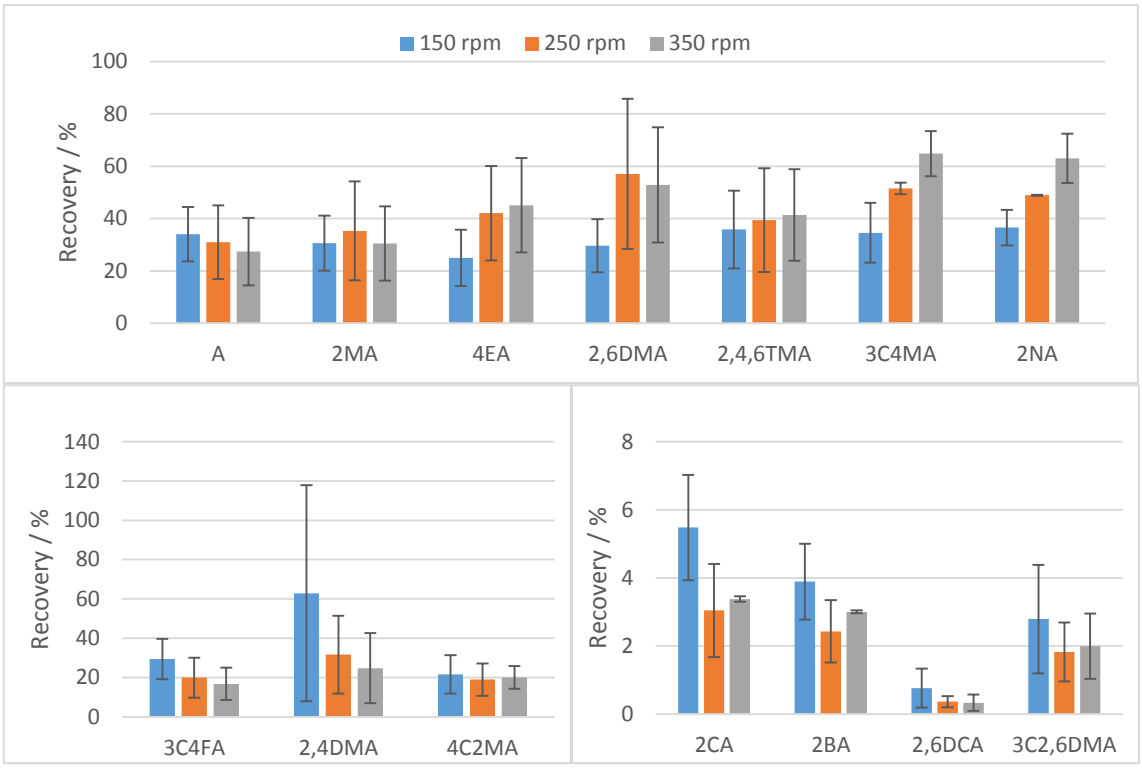


Fig. S 4.8. Influence of agitation speeds during HF-LPME on the recovery of the aromatic amines studied. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered.

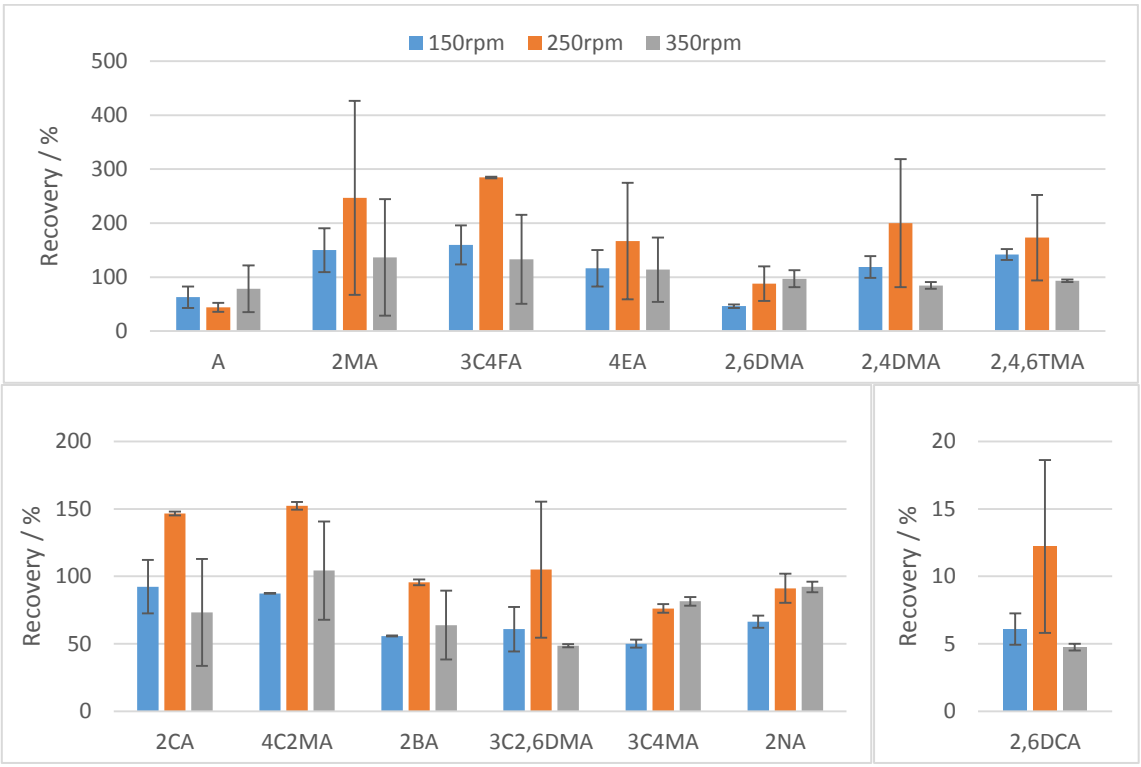


Fig. S 4.9. Influence of agitation speeds during PALME on the recovery of the aromatic amines studied. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered. 500 rpm results are not shown as they were compared to 250 rpm in a different experiment.

Acceptor pH optimization

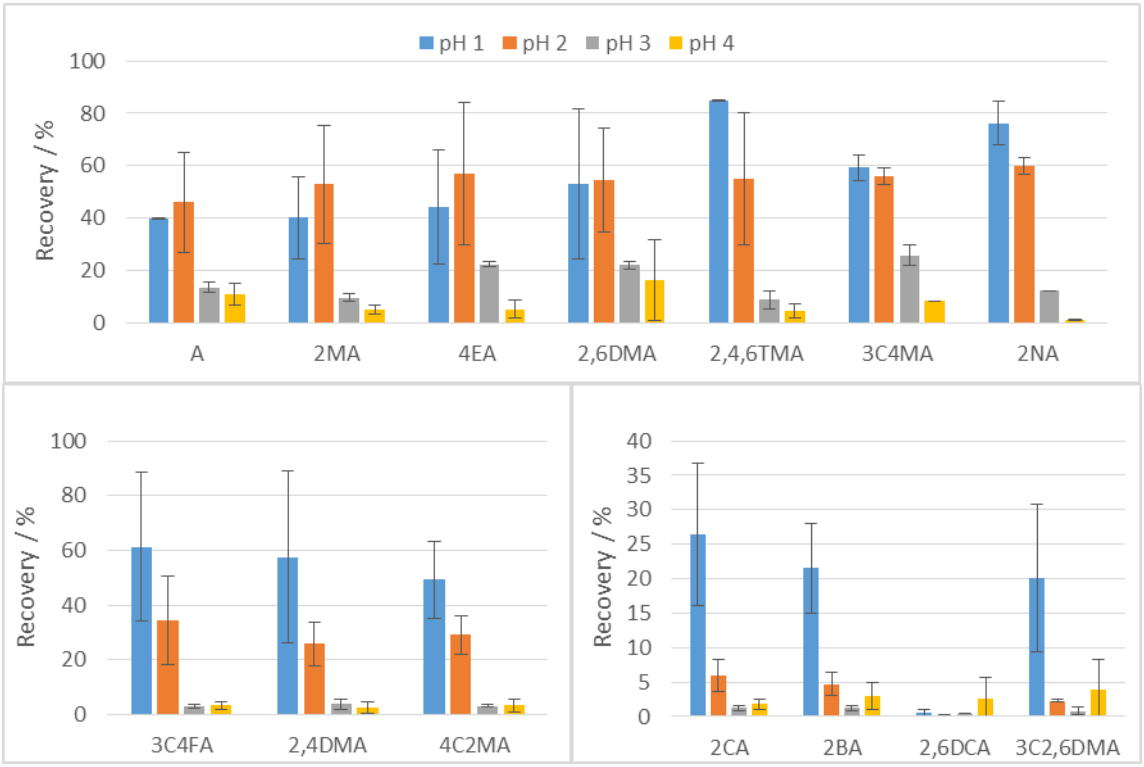


Fig. S 4.10. Influence of acceptor pH during HF-LPME on the recovery of the aromatic amines studied. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered.

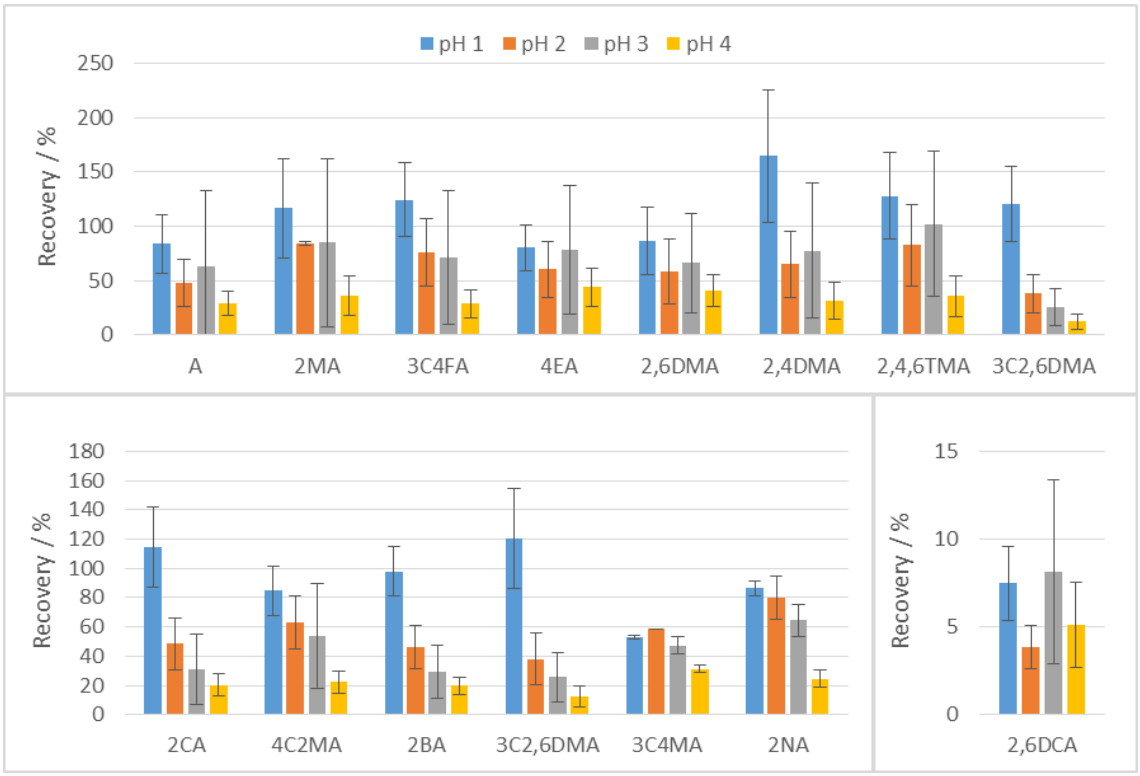


Fig. S 4.11. Influence of acceptor pH during PALME on the recovery of the aromatic amines studied. The experiments were done in triplicate and outliers determined by the Dean Dixon outlier test were not considered.

4.7 References

1. International Agency for Research on Cancer (2022) Agents Classified by the IARC Monographs, Volumes 1-132. https://monographs.iarc.who.int/list-of-classifications. Accessed 05 Jul. 2022.

2. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Some Aromatic Amines, Organic Dyes, and Related Exposures. Lyon, France: International Agency for Research on Cancer; 2008.

3. Guo J, Villalta PW, Weight CJ, Bonala R, Johnson F, Rosenquist TA, Turesky RJ. Targeted and Untargeted Detection of DNA Adducts of Aromatic Amine Carcinogens in Human Bladder by Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry. Chem Res Toxicol. 2018;31:1382-97. https://doi.org/10.1021/acs.chemrestox.8b00268.

4. Bartsch H. Metabolic activation of aromatic amines and azo dyes. IARC Scientific Publications. 1981:13-30.

5. Land SJ, Zukowski K, Lee MS, Debiec-Rychter M, King CM, Wang CY. Metabolism of aromatic amines: relationships of N-acetylation, O-acetylation, N,O-acetyltransfer and

deacetylation in human liver and urinary bladder. Carcinogenesis. 1989;10:727-31. https://doi.org/10.1093/carcin/10.4.727

6. Daryanavard SM, Zolfaghari H, Abdel-Rehim A, Abdel-Rehim M. Recent applications of microextraction sample preparation techniques in biological samples analysis. Biomed Chromatogr. 2021;35. https://doi.org/10.1002/bmc.5105.

7. Dean JR. Extraction Techniques in Analytical Science. 1 ed. Ando DJ, editor. Chichester, West Sussex, England: John Wiley & Sons Ltd; 2009.

8. Pedersen-Bjergaard S, Rasmussen KE. Liquid-Liquid-Liquid Microextraction for Sample Preparation of Biological Fluids Prior to Capillary Electrophoresis. Anal Chem. 1999;71:2650-6. https://doi.org/10.1021/ac990055n.

9. Gjelstad A. Three-phase hollow fiber liquid-phase microextraction and parallel artificial liquid membrane extraction. Trends Anal Chem. 2019;113:25-31. https://doi.org/10.1016/j.trac.2019.01.007.

10. Gjelstad A, Rasmussen KE, Parmer MP, Pedersen-Bjergaard S. Parallel artificial liquid membrane extraction: micro-scale liquid-liquid-liquid extraction in the 96-well format. Bioanalysis. 2013;5:1377-85. https://doi.org/10.4155/bio.13.59.

11. Soares da Silva Burato J, Vargas Medina DA, de Toffoli AL, Vasconcelos Soares Maciel E, Mauro Lanças F. Recent advances and trends in miniaturized sample preparation techniques. J Sep Sci. 2020;43:202-25. https://doi.org/10.1002/jssc.201900776.

12. Gjelstad A, Andresen AT, Dahlgren A, Gundersen TE, Pedersen-Bjergaard S. High-throughput liquid-liquid extraction in 96-well format: Parallel artificial liquid membrane extraction. LC GC Europe. 2017;30:10-7.

13. ChemicalBook https://www.chemicalbook.com/. Accessed 21 Nov. 2021.

14. Kim S, Chen J, Cheng T, Gindulyte A, He J, He S, Li Q, Shoemaker BA, Thiessen PA, Yu B, Zaslavsky L, Zhang J, Bolton EE. PubChem in 2021: new data content and improved web interfaces. Nucleic Acids Res. 2021;49:D1388-D95. https://doi.org/https://doi.org/10.1093/nar/gkaa971.

15. Majors RE, Pedersen-Bjergaard S, Taherkhani H, Rasmussen KE, Gjelstad A. Hollow-fibre liquid-phase microextraction in the three-phase mode - practical considerations. LC GC Europe. 2011;24:1-9.

16. Schmidt TC, Less M, Haas R, von Löw E, Steinbach K, Stork G. Gas chromatographic determination of aromatic amines in water samples after solid-phase extraction and derivatization with iodine: I. Derivatization. J Chromatogr A. 1998;810:161-72. https://doi.org/http://doi.org/10.1016/S0021-9673(98)00233-7.

17. Otto M. Chemometrics Statistics and Computer Application in Analytical Chemistry. 3 ed. Weinheim, Germany: Wiley-VCH; 2016.

18. Gjelstad A, Pedersen-Bjergaard S. Perspective: Hollow fibre liquid-phase microextraction – principles, performance, applicability, and future directions. Scientia Chromatographica. 2013;5:181-9.

19. Saraji M, Ghambari H. Comparison of three different dispersive liquid–liquid microextraction modes performed on their most usual configurations for the extraction of phenolic, neutral aromatic, and amino compounds from waters. J Sep Sci. 2018;41:3275-84. https://doi.org/https://doi.org/10.1002/jssc.201800133.

20. Magnusson B, Örnemark U. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics; 2014. Available from: http://www.eurachem.org. Accessed 11.12.2022

21. Tao Y, Liu J-F, Wang T, Jiang G-B. Simultaneous conduction of two- and three-phase hollow-fiber-based liquid-phase microextraction for the determination of aromatic amines in environmental water samples. J Chromatogr A. 2009;1216:756-62. https://doi.org/10.1016/j.chroma.2008.11.094.

22. Lin Z, Zhang J, Cui H, Zhang L, Chen G. Determination of aromatic amines in environmental water sample by hollow fiber-liquid phase microextraction and microemulsion electrokinetic chromatography. J Chromatogr A. 2010;1217:4507-10. https://doi.org/10.1016/j.chroma.2010.04.063.

23. Hou L, Lee HK. Dynamic three-phase microextraction as a sample preparation technique prior to capillary electrophoresis. Anal Chem. 2003;75:2784-9. https://doi.org/10.1021/ac020753z

24. Sarafraz-Yazdi A, Es'haghi Z. Two-step hollow fiber-based, liquid-phase microextraction combined with high-performance liquid chromatography: A new approach to determination of aromatic amines in water. J Chromatogr A. 2005;1082:136-42. https://doi.org/https://doi.org/10.1016/j.chroma.2005.05.102.

25. Zhao L, Zhu L, Lee HK. Analysis of aromatic amines in water samples by liquid-liquid-liquid microextraction with hollow fibers and high-performance liquid chromatography. J Chromatogr A. 2002;963:239-48. https://doi.org/10.1016/S0021-9673(02)00544-7.

26. Sarafraz-Yazdi A, Mofazzeli F, Es'haghi Z. A new high-speed hollow fiber based liquid phase microextraction method using volatile organic solvent for determination of aromatic amines in environmental water samples prior to high-performance liquid chromatography. Talanta. 2009;79:472-8. https://doi.org/10.1016/j.talanta.2009.04.015.

27. Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S. Hollow fiber liquid-phase microextraction. In: Pawliszyn J, Lord HL, editors. Comprehensive Sampling and Sample Preparation. 2: Elsevier; 2012. pp. 475-96.

28. Chinthakindi S, Kannan K. A liquid chromatography–tandem mass spectrometry method for the analysis of primary aromatic amines in human urine. J Chromatogr B. 2021;1180:122888-90. https://doi.org/10.1016/j.jchromb.2021.122888.

29. Fuller TW, Acharya AP, Meyyappan T, Yu M, Bhaskar G, Little SR, Tarin TV. Comparison of Bladder Carcinogens in the Urine of E-cigarette Users Versus Non E-cigarette Using Controls. Sci Rep. 2018;8:507. https://doi.org/https://doi.org/10.1038/s41598-017-19030-1.

30. Shepperd CJ, Newland N, Eldridge A, Haswell L, Lowe F, Papadopoulou E, Camacho O, Proctor CJ, Graff D, Meyer I. Changes in levels of biomarkers of exposure and biological effect in a controlled study of smokers switched from conventional cigarettes to reduced-toxicant-prototype cigarettes. Regul Toxicol Pharm. 2015;72:273-91. https://doi.org/10.1016/j.yrtph.2015.04.016.

31. Krautter GR, Chen PX, Borgerding MF. Consumption patterns and biomarkers of exposure in cigarette smokers switched to Snus, various dissolvable tobacco products, Dual use, or tobacco abstinence. Regul Toxicol Pharm. 2015;71:186-97. https://doi.org/10.1016/j.yrtph.2014.12.016.

32. Riedel K, Scherer G, Engl J, Hagedorn HW, Tricker AR. Determination of three carcinogenic aromatic amines in urine of smokers and nonsmokers. J Anal Toxicol. 2006;30:187-95. https://doi.org/https://doi.org/10.1093/jat/30.3.187.

33. Weiss T, Angerer J. Simultaneous determination of various aromatic amines and metabolites of aromatic nitro compounds in urine for low level exposure using gas chromatography–mass spectrometry. J Chromatogr B. 2002;778:179-92. https://doi.org/http://doi.org/10.1016/S0378-4347(01)00542-4.

34. DIN ISO 32645:2008-11, Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation. 2008:28.

35. Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S. Electrokinetic migration across artificial liquid membranes Tuning the membrane chemistry to different types of drug substances. J Chromatogr A. 2006;1124:29-34. https://doi.org/https://doi.org/10.1016/j.chroma.2006.04.039.

36. Wojnowski W, Tobiszewski M, Pena-Pereira F, Psillakis E. AGREEprep – Analytical greenness metric for sample preparation. Trends Anal Chem. 2022;149:116553. https://doi.org/10.1016/j.trac.2022.116553.

37. Olsen KN, Ask KS, Pedersen-Bjergaard S, Gjelstad A. Parallel artificial liquid membrane extraction of psychoactive analytes: a novel approach in therapeutic drug monitoring. Bioanalysis. 2018;10:385-95. https://doi.org/10.4155/bio-2017-0250.

38. Rorabacher DB. Statistical Treatment for Rejection of Deviant Values: Critical Values of Dixon's "Q" Parameter and Related Subrange Ratios at the 95% Confidence Level. Analytical Chemistry. 1991;63:139-46.

Chapter 5. Comparison of gas chromatographic techniques for the analysis of iodinated derivatives of aromatic amines

This chapter was adapted from: Lorenzo-Parodi N, Leitner E, Schmidt TC. Comparison of gas chromatographic techniques for the analysis of iodinated derivatives of aromatic amines. Anal Bioanal Chem. 2023;415:3313-25. https://doi.org/10.1007/s00216-023-04713-8.

5.1 Abstract

Some aromatic amines (AA) have been classified as carcinogens to humans. After entering the body, mainly through tobacco smoke, they can be detected in urine. Thus, their trace analysis as biomarkers in biofluids is of high relevance and typically achieved with gas chromatography (GC-MS), usually after derivatization. This study compares three gas chromatographic methods for the analysis of ten iodinated derivatives of the AA: GC-MS in single-ion monitoring (SIM) mode with (1) electron ionization (GC-EI-MS) and (2) negative chemical ionization (GC-NCI-MS), and (3) GC-EI-MS/MS in multiple reaction monitoring (MRM) mode using electron ionization.

All methods and most analytes showed good coefficients of determination ($R^2 > 0.99$) for broad linear ranges covering three to five orders of magnitude in the pg/L to ng/L range, with one and two exceptions for (1) and (2) respectively. Excellent limits of detection (LODs) of 9-50, 3.0-7.3, and 0.9-3.9 pg/L were observed for (1), (2), and (3) respectively; and good precision was achieved (intra-day repeatability < 15 % and inter-day repeatability < 20 % for most techniques and concentration levels). On average, recoveries between 80 and 104 % were observed for all techniques. Urine samples of smokers and never smokers were successfully analyzed, and p-toluidine (or 4-methylaninline) and 2-chloroaniline could be found at significantly ($\alpha = 0.05$) higher concentrations among smokers.

5.2 Introduction

Several aromatic amines (AA) have been classified as possible, probable, or certain carcinogens by the International Agency for Research on Cancer (IARC) [1], and most, if not all AA, are believed to have carcinogenic potential [2]. However, they are still widely used, for example, for the production of pharmaceuticals, pesticides, dyes, or rubber [1]. Unfortunately, not only the workers in these industries can get in contact with these substances, but also the general public is at risk: the main source of exposure to some aromatic amines, such as the carcinogenic 2-naphthylamine and ortho-toluidine, and the probable carcinogenic aniline and 4-chloro-o-toluidine, is cigarette smoke [2].

AA enter the blood during the smoking process and are transported into the liver, where they can be metabolized and further transported, for example, to the bladder, where they can react with DNA and proteins to form adducts that can lead to cancer, or can be excreted in the urine [3]. AA have been suggested as the main cause for the excess risk of bladder cancer in smokers [4].

The concentrations of AA in different matrices have been studied in several steps of the aforementioned process, for example, in smoke [5-9] (e.g., aniline, toluidines or dimethylanilines), as DNA [10-13] and protein adducts [12, 14-17] in cells/blood (e.g., 4-aminobiphenyl), or as free AA and metabolites in urine [3, 18-31] (e.g., naphthylamines, chloroanilines). Because the intake of substances during smoking varies depending on the individual smoking topography [32], and the amount of DNA and protein adducts is typically extremely small [33], this study focuses on urine samples. There, not only free aromatic amines can be found but also their metabolites, such as N-acetylaryl-amines, N-glucuronide arylamines, or hemoglobin and DNA adducts, which can be hydrolyzed and converted back to the free aromatic amines [14, 34].

Direct analysis of AA is possible using liquid chromatography (LC) [19, 21, 24, 25, 35-37]. However, its low peak capacity [38, 39] hinders its use for the analysis of complex urine samples. Due to its high sensitivity, short analysis time, and high resolving power [40], gas chromatography (GC) was used for this study.

In order to reduce the polarity of the AA and facilitate their analysis, they are typically derivatized. In this study, they were iodinated via a Sandmeyer-like reaction as reported by [18,

22, 39]. This derivatization procedure offers the advantage that the reagents used do not need strictly anhydrous conditions, as is the case for the commonly used acylation [39] and silylation derivatizations [40].

This derivatization step enables their analysis with different types of GC systems, such as GC-MS [18, 27, 30, 31, 41, 42], GCxGC-MS [22], GC-NCI-MS [8, 29, 43-45], or GC-MS/MS [3, 9]. However, a comparison of the different techniques, namely GC-MS, GC-NCI-MS, and GC-MS/MS, has not been previously reported for these analytes.

The aim of this study is, therefore, the comparison of different GC detection techniques for the determination of aromatic amines in urine after derivatization to the corresponding iodinated benzenes, namely GC-EI-MS, GC-NCI-MS, and GC-EI-MS/MS. To that end, all studied methods were validated and used for the analysis of real urine samples from smokers and never smokers.

5.3 Materials and methods

5.3.1 Chemicals and reagents

Methanol ≥ 99.9 %, HiPerSolv Chromanorm for LC-MS (VWR International GmbH, Darmstadt, Germany), was used for the preparation of standard solutions. Iodinated aromatic compounds (Table 5.1), with a purity of 97 % or more, were purchased from Merck KGaA (Darmstadt, Germany).

Table 5.1. Iodinated compounds used, including the abbreviation by which they are referred to in the text, their corresponding aromatic amine precursor, CAS Number (CAS Nr), and purity.

Analyte	Abbreviation	Aromatic amine precursor	CAS Nr	Purity (%)
4-iodotoluene	4IMB	p-toluidine	624-31-7	99
iodopentafluorobenzene	IPFB	pentafluoroaniline	827-15-6	99
2-iodo-1,3-dimethylbenzene	2I13DMB	2,6-dimethylaniline	608-28-6	97
iodobenzene	IB	aniline	591-50-4	98

Analyte	Abbreviation	Aromatic amine precursor	CAS Nr	Purity (%)
1-chloro-2-iodobenzene	1C2IB	2-chloroaniline	615-41-8	99
3-chloro-4-fluoroiodobenzene	3C4FIB	3-chloro-4-fluoroaniline	156150-67-3	98
2,4,5-trichloroiodobenzene	245TCIB	2,4,5-trichloroaniline	7145-82-6	98
2,4-dichloroiodobenzene	24DCIB	2,4-dichloroaniline	29898-32-6	98
1-bromo-4-iodobenzene	1B4IB	4-bromoaniline	589-87-7	98
2,4-difluoroiodobenzene	24DFIB	2,4-difluoroaniline	2265-93-2	98

Concentrated hydrochloric acid (HCl, 37 %) from VWR; ethyl acetate (99.9 %) from Carl Roth (Karlsruhe, Germany); diethyl ether (99.5 %) from ChemLab (Zedelgem, Belgium); sodium hydroxide (NaOH, 99 %), alizarin red S (98 %), hydriodic acid (unstabilized, 55 %), and sodium nitrite (99 %) from Merck KGaA; and sodium sulfite (≥ 98 %) and sulfamic acid (≥ 99 %) from Fluka (Buchs, Switzerland) were used.

5.3.2 Preparation of stock and standard solutions

All the stock and intermediate solutions were prepared in methanol. Individual stock solutions of each of the analytes were prepared at 1 g/L. An intermediate standard solution was prepared at 1 mg/L for the iodinated aromatic compounds. Working solutions were prepared by diluting the intermediate standard solutions in methanol and were used within 1 month. One working solution was prepared for each concentration tested. All the solutions were stored at 7 °C.

5.3.3 Sample preparation

Glass-covered stirring bars (VWR International GmbH) were placed in 20-mL crimp vials, which were then filled with 5 mL of the samples and closed with magnetic caps with Silicone/PTFE septa.

For the validation experiments, the samples were prepared by adding 10 µL of the corresponding iodinated working solution to 5 mL deionized water.

Urine samples

Urine samples from seven donors (four smokers and three never smokers) were collected in 1-L Schott bottles and stored at 7 °C for up to 1 month. It is believed that the samples are likely stable under those conditions based on Mazumder et al. [46], who found no marked decrease in concentration with samples at similar temperatures during the total time studied. However, more research is needed to confirm this hypothesis, since only 10 days were studied. The urine samples were prepared according to Lamani et al. [22], with a few modifications. First, 20 mL of urine was hydrolyzed with 10 mL of HCl (37 %) at 80 °C and 200 rpm stirring speed for 12 h in order to convert metabolized AA into free AA. All heating and stirring steps were done on an MR 3001 K stirring plate from Heidolph Instruments GmbH & Co. KG (Schwabach, Germany). Once the sample reached room temperature, it was basified by adding 20 mL of 10 M NaOH to the solution. Afterward, the amines were extracted two times into 5 mL of diethyl ether. The organic fractions were then mixed and cleaned with 2 mL of a 0.1 M NaOH solution. The amines were subsequently back-extracted into 10 mL of water, previously acidified with 200 µL concentrated HCl (37 %). Any remaining diethyl ether in the aqueous fraction was evaporated by nitrogen blowing on the samples for 20 min.

The aqueous extracts were then derivatized by substituting the nitrogen for an iodine atom in order to decrease the polarity of the extracted amines (see supplementary information (SI), Fig. S 5.1). This was achieved by adding to the 10-mL sample: 200 µL hydriodic acid (55 %) and 400 µL sodium nitrite (50 g/L) and stirring at 200 rpm for 20 min (step 1), adding 1 mL of sulfamic acid (50 g/L) and stirring at 200 rpm for another 45 min (step 2), then heating the sample to 95 °C for 5 min (step 3), and finally adding 800 µL sodium sulfite (120 g/L) (step 4) and 200 µL of alizarin red S (1 % w/v) (step 5), and adjusting the pH to 5 with NaOH and HCl solutions (step 6). This way, (step 1) the aromatic amines are diazotized and the diazonium ions are further substituted by iodine; (step 2) the surplus of nitrite is destroyed: (step 3) the unreacted diazonium ions are transformed into phenols, and the excess sulfamic acid is destroyed; (step 4) the iodine residue is reduced; (step 5) a pH indicator is added for easy identification of the correct pH; and (step 6) a pH value suitable for subsequent SPME is achieved.

Finally, 5 mL was transferred into a 20-mL vial with a stirrer, crimped, and then placed in the autosampler for further treatment, namely SPME and injection into the GC. For GC-NCI-MS and GC-EI-MS/MS, the samples were diluted 1:10.

Solid-phase microextraction

All the SPME fibers were conditioned prior to their first use, as recommended by the supplier (i.e., 250 °C for 30 min). The SPME extraction was done automatically by different autosamplers, namely HTX PAL (CTC Analytics, Zwingen, Switzerland) for the GC-EI-MS measurements, AOC-6000 (Shimadzu, Kyoto, Japan) for the measurements with the GC-EI-MS/MS, and AOC-5000 Plus for GC-NCI-MS (Shimadzu). All the autosamplers were controlled with the PAL Cycle Composer software except for the autosampler used in combination with the GC-EI-MS/MS, which was directly controlled by the GCMS Real Time Analysis software (Shimadzu). The samples were incubated for 5 min at 60 °C and 500 rpm in a single magnet mixer (SMM). Afterward, they were extracted from the headspace with a 65 µm PDMS/DVB SPME fiber (1 cm length, stableflex, 23 Ga, Merck KGaA) for 30 min before injection into the GC system. The SPME fiber remained at least for 5 min in the injector in order to condition the fiber after injection, except for the GC-EI-MS/MS measurements, where the fiber was pre-conditioned for 2 min in a dedicated conditioning station at 280 °C, and remained for 2 min in the injector.

The extraction efficiencies of the three SPME fibers used (one for each technique) were compared after the methods were validated, using GC-NCI-MS and a 1-ng/L solution of the iodinated derivatives (Fig. S 5.2, SI). Furthermore, a SPME test mix was analyzed regularly in order to ensure the integrity of the fiber and the performance of the system, by adding 20 µL of a 200-ng/L stock solution in a vial with a stirrer. A list of the analytes included in the mix (minimum purity 95 %, different providers) can be seen in Table S 5.1 (SI). Because the mix included mostly analytes that are not ionizable by GC-NCI-MS, it was not used for this technique.

A significantly lower intensity was observed with GC-EI-MS/MS during the first use of the corresponding SPME fiber (see Fig. S 5.3, SI). Although the manufacturer instructions were followed, it has been previously reported that it might be insufficient conditioning [47, 48]. Therefore, the GC-EI-MS/MS results were normalized according to the SPME Mix intensities, which, as expected, also showed a similar trend (see Table S 5.1, SI).

5.3.4 GC-MS analysis

In order to facilitate the comparison of the different techniques, as many parameters as possible were kept constant throughout the different devices. Helium (99.999 %, Linde, Höllriegelskreuth, Germany) was used as carrier gas for all techniques.

A GCMS-QP2010 Ultra (Shimadzu) equipped with a ZB-Wax 20 m x 0.18 mm x 0.18 µm (Phenomenex, CA, USA) was used for the GC-EI-MS analysis. The linear velocity was set to 45 cm/s, which corresponds to a column flow of 1.03 mL/min. The samples were injected in splitless mode, and after a sampling time of 1 min, the split ratio was set to 10. The injector temperature was set to 250 °C, the interface temperature to 230 °C, and the ion source temperature to 200 °C. The oven program started at a temperature of 40 °C, was held for 1 min, ramped at a rate of 10 °C/min to 240 °C, and held for 1 min. The final oven temperature was lower than in other instruments due to the different column used. The acquisition was made in SIM mode, with an event time of 0.2 s. Twenty channels were looked into (Table S 5.2 (SI)) typically corresponding to the molecular ions and the fragment resulting from the loss of iodine.

A GCMS-TQ8050 (Shimadzu) with a 30 m x 0.25 mm x 0.25 µm Rxi-5MS (Restek, PA, USA) was used for the GC-EI-MS/MS analysis. The injector, interface, and ion source temperatures were set to 270, 280, and 200 °C respectively. The injection was done in splitless mode, and a split ratio of 10 was applied after a sampling time of 1 min. The linear velocity was 35 cm/s. The oven starting temperature was 40 °C, which was held for 1 min, ramped to 280 °C at a rate of 10 °C/min, and held for 1 min. The MS was operated in multiple reaction monitoring (MRM) mode, using argon 5.0 (Linde) as the collision gas. The optimal collision energies (CE) were found by directly injecting 1 µL of a 0.5 mg/L (50:50 methanol:ethyl acetate) mixture of the iodinated analytes, at different CE, and comparing the intensities. The transitions monitored, and their corresponding CE, can be seen in Table 5.3 (SI). The event time was set to 0.3 s for all transitions.

GC-NCI-MS measurements were done on a GCMS-QP2010 Plus system (Shimadzu) equipped with a 30 m x 0.25 mm x 0.25 µm Rxi-5Sil MS column (Restek). The interface temperature was set to 250 °C and the ion source temperature to 160 °C. All other GC parameters were the same as for the GC-MS/MS. MS acquisition was performed in SIM mode, with an event time of 0.3 s, and monitoring the ions corresponding to chlorine (35, 37), bromine

(79, 81), and iodine (127). The ionization gas was isobutane 3.5 (Linde), set to a pressure of 0.7 bar.

A comparison of the chromatograms obtained with the aforementioned parameters can be seen in Fig. S 5.4 (SI).

5.3.5 Method validation

The validation was done mostly according to the Eurachem Guide [49]. The raw data were evaluated with GCMSsolution (Shimadzu) without applying smoothing, and the calculations were performed in Excel (Microsoft).

First of all, the linear ranges were studied with the aim of seeing not only how sensitive the instruments can be, but also whether they have a linear response at the concentration levels expected for real samples. Therefore, a very broad range was studied, and, subsequently, a logarithmic scale was used in order to have equidistant calibration levels, as recommended by the DIN 38402-51 [50]. Concentrations from 1 pg/L to 500 ng/L were tested, with three concentration levels per order of magnitude. Exemplary calibration curves for each technique can be seen in Fig. S 5.5 (SI).

Afterward, the limits of detection (LODs) and of quantification (LOQs) were studied by repeating ten times the analysis of a calibration level where most of the analytes showed a signal to noise ratio (S/N) between 6 and 15 (200 pg/L for GC-EI-MS, 100 pg/L for GC-NCI-MS, and 10 pg/L for GC-EI-MS/MS). This was done so that the concentrations used for the calculation of the limits were not extremely high in comparison with the limits themselves, and to consequently avoid obtaining overestimated sensitivities. Because the calibration curves were up to five orders of magnitude (1 – 100,000 pg/L) broad, and the points were equidistant only in the logarithmic scale, a normal linear fit would be very heavily influenced by the higher calibration levels. Therefore, in order to accurately determine lower concentrations, we limited the number of calibration levels in this and the following sections, so that there would be at least 5 points per curve and up to 7 levels. Furthermore, the concentration to be determined was, if possible, kept in the middle of the levels selected. Afterward, the limits were calculated according to the Eurachem Guide [49], with a constant (k, equal to 3 for LODs and 10 for

LOQs) multiplied by the standard deviation of the replicate concentrations and divided by the degrees of freedom (n – 1 = 9).

The next step was to calculate the intra-day and inter-day repeatability. In order to do that, at least three calibration points (equidistant in the logarithmic scale and well distributed within the linear range) were measured in triplicate over three consecutive days. The concentration levels tested were 1, 10, and 100 ng/L for GC-EI-MS; 0.1, 1, and 10 ng/L for GC-NCI-MS, and, because of the broad range that could be analyzed with GC-EI-MS/MS, four concentrations, namely 0.01, 0.1, 1, and 10 ng/L, were studied with that technique.

The recovery was calculated from the repeatability experiments. For each instrument and concentration level, the recovery was calculated by dividing the average of the concentrations obtained (n = 9) by the expected theoretical concentration and multiplying the result by 100.

5.4 Results and discussion

Ten aromatic amine derivatives (i.e., iodinated aromatic compounds) were measured directly, without further sample treatment, in order to facilitate the direct comparison of the methods. The studied analytes were selected as model compounds due to their diverse chemical structures and properties. Furthermore, most of them have been previously studied and found in smoke [5-9], blood/tissue [10, 14-16], and/or urine matrixes [3, 21-23, 26, 28-30]. Three of the most comprehensive papers in terms of AA studied [8, 22, 28] found aniline, p-toluidine, 2,6-dimethylaniline, 2-chloroaniline, 2,4,5-trichloroaniline and 2,4-dichloroaniline, which are also included in this research.

5.4.1 Linear range

The linear ranges observed can be found in Table 5.2. For the GC-NCI-MS experiments, a plateau in the linear curve could be observed at concentrations of 50 or 100 ng/L, depending on the compound. Excellent goodness of fit was achieved for all the methods tested, with coefficients of determination (R^2) above 0.99 for all cases except for 1B4IB and 245TCIB when measured with GC-NCI-MS (0.988 and 0.989 respectively, data not shown).

The determination of 1B4IB with the GC-EI-MS method was hindered by an interfering signal covering the peak (see Fig. S 5.6, SI), which led to the analyte being identified only in concentration levels of 10 ng/L or above. A different column was used with this instrument, which could have led to a different elution pattern and may explain why the interference was not observed in the other systems. A different set of m/z may be used to study this compound, such as 155 and 157, which corresponds to the fragment without iodine.

When compared with literature (Table 5.3), the results are similar or better than those typically reported. In most cases, a linear range of approximately 3 orders of magnitude is reported [20-24, 27, 29, 30]. The results presented here show a linear range of 4 orders of magnitude for GC-EI-MS and GC-NCI-MS and of 5 for GC-EI-MS/MS. The broader the linear range, the higher the likelihood that analytes at very low concentrations can be accurately quantified, and that there is no further dilution needed for samples with very high concentrations, saving both sample volume and time.

5.4.2 LODs and LOQs

As expected, GC-EI-MS/MS shows the lowest LODs and LOQs, followed by GC-NCI-MS and GC-EI-MS (see Table 5.2) which on average have 3 and 12 times higher LODs, respectively. The high sensitivity achieved with GC-NCI-MS can be attributed to the high selectivity of this technique for halogenated compounds, which have a high electron affinity. The even better results obtained with GC-EI-MS/MS can be explained by the fact that in the first quadrupole, only the ions selected are trapped, which decreases the background noise, and consequently increases the sensitivity significantly.

Table 5.2. Limits of detection (LODs), quantification (LOQs), and linear ranges in pictograms per liter, obtained for the iodinated aromatic compounds with the studied GC methods. The concentration ranges tested were 20-500,000 pg/L for GC-EI-MS, 2-100,000 pg/L for GC-NCI-MS, and 1-100,000 pg/L for GC-EI-MS/MS. LODs and LOQs were calculated with concentrations where most analytes had S/N between 6 and 15, namely 200 pg/L for GC-EI-MS, 100 pg/L for GC-NCI-MS, and 10 pg/L for GC-EI-MS/MS.

	GC-EI-MS			**GC-NCI-MS**			**GC-EI-MS/MS**		
	LOD (pg/L)	LOQ (pg/L)	Linear range (pg/L)	LOD* (pg/L)	LOQ* (pg/L)	Linear range (pg/L)	LOD (pg/L)	LOQ (pg/L)	Linear range (pg/L)
IPFB	30	99	100 – 500,000	7.3	19	50 – 50,000	0.9	2.9	5 – 100,000
24DFIB	50	167	200 – 500,000	4.7	31	10 – 20,000	0.9	2.9	2 – 100,000
IB	25	84	100 – 500,000	6.8	23	20 – 50,000	2.1	7	5 – 100,000
4IMB	21	71	100 – 500,000	5.2	31	20 – 50,000	1.1	3.5	1 – 100,000
3C4FIB	14	47	100 – 500,000	6.3	21	10 – 20,000	1.3	4	2 – 100,000
1C2IB	26	86	100 – 500,000	3.0	15	5 – 20,000	0.8	2.5	2 – 100,000
2I13 DMB	21	71	50 – 500,000	5.6	28	10 – 50,000	0.5	1.7	1 – 100,000
1B4IB	-	-	10000 – 500,000	4.9	14	20 – 50,000	2.0	7	10 – 100,000
24DCIB	9*	29*	50 – 500,000	4.8	20	10 – 20,000	1.2	4.0	2 – 100,000
245 TCIB	28	93	200 – 500,000	6.3	13	10 – 50,000	3.9	13	10 – 100,000

*Outliers found with Dixon's Q test ($\alpha = 0.05$, $Q_{Critical} = 0.412$) not included in the calculations. LODs and LOQs were calculated according to the Eurachem Guide [49], as a constant (3 and 10, respectively) multiplied by the standard deviation of the concentration from tenfold replicates, and divided by the degrees of freedom (n - 1 = 9). Smoothing was set to "none".

LODs and LOQs for the analysis of aromatic amines in urine are generally reported in the nanogram-per-liter range (see Table 5.3). The best LODs reported (< 5 ng/L) were achieved with MS/MS detectors [20, 24, 27] and GC-NCI-MS [29] systems, while the worst (> 50 ng/L) were observed with EI-MS detectors [21, 30]. A similar trend can be observed in this study (Table 5.2), where GC-EI-MS shows worse limits than the other methods tested. Nonetheless, the results obtained were better than most of those found in literature, with LODs of 9-50 pg/L for GC-EI-MS, 3.0-7.3 pg/L for GC-NCI-MS, and 0.5-3.9 pg/L for GC-EI-MS/MS. The lowest limit reliably reported for aromatic amines in urine is 0.89 ng/L [27], which is between 120 and 1800 times worse than those reported here for the iodinated derivatives with GC-NCI-MS and GC-EI-MS/MS. The reason for the higher sensitivity achieved here is most likely the combination of a pre-concentration step like SPME with very sensitive measurement techniques and the fact that the iodinated derivatives were measured directly. Taking into account that during a similar derivatization procedure, for most analytes an estimated loss of 10 % was observed [38], it would be expected that the limits found with these instruments, including the complete sample preparation, would still be comparable if not better than those found in literature.

Other factors can affect the sensitivity of the method, such as the amount of sample used (typically within 5-20 mL), the concentration level studied, the steps of the sample preparation procedure included, the use of matrix-matched calibrations, and the equations used for the calculations (signal to noise ratio, standard deviation, etc.). Unfortunately, on several occasions, information was lacking for a proper interpretation of the results. For example, when the limits were calculated based on the S/N ratio, the concentrations used or if smoothing was applied was usually not reported. If too-high concentrations are used, this can lead to too-low LODs, which seems to be the case for the lowest limit found in literature [20], where the extrapolated limit reported is more than three orders of magnitude lower than the linear range.

Table 5.3. Figures of merit of most recent literature regarding the analysis of aromatic amines from urine samples. Ranges reported correspond to the minimum and maximum values from different analytes and/or concentration levels. Data in parentheses indicate missing experimental information needed for its interpretation.

Der. reagent	Injection technique	Volume/ SPME Fiber	Instrument	Calibration range (ng/L)	LOD (ng/L)	Recovery (%)	Intra-day Precision (%)	Inter-day Precision (%)	Concentration in real samples	Ref.
HI	HS-SPME	110 µm PDMS/DVB	GC-MS	100–1,200	3-12[2]	n.r.	3-12	n.r.	NS: n.d., S: n.d.-243 ng/L	[18]
No	LI	5 µL	LC-MS/MS (MRM)	100-50,000	25-500[2]	75-114[a]	1.6-11.7	2.1-15.9	U: n.d.-1.5, S: n.d.-3.47 µg/L	[19]
TMA-HCl, PFPA	LI	1 µL	GC-MS/MS (EI,MRM)	482-1,280	1.8-111.2[1]	> 85	1.1-6.3	2.6-6.3	n.r.	[3]
No	HS-SPME	80 µm, JUC-Z2	GC–MS/MS (MRM)	50-100,000	(0.010-0.012)[2]	95-101	(7.1-7.7)	n.r.	NS: n.d., S: 68.4-123.1 ng/L	[20]
PFPA, Pyr	LI	5 µL	LC-MS (SIM)	1,000-1,000,000	10,003	n.r.	n.r.	n.r.	NS: 1-1.13, S: 1.46-2.33 µg/L	[21]
HI	HS-SPME	65 µm PDMS/DVB	GCxGC-MS	1-500	5.2-24.4[4]	n.r.	n.r.	n.r.	n.r.	[22]

Der. reagent	Injection technique	Volume/ SPME Fiber	Instrument	Calibration range (ng/L)	LOD (ng/L)	Recovery (%)	Intra-day Precision (%)	Inter-day Precision (%)	Concentration in real samples	Ref.
No	LI	1 µL	GC-FID	30–100,000	(7–10)[2]	93.0-99.9	2.5-5.9	4.7-7.3	NS: n.d.-1.2, S: 2-14.5 µg/L	[23]
No	LI	10 µL	LC-MS/MS (MRM)	5-10,000	(1.5-5)[5]	88-111	6.1-8.9	9.0-9.9	NS: 1.11-12.32, S: 5.39-67.02 ng/24 h	[24]
No	LI	3 µL	UFLC (UV-Vis)	5,000-500,000	(DI:39,600-94,400, AE:880-1,300)[2]	89-105	0.6-7.9	2.4-10	U: n.d.-12.8 µg/L	[25]
No	HS-SPME	PEG/CNTs	GC-FID	1-105	(0.5-50)[2]	63.7-97.0	3.2-9.1	5.5-12.0[a]	NS: n.d.-940, S: 1140-50960 ng/L	[26]
TMA-HCl, PFPA	LI	1 µL	GC-MS/MS (EI, MRM)	50-25,000	0.896	(20-25)	1.7-6.7	7.5-8.4	NS: 1.30-2.07, S: 7.43-10.16 pg/mg Cr	[27]
No	LI	1 µL	GC-MS (SIM)	5-60,000	2-26[7]	94-104	4.5-6.2	6.0-6.8	U: n.d.-690 ng/L	[28]
PFPA, Pyr	LI	0.2 µL	GC-MS (NCI, SIM)	10-2,500	(1-4)[2]	94-107	(2.7-4.6)	(5.1-7.0)	NS: 9.6-105.2, S: 15.3-204.2 ng/24 h	[29]

Der. reagent	Injection technique	Volume/ SPME Fiber	Instrument	Calibration range (ng/L)	LOD (ng/L)	Recovery (%)	Intra-day Precision (%)	Inter-day Precision (%)	Concentration in real samples	Ref.
PFPA, Pyr	LI	1 µL	GC-MS (SIM)	100-100,000	(50-2,000)[2]	70–125	1.8-14	7.5–19	U: n.d.-3.5 µg/L	[30]
PFPI	LI	1 µL	GC-MS (SIM)	n.r.	(0.05 ng)[3]	(82.3-96.8)	n.r.	n.r.	NS: n.d.-1073.4, S: 3.6-2119.8 ng/24 h	[31]
HI	HS-SPME	65 µm PDMS/DVB	GC-EI-MS	0.05-500	0.009-0.05[8]	93-116	2.8-11	1.8-46	NS: n.d.-64, S: n.d.-173 ng/L	This study
			GC-NCI-MS	0.005-50	0.003-0.007[8]	71-104	2.1-12	3.7-40		
			GC-EI-MS/MS	0.001-100	0.001-0.004[8]	66-117	0.2-13[c]	4.0-35		

Abbreviations: AE, after extraction; Cr, creatinine; Der., derivatization; HS, headspace; JUC-Z2, porous organic framework; LI, liquid injection; n.d., not detected; n.r., not reported; NS, never smoker; PDMS/DVB, polydimethylsiloxane/divinylbenzene; PEG/CNTs, poly(ethylene glycol) modified with multi-walled carbon nanotubes; PFPA, pentafluoropropionic anhydride; PFPI, pentafluoro propionyl-imidazol; Pyr, pyridine; Ref., reference; S, smoker; TMA-HCl, trimethylamine hydrochloride; U, unknown smoking status.

[1]according to CLSI EP17-A; [2]S/N (signal to noise ratio) = 3; [3]not reported; [4]according to DIN 32645; [5]according to FDA guideline; [6]$SD_{low\ quality\ control\ samples}$; [7]LOD = 3*$SD_{y/x}$/b ($_{y/x}$ = regression); [8]LOD = 3*SD/(n - 1), (n = degrees of freedom), according to Eurachem Guide [49].

[a]Considering 37 of the 41 analytes studied; [b]Batch-to-batch precision; [c]Excluding 24TCIB (28 %) and IB (19 %) at 10 pg/L.

5.4.3 Precision: intra-day and inter-day repeatability

Intra-day repeatability (reported as relative standard deviations or RSDs) values were on average below 15 % for all analytes, concentration levels, and measuring techniques (Table 5.4). These results are in agreement with literature, as seen in Table 5.3, despite the fact that generally lower concentrations are used in this study, and a decrease in precision can be expected at lower concentration levels.

For all three methods, as expected, the repeatability improves with increased concentration. For GC-EI-MS, it is not apparent at first; however, the method is not sensitive enough to detect 1B4IB at the lower concentration, which would significantly worsen the average repeatability of that concentration. If the same concentration level is compared across methods, for example, 1 ng/L, the average intra-day repeatabilities of the methods are 7.1 % for GC-EI-MS (without IB4IB), 5.5 % for GC-NCI-MS and 2.8 % for GC-EI-MS/MS. The individual intra-day repeatabilities of each analyte can be seen in Table S 5.4, SI.

The majority of the inter-day repeatability results (reported as RSDs) are below 20 %; however, there are some exceptions. This could be due to the fact that n is smaller (3 vs 9), and the typical errors introduced during sample preparation and measurement have a bigger effect the smaller the number of samples measured.

If the different replicates are studied over time (as exemplified for 10 ng/L in Fig. S 5.7, SI), a clear pattern appears for the GC-NCI-MS results. This decrease over time can be explained by the fact that the ionization gas used (isobutane 3.5) is not as pure as the gases typically used for gas chromatography (5.0 or above), leading to the ion source becoming dirty with a corresponding decrease of the resulting signals. Unfortunately, to the best of our knowledge there is no purer isobutane commercially available, and the other gases that are typically used present other disadvantages (namely, methane induces harder ionization, and ammonia results in more maintenance needed). Therefore an equivalent to an internal standard correction, based on the averaged response of all the analytes instead of one specific standard, was made (as explained in SI and exemplified in Table S 5.5), and much better precision results (below 15 % in all cases) were achieved (Table 5.4 and Fig. S 5.8, SI). Because of how fast the intensity decreases, GC-NCI-MS would not be recommended, even if an internal standard is used, for larger sample batches.

Table 5.4. Average intra-day and inter-day repeatability, and recovery (%) results obtained for each of the techniques studied. The concentration levels tested were as follows: for GC-EI-MS, L (low) = 1 ng/L, M (medium) = 10 ng/L, and H (high) = 100 ng/L; for GC-NCI-MS, L = 0.1 ng/L, M = 1 ng/L, and H = 10 ng/L; and for GC-EI-MS/MS, L = 0.01 ng/L, M-L = 0.1 ng/L, M-H = 1 ng/L, and H = 10 ng/L.

	GC-EI-MS			**GC-NCI-MS**			**GC-EI-MS/MS**			
	L	M	H	L	M	H	L	M-L	M-H	H
Intra-day repeatability (%, n = 9)	7.1	7.8	5.2	5.6	5.5	3.7	12	4.0	2.8	2.1
Intra-day repeatability (%, n = 9)*	3.8	5.7	2.0	3.9	2.9	1.6	10.3	3.5	1.5	1.0
Inter-day repeatability (%, n = 3)	25	15	7.7	13	27	24	15	16	21	15
Inter-day repeatability (%, n = 3)*	12.6	8.7	4.5	5.3	7.4	2.9	13.2	9.7	9.6	7.7
Recovery (%, n = 9)	102	104	96	83	94	80	92	89	88	80

* Results obtained after internal standard-equivalent correction (explained in SI).

5.4.4 Recoveries

On average, recoveries between 80 and 120 % were obtained for all techniques and concentration levels studied, with RSDs between 3 and 14 % (see Table 5.4 and Table S 5.6 (SI) for a more detailed table with recoveries for each analyte).

As mentioned in section 5.3.2, it was not possible to always select the calibration curve used so that the concentrations studied were in the middle. This could explain why some recoveries for the lower and higher levels appear to be worse. However, it needs to be kept in mind that up to 4 different levels were tested for recoveries, when typically, only one is reported. This was the case because the overall performance of the three instruments was to be

compared. In the case of real samples, it is recommended that a smaller calibration curve is used, with more points per order of magnitude.

During the method optimization for GC-EI-MS/MS, the measuring windows were set relatively narrow in order to have better selectivity. However, because during later experiments the intensity of the peaks increased, as explained in the section "Solid-phase microextraction" and the SI, a higher tailing than expected was observed, and, in some cases, cut off due to the window length (see Fig. S 5.9, SI). Therefore, especially for the higher concentration levels, a worse recovery can be observed. This could be avoided by increasing the observed window, or, alternatively, by using a higher split ratio.

The overall recovery range found in the literature is between 64 and 125 %, although generally, it is between 80 and 110 % (Table 5.3). Despite the fact that the concentration levels used in the literature are typically higher than in this study, the recoveries observed are in agreement.

5.4.5 Real Samples

GC-NCI-MS and GC-EI-MS/MS show extremely good sensitivities, which allows for the analysis of derivatized AA in the pictogram-per-liter range. A few derivatized AA can be often found in higher concentrations, which could lead to some analytes being outside of the calibration curves. If these AA were the main interest, this could be easily solved by diluting the samples before measuring them, which would provide the added advantage of reducing the matrix interference and therefore increasing the robustness of the analysis. This could also be an advantage when measuring archived samples, since instead of diluting after the sample preparation is done, less urine sample could be used to start with. Alternatively, if high- and low-concentration AA need to be analyzed, the GC-EI-MS/MS method could be adjusted by changing the Q1 or Q3 resolutions so that the sensitivity in those highly concentrated compounds is lower compared to those of the rest of the compounds.

In this study, the validated methods were used and the samples were diluted to avoid saturation. In most cases, IB still showed concentrations above the highest calibration point. This analyte is typically found in both smokers and never smokers in high concentrations, which means there is another source of exposure besides tobacco smoke. Therefore, when analyzing

the concentrations of aromatic amines in relation to smoking status, and in order to avoid saturation of the detector in scan methods, this analyte could be left out.

With all three techniques, more aromatic amines could be tentatively identified in the samples from smokers than never smokers (see Table 5.5). As expected, with GC-NCI-MS, the most aromatic amines could be tentatively identified. This is due to the fact that with this technique, m/z = 127 was one of the monitored ions. This ion corresponds to the loss of iodine and, due to the derivatization process, is to be expected in all the aromatic amines in the sample. Because the GC-EI-MS analysis was done in SIM mode, only those compounds with the studied m/z (Table S 5.2, SI) could be detected. This technique is the least specific, as also non-aromatic compounds are detected, and therefore have to be filtered out manually. Finally, GC-EI-MS/MS in MRM mode is the most selective technique, and the best option among those tested for target screening, as it only detects molecules with defined transitions within defined measuring windows. Nonetheless, a few isomers could still be detected. An exemplarily chromatogram from a smoker's and never smoker's sample can be seen in Fig. 5.1.

Table 5.5. Total number of tentatively identified derivatized aromatic amines with each technique, in urine samples from three NS = never smokers and four S = smokers. All peaks found were taken into account for the GC-NCI-MS and GC-EI-MS/MS techniques, and only peaks with a loss of 127 were included in the GC-EI-MS calculations.

	NS1	**NS2**	**NS3**	**S1**	**S2**	**S3**	**S4**
GC-EI-MS	37	39	38	41	41	42	45
GC-NCI-MS	55	55	49	61	79	74	68
GC-EI-MS/MS	13	15	14	16	16	16	16

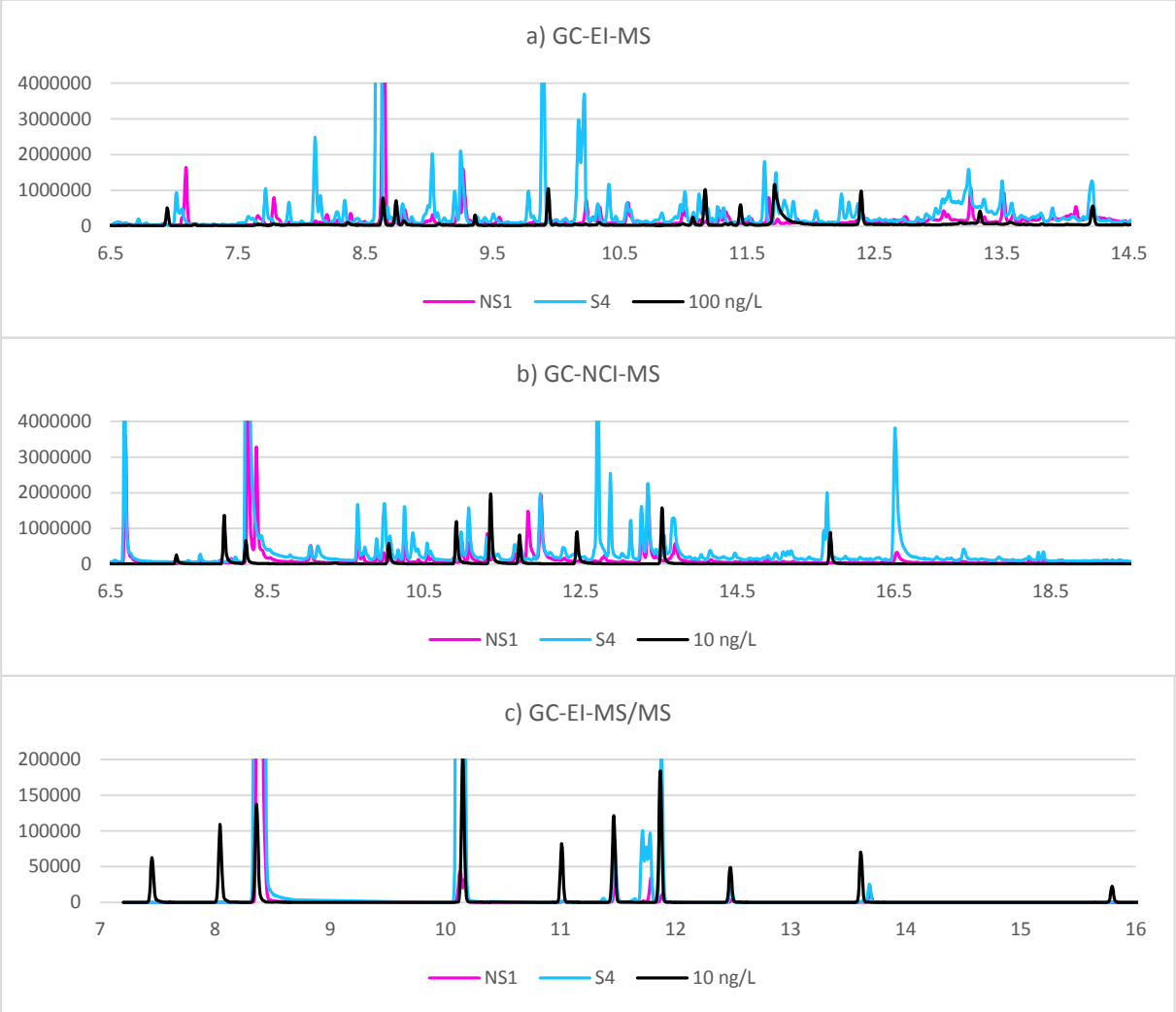


Fig. 5.1. Chromatogram comparison of pink = NS1, blue = S4 and black = 100 ng/L for a) GC-EI-MS or 10 ng/L for b) GC-NCI-MS, and c) GC-EI-MS/MS, zoomed. The m/z shown are a) the quantifier and qualifier ions reported in Table S 5.2 (SI), b) 127 and c) the transitions reported in Table 5.3 (SI). NS1 and S4 were diluted 1:10 for b) and c).

Six of the analytes studied could also be quantified with at least two techniques in most samples (Table 5.6). Great variability could be observed, as expected due to the nature of the samples, which could be partially accounted for by normalizing to creatinine and thereby correcting urinary output differences. Nonetheless, the averaged concentrations in samples from smokers were higher than in samples from never smokers for all six analytes. A similar trend can be observed in literature (Table 5.3). Furthermore, 4IMB and 1C2IB were found at significantly higher concentrations in smokers' samples, as determined by either Welch's two-

sided t-test or the two-variable t-test ($\alpha = 0.05$) [51, 52] and thus may be good candidates for future biomarker studies.

The three techniques show comparable results, most of the time within the same order of magnitude. Despite the extra dilution step, and because of the high sensitivity of the technique, IPFB, 24DFIB, and 1B4IB could only be detected with GC-EI-MS/MS in most samples (Table S 5.7, SI). 1C2IB shows the highest similarities between the three techniques, with RSDs below 20 % for all samples. 2,4DCIB could not be detected with GC-NCI-MS, but also showed RSDs below 20 % for the other two techniques. In several cases, the higher deviation was due to co-elutions present with some of the techniques (see SI). Depending on the analytical requirements, the GC parameters could be optimized to resolve specific co-elutions. Furthermore, the use of internal standards could have a positive effect minimizing the deviations between the techniques.

Table 5.6. Calculated concentrations in urine samples from three NS = never smokers and four S = smokers, in nanograms per liter, based on the average of the three techniques studied. Average NS and S concentrations are presented in bold.

	NS1	**NS2**	**NS3**	**NS**	**S1**	**S2**	**S3**	**S4**	**S**
4IMB	31	64	25	**40**	78	173	130	145	**132**
3C4FIB	0.6	0.6	0.5	**0.6**	0.6	0.4	1.0	0.9	**0.8**
1C2IB	13	17	13	**15**	22	19	34	29	**26**
2I13DMB	2.5	3.9	1.6	**3**	5.2	24	21	46	**24**
24DCIB	0.2	0.2	0.3	**0.3**	0.3	0.4	0.5	0.8	**0.5**
245TCIB	0.5	0.7	1.2	**0.8**	2.9	0.8	0.8	0.5	**1.2**

5.5 Conclusion

The most promising technique for the analysis of the iodinated derivatives of aromatic amines in urine is GC-EI-MS/MS. Despite showing slightly worse recoveries than GC-EI-MS, the obtained results are still within acceptable ranges. Furthermore, as expected, the sensitivity and selectivity of the method are significantly better, so that GC-EI-MS/MS would be the method of choice for further analysis. GC-NCI-MS shows a slightly worse behavior than GC-EI-MS/MS, with the addition of the significant loss in sensitivity over time due to the ionization gas purity. Nonetheless, for qualitative/non-target analysis, GC-NCI-MS offers the advantage that all the derivatized iodinated amines can be easily identified. Finally, GC-EI-MS shows the worst results in terms of sensitivity and selectivity. However, it has the advantage of being the most widespread and least expensive of the three techniques studied. This technique could therefore be especially interesting when low concentrations are not of interest, or for screening purposes.

One of the main drawbacks of GC-EI-MS/MS in MRM mode is that the analytes need to be defined in advance. This could be problematic when measuring real samples, since approximately 150 different AA have previously been identified in smokers' urine [22]. Most GC-EI-MS/MS offer the possibility of doing scan/MRM, which could enable qualitative non-target screening and the sensitive and selective quantification of specific target compounds. Another alternative would be to combine the derivatization method presented here with GC-EI-MS/MS in neutral loss mode.

Finally, the high sensitivity and selectivity obtained for the analysis of the iodinated derivatives with HS-SPME GC-EI-MS/MS are a great advantage over other methods found in literature. Especially for the analysis of valuable samples, such as archived samples (for example, from cohort studies), since it can enable a considerable reduction of sample volume needed. This could be used to foster our understanding of the interactions between the smoking status, the concentration of aromatic amines, and the risk of developing smoking-related diseases.

5.6 Supplementary information

5.6.1 Materials and methods

Derivatization procedure

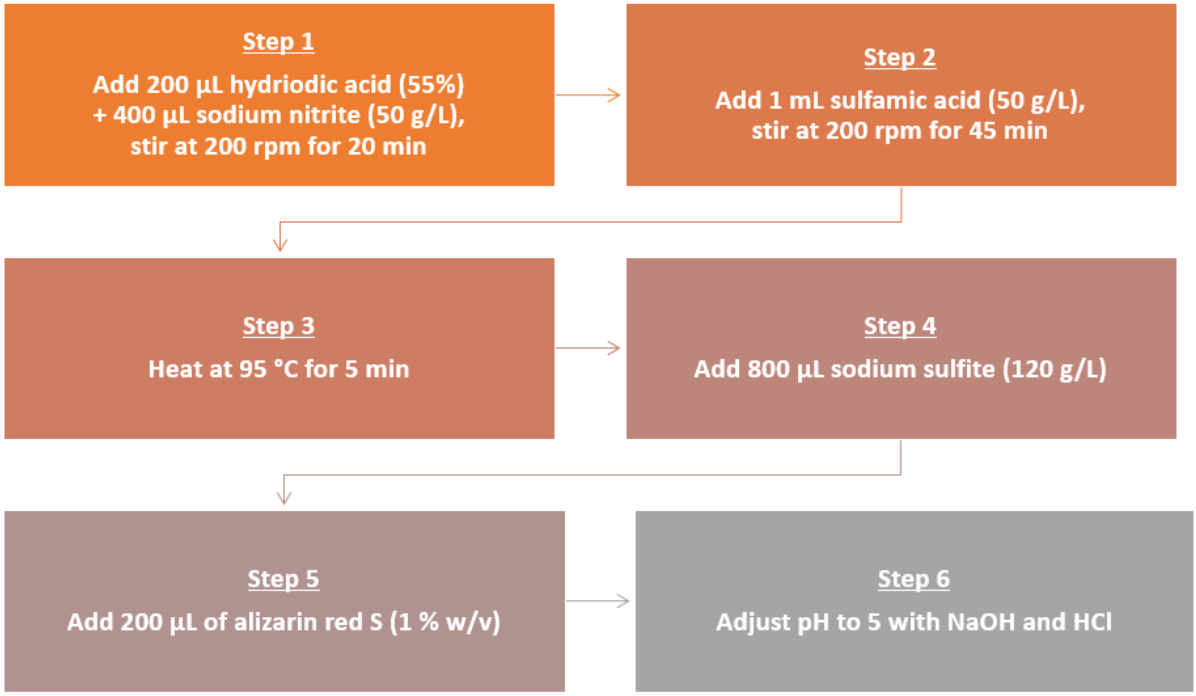


Fig. S 5.1. Derivatization procedure followed during sample preparation.

Fiber comparison

1 ng/L solutions of the iodinated derivatives were measured in triplicate with GC-NCI-MS, with each of the fibers used in this study. The results show that the fibers were comparable for most of the analytes, however, for 4IMB, 3C4FIB, and 1B4IB, the fiber used for the GG-MS/MS measurements showed significantly worse results (tested with one-way ANOVA, and shown in Fig. S 5.2). This could have an effect on the linear ranges and limits presented for those analytes when measured with GG-MS/MS. It would be expected that if the other fibers had been used, the results for these three analytes with GC-EI-MS/MS technique would have been even more sensitive.

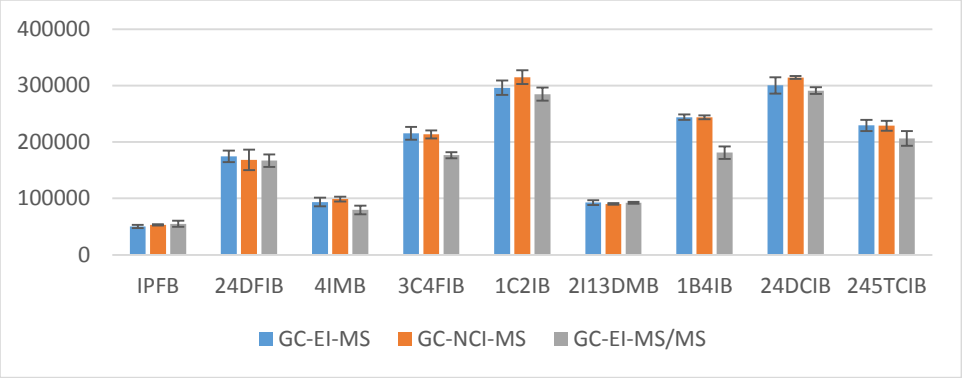


Fig. S 5.2. Comparison of the fibers used for each of the three techniques, by measuring 1 ng/L with GC-NCI-MS at the end of the experiments.

SPME Mix corrections for GC-EI-MS/MS

The day the calibration curve was measured (08. Feb), the intensities of the SPME Mix were much smaller than for the rest of the days, see Fig. S 5.3. Which might indicate insufficient conditioning [47, 48], despite following the manufacturer instructions.

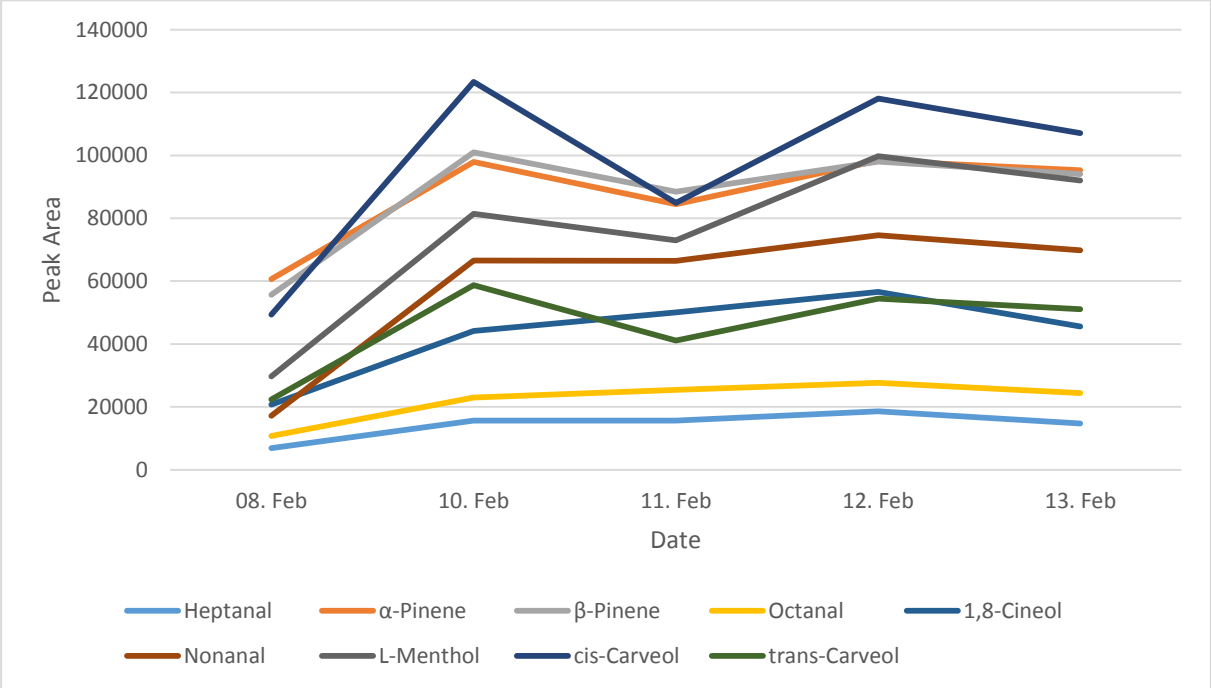


Fig. S 5.3. Peak areas of a selected number of analytes from the SPME mix over time, measured with GC-EI-MS/MS.

In order to correct for the significantly smaller intensity observed the first day, the results obtained in each of the experiments where GC-EI-MS/MS was used were normalized based on the SPME Mix intensity by correcting each day with the factor shown in Table S 5.1.

Table S 5.1. Relative peak areas of the SPME mix analyzed, normalized with the areas of the day the calibration curve was measured (08. Feb), and the correction factors used, which were calculated by normalizing the sum of the relative areas of each day with that of the 08. Feb.

	08. Feb	**10. Feb**	**11. Feb**	**12. Feb**	**13. Feb**
Heptanal	1.00	2.26	2.26	2.70	2.14
α-Pinene	1.00	1.61	1.39	1.62	1.57
β-Pinene	1.00	1.82	1.59	1.76	1.69
Octanal	1.00	2.14	2.37	2.57	2.27
n-Decane	1.00	2.00	1.83	2.11	2.12
p-Cymene	1.00	1.90	1.86	2.09	1.91
1,8-Cineol	1.00	2.13	2.42	2.73	2.20
Nonanal	1.00	3.87	3.86	4.34	4.06
L-Menthol	1.00	2.74	2.46	3.36	3.09
n-Dodecane	1.00	2.50	2.56	3.03	2.97
cis-Carveol	1.00	2.50	1.72	2.39	2.17
trans-Carveol	1.00	2.63	1.84	2.44	2.29
Carvone	1.00	2.83	2.74	3.39	2.97
1-Decanol	1.00	3.08	2.32	3.74	3.38
1-Undecanol	1.00	2.70	2.01	3.07	2.63
n-Tetradecane	1.00	2.50	3.08	3.22	3.10
1-Dodecanol	1.00	2.80	2.03	2.87	2.34
Sum	17	42.0	38.3	47.4	42.9
Correction factor	1.00	2.47	2.26	2.79	2.52

Mass spectrometric parameters

Table S 5.2. Mass spectrometric parameters for the GC-EI-MS analysis, including the quantifier and qualifier ion recorded for each of the analytes studied, and the corresponding monoisotopic masses calculated based on [53].

Analyte	Monoisotopic mass (Da)	Quantifier ion (m/z)	Qualifier ion (m/z)
IPFB	293.8965	294	117
24DFIB	239.9248	240	113
IB	203.9436	204	77
4IMB	217.9592	218	91
3C4FIB	255.8952	256	129
1C2IB	237.9046	238	111
2I13DMB	231.9749	232	105
1B4IB	281.8541	282	284
24DCIB	271.8657	272	145
245TCIB	305.8267	306	308

Table S 5.3. Mass spectrometric parameters for the GC-EI-MS/MS analysis in MRM mode, including start and end recording times, precursor and product ions, and collision energies (CE) for each of the analytes. The event time was set to 0.3 s for all transitions.

Analyte	Start time (min)	End time (min)	Precursor ion (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)	CE 1 (V)	CE 2 (V)
IPFB	7.20	7.65	294	167	117	26	33
24DFIB	7.80	8.20	240	113	63	21	33
IB	8.20	8.70	204	77	204	18	1
4IMB	9.90	10.40	218	91	65	18	30
3C4FIB	10.80	11.20	256	129	109	20	31
1C2IB	11.25	11.60	238	111	75	18	32
2I13DMB	11.65	12.10	232	105	77	18	32

Analyte	Start time (min)	End time (min)	Precursor ion (m/z)	Product ion 1 (m/z)	Product ion 2 (m/z)	CE 1 (V)	CE 2 (V)
1B4IB	12.25	12.65	282	155	157	20	18
24DCIB	13.40	13.90	272	145	109	19	30
245TCIB	15.60	16.10	308	181	179	21	32

5.6.2 Results and discussion

Chromatogram comparison

In Fig. S 5.4 the chromatograms of the 50 ng/L standard measured with the three techniques can be seen.

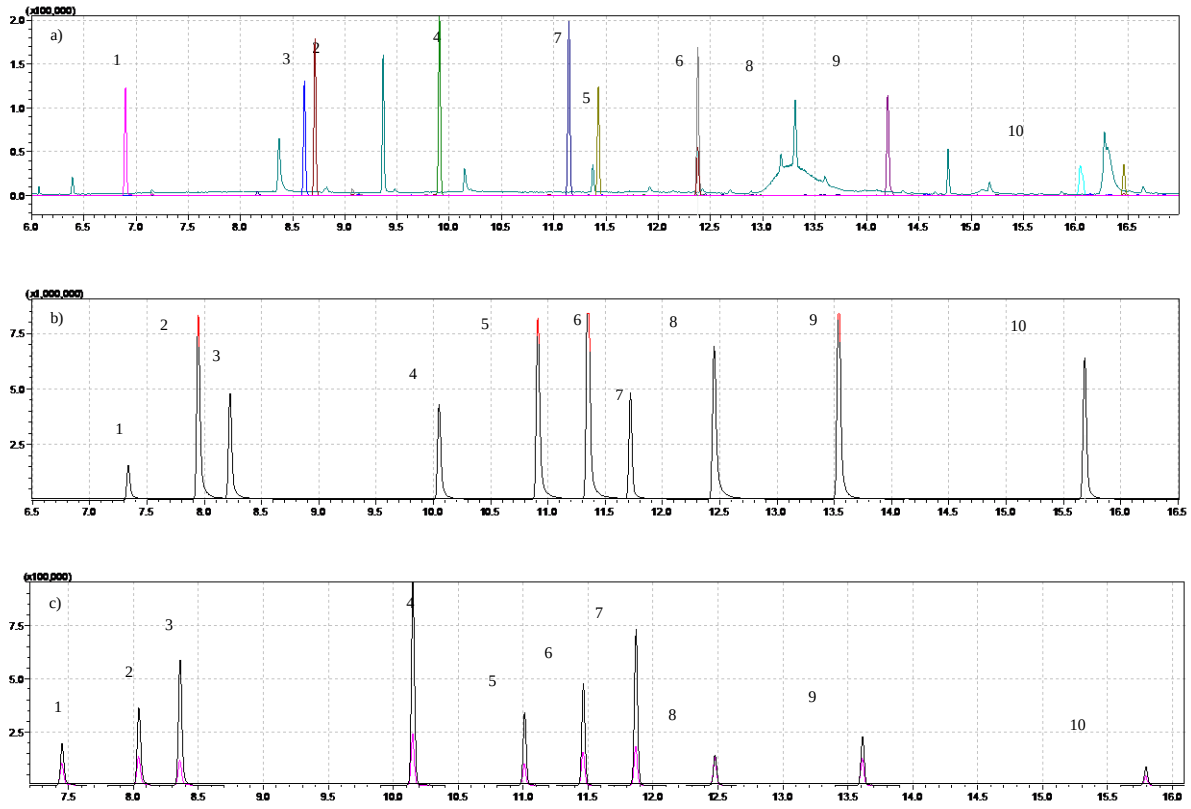


Fig. S 5.4. Chromatogram comparison of the 50 ng/L level measured with a) GC-EI-MS, b) GC-NCI-MS, and c) GC-EI-MS/MS. The m/z shown are a) the quantifier ions reported in Table S 5.2, b) 127 and c) the transitions reported in Table S 5.1. The peaks correspond to: 1. IPFB, 2. 24DFIB, 3. IB, 4. 4IMB, 5. 3C4FIB, 6. 1C2IB, 7. 2I13DMB, 8. 1B4IB, 9. 24DCIB, 10. 245TCIB.

As expected, GC-EI-MS is the least selective technique, since not only AA derivatives can be seen. Saturation of several compounds (marked with red) and tailing can also be observed in the GC-NCI-MS chromatogram. This is in agreement with the upper limit of the linear ranges found for this technique, which were ≤ 50 ng/L for all compounds. If concentrations in this order of magnitude are of interest, doing split injections or diluting the samples would reduce and/or eliminate the tailing and detector saturation.

Exemplary calibration curves

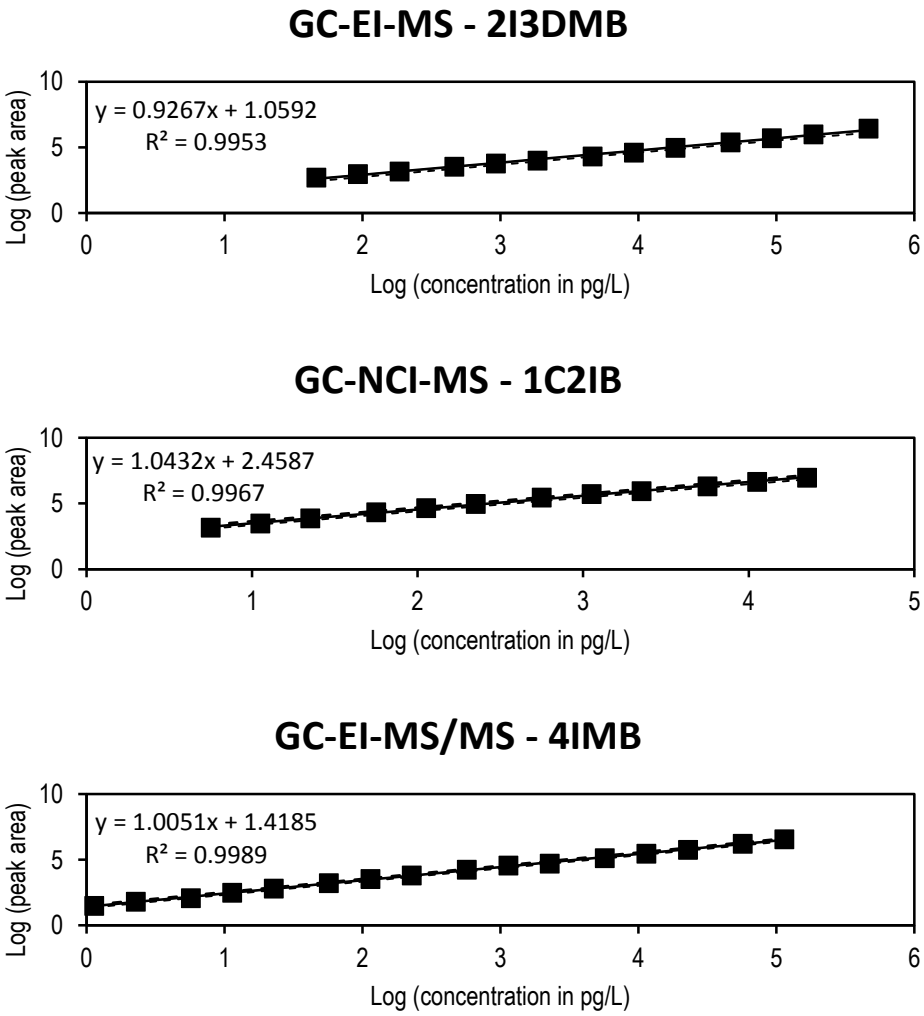


Fig. S 5.5. Concentration curves obtained with each of the techniques studied, for some of the analytes with the biggest linear range for each technique. The linear equations and the regression coefficients can be found within each graph. The prediction bands were calculated according to DIN 32645[54].

1B4IB with GC-EI-MS: problematic analyte

The high background noise observed (Fig. S 5.6) hinders the proper identification of IB4IB in lower calibration levels (< 10 ng/L).

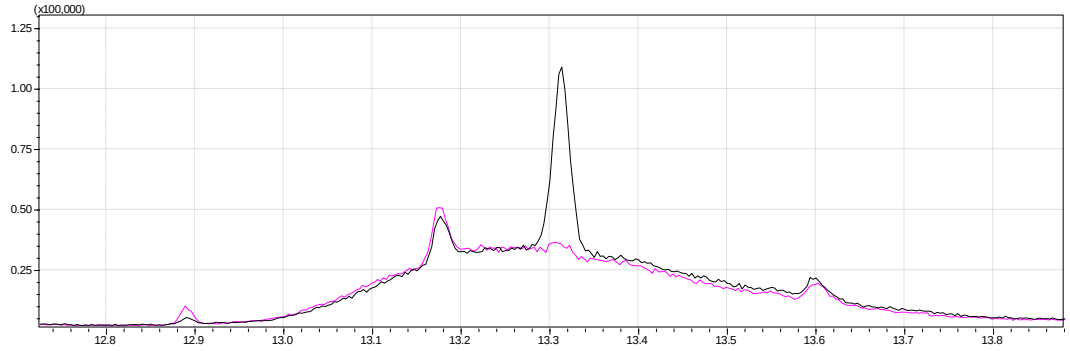


Fig. S 5.6. Chromatogram comparison of the m/z = 282, corresponding to the quantifier ion of 1B4IB, of the 5 ng/L (pink) and 50 ng/L (black) calibration standards, measured with GC-EI-MS.

Detailed intra-day and inter-day repeatability results

Intra-day repeatability values below 15 % were obtained for all analytes, concentration levels and measuring techniques with three exceptions, all in the lower concentration range: 1B4IB with GC-EI-MS, IB with GC-EI-MS/MS and 245TCIB with the same instrument (see Table S 5.4). The concentration level tested for IB4IB (1 ng/L) was below the linear range of the analyte for that technique. In the case of GC-EI-MS/MS, the concentration levels used were 10 pg/L, well below any previously reported limit (Table 5.3). In the case of 245TCIB, the low intra-day repeatability could be attributed to the fact that the concentration tested was the lowest concentration that could be detected.

Before the second repetition of the repeatability experiments with GC-EI-MS, real samples with relatively high concentrations were measured. This led to contaminated blanks for 24DFIB and IB, which had to be corrected for in the lower concentration level and could account for the worse precision observed for those analytes. In the case of GC-EI-MS/MS, IB and 4IMB were found in all the blanks from the precision experiments and also had to be corrected for in the lower calibration level. In this case, that could be because the concentration range tested for these experiments is four orders of magnitude broad, and the fiber might need

extra conditioning to avoid carryover for subsequent measurements at extremely low concentrations (10 pg/L). This can also be observed in the results, especially for IB. Furthermore, in one of the repetitions the syringe blank (where pure methanol was added, instead of a stock solution, after cleaning the syringe 10-20 times) appeared contaminated, which emphasizes the difficulty of working with such small concentrations and the importance of taking extra steps to make sure everything is clean.

Table S 5.4. Intra-day and inter-day repeatability (%) results obtained for the iodinated aromatic compounds. The concentration levels tested were: for GC-EI-MS, L (low) = 1 ng/L, M (medium) = 10 ng/L, H (high) = 100 ng/L; for GC-NCI-MS, L = 0.1 ng/L, M = 1 ng/L, H = 10 ng/L; and for GC-EI-MS/MS, L = 0.01 ng/L, M-L = 0.1 ng/L, M-H = 1 ng/L, H = 10 ng/L. Results in bold are above 20 %. GC-MS/MS results are normalized according to the SPME Mix intensities over time (described in SI).

Intra-day repeatability (%, n = 9)										
	GC-EI-MS			**GC-NCI-MS**			**GC-EI-MS/MS**			
	L	M	H*	L	M	H	L	M-L	M-H	H
IPFB	10*	11	6.8	4.8	5.0*	5.0	6.1*	2.5	1.4*	1.3
24DFIB	11	10	4.6	2.4	5.0*	5.3	13	2.5	2.2	1.8*
IB	9.9	5.5*	6.4	11	3.4*	3.8	19	3.2	4.1	1.9
4IMB	5.0*	10	4.5	12	8.8*	3.3*	8*	1.9*	3.3	1.9
3C4FIB	2.8*	6.6	6.0	3.7	7.3	4.3	8.9	5.2*	0.2*	2.4*
1C2IB	7.5	9.3	5.1	2.9	4.8	3.3*	12	2.0	3.0	2.3
2I13DMB	6.9*	5.5	5.5	2.9	3.3	2.1	7.7	3.2	2.5	1.3
1B4IB	-	7.6*	4.2	3.8	8.4	3.7	11*	5.4	4.3	2.6
24DCIB	3.8	5.7	5.2	3.0*	3.4	4.0	11	4.6*	3.2	2.4
245TCIB	6.8	5.3	3.9	9.2*	5.5	2.7	**28**	10*	3.5	2.9

Inter-day repeatability (%, n = 3)										
	GC-EI-MS			**GC-NCI-MS**			**GC-EI-MS/MS**			
	L	M	H*	L	M	H	L	M-L	M-H	H
IPFB	1.8*	4.0	9.1	12	20*	20	8.9*	12	13*	12
24DFIB	12	17	6.6	18	17*	**28**	**21**	10	19	12*
IB	16	8.9*	6.2	14	16*	**21**	**25**	**22**	**27**	18
4IMB	**46***	**22**	6.9	12	**28***	19*	6.0*	20*	**30**	18
3C4FIB	8.0*	3.3	4.7	13	**26**	**25**	13	20*	15*	16*
1C2IB	**46**	**21**	10	12	**34**	**28***	4.0	7.2	19	12
2I13DMB	**25***	12	9.2	9.3	**40**	**32**	9.1	14	16	9.9
1B4IB	-	19*	11	3.7	**27**	19	**24***	20	**30**	**22**
24DCIB	**31**	13	6.9	16*	**34**	**31**	8	13*	**21**	16
245TCIB	**41**	**24**	5.9	19*	**31**	**23**	**35***	20*	**22**	18

*Outliers found with Dixon´s Q test ($\alpha = 0.05$, $Q_{Critical} = 0.436$ and 0.941), not included in the calculations.

Inter-day repeatability: repetitions over time

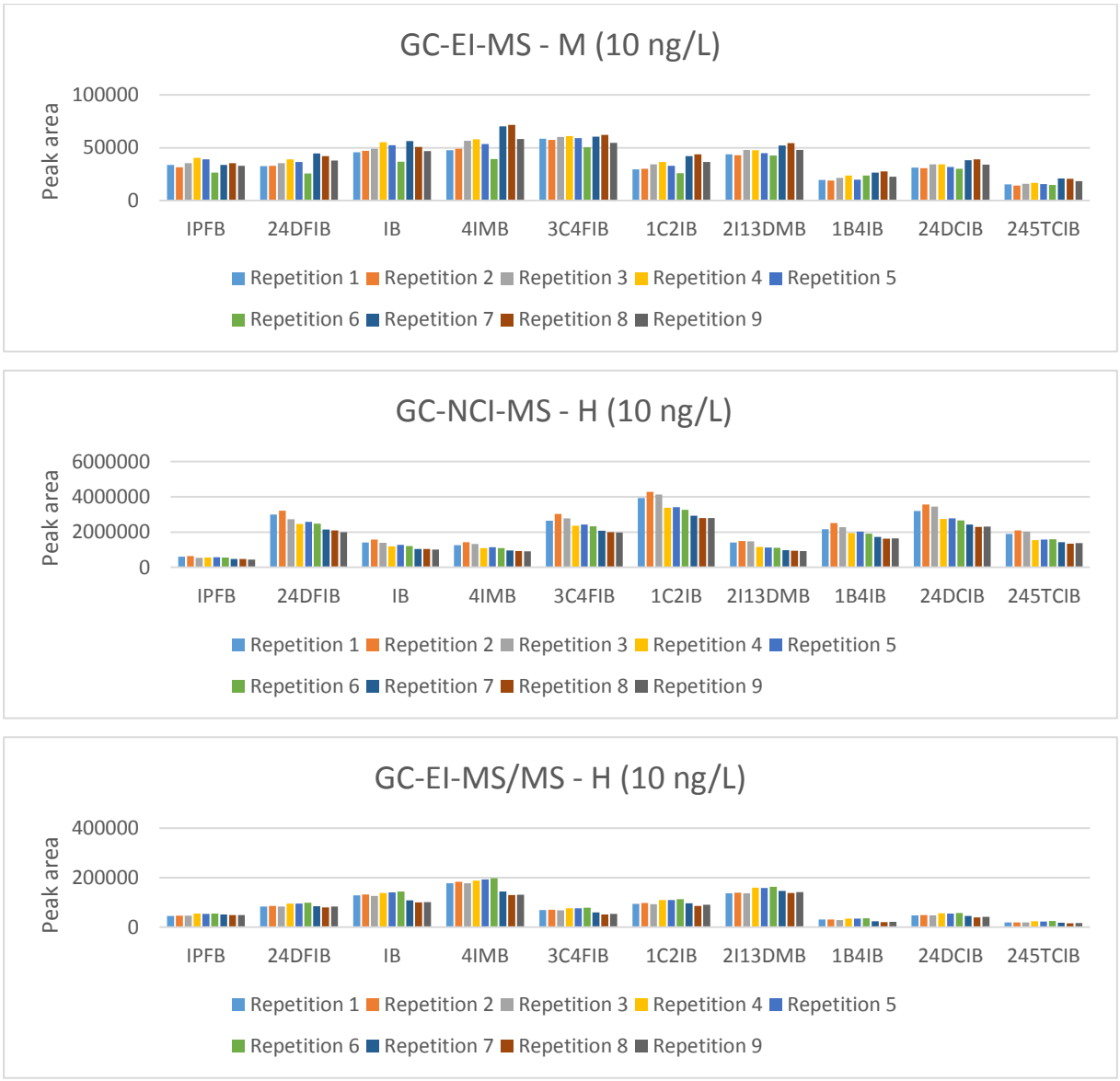


Fig. S 5.7. Peak area over time at the same concentration level for each method studied. Repetitions 1-3, 4-6 and 7-9 were performed in consecutive days. All outliers are included.

Internal standard-equivalent correction

Ideally, one isotopically labelled internal standard per analyte of interest is used both during the validation of the method, and the analysis of real samples. However, sometimes they are not commercially available, or their price is extremely high. In this study, an equivalent

correction was used during the LOD and precision/recovery experiments, but instead of based on a specific internal standard, it was based on the overall response of all the analytes.

First, the peak areas obtained for each analyte were normalized based on their average (analyte correction factor), afterwards, these results were added for each sample (sample correction factor) and the average was calculated, and finally, each sample correction factor was normalized by the average (internal standard equivalent) and used for the corresponding sample. In Table S 5.5 an example calculation can be seen for two analytes and two samples, and Fig. S 5.8 shows the corrected results for GC-NCI-MS (non-corrected results in Fig. S 5.7).

Table S 5.5. Exemplary calculation of the internal standard-equivalent correction.

Repetition	**1**	**2**	**Average**
Peak area IPFB	616126	650372	633249
Peak area 24DFIB	2888157	3129281	3008719
IPFB correction factor	0.97	1.03	-
24DFIB correction factor	0.96	1.04	-
Sample correction factor	1.93	2.07	2
Internal standard equivalent	0.97	1.03	-

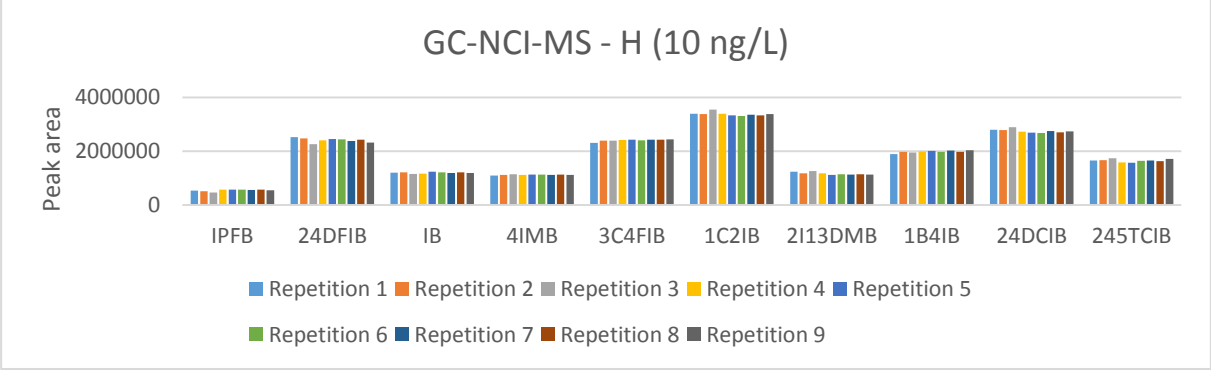


Fig. S 5.8. Peak area over time for 10 ng/L measured with GC-NCI-MS, after internal standard equivalent correction. Repetitions 1-3, 4-6 and 7-9 were performed in consecutive days. All outliers are included.

Detailed recovery results

The recoveries obtained for each of the analytes can be seen in Table S 5.6. Some analytes showed recoveries below 80 %. In the case of GC-MS, 1IB4IB could not be seen in the low concentration level (1 ng/L) as discussed in section 5.4.1, and shown in Fig. S 5.6. The lower recoveries obtained when not optimal calibration curves are used can be seen, for example, at the low concentration levels for 245TCIB measured with GC-MS, and 1B4IB measured with GC-NCI-MS and at the high concentration level for 24DFIB measured with GC-NCI-MS. The effect of the bigger tailing in the GC-MS/MS recovery results, as described in the main text, is more prominent for those analytes with a higher intensity than the rest, like 4IMB, 2I13DMB, and IB.

The GC-EI-MS results for the analytes 24DFIB and IB for the lower concentration levels show that the blank correction mentioned in the section "Detailed intra-day and inter-day repeatability results" had no adverse effect in the recoveries obtained and corroborating the need for such a correction. In the case of the GC-EI-MS/MS results, 4IMB might seem like it was over-corrected for, since the recovery obtained is only 81 %. However, this value is very similar in the other concentration levels, and, when compared with the other analytes within the same level, it is consistently in the lower range.

Table S 5.6. Recovery (%) results obtained for the iodinated aromatic compounds, including average and relative standard deviation (RSD). Results in bold are below 80 %. The concentration levels tested can be seen in Table S 5.4. GC-EI-MS/MS results are normalized according to the SPME Mix intensities over time.

	Recovery (%, n = 9)									
	GC-EI-MS			**GC-NCI-MS**			**GC-EI-MS/MS**			
	L	M	H*	L	M	H	L	M-L	M-H	H
IPFB	94	107	107	97	99*	84	102	103	88	**74**
24DFIB	94	106	100	**73**	85*	**79**	90	**78**	**76**	**69**
IB	99	110*	104	82	88*	80	92	87	**79**	**74**
4IMB	105	107	99	82	93	81	81	81	80	**79**

Recovery (%, n = 9)										
	GC-EI-MS			**GC-NCI-MS**			**GC-EI-MS/MS**			
	L	M	H*	L	M	H	L	M-L	M-H	H
3C4FIB	99*	99	99	91	104	84	98	87	90	83
1C2IB	111	107	99	82	90	80	**66**	80	85	80
2I13DMB	98	102	100	80	85	**74**	80	**68**	**75**	**75**
1B4IB	-	95	93	**71**	91	82	106	97	96	87
24DCIB	108	101	95	84	92	80	101	98	93	83
245TCIB	116	107	96	85	98	81	102	114	117	96
Average	102	104	96	83	94	80	92	89	88	80
RSD	8	4	4	8	6	3	13	14	12	8

*Outliers found with Dixon´s Q test ($\alpha = 0.05$, $Q_{Critical} = 0.436$), not included in the calculations.

High concentrations with GC-EI-MS/MS: tailing outside of measuring windows

It is believed that the SPME fiber was not sufficiently conditioned (despite following the manufacturer´s instructions) when the first experiments, namely the calibration curve, were measured with GC-EI-MS/MS. Therefore, a lower intensity can be observed for those experiments (Fig. S 5.9). Furthermore, because a narrow window was set for each analyte in the MS/MS parameters in order to increase the selectivity, when the intensities were higher and bigger tailing occurred, this was not always fully recorded (inset, Fig. S 5.9). This could lead to lower recoveries than expected, especially for those analytes with more tailing or narrower windows.

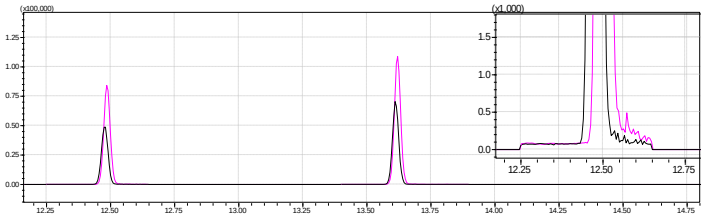


Fig. S 5.9. Chromatogram comparison of the 10 ng/L level measured with GC-EI-MS/MS during the calibration curve experiments (black) and the LOD, LOQ, and recovery experiments (exemplary from the first day, pink) (see Table 5.3 for recorded precursor/product ions). In contrast to 24DCIB (right), 1B4IB (left) shows some tailing (inset, exemplary from the third day) that continues past the recorded time.

Real samples concentrations

The values shown in Table S 5.7 were used for the calculations of the average concentrations of Table 5.5, with the exception of the values marked with *. These values were excluded for different reasons. In the case of 4IMB, we believe the slight differences in the columns used enabled the separation of isomers with GC-EI-MS/MS, but not with the other techniques, as seen in the comparison with GC-EI-MS in Fig. S 5.10.

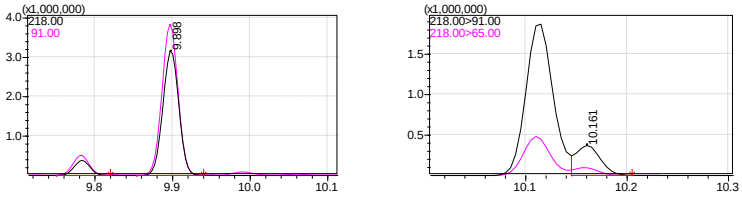


Fig. S 5.10. Chromatograms corresponding to 4IMB in S4 samples, measured with GC-EI-MS (left) and GC-EI-MS/MS (right).

In the case of 3C4FIB and 245TCB, it is believed that the analytes of interest might have co-eluted with other iodinated analytes when measured with GC-NCI-MS. Because with the other two techniques only analytes with a defined m/z ratio are detected, co-eluting compounds with different molecular ions can be successfully avoided. GC-NCI-MS does not provide information regarding the molecular ions and can therefore lead to higher concentrations. A possibility would be to combine GC-NCI-MS with one of the other techniques discussed here. Another alternative could be GCxGC-NCI-MS, since a significantly higher chromatographic resolution can be achieved, minimizing potential co-elutions.

Table S 5.7. Calculated concentrations in the urine samples from three NS = non-smokers and four S = smokers, in ng/L. The techniques are abbreviated: GC: GC-EI-MS; NCI: GC-NCI-MS; MSMS: GC-EI-MS/MS. nd = not detected, > C = results above the upper calibration curve limit. Results not considered for the average calculations marked with *italics*.

	NS1			**NS2**			**NS3**			**S1**			**S2**			**S3**			**S4**		
	GC	NCI	MS MS	GC	NCI	MS MS	GC	NCI	MS MS	GC	NCI	MS MS	GC	NCI	MS MS	GC	NCI	MS MS	GC	NCI	MS MS
IPFB	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.07	nd	nd	nd
24 DFIB	nd	nd	0.04	nd	nd	0.05	nd	nd	0.04	nd	nd	0.05	nd	nd	0.07	nd	nd	0.07	nd	nd	0.08
IB	>C	>C	823	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C	>C
4IMB	14	47	*5.0*	68	60	*7.1*	11.3	39	*1.6*	89	67	*4.1*	237	110	*19*	>C	130	*54*	>C	145	*79*
3C4 FIB	0.8	*63*	0.5	0.7	*69*	0.5	0.6	*63*	0.5	0.6	*63*	0.6	0.4	*62*	0.4	1.1	*83*	0.9	1.1	*78*	0.8
1C2IB	13	16	10	19	15	18	12	14	14	22	20	24	18	17	21	34	34	34	28	30	28
2I13 DMB	1.7	5	1.4	2.8	7	2.2	1.5	1.9	1.4	3.4	9	3	4.9	62	4.4	19	32	12	60	41	38
1B4IB	nd	nd	2.5	nd	nd	6.3	nd	nd	6.2	nd	nd	15	103	173	129	nd	nd	15	nd	nd	17
24 DCIB	0.3	nd	0.2	0.2	nd	0.2	0.4	nd	0.3	0.3	nd	0.3	nd	nd	0.4	0.5	nd	0.6	0.8	nd	0.9
245TC IB	nd	0.5	0.5	nd	*12*	0.7	nd	nd	1.2	nd	5.0	0.7	nd	*111*	0.8	nd	*9.3*	0.8	0.2	*168*	0.8

5.7 References

1. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Chemical Agents and Related Occupations. Lyon, France: International Agency for Research on Cancer; 2012.

2. Pereira L, Mondal PK, Alves M. Aromatic amines sources, environmental impact and remediation. In: Lichtfouse E, Schwarzbauer J, Robert D, editors. Pollutants in Buildings, Water and Living Organisms. Environmental Chemistry for a Sustainable World. Cham: Springer International Publishing; 2015. pp. 297-346. https://doi.org/10.1007/978-3-319-19276-5_7.

3. Mazumder S, Ahamed RA, McGahee E, Wang L, Seyler TH. A new automated method for the analysis of aromatic amines in human urine by GC-MS/MS. J Anal Toxicol. 2019;43:25-35. https://doi.org/10.1093/jat/bky045.

4. Besaratinia A, Tommasi S. Genotoxicity of tobacco smoke-derived aromatic amines and bladder cancer: Current state of knowledge and future research directions. FASEB J. 2013;27:2090-100. https://doi.org/10.1096/fj.12-227074.

5. Deng H, Yang F, Li Z, Bian Z, Fan Z, Wang Y, Liu S, Tang G. Rapid determination of 9 aromatic amines in mainstream cigarette smoke by modified dispersive liquid liquid microextraction and ultraperformance convergence chromatography tandem mass spectrometry. J Chromatogr A. 2017;1507:37-44. https://doi.org/10.1016/j.chroma.2017.05.056.

6. Bie Z, Lu W, Zhu Y, Chen Y, Ren H, Ji L. Rapid determination of six carcinogenic primary aromatic amines in mainstream cigarette smoke by two-dimensional online solid phase extraction combined with liquid chromatography tandem mass spectrometry. J Chromatogr A. 2017;1482:39-47. https://doi.org/10.1016/j.chroma.2016.12.060.

7. Zhang J, Bai R, Zhou Z, Liu X, Zhou J. Simultaneous analysis of nine aromatic amines in mainstream cigarette smoke using online solid-phase extraction combined with liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2017;409:2993-3005. https://doi.org/10.1007/s00216-017-0245-6.

8. Smith CJ, Dooly GL, Moldoveanu SC. New technique using solid-phase extraction for the analysis of aromatic amines in mainstream cigarette smoke. J Chromatogr A. 2003;991:99-107. https://doi.org/10.1016/S0021-9673(03)00205-X.

9. Stabbert R, Schäfer KH, Biefel C, Rustemeier K. Analysis of aromatic amines in cigarette smoke. Rapid Commun Mass Spectrom. 2003;17:2125-32. https://doi.org/10.1002/rcm.1161.

10. Guo J, Villalta PW, Weight CJ, Bonala R, Johnson F, Rosenquist TA, Turesky RJ. Targeted and Untargeted Detection of DNA Adducts of Aromatic Amine Carcinogens in Human Bladder by Ultra-Performance Liquid Chromatography-High-Resolution Mass Spectrometry. Chem Res Toxicol. 2018;31:1382-97. https://doi.org/10.1021/acs.chemrestox.8b00268.

11. Yun BH, Bellamri M, Rosenquist TA, Turesky RJ. Method for Biomonitoring DNA Adducts in Exfoliated Urinary Cells by Mass Spectrometry. Anal Chem. 2018;90:9943-50. https://doi.org/10.1021/acs.analchem.8b02170.

12. Cai T, Bellamri M, Ming X, Koh WP, Yu MC, Turesky RJ. Quantification of Hemoglobin and White Blood Cell DNA Adducts of the Tobacco Carcinogens 2-Amino-9H-pyrido[2,3-b]indole and 4-Aminobiphenyl Formed in Humans by Nanoflow Liquid Chromatography/Ion Trap Multistage Mass Spectrometry. Chem Res Toxicol. 2017;30:1333-43. https://doi.org/10.1021/acs.chemrestox.7b00072.

13. Lee HW, Wang HT, Weng MW, Hu Y, Chen WS, Chou D, Liu Y, Donin N, Huang WC, Lepor H, Wu XR, Wang H, Beland FA, Tang MS. Acrolein- and 4-Aminobiphenyl-DNA adducts in human bladder mucosa and tumor tissue and their mutagenicity in human urothelial cells. Oncotarget. 2014;5:3526-40. https://doi.org/10.18632/oncotarget.1954.

14. Sabbioni G. Hemoglobin Adducts and Urinary Metabolites of Arylamines and Nitroarenes. Chem Res Toxicol. 2017;30:1733-66. https://doi.org/10.1021/acs.chemrestox.7b00111.

15. Pathak KV, Chiu TL, Amin EA, Turesky RJ. Methemoglobin Formation and Characterization of Hemoglobin Adducts of Carcinogenic Aromatic Amines and Heterocyclic Aromatic Amines. Chem Res Toxicol. 2016;29:255-69. https://doi.org/10.1021/acs.chemrestox.5b00418.

16. Richter E, Rösler S, Scherer G, Gostomzyk JG, Grübl A, Krämer U, Behrendt H. Haemoglobin adducts from aromatic amines in children in relation to area of residence and exposure to environmental tobacco smoke. Int Arch Occup Environ Health. 2001;74:421-8. https://doi.org/10.1007/s004200100243.

17. Bernert JT, Jain RB, Pirkle JL, Wang L, Miller BB, Sampson EJ. Urinary tobacco-specific nitrosamines and 4-aminobiphenyl hemoglobin adducts measured in smokers of either regular or light cigarettes. Nicotine Tobacco Res. 2005;7:729-38. https://doi.org/10.1080/14622200500259762.

18. Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

19. Chinthakindi S, Kannan K. A liquid chromatography–tandem mass spectrometry method for the analysis of primary aromatic amines in human urine. J Chromatogr B. 2021;1180:122888-90. https://doi.org/10.1016/j.jchromb.2021.122888.

20. Niu J, Zhao X, Jin Y, Yang G, Li Z, Wang J, Zhao R, Li Z. Determination of aromatic amines in the urine of smokers using a porous organic framework (JUC-Z2)-coated solid-phase microextraction fiber. J Chromatogr A. 2018;1555:37-44. https://doi.org/10.1016/j.chroma.2018.04.059.

21. Fuller TW, Acharya AP, Meyyappan T, Yu M, Bhaskar G, Little SR, Tarin TV. Comparison of Bladder Carcinogens in the Urine of E-cigarette Users Versus Non E-cigarette Using Controls. Sci Rep. 2018;8:1-6. https://doi.org/10.1038/s41598-017-19030-1.

22. Lamani X, Horst S, Zimmermann T, Schmidt TC. Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GCxGC-qMS). Anal Bioanal Chem. 2015;407:241-52. https://doi.org/10.1007/s00216-014-8080-5.

23. Amiri A, Baghayeri M, Nori S. Magnetic solid-phase extraction using poly(para-phenylenediamine) modified with magnetic nanoparticles as adsorbent for analysis of monocyclic aromatic amines in water and urine samples. J Chromatogr A. 2015;1415:20-6. https://doi.org/10.1016/j.chroma.2015.08.046.

24. Yu J, Wang S, Zhao G, Wang B, Ding L, Zhang X, Xie J, Xie F. Determination of urinary aromatic amines in smokers and nonsmokers using a MIPs-SPE coupled with LC-MS/MS method. J Chromatogr B. 2014;958:130-5. https://doi.org/10.1016/j.jchromb.2014.03.023.

25. Jiang C, Sun Y, Yu X, Gao Y, Zhang L, Wang Y, Zhang H, Song D. Application of C18-functional magnetic nanoparticles for extraction of aromatic amines from human urine. J Chromatogr B. 2014;947-948:49-56. https://doi.org/10.1016/j.jchromb.2013.12.008.

26. Sarafraz-Yazdi A, Ardaki MS, Amiri A. Determination of monocyclic aromatic amines using headspace solid-phase microextraction based on sol-gel technique prior to GC. J Sep Sci. 2013;36:1629-35. https://doi.org/10.1002/jssc.201200940.

27. Seyler TH, Bernert JT. Analysis of 4-aminobiphenyl in smoker's and nonsmoker's urine by tandem mass spectrometry. Biomarkers. 2011;16:212-21. https://doi.org/10.3109/1354750X.2010.544755.

28. Jurado-Sánchez B, Ballesteros E, Gallego M. Continuous solid-phase extraction method for the determination of amines in human urine following on-line microwave-assisted acid hydrolysis. Anal Bioanal Chem. 2010;396:1929-37. https://doi.org/10.1007/s00216-009-3395-3.

29. Riedel K, Scherer G, Engl J, Hagedorn H-W, Tricker AR. Determination of three carcinogenic aromatic amines in urine of smokers and nonsmokers. J Anal Toxicol. 2006;30:187-95. https://doi.org/10.1093/jat/30.3.187.

30. Weiss T, Angerer J. Simultaneous determination of various aromatic amines and metabolites of aromatic nitro compounds in urine for low level exposure using gas chromatography-mass spectrometry. J Chromatogr B. 2002;778:179-92. https://doi.org/10.1016/S0378-4347(01)00542-4.

31. Grimmer G, Dettbarn G, Seidel A, Jacob J. Detection of carcinogenic aromatic amines in the urine of non-smokers. Sci Total Environ. 2000;247:81-90. https://doi.org/10.1016/S0048-9697(99)00471-4.

32. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Tobacco smoke and involuntary smoking. Lyon, France: International Agency for Research on Cancer; 2004.

33. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer. 2003;3:733-44. https://doi.org/10.1038/nrc1190.

34. Yih Hui B, Mohamad Zain NN, Mohamad S, Mohamed Fauzi H, Alias Y, Chandrasekaram K, Rahim NY, Yahaya N, Raoov M. Determination of Aromatic Amines in Urine using Extraction and Chromatographic Analysis: A Minireview. Anal Lett. 2019;52:2974-92. https://doi.org/10.1080/00032719.2019.1636057.

35. Sanchis Y, Coscollà C, Yusà V. Comprehensive analysis of photoinitiators and primary aromatic amines in food contact materials using liquid chromatography High-Resolution Mass Spectrometry. Talanta. 2019;191:109-18. https://doi.org/10.1016/j.talanta.2018.08.047.

36. Muz M, Ost N, Kühne R, Schüürmann G, Brack W, Krauss M. Nontargeted detection and identification of (aromatic) amines in environmental samples based on diagnostic derivatization and LC-high resolution mass spectrometry. Chemosphere. 2017;166:300-10. https://doi.org/10.1016/j.chemosphere.2016.09.138.

37. Patsias J, Papadopoulou-Mourkidou E. Development of an automated on-line solid-phase extraction-high-performance liquid chromatographic method for the analysis of aniline, phenol, caffeine and various selected substituted aniline and phenol compounds in aqueous matrices. J Chromatogr A. 2000;904:171-88. https://doi.org/10.1016/S0021-9673(00)00927-4.

38. Zimmermann T, Ensinger WJ, Schmidt TC. In Situ Derivatization/Solid-Phase Microextraction: Determination of Polar Aromatic Amines. Anal Chem. 2004;76:1028-38. https://doi.org/10.1021/ac035098p.

39. Schmidt TC, Less M, Haas R, von Löw E, Steinbach K, Stork G. Gas chromatographic determination of aromatic amines in water samples after solid-phase extraction and derivatization with iodine. I. Derivatization. J Chromatogr A. 1998;810:161-72. https://doi.org/10.1016/S0021-9673(98)00233-7.

40. Kataoka H. Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. J Chromatogr A. 1996;733:19-34. https://doi.org/10.1016/0021-9673(95)00726-1.

41. Torbati M, Mohebbi A, Farajzadeh MA, Afshar Mogaddam MR. Simultaneous derivatization and air–assisted liquid–liquid microextraction based on solidification of lighter than water deep eutectic solvent followed by gas chromatography–mass spectrometry: An efficient and rapid method for trace analysis of aromatic amines in aqueous samples. Anal Chim Acta. 2018;1032:48-55. https://doi.org/10.1016/j.aca.2018.06.025.

42. Akyüz M. Simultaneous determination of aliphatic and aromatic amines in ambient air and airborne particulate matters by gas chromatography-mass spectrometry. Atmos Environ. 2008;42:3809-19. https://doi.org/10.1016/j.atmosenv.2007.12.057.

43. Akyüz M, Ata S. Determination of aromatic amines in hair dye and henna samples by ion-pair extraction and gas chromatography-mass spectrometry. J Pharm Biomed Anal. 2008;47:68-80. https://doi.org/10.1016/j.jpba.2007.12.011.

44. Turesky RJ, Freeman JP, Holland RD, Nestorick DM, Miller DW, Ratnasinghe DL, Kadlubar FF. Identification of aminobiphenyl derivatives in commercial hair dyes. Chem Res Toxicol. 2003;16:1162-73. https://doi.org/10.1021/tx030029r.

45. Longo M, Cavallaro A. Determination of aromatic amines at trace levels by derivatization with heptafluorobutyric anhydride and gas chromatography-electron-capture negative-ion chemical ionization mass spectrometry. J Chromatogr A. 1996;753:91-100. https://doi.org/10.1016/S0021-9673(96)00548-1.

46. Mazumder S, Ahamed RA, Seyler TH, Wang L. Short- and Long-Term Stability of Aromatic Amines in Human Urine. Int J Env Res Public Health. 2023;20:4135.

47. Domínguez I, Arrebola FJ, Gavara R, Martínez Vidal JL, Frenich AG. Automated and simultaneous determination of priority substances and polychlorinated biphenyls in wastewater using headspace solid phase microextraction and high resolution mass spectrometry. Anal Chim Acta. 2018;1002:39-49. https://doi.org/10.1016/j.aca.2017.11.056.

48. de Perre C, Le Ménach K, Ibalot F, Parlanti E, Budzinski H. Development of solid-phase microextraction to study dissolved organic matter—Polycyclic aromatic hydrocarbon interactions in aquatic environment. Anal Chim Acta. 2014;807:51-60. https://doi.org/10.1016/j.aca.2013.11.026.

49. Magnusson B, Örnemark U. Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics; 2014. Available from: http://www.eurachem.org. Accessed 11.12.2022

50. International Organization for Standardization, DIN 38402-51:2017-05, German standard methods for the examination of water, waste water and sludge - General information (group A) - Part 51: Calibration of analytical methods - Linear calibration (A 51). (2017).

51. Otto M. Chemometrics. Statistics and Computer Application in Analytical Chemistry. 3 ed. Weinheim, Germany: Wiley-VCH; 2016. 400 p.

52. Miller JN, Miller JC. Statistics and Chemometrics for Analytical Chemistry. 6 ed. Harlow, England: Pearson Education Limited; 2010.

53. Bienfait B, Ertl P. JSME: a free molecule editor in JavaScript. Journal of Cheminformatics. 2013;5:24. https://doi.org/10.1186/1758-2946-5-24.

54. International Organization for Standardization, DIN EN ISO 32645:2008-11, Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation. (2008).

Chapter 6. Automation and optimization of the sample preparation of aromatic amines for their analysis with gas chromatography-mass spectrometry (GC-MS)

This chapter was adapted from: Lorenzo-Parodi N, Kaziur-Cegla W, Schmidt TC. Automation and optimization of the sample preparation of aromatic amines for their analysis with GC–MS. Green Anal Chem. 2023;6:100071. https://doi.org//10.1016/j.greeac.2023.100071.

6.1 Abstract

Aromatic amines (AAs) in urine typically require complex, labor-intensive, and time-consuming sample preparation procedures before they can be analyzed. Usually it consists of hydrolysis, extraction and, especially when analyzed with GC, derivatization. Traditionally, these steps are done manually, significantly contributing to the total analysis time, and providing opportunities for human errors. Automation presents several advantages, such as minimized human intervention and errors, and an overall greener analytical procedure.

In this study, the automation of the AAs sample preparation procedure for urine samples was investigated. Problems encountered during the automation and adjustments made to the original protocol are discussed in detail. Some examples include volume limitations or needle penetration depth adjustments. Taking advantage of the automation, several steps of the sample preparation procedure could be further optimized, such as reaction/extraction times or some of the reagents which were not optimal for the automated set-up.

The automated procedure presented here enables a user-friendly and green approach for the analysis of AA in urine, which could be used to gain a better understanding between smoking, AA concentrations in urine, and the risk of developing smoking related diseases.

6.2 Introduction

Aromatic amines (AAs) are used as raw materials and intermediates in several industries, such as those manufacturing dyes, rubber, pharmaceuticals, or pesticides [1], which can result in occupational exposure. The general public can also come in contact with AAs thanks to tobacco smoke, which may be the major source of exposure [2].

After smoke inhalation, AAs enter the bloodstream and are eventually transported, together with their metabolites, to the bladder, where they can be excreted with urine [3]. Most AAs are believed to have carcinogenic potential [2], several have been classified as carcinogenic or probably carcinogenic to humans [1], and they have been suggested as the main cause of the excess risk of bladder cancer in smokers [4].

AAs in urine are typically analyzed after a complex, labor-intensive, and time-consuming sample preparation procedure. First, a hydrolysis step, which is typically acidic, but can also be basic or enzymatic, transforms metabolized AAs into their free form [5]. Afterward, an extraction step, typically liquid-liquid extraction (LLE) or solid-phase extraction (SPE), minimizes interferences with matrix compounds and acts as a clean-up step [5-7]. The extract can be directly analyzed with liquid chromatography (LC), however, it is generally not recommended for highly complex samples such as urine due to its low peak capacity [8, 9]. Alternatively, AAs can be analyzed with GC after a derivatization step, which helps to increase selectivity, sensitivity, and specificity [10, 11] and minimizes and/or eliminates problems that arise due to their physicochemical characteristics, such as their polarity or high water solubility [10-13]. The most common derivatization techniques are acylation and silylation, however, one of the biggest disadvantages is the need to be performed in anhydrous conditions [9-13]. A promising alternative is iodination via a Sandmeyer-like reaction as reported by [9, 14-16], which consists of two steps (see Fig. 6.1). First, the amino group is diazotized with nitrite in an acidic medium, followed by a substitution of the diazo group with iodine at elevated temperatures. After derivatization, the AAs can be analyzed with GC-MS, however, an enrichment step like SPME is recommended to increase sensitivity.

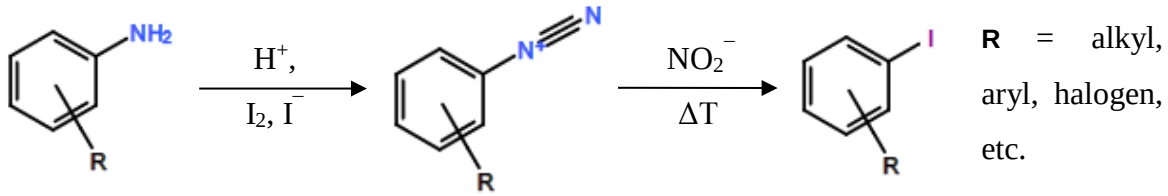


Fig. 6.1. Reaction scheme for the iodination of aromatic amines, after Schmidt et al. [9].

Traditionally, sample preparation is done manually, significantly contributing to the analysis time and error generation. It has been reported that two-thirds of the analysis time [6, 17] is spent on sample preparation and 50 % of the error can be attributed to manual sample preparation [17]. Automation presents not only the advantages of minimized human intervention and errors, but is also considered a key step towards green chemistry [18].

Mazumder et al. [3] have been, to the best of our knowledge, the first and only ones to report an automated sample preparation method for the analysis of aromatic amines with GC. However, the sample preparation followed has no overlap with the one proposed here, and a few steps seem to have been done manually. A semiautomatic flow-base method was reported by Jurado-Sánchez et al. [19], which was based on an on-line microwave-assisted acid hydrolysis step and continuous solid-phase extraction, neither of which are used in this study.

The aim of this study is therefore, to automate and optimize the sample preparation of AA for their analysis with GC-MS, including the acidic hydrolysis, LLE, and Sandmeyer-like derivatization steps. This would be the first fully automatic method for the analysis of aromatic amines in urine with GC-MS, and not only would allow for optimization possibilities previously considered too resource-consuming, but it would also facilitate the analysis of high numbers of samples, which is key to a better understanding of the relationship between smoking status, AA present in the urine, and the risk of developing smoking-related diseases. Furthermore, the automation discussion presented here could be applied to other analytical questions, including different analytes or matrices.

6.3 Materials and methods

6.3.1 Chemicals and reagents

The aromatic amines used were purchased from Sigma-Aldrich (Steinheim, Germany), except for 4-chloro-2-methylaniline (4C2MA) and 3-chloro-2,6-dimethylaniline (3C2,6DMA) which were purchased from Fluka (Darmstadt, Germany) and Alfa Aesar (Karlsruhe, Germany), respectively, see Table 6.1.

Table 6.1. List of aromatic amines used, their abbreviation, CAS-number and purity.

Compound	Abbreviation	CAS-number	Purity
Aniline	A	62-53-3	≥ 99.5 %
2-Methylaniline	2MA	95-53-4	≥ 99.0 %
3-Chloro-4-fluoroaniline	3C4FA	367-21-5	98.0 %
2-Chloroaniline	2CA	95-51-2	≥ 99.5 %
4-Ethylaniline	4EA	589-16-2	98.0 %
2,6-Dimethylaniline	2,6DMA	87-62-7	99.0 %
2,4-Dimethylaniline	2,4DMA	95-68-1	≥ 99.0 %
4-Chloro-2-methylaniline	4C2MA	95-69-2	≥ 98.0 %
2-Bromoaniline	2BA	615-36-1	98.0 %
2,4,6-Trimethylaniline	2,4,6TMA	88-05-1	98.0 %
2,6-Dichloroaniline	2,6DCA	608-31-1	98.0 %
3-Chloro-2,6-dimethylaniline	3C2,6DMA	26829-77-6	99.0 %
3-Chloro-4-methoxyaniline	3C4MA	5345-54-0	97.0 %
2-Naphthylamine	2NA	91-59-8	≥ 95.0 %

For the derivatization, hydriodic acid (HI, ACS reagent, unstabilized, 55 %), sodium nitrite (99 %), and alizarin red S (98 %) were purchased from Sigma-Aldrich, sodium sulfite (puriss. p.a., ACS reagent, RT, ≥ 98 %), and sulfamic acid (T, ≥ 99 %) from Fluka, and sodium acetate (NaOAc, ≥ 99 %) from AppliChem (Darmstadt, Germany).

Ultrapure water was obtained from a PureLab Ultra water system from ELGA LabWater (Celle, Germany), hydrochloric acid (HCl, 37 %) and sodium hydroxide (98 %, NaOH) from Bernd Kraft (Duisburg, Germany), and diethyl ether (DEE) and HPLC grade methanol from Fisher Scientific (Schwerte, Germany).

6.3.2 Sample preparation

The sample preparation method used was based on [9, 14-16]. Several parameters were further optimized and/or had to be adjusted for automation. These changes are described in the results and discussion section.

Stock solution and samples

Individual stock solutions of 1 g/L in methanol were prepared for each of the AA studied and were mixed in a stock solution of 20 mg/L in methanol. The samples had a final concentration of 20 µg/L and were prepared in either ultrapure water or, for the derivatization tests, in 0.24 M HCl, prepared by acidifying 25 mL of ultrapure water with 490 µL 37 % HCl. Unless stated otherwise, the measurements were done in triplicates.

Hydrolysis

The original hydrolysis procedure used 20 mL of urine sample, to which 10 mL concentrated hydrochloric acid (37 %) were added. In order to cleave aromatic amine adducts, the mixture was heated for 12 h at 110 °C. After the samples had cooled to room temperature, 20 mL of a 10 M NaOH solution was added to make sure the amines were present in their neutral form.

As an alternative option, a Mars 5 digestion microwave system (CEM Corporation, North Carolina, USA) was also tested (results not shown), and the only significant advantage observed was a reduction in the overall time needed. Because the labor required was the same with both approaches, and because automation was not possible, it was not investigated further.

Liquid-liquid extraction

The original LLE procedure consisted of extracting the neutral aromatic amines into 5 mL DEE, twice. Afterward, the organic fractions were mixed and cleaned with 2 mL of a 0.1 M NaOH solution, and the amines were back-extracted into 10 mL of acidified water with 200 µL concentrated HCl (37 %). Nitrogen (N_2) was used to eliminate any remaining DEE in the aqueous fraction.

Derivatization

During manual derivatization, 100 µL hydriodic acid (55 %, reagent 1) and 200 µL sodium nitrite (50 g/L, reagent 2) were added to the sample, which was shaken for 20 min at 300 rpm on a KS 260 control shaker (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The surplus of nitrite was destroyed by the addition of 500 µL sulfamic acid (50 g/L, reagent 3). The sample was then shaken for another 45 min at 300 rpm and then placed in a 95 °C hot water bath for 5 min to facilitate the substitution of the diazo groups by iodine. After cooling the sample down, 125 µL sodium sulfite (supersaturated, reagent 4) was added to reduce residual iodine, which could be observed with the discoloration of the initially brown solution. 100 µL alizarin red S (1 % w/v, reagent 5) was added to enable the identification of the correct pH. Finally, 500 µL supersaturated NaOAc (reagent 6) was added to adjust the pH to 5. The reagents were shaken by hand before adding them to the samples with Eppendorf pipettes. Once they were added, the vial containing the sample and the reagent was also shaken.

Some steps of the derivatization procedure were adapted for their automation. Shaking steps previously done in the shaker took place in the agitator at 400 rpm and 40 °C. Instead of manual shaking of reagents and samples, they were vortexed for 5 s at 1250 rpm. And since liquid syringes were used to add reagents, pre-/post-cleaning steps (1/≥6 cycles) were added. More information regarding the automation can be found in the results and discussion section and the supplementary information (SI).

Solid-phase microextraction

A 110 µm DVB/PDMS SPME fiber (BGB Analytik Vertrieb GmbH, Rheinfelden, Germany) was used together with an IP-deactivated SPME liner from Restek (Bad Homburg, Germany).

The samples were incubated at 60 °C for 10 min and under 500 rpm agitation. Simultaneously, the fiber was conditioned in the SPME conditioning station at 230 ºC for 8 min. The SPME fiber was afterward injected into the headspace of the vial (still at 60 °C and under 250 rpm agitation) for 25 min, and the analytes were desorbed into the injection port for 5 min.

6.3.3 Instrumentation

The sample preparation process was automated with the PAL RTC autosampler (CTC, Zwingen, Switzerland), which was controlled by the software Chronos (Axel Semrau, Sprockhövel, Germany), see the SI for more information. The following modules were used: parking stations for up to six tools (liquid syringes and SPME fiber), tray holders for the samples and reagents, wash stations for cleaning syringes, a vortex unit to quickly mix the reagents/samples, an agitator for longer and/or heated shaking steps and a SPME conditioning station. The final set-up used can be seen in the SI.

After derivatization and SPME, the SPME fiber was injected into a GC-2010 Plus gas chromatograph coupled to a GCMS-QP2010 Ultra mass spectrometer, and controlled with GCMS Real Time Analysis from Shimadzu GmbH (Duisburg, Germany).

6.3.4 GC-MS analysis

Separation of the analytes was performed with a BPX5 capillary column, which was 30 m x 0.25 mm ID x 0.25 µm df (SGE). The carrier gas was helium (99.999 % from Air Liquide, Krefeld, Germany) at a constant flow rate of 1 mL/min, and injection was done in splitless mode with a sampling time of 5 min, and using a split ratio of 10:1 afterward. The column oven was set to 40 °C for 1 min hold time, increased with a 10 °C/min ramp until 230 °C, and held for 1 min. The temperature used for the injection port, the MS interface, and the ion source was 230 °C. Mass-to-charge ratios between m/z 74 and 470 were scanned, using a detector voltage of 1 kV. The quantifier and qualifier ions used can be seen in Table S 6.1, SI.

6.3.5 Data evaluation

The data was processed with GCMS Post Run Analysis (Shimadzu GmbH) and evaluated using Excel (Microsoft) as described by [14, 15]. In summary, outliers were detected using the Dixon test [20] and statistical differences were determined with Welch's two-sided t-test or the two-variable t-test, depending on the results from the Fisher's F-test [20].

6.4 Results and discussion

6.4.1 Hydrolysis

One of the critical points taken into consideration while automating the hydrolysis step was the volume limitation due to the commercially available vials and autosampler racks. It is possible to print or order custom vials and racks, however, this not only leads to an increased cost, but also limits its applicability in other laboratories. Therefore, an alternative route was investigated, namely, the miniaturization of the experiment. For this step, 1:10 of the volumes from the original protocol were used, as described in Table S 6.2 (SI). This approach provides an additional advantage for samples where the total available volume is limited, like archived samples, and contributes to an overall greener analytical method thanks to the decreased amounts of reagents needed.

Fig. 6.2 includes a scheme of the steps needed for the automatic hydrolysis, and the optional addition of an internal standard. In the original protocol, a filtration step after the addition of NaOH is recommended. This is the case because, in a very small number of cases, a black precipitate appears, which is believed to interfere with the extraction. However, there is no data available comparing the filtered and unfiltered samples and we were not able to observe such precipitates during our experiments. Nonetheless, if needed, this step could be done manually or alternative hydrolysis/extraction techniques could be studied.

This automated step could be very easily adapted to other sample preparation procedures by exchanging the reagents used and modifying the reaction times, and even basic hydrolysis could be directly done by using NaOH as the first reagent and adjusting the pH with HCl.

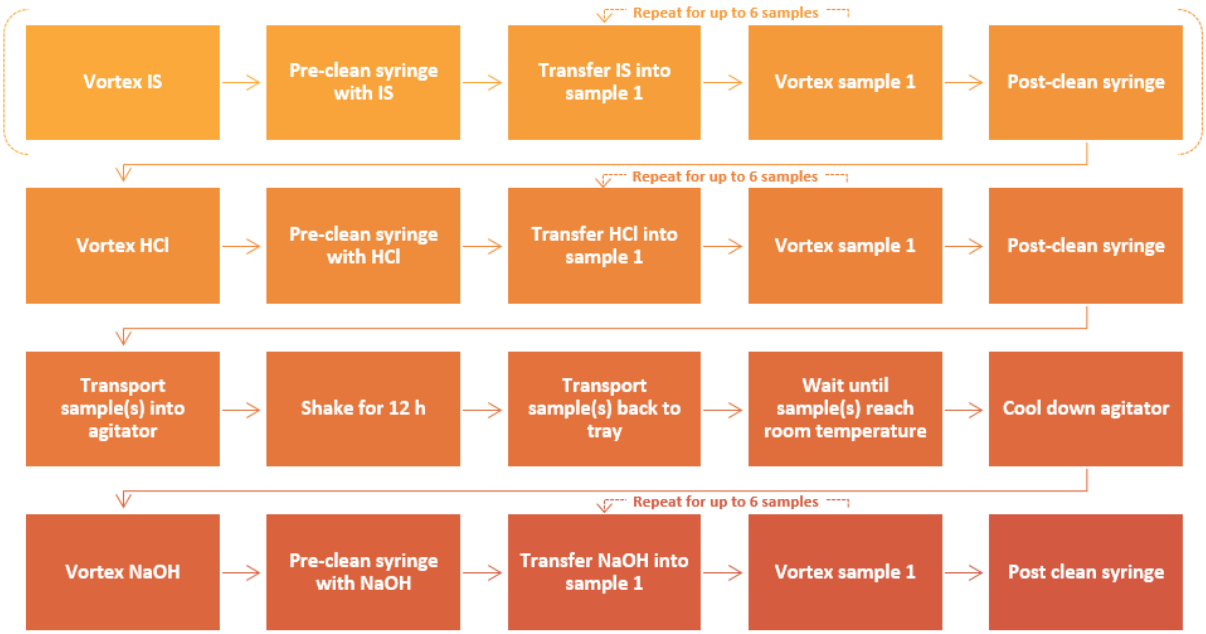


Fig. 6.2. Simplified overview of the hydrolysis steps, and, in parentheses, the steps corresponding to the optional addition of an internal standard (IS). Optional steps and repetitions denoted with dotted lines.

6.4.2 Liquid-liquid extraction

When automating the LLE steps, there were a few points taken into consideration.

Volume of reagents

Similar to the modifications needed for the hydrolysis steps, the volumes of the reagents used during LLE also need to be miniaturized. Originally, it was planned to use 1:5 of the original volumes, however, when trying to aspirate the DEE after the addition of NaOH, a portion of the DEE remained in the "waste". This happened because the needle of the syringe was too short (ca. 57 mm) and could not go deep enough into the vial (ca. 75 mm) to aspirate all DEE. In order to avoid that, and because no negative effects were expected, it was decided to increase the volume of NaOH used for washing so that the needle could reach the organic phase. An alternative would be to buy longer syringes and the corresponding tool, which are commercially available. In Table S 6.2 (SI) the original and adapted volumes used can be seen.

Acidifying reagent

HCl is typically used to back-extract the aromatic amines from the organic solvent into the aqueous phase [16]. In Schmidt et al. [9], it was reported that chloride ions from HCl might compete with iodide ions during the substitution reaction and create chlorinated by-products. Therefore, HI and HCl were studied as acidifying reagents. As discussed in the SI, HCl was chosen due to the significantly higher intensities obtained for most AA, and presented the added benefit that it is cheaper, and it does not need to be kept refrigerated.

Vortexing

Vortexing was selected instead of shaking to mix the aqueous and organic phases, since it is more vigorous and resembles manual shaking the most. After vortexing the sample, a waiting time was set to allow the phases to separate.

Needle penetration

The biggest problem encountered during the automation of the LLE step was establishing the needle penetration depth for the different steps, since the needle had to be placed in order to ensure the total collection of the desired volume, and at the same time avoid collecting the other phase. To make sure that the extraction was as efficient as possible and no "wanted" phase or analyte was lost, a small volume of the "waste" phase was aspirated together with the "wanted" phase, with one exception -see below-.

Purging the sample with N2

During the manual procedure, the last step before derivatization is to purge the samples with N_2 in order to evaporate any DEE left. The only commercially available tool found, compatible with the PAL RTC, and potentially capable of such step was the multi-headspace extraction (MHE) tool. However, the needle of the MHE tool is very small and it is too far away from the sample to resemble the manual step. Furthermore, this step should be done under a hood to avoid "purging" DEE into the laboratory air, which was not possible due to the dimensions of the system and the hood. Therefore, it was decided to eliminate all the DEE by adjusting the needle penetration depth of the last step instead. The drawback of this approach is that by doing that a droplet of the aqueous phase is also discarded, however, it was considered better than leaving DEE in the sample, which could interfere with the derivatization.

A scheme of the steps followed during the automated LLE can be seen in Fig. 6.3. In general, two to three extractions are considered to be a good compromise between the yields obtained and the resources needed. Because the needle was 57 mm long, only the supernatant could be removed from the vial (75 mm). Therefore, whenever the supernatant was the fraction of interest, a new vial was needed for its collection. When the infranatant was the fraction of interest, the supernatant could be discarded, and no new vial was needed. This was the case for the back-extraction in the last step, where the supernatant, the DEE fraction, was discarded. In total, three vials were needed per sample, the original one, and two empty ones. Mistakes were avoided by designating a tray for each step, namely, tray holder 2 – slot 1 for the original samples, tray holder 2 - slot 2 for the intermediate empty vials, tray holder 2 –slot 3 for the empty vials where the samples will be derivatized, and tray holder 1 – slot 3 for the reagents. The same was done for the different wastes. The small wash station – position 1 was used for strongly basic waste (for example, when cleaning the syringe with NaOH), position 2 for DEE (when cleaning the syringe with DEE), and position 3 for strongly acidic waste (for example, when cleaning the syringe with acidified water). The washing solvents were placed in position 4 and the large wash station. In order not to overfill the DEE waste vial, after the final extraction step (i.e., the back-extraction into acidified water), the DEE waste was disposed of in the vials where the samples had been (V0), see Fig. 6.3.

Fig. 6.3. Simplified overview of the liquid-liquid extraction steps. Several vials are needed for the same sample, denoted as (Vn). Optional steps denoted with dotted lines.

The automation of the extraction step for the analysis of AAs in urine samples has previously been addressed. In Mazumder et al. [3], a supported liquid extraction (SLE) was done instead of LLE. While the method presented there was thoroughly validated and provided several advantages to the equivalent manual method, the hydrolysis and derivatization steps were done manually. In Jurado-Sánchez et al. [19], the HCl needed for the acidic hydrolysis was added manually, but the hydrolysis step took place on-line, together with the neutralization and subsequent SPE, which were done automatically. The method was also validated and used to calculate biological half-lives ($t_{1/2}$) of several AA, however, it was unclear whether the SPE column was conditioned manually or if that could also be automatically done.

The automated LLE presented here could also be used for different applications, and different organic solvents and reagents could be easily exchanged accordingly. However, it is important to keep in mind that if different volumes are needed, the needle penetration depth has to be carefully adjusted.

6.4.3 Derivatization

During the automation of the derivatization steps, several iterations were tested, and multiple improvements were done. The following points were deemed the most important to pay especial attention to.

Agitator temperature

Since the same agitator is used for the heating step at 95 °C and the shaking steps, which are carried out at room temperature during manual derivatization, the times needed for heating and cooling of the agitator need to be considered. Although it is possible to set the agitator to lower temperatures, the time needed to reach those is relatively long, so a compromise was found by shaking at 40 °C.

Reaction times

Since during automatic derivatization a higher temperature is used (40 °C) in comparison to the manual derivatization (room temperature), the required reaction times may be shorter, which would allow for higher throughputs. Therefore, the possibility of decreasing these times was studied (see SI), and it was concluded that the reaction time waited after adding hydriodic acid and sodium nitrite could be decreased to 15 min.

Supersaturated solutions

When the derivatization is done manually, the addition of the reagents to the sample is typically done with pipettes. With the automatic derivatization these were replaced by syringes. Because two of the reagents were supersaturated solutions, there was a risk that in contact with the cold syringe some precipitates would appear in the barrel and the needle, and could potentially block the syringe. Therefore, alternatives were considered, and are discussed in detail in the SI. Based on those results, a larger volume (250 µL) of sodium sulfite at a lower

concentration (120 g/L), was used, and supersaturated NaOAc was replaced with 185 µL 10 M NaOH.

Aspiration speed

When adding NaOH automatically, it could be seen that bubbles were formed during aspiration, most likely due to the higher viscosity in comparison with the previously used NaOAc. This could be easily corrected by decreasing the aspiration speed from 5 µL/s to 1 µL/s.

Mixing

Initially, after the addition of sodium sulfite, particles could still be seen in the vials, indicating insufficient mixing. Therefore, the vortex speed was increased from 750 rpm to 1250 rpm. Furthermore, this step was repeated a second time after the addition of sodium sulfite to make sure no particles remained.

Miniaturization

As part of the optimization, the possibility of further miniaturizing the analysis was also studied. Not only because it would enable the use of samples with limited volumes, such as archived samples from population studies, but also as a step towards a greener analytical method. This was done in combination with an alternative extraction procedure where the final volume after extraction was 100 µL instead of 5 mL, as reported in [15]. Because, ideally, the absolute amount of AA remained constant, the volumes of the reactants used for derivatization were kept constant. The only exception was the 10 M NaOH solution used in the final step to adjust the pH, since due to the lower volume of acidified sample used, the final pH was less acidic, and only 92 µL of 10 M NaOH were needed instead of 185 µL. While this procedure would enable a further miniaturization, it was not implemented in the final set-up due to the lack of tools available for its full automation.

A scheme of the final automatic derivatization method can be seen in Fig. 6.4.

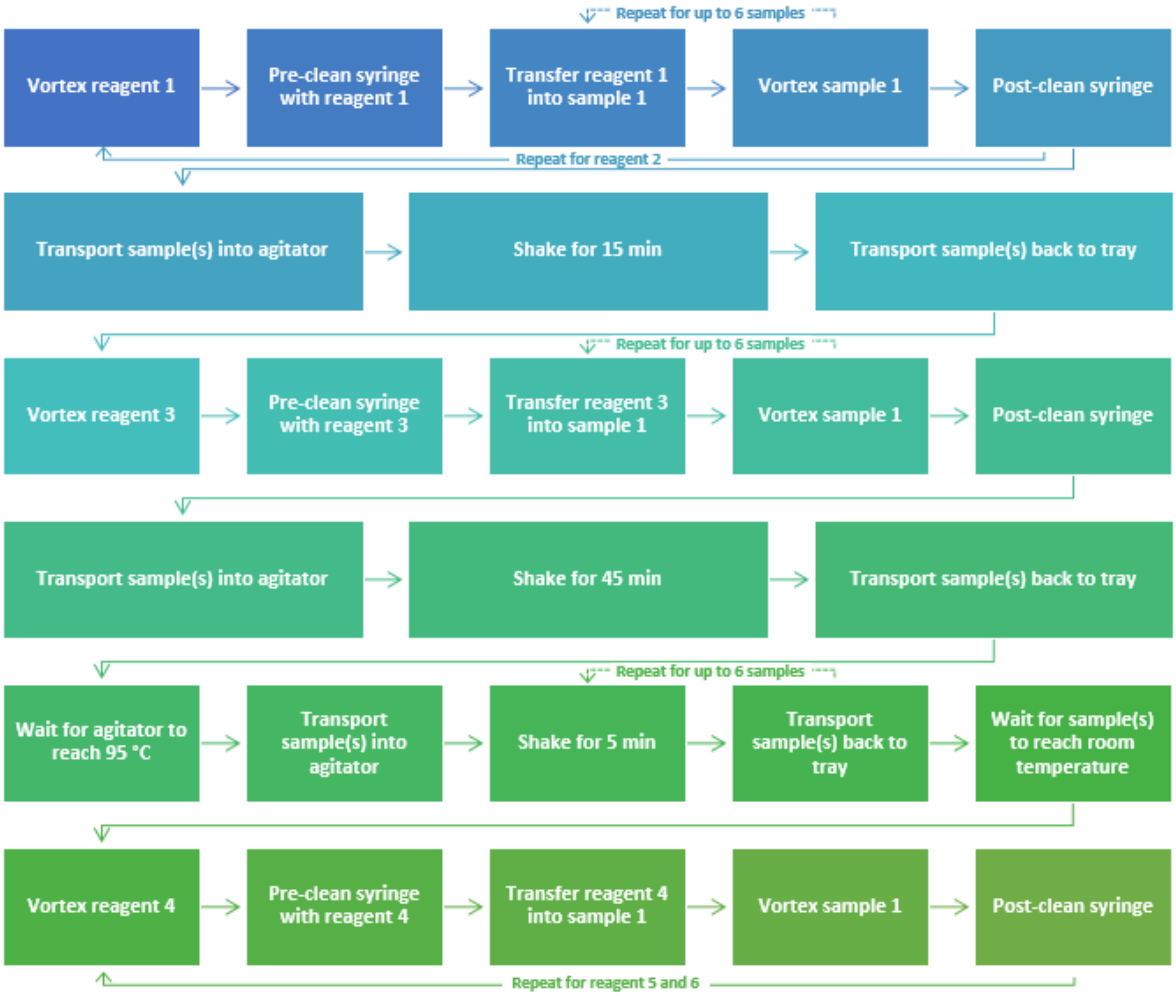


Fig. 6.4. Simplified overview of the derivatization steps. Optional repetitions denoted with dotted lines.

While the discussions and conclusions from this section are applicable to the automation of many other analytical procedures, this step is the trickiest to use without further modifications of the script. This is due to the fact that the derivatization steps are targeted to a specific derivatization protocol. Nonetheless, some sections of the script (like the sequence vortex reagent, pre-clean syringe, transfer reagent, vortex sample and post clean syringe) could be used as building blocks for many different derivatization procedures.

6.4.4 Solid-phase microextraction

Extraction time

Taking advantage of the automated derivatization and the reduced workload, the SPME extraction time was further optimized (see SI). No significant loss in sensitivity could be observed when reducing the extraction time to 10 min, which enabled a higher throughput by decreasing the extraction time 60 %.

From all the steps included in the sample preparation of the AA, SPME is the only one that is routinely automated thanks to an autosampler. The typical steps involved can be seen in Fig. 6.5.

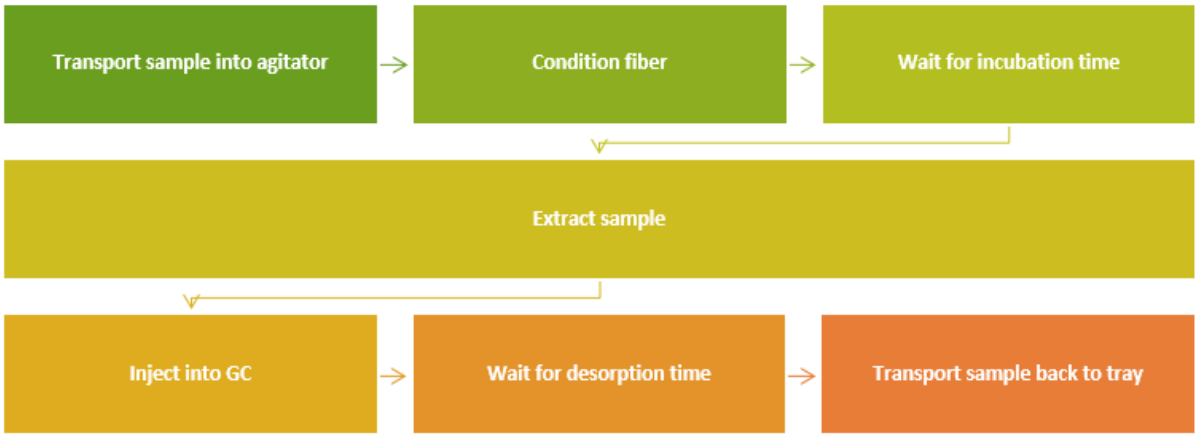


Fig. 6.5. Simplified overview of the solid-phase microextraction steps.

6.4.5 Analytical greenness

To evaluate the analytical greenness of the method, the AGREE and AGREEprep metrics were used (see Fig. 6.6), as described by [18, 21].

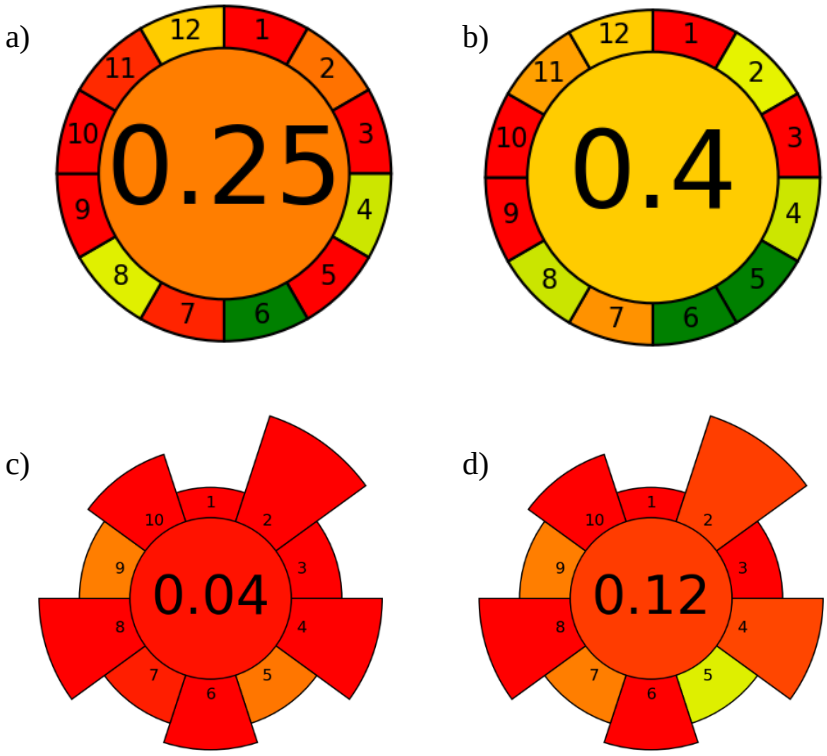


Fig. 6.6. Results of AGREE (a, b) and AGREEprep (c, d) analysis for the original manual method (a, c) and the new automated method proposed in this publication (b, d).

The AGREE metric almost doubled with the proposed changes, indicating a significant improvement of the greenness of the method. The biggest improvements can be seen in principle 5, thanks to the automation and miniaturization of the method, and 7 and 11, thanks to its miniaturization (see SI for more information). Some principles remained constant, including principles 1, 3, 9 and 10, corresponding to the sampling procedure, positioning of the analytical device, energy consumption and type of reagents, respectively. Due to the nature of the sample and analysis type, principles 1, 3 and 9 are not very likely to be improved upon. The use of less toxic, renewable, or bio-based reagents, corresponding to point 10, 11, and 12, could be studied further. When AGREEprep is considered, the overall scores are lower, but the difference between the two methods is more than doubled. The biggest difference can be seen in principle 5, thanks to the miniaturization of the method, followed by principle 7, which correlates to its automation. While a few principles remained constant, as was the case with AGREE, most principles remained in the red-orange scale, showing there is more room for

improvement. One possibility for improvement that would affect both principle 6, sample throughput, and principle 8, energy consumption, would be to further optimize the hydrolysis step with the aim of minimizing the time needed for this step. Furthermore, alternatives to the reagents/solvents used could also be studied, which could improve principle 2, hazardous materials, 3, sustainability, renewability, and reusability of materials, and 10, operator´s safety. To find less toxic alternatives (principle 2 and 10), databases like the ChlorTox Base could be used, which includes information on the "greenness" of the most popular reagents used in the chemical laboratory, based on the chemical hazards of each substance [22]. However, for certain analytical questions, and especially for the analysis of biological samples, the maximum achievable score is usually below 1 [23]. This is the case mainly due to the instrumentation typically used, namely the mass spectrometer, which equates to a score of 0 in AGREE and 0.25 in AGREEprep for principle 9, and the fact that such instrumentation is typically placed in specialized laboratories (ex situ), giving a score of 0 in principle 3 in AGREE and 1 in AGREEprep, as reported for AGREEprep by [23]. Furthermore, direct analysis of aromatic amines in urine is not recommended due, in part, to the complexity of the matrix, which limits the score of principle 1 in AGREE to 0.

6.5 Further optimization possibilities

The final method proposed here is fully automated, and only requires manual assistance to prepare the reagents, to place them, together with the samples, the solvents, and the needed vials in the autosampler, and to empty the waste stations when full. Usually, it is not needed to re-fill the solvents/empty the waste vials within the batch. If the batch is so long that this is the case, more washing stations could be added to the autosampler to avoid this. In Table 6.2, the most important proposed improvements, including the positive effect they had in terms of performance, greenness, and practicality, can be seen.

Table 6.2. Main improvements proposed to each automated sample preparation step, including the benefits thereby gained.

Step	Proposed improvements	Benefits
Hydrolysis	Miniaturization	Access to archived samples, cheaper and greener method
LLE	Reduced reagents volume	Cheaper and greener method
	Use of HCl instead of HI	Cheaper, no need to refrigerate
	Vortex instead of shaking	Needed for automation
Derivatization	Reactions at 40°C instead of room temperature	Faster derivatization, higher throughput, greener method
	Replacement of supersaturated reagents	Syringe less likely to clog, more robust method
SPME	Extraction time reduction	Faster SPME, higher throughput, greener method

Abbreviations: LLE, liquid-liquid extraction; HCl, hydrochloric acid; HI, hydriodic acid; SPME, solid-phase microextraction.

One logistical issue faced during the automation of the derivatization steps, was that hydriodic acid is to be kept refrigerated. This diminished the automation potential of the set-up, since HI had to be manually placed in the fridge immediately after it was automatically added. This could be easily solved by including a Tray Cooler Module or Peltier Stack Module in the configuration (see SI), since that way all reagents can be kept within reach of the autosampler. For the final procedure, it was replaced by HCl, which could be kept at room temperature.

Because the heat transfer in a water bath and an agitator could be expected to be different, it might be worth investigating if prolonging the heating step in the agitator has a positive effect on the derivatization efficiency.

With the current setup, up to 6 samples could be simultaneously derivatized. If needed, an extra agitator could be added to double the sample throughput. In this case, it might be interesting to study the stability of the derivatized samples over time and whether the addition of internal standards is strictly necessary.

Another improvement possibility would be the Pipette tool, which enables the use of disposable pipette tips instead of syringes. This offers the advantage of being able to eliminate the cleaning steps, saving time and solvents. Furthermore, it minimizes cross-contamination possibilities, which are especially critical during the LLE step, when the samples come in direct contact with the syringes. Because the pipette tips are placed instead of a rack, it would be necessary to assess if another tray holder would be needed, and if the space available is sufficient.

Alternatively, different extraction techniques could be integrated into the automated method, such as parallel artificial liquid membrane extraction (PALME), which has been proven a very promising alternative to LLE for the analysis of aromatic amines and has automation potential [15], SLE as discussed by [3], or SPE as reported by [19].

The use of PALME and the subsequent further miniaturization would have a positive effect in the overall greenness of the method. This could be further improved by decreasing the hydrolysis time, which would in turn increase the sample throughput and significantly decrease the overall energy consumption.

The automation of the sample preparation steps presented here enables a reliable, user-friendly, and green approach for the analysis of AA in urine. It minimizes not only the error possibilities, but also the analysis time, and increases the overall sample throughput. This allows for optimization possibilities previously deemed too resource-consuming to be worth investigating and offers the advantage that it is user independent and can be performed by users without extensive previous experience. Furthermore, it facilitates the analysis of high numbers of samples, which are essential to better understand the relationship between the smoking status of the donors, the concentrations of AA present in their urine, and the risk of developing smoking-related diseases like bladder cancer.

Finally, the proposed improvements and the discussion regarding automation can be applied to other sample preparation procedures, including different analytes, matrices, or analytical techniques. Some of the automated steps presented here can be directly used in many other applications, like the hydrolysis or SPME steps. Some may require a few more changes, like adjusting the needle penetration depth for the LLE step, or modifying the script for different derivatization procedures. All in all, the discussions and challenges addressed here can be extrapolated to the automation of many other analytical procedures.

6.6 Supplementary information

6.6.1 Materials and methods

Automation with Chronos

Chronos enables the user to design a method consisting of different tasks (Fig. S 6.1), such as “Transfer”, “Clean syringe”, “Vortex vial”. Each task has a set of properties (Fig. S 6.2), like temperatures, speeds, or volumes, which can be defined by the user. If these properties are repeated across different tasks, for example agitation speed, the software offers the opportunity to define “tokens”, which are configured in the “Columns” section (Fig. S 6.3). This enables the user to easily change the value of these properties, without the need to modify all tasks using it individually, speeding up the process and minimizing the possible errors.

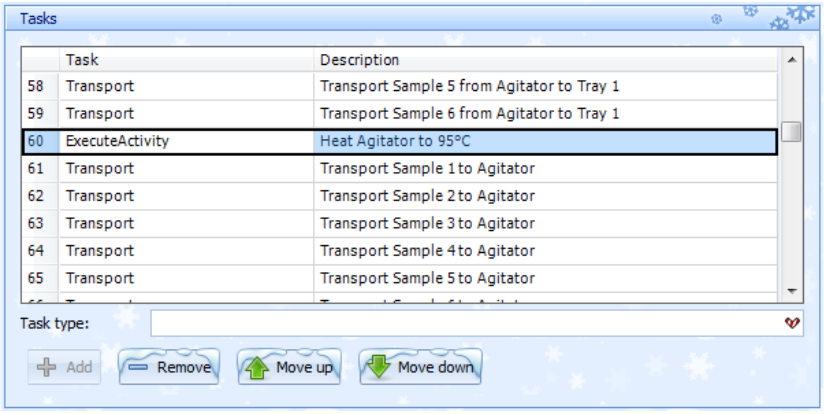


Fig. S 6.1. Examples of some Tasks used during the automatic derivatization with Chronos.

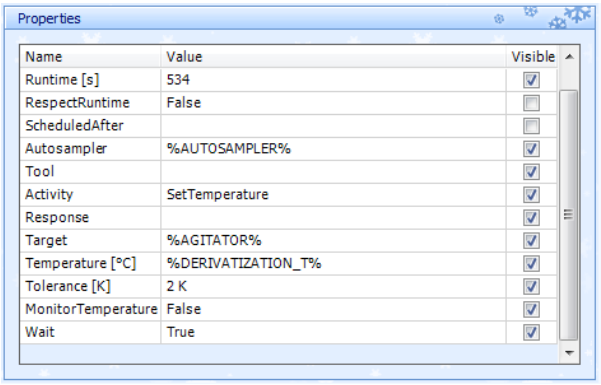


Fig. S 6.2. Examples of some Properties functions in Chronos.

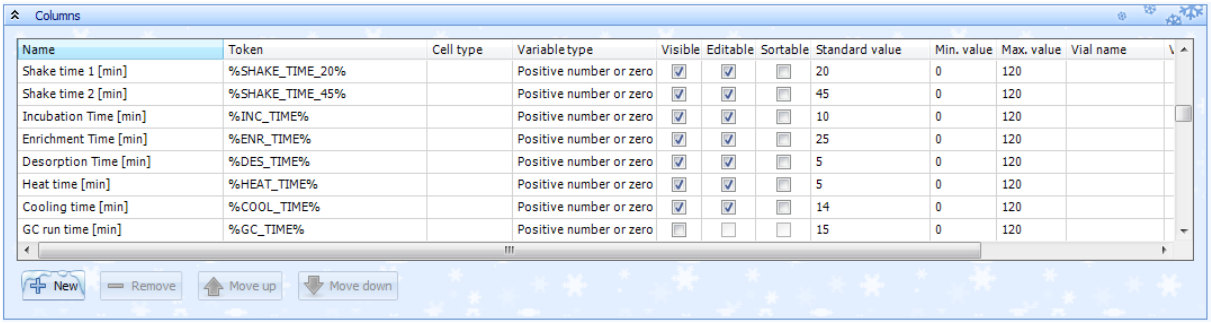


Fig. S 6.3. Example of the Columns section (Definition of tokens) in Chronos.

Most tasks are very intuitive and easily defined. For example, for the “Transport” task, which can be used to transport a vial from one position to another, the property “Source” (e.g. a tray position) and “Destination” (e.g. an agitator position) needs to be defined.

There was only one task in the derivatization process that could not be accomplished with the pre-defined tasks: heating up and changing the agitator´s temperature. In order to program those steps, the task “Execute activity” was used, which enables movements or actions of the autosampler which are normally not defined as a task. “Execute activity” should be used carefully as it can lead to errors during sample preparation. During this automation, a “Set temperature” of a “Target”, i.e. the agitator, was defined. The property “Wait” enabled to wait until the set temperatures were reached (“true”), to continue with the next sample preparation steps while it was cooling down (“false”), and to make sure that the lower temperature was reached before transporting the samples into the agitator (“true”).

Instrumentation

The set-up used for this study can be seen in Fig. S 6.4. The use of a small and a big wash station was critical for the analysis of multiple samples, since it allowed for a larger volume of waste to be generated without having to empty the waste vials. The small wash station was used for the more concentrated waste from the pre-cleaning steps with the reagents and from the first post-cleaning cycle. After that, the syringes were thoroughly cleaned using water from the big washing station which was disposed through the tubing into a Schott bottle.

Fig. S 6.4. Picture of the set-up used, including PAL RTC head, rail and controller, GC-MS QP2010 Ultra and computer. The PAL modules used in this study (from left to right) were: two parking stations, SPME Fiber Conditioning station; small and big washing stations, two tray holders, vortex mixer and agitator.

GC-MS analysis parameters

The quantifier and qualifier ions used for the automatic detection of the derivatized AA, generally corresponding to the molecular ion and the loss of iodine, can be seen in Table S 6.1.

Table S 6.1. Mass spectrometric parameters used for the detection of the derivatized aromatic amines, including the analyte abbreviation (Analyte abbr.), the corresponding iodinated derivative, and the quantifier and qualifier ion of each analyte studied.

Analyte abbreviation	Iodinated aromatic compound	Quantifier ion (m/z)	Qualifier ion (m/z)
A	Iodobenzene	204	77
2MA	1-Iodo-2-methylbenzene	218	91
3C4FA	3-Chloro-4-fluoro-1-iodobenzene	256	129
2CA	2-Chloro-1-iodobenzene	238	111
4EA	4-Ethyl-1-iodobenzene	232	217

Analyte abbreviation	Iodinated aromatic compound	Quantifier ion (m/z)	Qualifier ion (m/z)
2,6DMA	2,6-Dimethyl-1-iodobenzene	232	105
2,4DMA	2,4-Dimethyl-1-iodobenzene	232	105
4C2MA	4-Chloro-1-iodo-2-methylbenzene	252	125
2BA	2-Bromo-1-iodobenzene	282	155
2,4,6TMA	1-Iodo-2,4,6-trimethylbenzene	246	119
2,6DCA	2,6-Dichloro-1-iodobenzene	272	145
3C2,6DMA	3-Chloro-2,6-dimethyl-1-iodobenzene	266	139
3C4MA	3-Chloro-1-iodo-4-methoxybenzene	268	253
2NA	2-Iodonaphthalene	254	127

6.6.2 Results and discussion

Hydrolysis and LLE - Volumes of reagents used

In Table S 6.2 the original volumes as reported in Lamani et al. [16] and the adapted volumes used in this research can be seen.

Table S 6.2. Original volumes of reagents used in the sample preparation of urine samples by Lamani et al. [16] and the adapted volumes selected for automatic hydrolysis (H) and liquid-liquid extraction (LLE).

Reagent	Step	Original volume [mL]	Adapted volume [mL]
Sample	H	20	2
HCl	H	10	1
NaOH	H	20	2
DEE	LLE	5 (twice)	1 (twice)
NaOH Wash	LLE	2	1.5
Acidified H_2O	LLE	5	1

LLE - Acidifying reagent

As discussed in the main text, HCl and HI were studied as acidifying reagents. No chlorinated by-products were found in the chromatograms and the intensities observed were in most cases significantly better when using HCl as acidifying reagent (see Fig. S 6.5). This could be due to the fact that Schmidt et al. [9] observed most by-products with diamino aromatics, which were not present in this study, and that a smaller ratio of chloride to iodide ions was used. Furthermore, the same volume of reagent was used in both cases, which resulted in a lower pH for HCl, which could have facilitated the diazotization step of the derivatization. The only exception was found with 3C4MA, probably due to the methoxy group present in the para position, which might have a similar effect as the amino group in the diamino aromatics. Since their substituent constants in the Hammett equation [24] are relatively similar, it could be expected that the methoxy group would also facilitate the substitution by Cl.

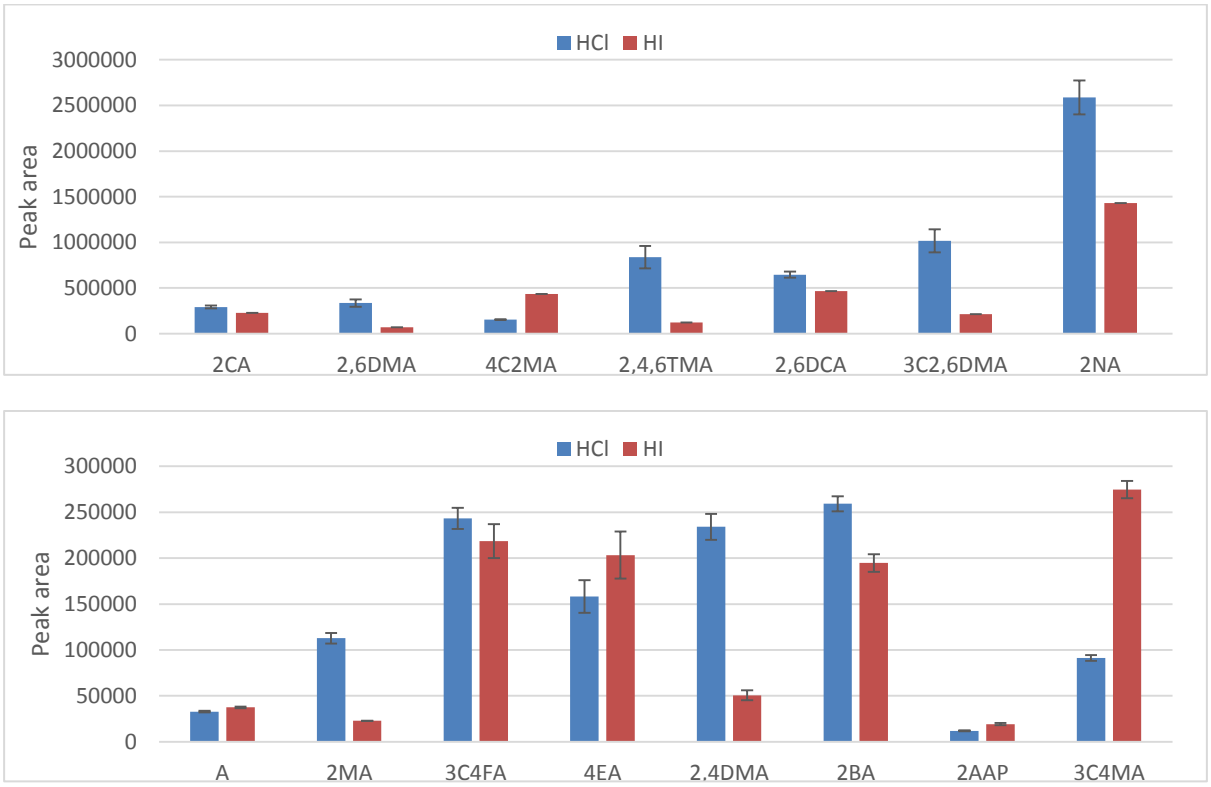


Fig. S 6.5. Averaged peak areas of the aromatic amines studied, prepared with the automated derivatization method described in this chapter, using samples acidified with HCl (blue) and with HI (red).

Because for most compounds HCl was significantly better, and in contrast with HI it is cheaper and it does not need to be kept refrigerated, HCl was used in all other experiments. If HI, or any other refrigerated reagent is needed, it is possible to store them in cooled modules between 4 and 40 °C within the PAL RTC autosampler, thanks to the Tray Cooler (Fig. S 6.6) and the Peltier Stack (Fig. S 6.7) modules.

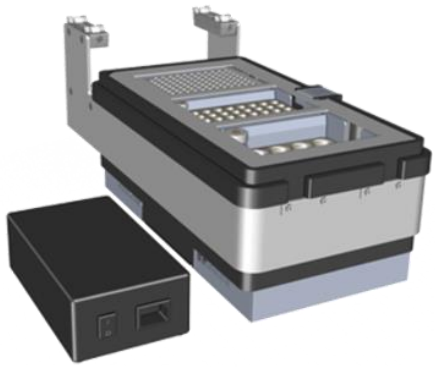


Fig. S 6.6. Tray Cooler Module, picture from https://www.palsystem.com/index.php?id=205, accessed 06.07.2023.

Fig. S 6.7. Peltier Stack Modules: 2DW (left) and 6DW (right), pictures from https://www.palsystem.com/index.php?id=205, accessed 06.07.2023.

Derivatization - Reaction times

During the derivatization procedure, there are two occasions in which the samples are shaken/agitated: after adding hydriodic acid and sodium nitrite for 20 min (reaction time 1, RT1), and after adding sulfamic acid for 45 min (RT2). With 5 min intervals, RT1 was tested between 20-10 min and RT2 between 45-30 min (Fig. S 6.8). For RT1, the best results can be

observed when waiting for 15 min, and in some cases, they were significantly better. For RT2, there is no significant difference for most analytes between 45 and 40 min, except for 2NA which shows significantly better results with 45 min, and 35, and 30 min generally show worse results. Therefore, the automatic derivatization was modified and the agitating times of 15 min for RT1 and 45 min for RT2 were used.

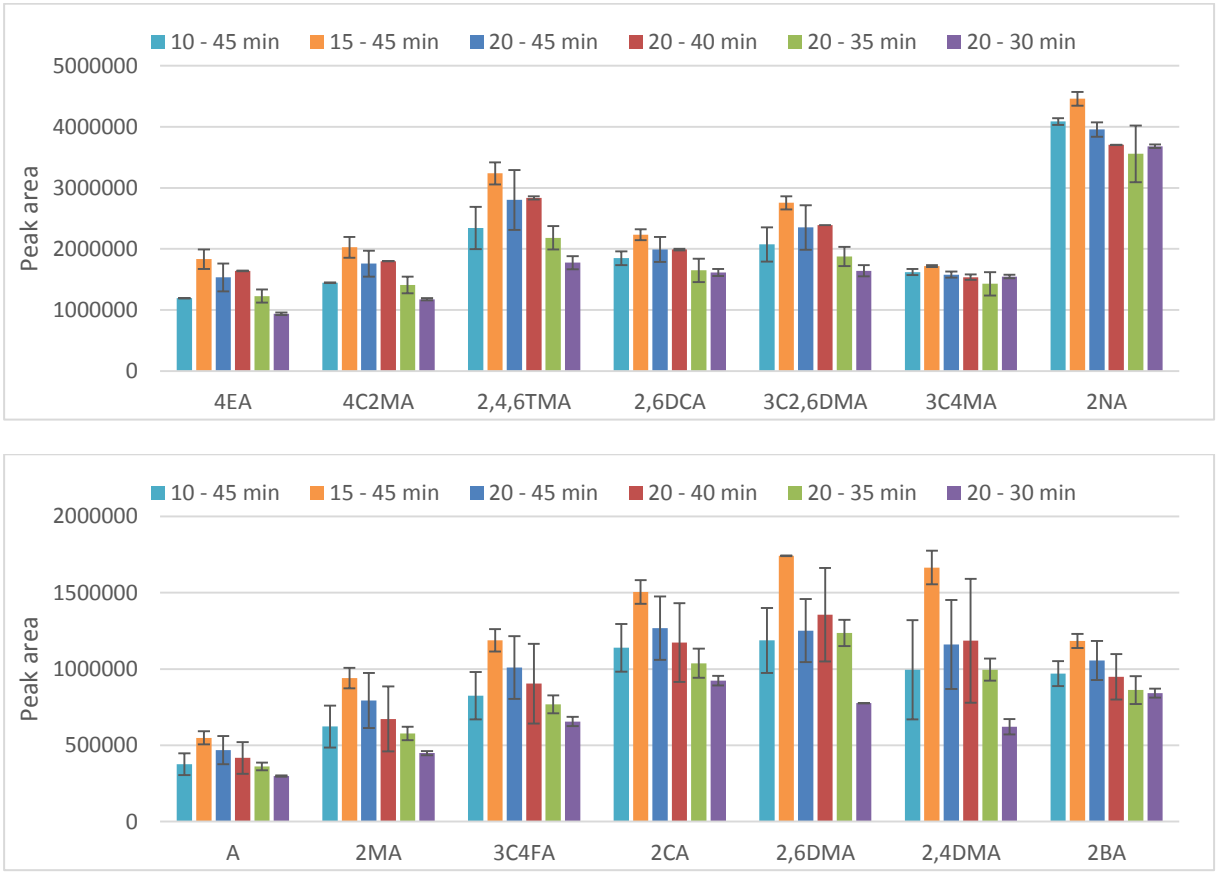


Fig. S 6.8. Average peak areas of the aromatic amines studied when waiting RT1–RT2 during derivatization: 10-45 min (light blue), 15-45 min (orange), 20-45 min (dark blue), 20-40 min (red), 20-35 min (green) and 20-30 min (purple).

Derivatization - Supersaturated solutions

Since there were two supersaturated solutions in the derivatization protocol that could potentially clog the syringe, the following alternatives were considered.

Instead of supersaturated sodium sulfite, a 120 g/L solution and double the volume, i.e. 250 µL, were used. Because the addition of this compound triggers a discoloration of the

solution, it could be visually confirmed that the amount and concentration of reagent was sufficient.

The addition of NaOAc had the objective of adjusting the final pH of the solution, so that it would be within a suitable range for SPME (2-11), and ideally between 4 and 6. Because 10 M NaOH is already used during the LLE step, it was studied as alternative to NaOAc. The pH of the solutions was checked after manually adding different volumes of 10 M NaOH, and the results with pH 3-9 were further analyzed with GC-MS (see Fig. S 6.9). Because a pH between 4 and 6 was desired and for most analytes 180 µL of 10 M NaOH gave the worst results, 185 µL 10 M NaOH was chosen.

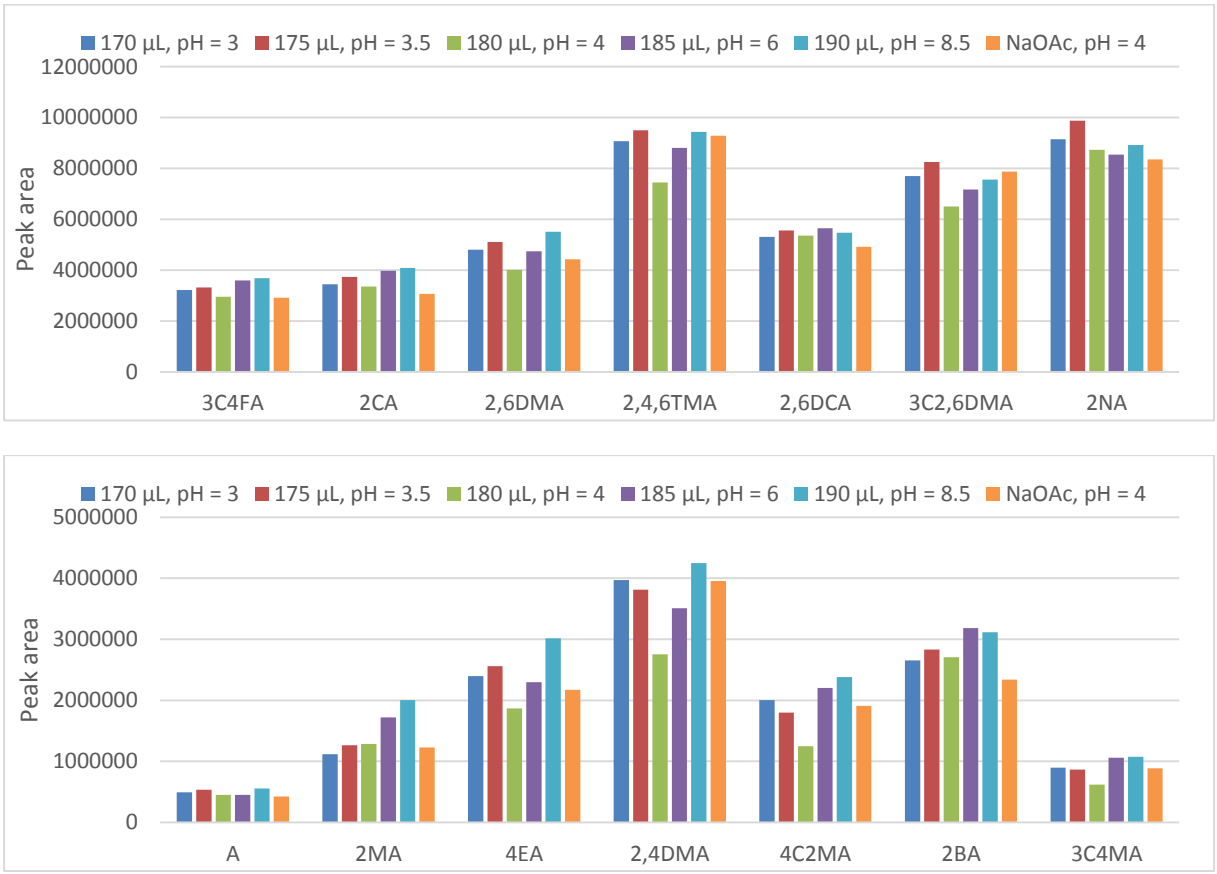


Fig. S 6.9. Peak areas of the aromatic amines studied, when the solution was basified with: 170 µL (dark blue, pH = 3), 175 µL (red, pH = 3.5), 180 µl (green, pH = 4), 185 µL (purple, pH = 6), and 190 µL (light blue, pH = 8.5) of 10 M sodium hydroxide (NaOH) and 500 µL supersaturated sodium acetate (NaOAc, orange, pH = 4), n=1.

SPME - Extraction time

The time needed for the SPME extraction step was also studied (see Fig. S 6.10). While 5 min extraction time showed significantly worse results for 3C4MA and 2NA, 10 min extraction time showed comparable or better results than the rest. The method was changed accordingly, which allowed to save 15 min per sample compared with the original 25 min reported by [16].

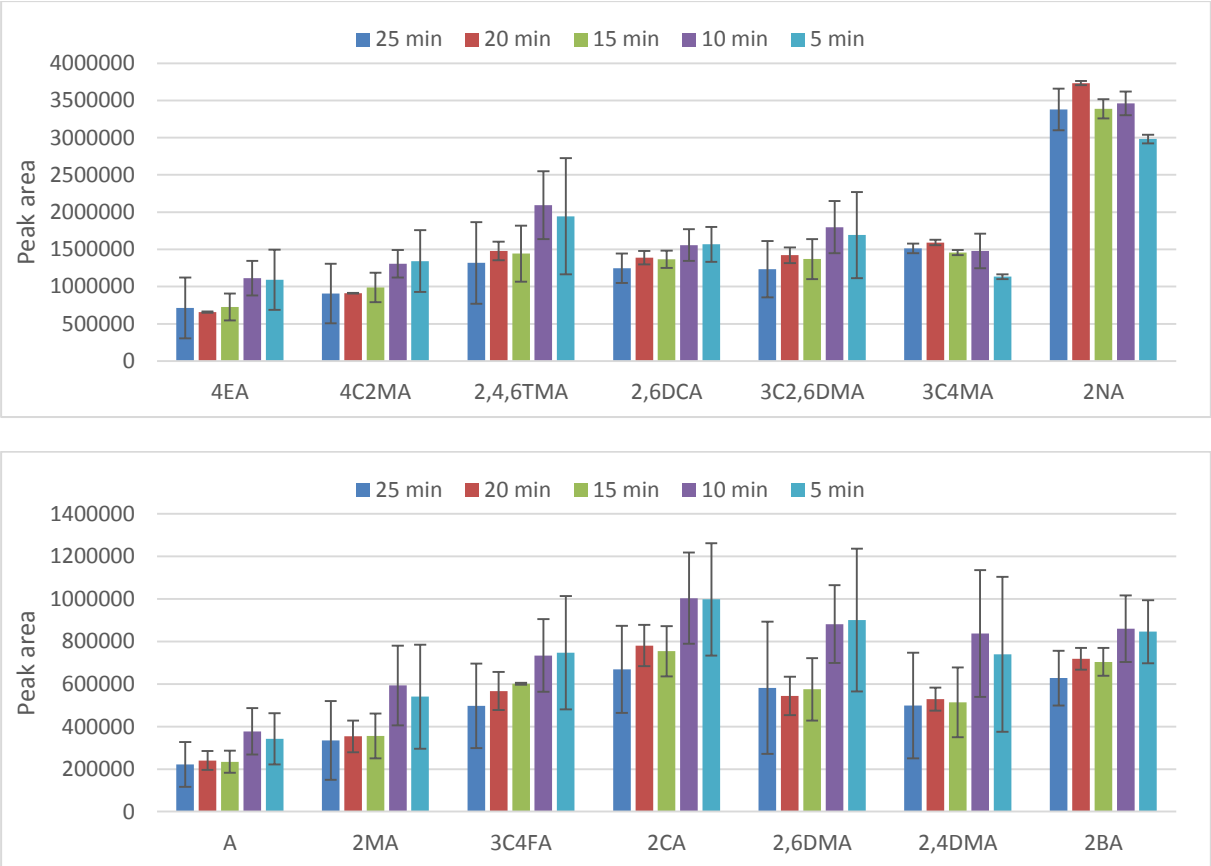


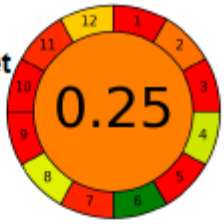
Fig. S 6.10. Average peak areas of the aromatic amines studied when extracted with SPME for: 25 min (dark blue), 20 min (red), 15 min (green), 10 min (purple), and 5 min (light blue).

Analytical greenness metrics – AGREE and AGREEprep

The reports created when evaluating the AGREE and AGREEprep metrics can be seen below. First, the report for the original method is shown, followed by the new automated method proposed here.

Analytical Greenness report sheet

02/06/2023 07:03:04

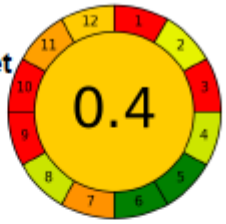


1. Sample treatment
2. Sample amount
3. Device positioning
4. Sample prep. stages
5. Automation, miniaturization
6. Derivatization
7. Waste
8. Analysis throughput
9. Energy consumption
10. Source of reagents
11. Toxicity
12. Operator's safety

Criteria	Score	Weight
1. Direct analytical techniques should be applied to avoid sample treatment.	0.0	2
2. Minimal sample size and minimal number of samples are goals.	0.22	2
3. If possible, measurements should be performed in situ.	0.0	2
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.	0.6	2
5. Automated and miniaturized methods should be selected.	0.0	2
6. Derivatization should be avoided.	1.0	2
7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.	0.07	2
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.	0.56	2
9. The use of energy should be minimized.	0.0	2
10. Reagents obtained from renewable sources should be preferred.	0.0	2
11. Toxic reagents should be eliminated or replaced.	0.08	2
12. Operator's safety should be increased.	0.4	2

Analytical Greenness report sheet

02/06/2023 07:12:21



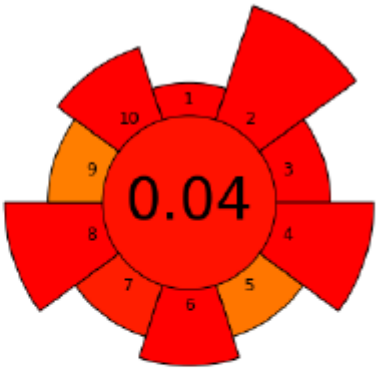
1. Sample treatment
2. Sample amount
3. Device positioning
4. Sample prep. stages
5. Automation, miniaturization
6. Derivatization
7. Waste
8. Analysis throughput
9. Energy consumption
10. Source of reagents
11. Toxicity
12. Operator's safety

Criteria	Score	Weight
1. Direct analytical techniques should be applied to avoid sample treatment.	0.0	2
2. Minimal sample size and minimal number of samples are goals.	0.55	2
3. If possible, measurements should be performed in situ.	0.0	2
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.	0.6	2
5. Automated and miniaturized methods should be selected.	1.0	2
6. Derivatization should be avoided.	1.0	2
7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.	0.29	2
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.	0.61	2
9. The use of energy should be minimized.	0.0	2
10. Reagents obtained from renewable sources should be preferred.	0.0	2
11. Toxic reagents should be eliminated or replaced.	0.32	2
12. Operator's safety should be increased.	0.4	2

AGREEprep

Analytical Greenness Metric for Sample Preparation

02/06/2023 07:24:23

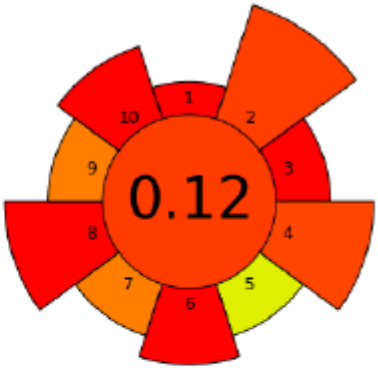


#	Criterion	Score	Weight
1.	**Sample preparation placement** Sample preparation placement: Ex situ	0.0	1
2.	**Hazardous materials** Mass [g] or volume [mL] of problematic materials: 25.2	0.0	5
3.	**Sustainability, renewability, and reusability of materials** < 25% of reagents and materials are sustainable or renewable, but can only be used ONCE	0.0	2
4.	**Waste** Mass [g] or volume [mL] of waste: 104.7	0.0	4
5.	**Size economy of the sample** Mass [g] or volume [mL] of the sample: 20	0.23	2
6.	**Sample throughput** Hourly sample throughput: 0.25	0.0	3
7.	**Integration and automation** No. of sample prep. steps: 5 steps; degree if automation: Manual systems	0.06	2
8.	**Energy consumption** Approximate energy consumption per analysis [W]: 3051	0.0	4
9.	**Post-sample preparation configuration for analysis** Liquid chromatography, gas chromatography with quadrupole detection, etc.	0.25	2
10.	**Operator's safety** No. of distinct hazards: 4 or more hazards	0.0	3

AGREEprep

Analytical Greenness Metric
for Sample Preparation

02/06/2023 07:27:36



#	Criterion	Score	Weight
1.	**Sample preparation placement** Sample preparation placement: Ex situ	0.0	1
2.	**Hazardous materials** Mass [g] or volume [mL] of problematic materials: 4.2	0.13	5
3.	**Sustainability, renewability, and reusability of materials** < 25% of reagents and materials are sustainable or renewable, but can only be used ONCE	0.0	2
4.	**Waste** Mass [g] or volume [mL] of waste: 20.8	0.14	4
5.	**Size economy of the sample** Mass [g] or volume [mL] of the sample: 2	0.57	2
6.	**Sample throughput** Hourly sample throughput: 0.3	0.0	3
7.	**Integration and automation** No. of sample prep. steps: 5 steps; degree if automation: Fully automated systems	0.25	2
8.	**Energy consumption** Approximate energy consumption per analysis [W]: 666	0.0	4
9.	**Post-sample preparation configuration for analysis** Liquid chromatography, gas chromatography with quadrupole detection, etc.	0.25	2
10.	**Operator's safety** No. of distinct hazards: 4 or more hazards	0.0	3

6.7 References

1. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Chemical Agents and Related Occupations. Lyon, France: International Agency for Research on Cancer; 2012.

2. Pereira L, Mondal PK, Alves M. Aromatic amines sources, environmental impact and remediation. In: Lichtfouse E, Schwarzbauer J, Robert D, editors. Pollutants in Buildings, Water and Living Organisms. Environmental Chemistry for a Sustainable World. Cham: Springer International Publishing; 2015. pp. 297-346. https://doi.org/10.1007/978-3-319-19276-5_7.

3. Mazumder S, Ahamed RA, McGahee E, Wang L, Seyler TH. A new automated method for the analysis of aromatic amines in human urine by GC-MS/MS. J Anal Toxicol. 2019;43:25-35. https://doi.org/10.1093/jat/bky045.

4. Besaratinia A, Tommasi S. Genotoxicity of tobacco smoke-derived aromatic amines and bladder cancer: Current state of knowledge and future research directions. FASEB J. 2013;27:2090-100. https://doi.org/10.1096/fj.12-227074.

5. Yih Hui B, Mohamad Zain NN, Mohamad S, Mohamed Fauzi H, Alias Y, Chandrasekaram K, Rahim NY, Yahaya N, Raoov M. Determination of Aromatic Amines in Urine using Extraction and Chromatographic Analysis: A Minireview. Anal Lett. 2019;52:2974-92. https://doi.org/10.1080/00032719.2019.1636057.

6. Daryanavard SM, Zolfaghari H, Abdel-Rehim A, Abdel-Rehim M. Recent applications of microextraction sample preparation techniques in biological samples analysis. Biomed Chromatogr. 2021;35. https://doi.org/10.1002/bmc.5105.

7. Dean JR. Extraction Techniques in Analytical Science. 1 ed. Ando DJ, editor. Chichester, West Sussex, England: John Wiley & Sons Ltd; 2009.

8. Zimmermann T, Ensinger WJ, Schmidt TC. In Situ Derivatization/Solid-Phase Microextraction: Determination of Polar Aromatic Amines. Anal Chem. 2004;76:1028-38. https://doi.org/10.1021/ac035098p.

9. Schmidt TC, Less M, Haas R, von Löw E, Steinbach K, Stork G. Gas chromatographic determination of aromatic amines in water samples after solid-phase extraction and derivatization with iodine. I. Derivatization. J Chromatogr A. 1998;810:161-72. https://doi.org/10.1016/S0021-9673(98)00233-7.

10. Płotka-Wasylka JM, Morrison C, Biziuk M, Namieśnik J. Chemical Derivatization Processes Applied to Amine Determination in Samples of Different Matrix Composition. Chem Rev. 2015;115:4693-718. https://doi.org/10.1021/cr4006999.

11. Kataoka H. 2.1.2. Gas chromatography of amines as various derivatives. In: Molnár-Perl I, editor. Quantitation of amino acids and amines by chromatography: methods and protocols.

Journal of Chromatography Library. 70: Elsevier; 2005. pp. 364-404. https://doi.org/10.1016/S0301-4770(05)80016-8.

12. Ferreira AMC, Laespada MEF, Pavón JLP, Cordero BM. In situ aqueous derivatization as sample preparation technique for gas chromatographic determinations. J Chromatogr A. 2013;1296:70-83. https://doi.org/10.1016/j.chroma.2013.04.084.

13. Farajzadeh MA, Nouri N, Khorram P. Derivatization and microextraction methods for determination of organic compounds by gas chromatography. Trends Anal Chem. 2014;55:14-23. https://doi.org/10.1016/j.trac.2013.11.006.

14. Lorenzo-Parodi N, Leitner E, Schmidt TC. Comparison of gas chromatographic techniques for the analysis of iodinated derivatives of aromatic amines. Anal Bioanal Chem. 2023;415:3313-25. https://doi.org/10.1007/s00216-023-04713-8.

15. Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

16. Lamani X, Horst S, Zimmermann T, Schmidt TC. Determination of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GCxGC-qMS). Anal Bioanal Chem. 2015;407:241-52. https://doi.org/10.1007/s00216-014-8080-5.

17. Majors RE. Overview of Sample Preparation. LCGC. 2015;33:46-51.

18. Wojnowski W, Tobiszewski M, Pena-Pereira F, Psillakis E. AGREEprep – Analytical greenness metric for sample preparation. Trends Anal Chem. 2022;149:116553. https://doi.org/10.1016/j.trac.2022.116553.

19. Jurado-Sánchez B, Ballesteros E, Gallego M. Continuous solid-phase extraction method for the determination of amines in human urine following on-line microwave-assisted acid hydrolysis. Anal Bioanal Chem. 2010;396:1929-37. https://doi.org/10.1007/s00216-009-3395-3.

20. Otto M. Chemometrics Statistics and Computer Application in Analytical Chemistry. 3 ed. Weinheim, Germany: Wiley-VCH; 2016.

21. Pena-Pereira F, Wojnowski W, Tobiszewski M. AGREE—Analytical GREEnness Metric Approach and Software. Anal Chem. 2020;92:10076-82. https://doi.org/10.1021/acs.analchem.0c01887.

22. Nowak PM, Bis A, Zima A. ChlorTox Base – a useful source of information on popular reagents in terms of chemical hazards and greenness assessment. Green Anal Chem. 2023;6:100065. https://doi.org/10.1016/j.greeac.2023.100065.

23. Hay AO, Hansen FA, Psillakis E, Pedersen-Bjergaard S. Liquid-phase microextraction in bioanalysis – How green can it be? Green Anal Chem. 2022;3:100028. https://doi.org/10.1016/j.greeac.2022.100028.

24. Hansch C, Leo A, Taft RW. A survey of Hammett substituent constants and resonance and field parameters. Chem Rev. 1991;91:165-95. https://doi.org/10.1021/cr00002a004.

Chapter 7. General conclusion and outlook

7.1 General conclusion and outlook

Due to the adverse health and safety concerns linked to AA, some of which have been classified as carcinogenic, their manufacture and/or use in some industrial settings has seen a decline in developed countries. Unfortunately, it has shifted to developing countries, where occupational safety systems are often weaker [1, 2]. They can also be found in tobacco smoke and therefore about a quarter of the global population (as of 2020 [3]) are regularly exposed to them. In the human body, these AA can be metabolized and/or be excreted with urine. The analysis of AA in urine typically involves very complex, labor-intensive and time-consuming sample preparation procedures [4]. After an initial proof-of concept evaluation, a very promising analytical protocol was further optimized and automated.

LLE is one of the most commonly used clean-up technique for the extraction of compounds from aqueous samples [5]. It is however, a very time-consuming and labor-intensive technique that uses large sample volumes and high amounts of often toxic organic solvents [6, 7]. As a result, it is slowly being replaced by newer and greener techniques, such as microextraction techniques, which require less solvents, time, and labor [8, 9]. An overview of different microextraction techniques and an evaluation of their analytical greenness and sustainability can be seen in [10]. HF-LPME and PALME have been proven especially suitable alternatives for biological matrixes, thanks to, among others, the extra physical barrier that aids in the matrix clean-up [11]. While HF-LPME has been studied for the analysis of aromatic amines, for example [12-16], the use of PALME for this analysis was first tested in the context of this thesis (see Chapter 4). PALME was proven far superior to HF-LPME in terms of the recoveries observed, and it offered the advantages of a simpler and less error-prone set-up. While comparisons of these techniques are common within review articles and book chapters [11, 17, 18], this was also, to the best of our knowledge, the first experimental study in which both techniques were used for exactly the same analytical question, enabling a direct comparison.

While the equipment needed for PALME is commercially available, the available sizes and membrane materials are extremely limited. For example, commercially available 96-well acceptor plates have a polyvinylidene fluoride, or PVDF, membrane which can lead to non-

specific binding of some analytes, as described by [19]. This was solved in-house by replacing the PVDF membrane with a polypropylene (PP) one [19]. However, that solution is not commercially available. Furthermore, the variability of plate sizes is also limited, and therefore so are the volumes of sample/acceptor that can be used. Both issues could be problematic for certain applications and would also need to be improved before this technique can be more commonly seen in laboratories.

One key point towards green analytical chemistry is automation [20]. Several successful automation attempts for LPME have been summarized by [21-23], and while HF-LPME has been successfully automated using robots similar to the PAL RTC, for PALME, 96-well plate specific robots are typically used instead [21-23]. Because for the analysis of AA with GC-MS a derivatization procedure and SPME were performed, and they cannot be done with the 96-well plate robots, an operator would have to manually place the extracted samples into the PAL RTC for the following steps. More research is needed in order to evaluate if an automated miniaturized LLE as discussed in Chapter 6 could be comparable and as beneficial as PALME in terms of analytical greenness and performance. Further automation possibilities of PALME should also be studied, for example, by taking advantage of the automated set-up used for µ-SPE with the PAL RTC. Based on the latest developments regarding green analytical techniques and particularly the use of green solvents [24], PALME has the potential to follow the path of SPME, and become fully integrated in the PAL RTC.

In general, automation is a research area that has been gaining a lot of interest in the past years. Specifically for the analysis of AA, two publications reported (semi)-automatic sample preparation methods [25, 26], however, they have little overlap with the one used in this thesis, both in terms of the sample preparation followed, and the means by which it was automated, i.e. a Hamilton Microlab STAR™ Liquid Handling Workstation [25] and a continuous flow system [26]. Thanks to the automated procedure developed here (see Chapter 6), several steps could be successfully further optimized, with significantly lower costs, especially in terms of labor. There are, however, unexplored areas that could be worth investigating, for example, the use of the pipette tool for the PAL RTC, which was released after the experimental part of the thesis was finished and could therefore not be tested. It would enable the use of disposable pipette tips instead of syringes and would not only minimize cross-contamination possibilities but also eliminate the need for cleaning steps, saving time and toxic

solvents. Another point that could be systematically studied is to which degree particles appear after hydrolysis, whether they interfere with the analysis, and, if needed, how to best include a filtering step in the automated procedure to remove them.

The knowledge gained through this thesis and the automated procedure developed could be used not just to further optimize the method, but also as template to automate other analytical procedures, including different analytes or matrices. Especially interesting in the context of AA analysis and this thesis, it could be used to do a systematic comparison of some of the most common and/or promising derivatization techniques used for the analysis of AA. For a more comprehensive comparison, the RGB model could be used, which evaluates the analytical (red), ecological (green), and practical (blue) aspects of the methods and combines them into a "whiteness" parameter. The concept of White Analytical Chemistry (WAC) was recently presented as an extension of Green Analytical Chemistry (GAC) and argued to be "closer to the idea of sustainable development due to a more holistic view, as it strives for a compromise that avoids an unconditional increase in greenness at the expense of functionality" [27].

While the optimization and automation of the sample preparation of AA in urine was one of the core focuses of this thesis, the analytical efficiency and user friendliness were given the highest priorities. With the automated procedure developed here, a detailed study on how to improve its greenness could be carried out, for example by evaluating green reagent/solvent alternatives. Databases like the ChlorTox Base could be used as a starting point to find a selection of greener solvent and reagent alternatives [28].

One last area where more research would be recommended is related to the detection step (see Chapter 5). While MRM was proven most successful for target analytes and could be used to monitor the carcinogenic and probable/possible carcinogenic AA, the list is being constantly evaluated and it is likely new AA will be added. Therefore, to have a better understanding of the relationship of smoking status, AA contents and smoking-related diseases, and be able to retrospectively monitor new AA, non-target analysis would be essential. There are a few promising solutions, such as the simultaneous scan and MRM that would enable sensitive detection of the target AA, while simultaneously scanning for non-target analytes. Neutral loss is also an interesting technique that could both be extremely selective towards the derivatized AA, since it could detect all compounds where an iodine is lost and enable simultaneous target and non-targeted analysis. These techniques could be combined with

GCxGC in order to gain extra resolving power. However, typically GCxGC is performed using single quadrupoles, in which case several data evaluation software, like ChromSpace, could enable the filtering of the analytes according to neutral loss characteristics.

Having a green, user-friendly, sensitive, and selective method would make the analysis of thousands of real samples possible. Having big data sets could in turn enable the successful use of more advanced data evaluation methods like random forests or neural networks, among others. Furthermore, if health information is available, like in the case of cohort studies such as the Heinz Nixdorf Recall Study [29], the relationship between AA, smoking status and smoking-related diseases could also be studied. The analysis of individual samples from specific donors could therefore provide us with the information needed to establish these relationships and find an AA that could be a biomarker for the development of bladder cancer and other smoking related diseases.

On the other hand, the analysis of collective samples could provide answers to a completely new set of questions. Several articles have been published describing occupational exposure to AA and an increase in bladder cancer risk for exposed workers, such as [30-33]. The International Agency for Research on Cancer (IARC) has found substantial data gaps regarding the occupational exposure levels [2]. Often, exposure to AA is reported in mg/m^3, but many factors can affect how much a specific worker is exposed, such as the use or lack thereof of personal protective equipment. Furthermore, dermal exposure might contribute significantly to the total AA uptake, as it has been shown for aniline in certain situations [2]. Therefore, an alternative solution would be to take a look at urinary concentrations instead. Ideally, each worker could be monitored regularly. However, a possible cost-efficient alternative would be to analyze urine collected from the buildings were occupational exposure might occur and compare it to collected urine from the surrounding population. In this way, a cost-efficient real time monitoring of occupational exposure could take place, and if differences to the general population are observed, corresponding measures to minimize/prevent adverse health effects could be directly taken.

The adverse health effects of smoking are well documented [34-39], and multiple governments have tried different measures to minimize tobacco consumption [3, 34, 40]. However, some of the strategies, like increased taxation, could lead to illegal smuggling from

other regions, and an underestimation of the smoking population calculated based on those taxes. A possible alternative evaluation strategy of the success of such measures would be to collect and analyze the population urine before and after the implementation of such measures and see if a decrease in AA concentration can be observed. A similar strategy was carried out by Boogaerts et al. [41], but instead of AA they measured cotinine and hydroxycotinine, metabolites of nicotine. AA like 4-ABP, which has been proven a reliable marker for tobacco exposure [42], could be studied as complementary or alternative markers. Furthermore, AA concentration in wastewater could also be tracked over time to see the general population exposure to tobacco smoke and evaluate the health status of the population, in what could be considered wastewater-based epidemiology [43, 44].

7.2 References

1. OECD. Limits on Aromatic Amines in Textiles Coloured with Azo Dyes2006.

2. IARC Working Group on the Identification of Carcinogenic Hazards to Humans. Some Aromatic Amines and Related Compounds. Lyon, France: International Agency for Research on Cancer; 2021.

3. WHO global report on trends in prevalence of tobacco use 2000–2025. Geneva: World Health Organization; 2021.

4. Lorenzo-Parodi N, Kaziur-Cegla W, Schmidt TC. Automation and optimization of the sample preparation of aromatic amines for their analysis with GC–MS. Green Anal Chem. 2023;6:100071. https://doi.org/10.1016/j.greeac.2023.100071.

5. Dean JR. Extraction Techniques in Analytical Science. 1 ed. Ando DJ, editor. Chichester, West Sussex, England: John Wiley & Sons Ltd; 2009.

6. Majors RE. Overview of Sample Preparation. LCGC. 2015;33:46-51.

7. Vas G, Nagy K, VÉKey K. Chapter 4 - Biomedical sampling. In: Vékey K, Telekes A, Vertes A, editors. Medical Applications of Mass Spectrometry. Amsterdam: Elsevier; 2008. pp. 37-59. https://doi.org/10.1016/B978-044451980-1.50006-9.

8. Płotka-Wasylka J, Szczepańska N, de la Guardia M, Namieśnik J. Miniaturized solid-phase extraction techniques. Trends Anal Chem. 2015;73:19-38. https://doi.org/10.1016/j.trac.2015.04.026.

9. Kędziora-Koch K, Wasiak W. Needle-based extraction techniques with protected sorbent as powerful sample preparation tools to gas chromatographic analysis: Trends in application. J Chromatogr A. 2018;1565:1-18. https://doi.org/10.1016/j.chroma.2018.06.046.

10. Tintrop LK, Salemi A, Jochmann MA, Engewald WR, Schmidt TC. Improving greenness and sustainability of standard analytical methods by microextraction techniques: A critical review. Anal Chim Acta. 2023;1271:341468. https://doi.org/10.1016/j.aca.2023.341468.

11. Gjelstad A. Three-phase hollow fiber liquid-phase microextraction and parallel artificial liquid membrane extraction. Trends Anal Chem. 2019;113:25-31. https://doi.org/10.1016/j.trac.2019.01.007.

12. Lin Z, Zhang J, Cui H, Zhang L, Chen G. Determination of aromatic amines in environmental water sample by hollow fiber-liquid phase microextraction and microemulsion electrokinetic chromatography. J Chromatogr A. 2010;1217:4507-10. https://doi.org/10.1016/j.chroma.2010.04.063.

13. Tao Y, Liu J-F, Wang T, Jiang G-B. Simultaneous conduction of two- and three-phase hollow-fiber-based liquid-phase microextraction for the determination of aromatic amines in environmental water samples. J Chromatogr A. 2009;1216:756-62. https://doi.org/10.1016/j.chroma.2008.11.094.

14. Sarafraz-Yazdi A, Mofazzeli F, Es'haghi Z. A new high-speed hollow fiber based liquid phase microextraction method using volatile organic solvent for determination of aromatic amines in environmental water samples prior to high-performance liquid chromatography. Talanta. 2009;79:472-8. https://doi.org/10.1016/j.talanta.2009.04.015.

15. Hou L, Lee HK. Dynamic three-phase microextraction as a sample preparation technique prior to capillary electrophoresis. Anal Chem. 2003;75:2784-9. https://doi.org/10.1021/ac020753z

16. Zhao L, Zhu L, Lee HK. Analysis of aromatic amines in water samples by liquid-liquid-liquid microextraction with hollow fibers and high-performance liquid chromatography. J Chromatogr A. 2002;963:239-48. https://doi.org/10.1016/S0021-9673(02)00544-7.

17. Pedersen-Bjergaard S. 5 - Analytical microextraction with supported liquid membranes. In: Lucena R, Cárdenas S, editors. Analytical Sample Preparation With Nano- and Other High-Performance Materials: Elsevier; 2021. pp. 97-109. https://doi.org/10.1016/B978-0-12-822139-6.00013-4.

18. Pedersen-Bjergaard S. Chapter 8 - Microextraction With Supported Liquid Membranes. In: Poole CF, editor. Liquid-Phase Extraction: Elsevier; 2020. pp. 241-63. https://doi.org/10.1016/B978-0-12-816911-7.00008-6.

19. Gjelstad A, Andresen AT, Dahlgren A, Gundersen TE, Pedersen-Bjergaard S. High-throughput liquid-liquid extraction in 96-well format: Parallel artificial liquid membrane extraction. LC GC Europe. 2017;30:10-7.

20. Wojnowski W, Tobiszewski M, Pena-Pereira F, Psillakis E. AGREEprep – Analytical greenness metric for sample preparation. Trends Anal Chem. 2022;149:116553. https://doi.org/10.1016/j.trac.2022.116553.

21. Soares da Silva Burato J, Vargas Medina DA, de Toffoli AL, Vasconcelos Soares Maciel E, Mauro Lanças F. Recent advances and trends in miniaturized sample preparation techniques. J Sep Sci. 2020;43:202-25. https://doi.org/10.1002/jssc.201900776.

22. Alexovič M, Dotsikas Y, Bober P, Sabo J. Achievements in robotic automation of solvent extraction and related approaches for bioanalysis of pharmaceuticals. J Chromatogr B. 2018;1092:402-21. https://doi.org/10.1016/j.jchromb.2018.06.037.

23. Alexovič M, Horstkotte B, Solich P, Sabo J. Automation of static and dynamic non-dispersive liquid phase microextraction. Part 2: Approaches based on impregnated membranes and porous supports. Anal Chim Acta. 2016;907:18-30. https://doi.org/10.1016/j.aca.2015.11.046.

24. Hay AO, Hansen FA, Psillakis E, Pedersen-Bjergaard S. Liquid-phase microextraction in bioanalysis – How green can it be? Green Anal Chem. 2022;3:100028. https://doi.org/10.1016/j.greeac.2022.100028.

25. Mazumder S, Ahamed RA, McGahee E, Wang L, Seyler TH. A new automated method for the analysis of aromatic amines in human urine by GC-MS/MS. J Anal Toxicol. 2019;43:25-35. https://doi.org/10.1093/jat/bky045.

26. Jurado-Sánchez B, Ballesteros E, Gallego M. Continuous solid-phase extraction method for the determination of amines in human urine following on-line microwave-assisted acid hydrolysis. Anal Bioanal Chem. 2010;396:1929-37. https://doi.org/10.1007/s00216-009-3395-3.

27. Nowak PM, Wietecha-Posłuszny R, Pawliszyn J. White Analytical Chemistry: An approach to reconcile the principles of Green Analytical Chemistry and functionality. Trends Anal Chem. 2021;138:116223. https://doi.org/10.1016/j.trac.2021.116223.

28. Nowak PM, Bis A, Zima A. ChlorTox Base – a useful source of information on popular reagents in terms of chemical hazards and greenness assessment. Green Anal Chem. 2023;6:100065. https://doi.org/10.1016/j.greeac.2023.100065.

29. Hovanec J, Weiß T, Koch HM, Pesch B, Behrens T, Kendzia B, Arendt M, Dragano N, Moebus S, Schmidt B, Brüning T, Jöckel K-H. Smoking intensity and urinary nicotine metabolites by socioeconomic status in the Heinz Nixdorf Recall study. BMC Public Health. 2022;22:302. https://doi.org/10.1186/s12889-022-12609-y.

30. Chamot S, Delzard M, Leroy L, Marhic G, Ganry O. Occupational exposures in the rubber tire industry and risk of cancer: a systematic review. Int Arch Occup Environ Health. 2023. https://doi.org/10.1007/s00420-023-01972-x.

31. Hosseini B, Zendehdel K, Bouaoun L, Hall AL, Rashidian H, Hadji M, Gholipour M, Haghdoost AA, Schüz J, Olsson A. Bladder cancer risk in relation to occupations held in a nationwide case-control study in Iran. Int J Cancer. 2023;153:765-74. https://doi.org/10.1002/ijc.34560.

32. Alston F, Okorie O. Occupational exposures: chemical carcinogens and mutagens. 1st ed. Boca Raton: CRC Press; 2023.

33. Ciocan C, Godono A, Franco N, La Vecchia C, Negri E, Boffetta P, Pira E. Mortality from bladder cancer in dyestuff workers exposed to aromatic amines: A 73-year follow-up. La Medicina del lavoro. 2022;113:e2022017. https://doi.org/10.23749/mdl.v113i2.12893.

34. Kalkhoran S, Benowitz NL, Rigotti NA. Prevention and Treatment of Tobacco Use: JACC Health Promotion Series. J Am Coll Cardiol. 2018;72:1030-45. https://doi.org/10.1016/j.jacc.2018.06.036.

35. Schane RE, Ling PM, Glantz SA. Health Effects of Light and Intermittent Smoking. Circulation. 2010;121:1518-22. https://doi.org/10.1161/circulationaha.109.904235.

36. Yanbaeva DG, Dentener MA, Creutzberg EC, Wesseling G, Wouters EFM. Systemic Effects of Smoking. Chest. 2007;131:1557-66. https://doi.org/10.1378/chest.06-2179.

37. Saha SP, Bhalla DK, Whayne TF, Gairola CG. Cigarette smoke and adverse health effects: An overview of research trends and future needs. Int J Angiol. 2007;16:77-83. https://doi.org/10.1055/s-0031-1278254.

38. Das SK. Harmful health effects of cigarette smoking. Mol Cell Biochem. 2003;253:159-65. https://doi.org/10.1023/A:1026024829294.

39. Fagerström K. The Epidemiology of Smoking. Drugs. 2002;62:1-9. https://doi.org/10.2165/00003495-200262002-00001.

40. WHO report on the global tobacco epidemic, 2021: Addressing new and emerging products. Geneva: World Health Organization; 2021.

41. Boogaerts T, Jurgelaitiene L, Dumitrascu C, Kasprzyk-Hordern B, Kannan A, Been F, Emke E, de Voogt P, Covaci A, van Nuijs ALN. Application of wastewater-based epidemiology to investigate stimulant drug, alcohol and tobacco use in Lithuanian communities. Sci Total Environ. 2021;777:145914. https://doi.org/10.1016/j.scitotenv.2021.145914.

42. Habibagahi A, Alderman N, Kubwabo C. A review of the analysis of biomarkers of exposure to tobacco and vaping products. Anal Methods. 2020;12:4276-302. https://doi.org/10.1039/D0AY01467B.

43. Singer AC, Thompson JR, Filho CRM, Street R, Li X, Castiglioni S, Thomas KV. A world of wastewater-based epidemiology. Nat Water. 2023;1:408-15. https://doi.org/10.1038/s44221-023-00083-8.

44. O'Keeffe J. Wastewater-based epidemiology: current uses and future opportunities as a public health surveillance tool. Environ Health Rev. 2021;64:44-52. https://doi.org/10.5864/d2021-015.

Appendix

1 List of Figures

2 List of Figures S

3 List of Tables

4 List of Tables S

5 List of Abbreviations and Symbols

%	Percentage
<	Less than
=	Equal to
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
µ	Micro (10^{-6})
° C	Degree Celsius
1AND7	1-aminonaphthalene-d_7
1B4IB	1-bromo-4-iodobenzene

1C2IB	1-chloro-2-iodobenzene
^{1}D	First dimension
1O	1-octanol
2,3DMA	2,3-dimethylaniline
2,4,6TMA	2,4,6-trimethylaniline
2,4DMA	2,4-dimethylaniline
2,4DNA	2,4-dinitroaniline
2,6DC4NA	2,6-dichloro-4-nitroaniline
2,6DCA	2,6-dichloroaniline
2,6DMA	2,6-dimethylaniline
245TCIB	2,4,5-trichloroiodobenzene
24DCIB	2,4-dichloroiodobenzene
24DFIB	2,4-difluoroiodobenzene
2AAP	2-aminoacetophenone
2BA	2-bromoaniline
2CA	2-chloroaniline
^{2}D	Second dimension
2I13DMB	2-iodo-1,3-dimethylbenzene
2MA	2-methylaniline
2NA	2-naphthylamine
2O	2-octanone
3,4DCA	3,4-dichloroaniline
3C2,6DMA	3-chloro-2,6-dimethylaniline
3C4FA	3-chloro-4-fluoroaniline
3C4FIB	3-chloro-4-fluoroiodobenzene
3C4MA	3-chloro-4-methoxyaniline
3CA	3-chloroaniline
3NA	3-nitroaniline
4ABP	4-aminobiphenyl
4ABPD9	4-aminobiphenyl-d_9
4BA	4-bromoaniline
4C2MA	4-chloro-2-methylaniline

4CA	4-chloroaniline
4EA	4-ethylaniline
4IBPD9	4-iodobiphenyl-d9
4IMB	4-iodotoluene
4MA	4-methylaniline
4NA	4-nitroaniline
A	Aniline
a	Intercept
AA	Aromatic amines
Abbrev.	Abbreviation
ACS	American chemical society
AD5	Aniline-d_5
AE	After extraction
AG	Public limited company - Aktiengesellschaft
amu	Atomic mass unit
AOC	Automatic operation controller
B	Benzene
b	Slope
c	Centi (10^{-2})
CA	California
CAS	Chemical abstracts service
C.C.	Calibration curve
CE	Collision energy
CH	Switzerland
C_i	Average concentration
Cl	Chloride
Cmpd	Compound
Co. KG	Limited partnership - Compagnie Kommanditgesellschaft
Cr	Creatinine
CV	Coefficient of variation
df	Degrees of freedom
DAE	Diamylether

DDA	Dodecyl acetate
DE	Germany
DEE	Diethyl ether
Der.	Derivatization
df	Film thickness
DHE	Dihexyl ether
DI	Direct immersion
DIN	German institute for standardization - Deutsches Institut für Normung
DMA	Dimethylaniline
DNA	Deoxyribonucleic acid
DU	Undecane
DVB	Divinylbenzene
e.g.	Exempli gratia, or for example
EB	Ethylbenzene
EI	Electron ionization
F	Female
g	Gram
Ga	Gauge
GC	Gas chromatography
GmBH	Company with limited liability - Gesellschaft mit beschränkter Haftung
H	n-heptane
HCl	Hydrochloric acid
HF	Hollow fiber
HFn-LPME	n-phase hollow fiber–liquid-phase microextraction
Hi	High
HI	Hydriodic acid
HPLC	High-performance liquid chromatography
HS	Headspace
i.e.	Id est, or that is
IARC	International Agency for Research on Cancer
IB	Iodobenzene
ID	Internal diameter

IPFB	Iodopentafluorobenzene
IS	Internal standard
JUC-Z2	Two-dimensional porous organic framework
k	Kilo (10^3)
KGaA	Partnership limited by shares - Kommanditgesellschaft auf Aktien
L	Liter or Low
LC	Liquid chromatography
LI	Liquid injection
LLC	Limited liability company
LLE	Liquid-liquid extraction
LMCS	Longitudinally modulated cryogenic system
LOD	Limits of detection
log	Logarithm
LOQ	Limits of quantification
LPME	Liquid-phase microextraction
M	Male
m	Meter or Mili (10^{-3})
m/z	Mass to charge ratio
Me	Medium
MeOH	Methanol
M-H	Medium-high
MHE	Multi-headspace extraction
min	Minute
min.	Minimum
M-L	Medium-low
MRM	Multiple reaction monitoring
MS	Mass spectrometry
n	Nano (10^{-9}) or Number of repetitions
n.a.	Not available
n.d.	Not detected
n.r.	Not reported
N_2	Nitrogen

NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NCI	Negative chemical ionization
Nr	Number
NS	Never smoker
O	Octane
ºC	Degrees Celsius
OD	Outer diameter
pp	Pico (10^{-12}) or - $\log_{10}$
P	Octanol-water partition coefficient
Pa	Pascal
PA	Pennsylvania
PALME	Parallel artificial liquid membrane extraction
PDMS	Polydymethylsiloxane
PEG/CNTs	Poly(ethylene glycol) modified with multi-walled carbon nanotubes
PFPA	Pentafluoropropionic anhydride
PFPI	Pentafluoropropionyl-imidazol
pKa	Acid dissociation constant
PP	Polypropylene
PS	Past smoker
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
Pyr	Pyridine
QP	Quadrupole
R^2	Coefficient of determination
Ref.	Reference
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSD	Relative standard deviation
RTn	Reaction time number n
s	Second
S	Smoker

S.I.	Similarity Index
S/N	Signal to noise ratio
SD	Standard deviation
SI	Supplementary information
SIM	Single-ion monitoring
SLE	Supported liquid extraction
SLM	Supported liquid membrane
SMM	Single magnet mixer
SPE	Solid phase extraction
spect.	Spectra
SPME	Solid phase micro-extraction
std.	Standard
T	Toluene
t_{calc}	Calculated t value
t_{crit}	Critical t value
TMA-HCl	Trimethylamine hydrochloride
t_r	Retention time
U	Unknown smoking status
UFLC	Ultra-fast liquid-chromatography.
UK	United Kingdom
USA	United States of America
V	Vial or Volt
VF	Volumetric flask
w/v	Weight/volume
X	o-xylen
α	Alpha
puriss.	purissimum
p.a.	pro analysis

6 List of Publications

6.1 Publications in peer-reviewed journals as first author

Lorenzo-Parodi N, Moebus S, Schmidt TC. Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography-mass spectrometry (GCxGC-MS). Int. J. Hyg. Environ. Health. 2024;257:114343 https://doi.org/10.1016/j.ijheh.2024.114343

Lorenzo-Parodi N, Kaziur-Cegla W, Schmidt TC. Automation and optimization of the sample preparation of aromatic amines for their analysis with GC–MS. Green Anal Chem. 2023;6:100071. https://doi.org//10.1016/j.greeac.2023.100071.

Lorenzo-Parodi N, Leitner E, Schmidt TC. Comparison of gas chromatographic techniques for the analysis of iodinated derivatives of aromatic amines. Anal Bioanal Chem. 2023;415:3313-25. https://doi.org/10.1007/s00216-023-04713-8.

Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

Lorenzo-Parodi N, Kaziur W, Stojanović N, Jochmann MA, Schmidt TC. Solventless microextraction techniques for water analysis. TrAC, Trends Anal Chem. 2019;113:321-31. https:/doi.org/10.1016/j.trac.2018.11.013.

6.2 Publications in peer-reviewed journals as co-author

Lutz F, Lorenzo-Parodi N, Schmidt TC, Niemeyer J. Heteroternary cucurbit[8]uril complexes as supramolecular scaffolds for self-assembled bifunctional photoredoxcatalysts. Chem Commun. 2021;57:2887-90. https://doi.org/10.1039/D0CC08025J.

Dobaradaran S, Schmidt TC, Lorenzo-Parodi N, Kaziur-Cegla W, Jochmann MA, Nabipour I, Lutze HV, Telgheder U. Polycyclic aromatic hydrocarbons (PAHs) leachates from cigarette butts into water. Environ Pollut. 2020;259:113916. https://doi.org/10.1016/j.envpol.2020.113916.

Kalus M-R, Lanyumba R, Lorenzo-Parodi N, Jochmann MA, Kerpen K, Hagemann U, Schmidt TC, Barcikowski S, Gökce B. Determining the role of redox-active materials during laser-induced water decomposition. PCCP. 2019;21:18636-51. https://doi.org/10.1039/C9CP02663K.

Dobaradaran S, Schmidt TC, Lorenzo-Parodi N, Jochmann MA, Nabipour I, Raeisi A, Stojanović N, Mahmoodi M. Cigarette butts: An overlooked source of PAHs in the environment? Environ Pollut. 2019;249:932-9. https://doi.org/10.1016/j.envpol.2019.03.097.

6.3 Oral Presentations

Lorenzo-Parodi N, Schmidt TC, Aromatic amines in human urine: sample preparation using hollow fiber-liquid phase microextraction (HF-LPME).

- 19th International Symposium on Advances in Extraction Technologies (ExTech 2017), Santiago de Compostela, Spain, 27-30/06-2017

Lorenzo-Parodi N, Kaziur W, Gjelstad A, Schmidt TC, Aromatic amines in human urine: automation and optimization of the sample preparation.

- 29. Doktorandenseminar Hohenroda, Hohenroda, Germany, 06-09/01/2019
- Anakon 2019, Münster, Germany, 25-28/03/2019

6.4 Poster Presentations

Lorenzo-Parodi N, Schmidt TC, Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography mass spectrometry (GCxGC-qMS).

- Analytica Conference, München, Germany, 10-13/05/2016
- 40th International Symposium on Capillary Chromatography (ISCC) and 13th GCxGC Symposium, Riva del Garda, Italy, 01-03/06/2016

Lorenzo-Parodi N, Kaziur W, Gjelstad A, Schmidt TC, Aromatic amines in human urine: Sample preparation using Liquid Phase-Micro Extraction (LPME).

- 19th International Symposium on Advances in Extraction Technologies (ExTech2017), Santiago de Compostela, Spain, 27-30/06/2017.

- XIX Euroanalysis 2017, Stockholm, Sweden, 28/08-01/09/2017
- 42nd International Symposium on Capillary Chromatography (ISCC) and 15th GCxGC Symposium, Riva del Garda, Italy, 13-18/05/2018
- Analytica conference, München, Germany, 10-12/04/2018

7 Declaration of author contributions

This thesis includes work that was published in cooperation with co-authors. My own contributions are declared as follows:

Lorenzo-Parodi N, Kaziur W, Stojanović N, Jochmann MA, Schmidt TC. Solventless microextraction techniques for water analysis. TrAC, Trends Anal Chem. 2019;113:321-31. https:/doi.org/10.1016/j.trac.2018.11.013.

Nerea Lorenzo-Parodi: conceptualization, investigation, writing original draft, visualization. Wiebke Kaziur-Cegla: conceptualization, investigation, writing original draft, visualization. Nenad Stojanović: conceptualization, investigation, writing original draft, visualization. Maik A. Jochmann: writing – review and editing. Torsten C. Schmidt: writing – review and editing, supervision.

Lorenzo-Parodi N, Kaziur-Cegla W, Gjelstad A, Schmidt TC. Liquid-phase microextraction of aromatic amines: hollow fiber–liquid-phase microextraction and parallel artificial liquid membrane extraction comparison. Anal Bioanal Chem. 2023;415:1765-76. https://doi.org/10.1007/s00216-023-04579-w.

Nerea Lorenzo-Parodi: conceptualization, methodology, validation, investigation, writing original draft, visualization. Wiebke Kaziur-Cegla: investigation, writing – review and editing and visualization. Astrid Gjelstad: writing – review and editing. Torsten C. Schmidt: writing—review and editing, supervision.

Lorenzo-Parodi N, Leitner E, Schmidt TC. Comparison of gas chromatographic techniques for the analysis of iodinated derivatives of aromatic amines. Anal Bioanal Chem. 2023;415:3313-25. https://doi.org/10.1007/s00216-023-04713-8.

Nerea Lorenzo-Parodi: conceptualization, methodology, validation, investigation, writing the original draft, visualization. Erich Leitner: resources, writing – review and editing. Torsten C. Schmidt: writing – review and editing, supervision.

Lorenzo-Parodi N, Kaziur-Cegla W, Schmidt TC. Automation and optimization of the sample preparation of aromatic amines for their analysis with GC–MS. Green Anal Chem. 2023;6:100071. https://doi.org//10.1016/j.greeac.2023.100071.

Nerea Lorenzo-Parodi: conceptualization, methodology, validation, investigation, writing – original draft. Wiebke Kaziur-Cegla: investigation, writing – review and editing. Torsten C. Schmidt: writing – review and editing, supervision.

Lorenzo-Parodi N, Moebus S, Schmidt TC. Analysis of aromatic amines in human urine using comprehensive multi-dimensional gas chromatography-mass spectrometry (GCxGC-MS). Int. J. Hyg. Environ. Health. 2024;257:114343. https://doi.org/10.1016/j.ijheh.2024.114343

Nerea Lorenzo-Parodi: conceptualization, methodology, validation, investigation, writing original draft, visualization. Susanne Moebus: writing – review and editing. Torsten C. Schmidt: writing – review and editing, supervision.

8 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

The Curriculum Vitae is not included in the online version due to data protection reasons.

This page is left intentionally blank.

9 Erklärung

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

‚Aromatic amines in human urine: optimization and automation of the analytical method for their analysis as iodinated derivatives‘

selbst verfasst, keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet sind und die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.