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PhD thesis

Development and application of targeted and untargeted analytical assays for the human biomonitoring of volatile organic compounds and endocrine disrupting chemicals

SSD: MED/44 - Occupational Medicine

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Abstract

Biomonitoring is a useful approach to assess the exposure to pollutants in human subjects. The aim of this project was the development of new analytical methods for the biomonitoring of exposure to volatile and persistent organic pollutants, their application to selected groups of subjects, and a comparison between different approaches (untargeted vs targeted approaches).

A high-throughput isotope dilution tandem mass spectrometric method coupled with reversed-phase liquid chromatography (HPLC-MS/MS) was developed for the analysis of a total of 17 urinary mercapturic acids, as metabolites of several volatile organic compounds. A complete validation was carried out including precision, accuracy, linearity, sensibility, process efficiency, and external verification.

This method was applied to a group of 49 coke-oven workers and 49 individuals living in the same area. Active tobacco smoking was an exclusion criterion for both groups. Urinary levels of the metabolites of benzene, styrene, acrylonitrile, and 1,3-butadiene were 2–10 fold higher in workers than in controls.

The method was also applied to a group of subjects with different smoking habits, in particular: 38 non-smokers (NS), 7 electronic cigarette users (ECU), and 22 traditional tobacco smokers (TTS). Most of the measured mercapturic acids were 2 - 165 fold-higher in TTS compared to NS. The metabolites of acrylonitrile and acrolein were 1.8 and 4.9 fold-higher higher in ECU than NS, respectively. Furthermore, comparing smokers to non-smoking coke oven workers, the first were exposed to a greater amount of volatile organic compounds.

An untargeted metabolomic approach was applied to the same population of subjects with different smoking habits. Samples were analysed by liquid chromatography/time-of flight mass spectrometry. Among putatively annotated compounds there were the glucuronide conjugated of 3-hydroxycotinine and the sulfate conjugate of methoxyphenol. Considering mercapturic acids, the coherence

between the targeted and untargeted approach was found for a limited number of chemicals, typically the most abundant.

Finally, an HPLC-MS/MS method for the determination of some endocrine-disrupting persistent chemicals was developed. Targeted molecules were bisphenol A and metabolites of phthalates, including emergent terephthalates. A complete validation was carried out and the method was applied to 36 non-occupationally exposed adults.

Summary

Preface

Since birth (and also before birth, during the prenatal stage) we are continuously exposed to a mixture of physical, biological, and chemical agents. Among the latter, there are both natural and man-made compounds. Inhalation, skin contact, and ingestion are the main routes of exposure. Even though most of the chemicals we encounter are fundamental for our lives (as oxygen and nutrients), other can be dangerous and our organism would benefit if it were not exposed to such compounds. While there are several compounds (both natural and artificial) which can be toxic for us, the effectiveness of those chemicals to cause toxic effects is mediated by several factors and one of the most important is the quantity.

"All things are poison, and nothing is without poison; the dosage alone makes it so a thing is not a poison." - Paracelsus (1493-1541).

Indeed, even oxygen and some nutrients can be dangerous if taken in extremely large quantities in a short amount of time. Likewise, toxic compounds may not exert their adverse consequences if taken in low amounts. The impact is different for each compound and it also depends on the ability of our organism to metabolise and excrete harmful substances. For these reasons, the ability to measure to which compounds and in what quantities we are exposed in certain situations (e.g. occupationally) is of considerable importance. Indeed, a nowadays challenge is the study and the characterisation of the "exposome", defined as *"the measure of all the exposures of an individual in a lifetime and how those exposures relate to health"*. Specific analytical techniques are needed to perform these measurements and, during the last decades, impressive technological advancements, as in chromatography and in mass spectrometry techniques, have allowed to measure several chemicals and even in extremely small quantities.

The measurement of the exposure to pollutants can be performed through environmental monitoring (direct measurement of the toxic compounds) or through

biological monitoring (also referred as "biomonitoring"). Biomonitoring consists in the analysis of the toxic compounds, or their specific metabolites, in the subjects' accessible biological materials, such as biofluids (urine or blood). Even though it is an "indirect" measurement, its main advantage is the potential to assess the body burden of a given toxicant including all sources and exposure routes.

Biomonitoring has been widely used to assess the exposure to exogenous compounds: several analytical methods have been developed and they have been used to monitor the exposure to a specific chemical, or groups of chemicals, in different environmental and occupational conditions. However, biomonitoring is a field in continuous development for different reasons: first, thanks the constant improvements in analytical instrumentations, chemicals of interest can be determined in lower concentrations, thus allowing to quantify toxicant (or metabolite of toxicant) present in low concentrations in human biofluid; second, as specific dangerous man-made compounds are restricted by law, new alternatives are conceived and biomonitoring methods should be updated to be able to monitor even these emergent compounds; third, biomonitoring methods, in order to be sensitive and specific, are often limited to a restricted number of compounds, while humans are exposed to mixtures of compounds; forth, biological samples are complex matrices which should be removed through a suitable sample preparation in order to avoid signal suppressions and other matrix effects, while, at the same time, analytical assays tend to be as high-throughput as possible, in order to be able to process multiple samples per time; fifth, biomonitoring is usually performed analysing known compounds, while being able to work without an "a priori" knowledge would allow to find new compounds, i.e. new biomarkers, related to a specific condition or exposure.

All these considered, this thesis, and the work behind it, is intended to move a little step forward the field of biomonitoring, trying to address the lacks highlighted above.

Aims the thesis

The main objective of this thesis was the development and application of efficient and comprehensive analytical methods to biomonitor the occupational and environmental exposure to volatile and persistent organic pollutants thanking into account the needs for sensitive methods, in order to quantify those specific metabolites present at very low concentrations; the care in determining both legacy and emergent compounds; determining broad ranges of compounds; being as high-throughput as possible while still properly control for the matrix effect; and trying to implement novel non-targeted approaches to screen differences in exposure in potentially previous unknown compounds: in particular, an untargeted method was developed, and its strengths and limitations were highlighted in comparison to traditional targeted approaches. Another aim was to apply the developed method to selected group of subjects to evaluate the exposure to specific environmental and working conditions.

Five specific aims are addressed in each chapter of the thesis.

The first specific aim was the development of a liquid chromatography method coupled with tandem mass spectrometry (LC-MS/MS) for the sensitive and rapid determination of several mercapturic acids in urine samples. Mercapturic acids are specific biomarkers of volatile organic compounds, usually present at low concentrations in urine. The mercapturic acids included in the study were the metabolites of benzene, toluene, 1,3-butadiene, styrene, acrylonitrile, 4-chloronitrobenzene, acrylamide, acrolein, propylene oxide, N,N-dimethylformamide, crotonaldehyde, ethylene oxide, and methylating and ethylating agents. The developed method underwent a complete validation, including linearity, sensitivity, accuracy, precision, selectivity, stability, and process efficiency, along with an external verification (first chapter).

The second specific aim was to carry out a first biomonitoring study, using the method for analysing mercapturic acids in 49 coke-oven workers and 49 individuals

living in the same area were enrolled, while active tobacco smoking was an exclusion criterion for both groups (second chapter).

The third specific objective was to conduct a second biomonitoring study quantifying the considered mercapturic acids in sixty-seven healthy adult subjects with different smoking habits, among which non-smokers, electronic cigarette smokers, and traditional tobacco smokers (third chapter).

The fourth specific aim was to develop an untargeted metabolomic workflow, using the same urine samples from smoking and non-smoking subjects. A high-resolution time-of-flight mass spectrometer (QToF MS) interfaced with liquid chromatography was used. This instrument is able to detect ions with a high mass accuracy and mass resolution, therefore allowing identification of molecular formulae of compounds whose signals were significantly different between sample groups. A complete workflow, including sample preparation, HPLC-MS/MS analyses, statistical elaboration, and metabolite annotation was conducted (fourth chapter).

The fifth specific objective was the set-up of an analytical assay to determine bisphenol A and phthalate metabolites. Phthalates and bisphenol A are endocrine disrupter chemicals and the usage of some of them have been restricted. For this reason, novel compounds are expected to be used as alternatives. An analytical method was developed and validated for the simultaneous quantitation of urinary metabolites of bisphenol A and phthalates, including both recognised EDCs and an alternative plasticiser, which exposure may be on the raise: investigated analytes were bisphenol A (BPA), monobenzyl phthalate (MBzP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP), mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP), monoethyl phthalate (MEP), and mono-n/i-butyl phthalates (MnBP/MiBP) (fifth chapter).

Note

The results presented in each chapter are already published in international peer-reviewed journal articles. The content of each chapter is the same of the related paper with some additional results or extended discussion in some cases: i.e. while some additional content was cut during the revision of the manuscripts, it was included in this thesis.

The work was conducted in the Laboratory of Environmental and Industrial Toxicology, Department of Clinical Sciences and Community Health, University of Milan and Clinica del Lavoro “Luigi Devoto”, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico. The responsible of the laboratory is Professor Silvia Fustinoni.

The untargeted metabolomic analyses using the Qtof MS were conducted at the novel technological platform called “OMICS” of the University of Milan.

The training for the untargeted metabolomic approaches, along with part of the elaboration of the untargeted metabolomic data, were conducted at the Phenome Centre Birmingham and Birmingham Metabolomics Training Centre, School of Biosciences, University of Birmingham, which responsible is Professor Warwick Dunn.

1 Biomonitoring of volatile organic compounds: development of a method to quantify mercapturic acids in urine samples

Most of the content of this chapter has been published in the *Journal of Chromatography B.*

Frigerio G., Mercadante R., Polledri E., Missineo P., Campo L., Fustinoni S. (2019). An LC-MS/MS method to profile urinary mercapturic acids, metabolites of electrophilic intermediates of occupational and environmental toxicants. *J Chromatogr B Analyt Technol Biomed Life Sci* 1117:66-76. doi: 10.1016/j.jchromb.2019.04.015.

1.1 Introduction

Long-term exposure to some occupational and environmental toxicants, such as benzene, toluene, styrene, 1,3-butadiene, and acrylamide, has adverse health effects, including cancer development (Bahadar et al., 2014; Galbraith et al., 2010; Filley et al., 2004; Sielken and Valdez-Flores, 2015; Bein and Leikauf, 2011; Semla et al., 2017; IARC, 2012a; IARC, 2012d). Environmental exposure to some of these compounds is ubiquitous, although higher levels of exposure have been found in certain workplaces (IARC, 2012a). Tobacco smoke is a relevant non-occupational exposure source (Hecht, 2002; IARC, 2012d).

Exposure to toxic compounds can be assessed through environmental or biological monitoring. The latter can be conducted by using different specimens, including noninvasively collected urine samples, to determine metabolites of compounds of interest (Jakubowski, 2012).

After absorption and distribution, several toxic substances may undergo initial biotransformation (phase I metabolism), occurring mainly in the liver, to oxygenated and active electrophilic intermediates, due mostly to the oxidative action of cytochrome P450 enzymes. These intermediates are believed to be active species capable of reacting with DNA and responsible for the genotoxicity associated with parent compounds. Regardless of whether an electrophilic molecule has undergone such initial modification, it may be subjected to the "mercapturic acid pathway" (phase II metabolism; Figure 1.1). During this biotransformation, catalysed by the glutathione S-transferase enzyme, the electrophilic compound is deactivated by conjugation with glutathione, an endogenous tripeptide formed by glutamic acid, cysteine, and glycine. Through other enzymatic reactions, glutamic acid and, subsequently, glycine are removed. The remaining cysteine conjugate is N-acetylated and finally excreted in urine as a mercapturic acid (De Rooij et al., 1998; Parkinson and Ogilvie, 2010).

Urinary mercapturic acids may be useful for the assessment of exposure to occupational and environmental toxicants (Perbellini et al., 2002; De Rooij et al., 1998). Various methods have been developed for the analysis of this class of compounds (Mathias and B'hymer, 2016). Most of them permit consideration of only a small number of mercapturic acids. To our knowledge, only two papers contain descriptions of methods covering a wide range of analytes: Alwis and co-workers (Alwis et al., 2012) described a method suitable for the quantification of 28 metabolites related to 19 volatile organic compounds, and Pluym and coworkers (Pluym et al., 2015) used two methods to quantify 18 mercapturic acids. However, high-throughput methods covering large numbers of mercapturic acids with single chromatographic runs, with suitable sensitivity for application in occupationally exposed and unexposed populations, are needed. Our goal in this study was to develop and validate a simple and sensitive analytical method for the detection of several mercapturic acids in human urine in a single chromatographic run, to be used for the profiling of human exposure to several toxicants.

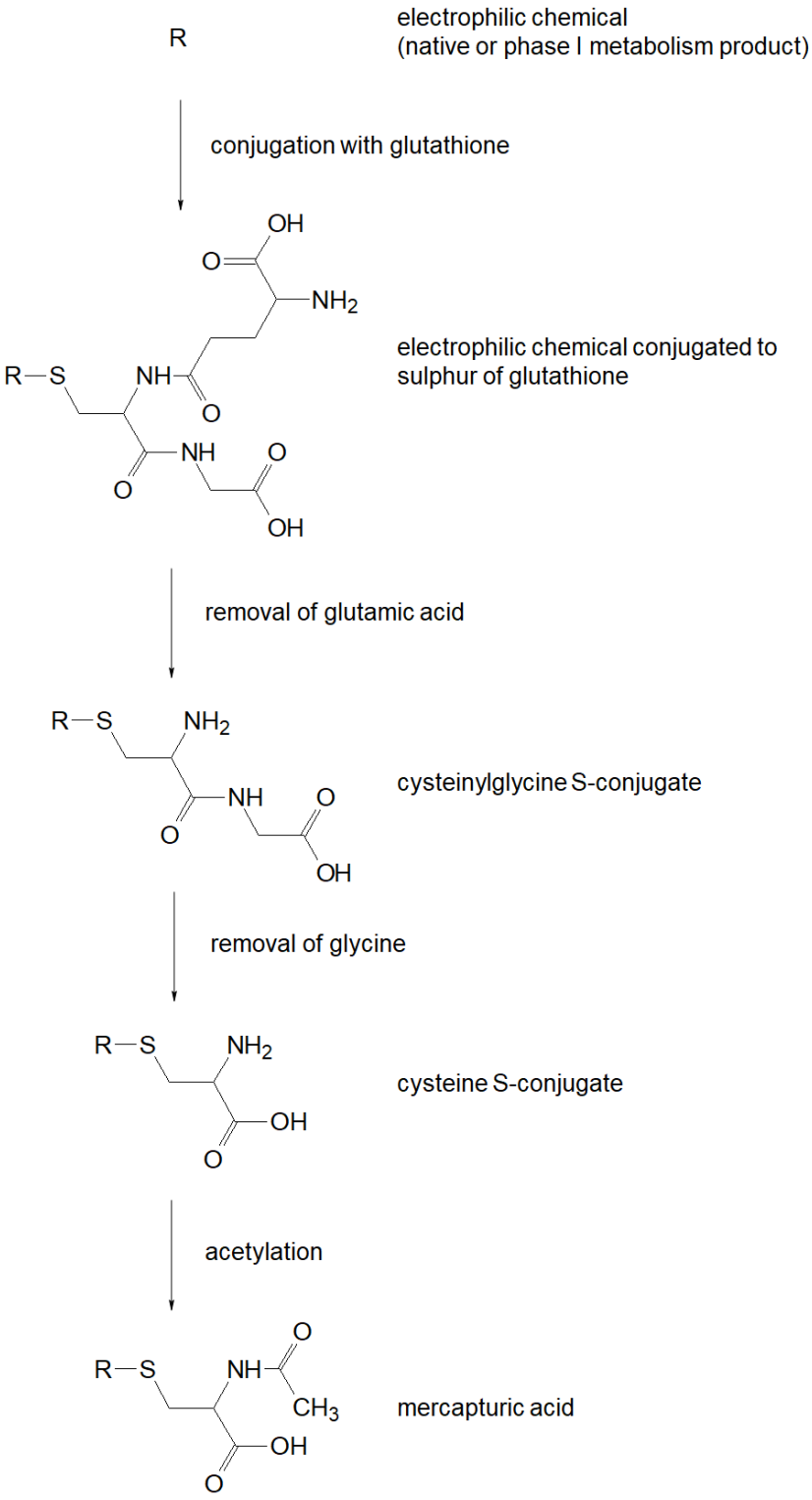


Figure 1.1 - Mercapturic acid pathway

1.2 Materials and methods

1.2.1 Chemicals

For the preparation of standard solutions, N-acetyl-S-(2-hydroxypropyl)cysteine dicyclohexylammonium salt (2-HPMA), N-acetyl-S-(2-hydroxypropyl)cysteine-D_3 dicyclohexylammonium salt (2-HPMA-D_3; isotopic purity, 95.7%), N-acetyl-S-(3-hydroxypropyl)cysteine dicyclohexylammonium salt (3-HPMA), N-acetyl-D_3-S-(3-hydroxypropyl)cysteine dicyclohexylammonium salt (3-HPMA-D_3; isotopic purity, 98.5%), N-acetyl-S-(carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(carbamoylethyl)-L-cysteine-D_3 (AAMA-D_3; isotopic purity, 98.7%), N-acetyl-S-(N-methylcarbamoyl)-L-cysteine (AMCC), N-acetyl-D_3-S-(N-methylcarbamoyl)-L-cysteine (AMCC-D_3; isotopic purity, 98.7%), N-Acetyl-S-(2-cyanoethyl)-L-cysteine ammonium salt (CEMA), N-Acetyl-S-(2-cyanoethyl)-L-cysteine-D_3 ammonium salt (CEMA-D_3; isotopic purity, 98.9%), N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine disodium salt (mixture of diastereomers; CMEMA), N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine-D_3 disodium salt (CMEMA-D_3; isotopic purity, 97.3%), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (mixture of diastereomers; DHBMA), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine-D_7 (mixture of diastereomers; DHBMA-D_7; isotopic purity not reported), N-acetyl-S-ethyl-L-cysteine (EMA), N-acetyl-S-ethyl-L-cysteine-D_5 (EMA-D_5; isotopic purity, 99.6%), N-acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine dicyclohexylammonium salt (GAMA), N-acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine-D_3 dicyclohexylammonium salt (GAMA-D_3; isotopic purity, 99.0%), N-acetyl-S-(2-hydroxyethyl)-L-cysteine dicyclohexylammonium salt (HEMA), N-acetyl-S-(2-hydroxyethyl-D_4)-L-cysteine dicyclohexylamine salt (HEMA-D_4; isotopic purity not reported), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine dicyclohexylammonium salt (mixture of diastereomers; HMPMA), N-acetyl-D_3-S-(3-hydroxypropyl-1-methyl)-L-cysteine dicyclohexylammonium salt (HMPMA-D_3; isotopic purity, 98.7%), (R,S)-N-acetyl-S-[1-(hydroxymethyl)-2-propen-1-yl]-L-cysteine + (R,S)-N-acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine

(approximately 1:1 mixture; MHBMA), (R,S)-N-acetyl-S-[1-(hydroxymethyl)-2-propenyl]-L-cysteine-D_6 + (R,S)-N-acetyl-S-[2-(hydroxymethyl)-3-propenyl)-L-cysteine-D_6 (mixture; MHBMA-D_6; isotopic purity not reported), N-acetyl-S-methyl-L-cysteine (MMA), N-acetyl-S-methyl-L-cysteine-D_3 (MMA-D_3; isotopic purity, 99.9%), S-(4-nitrophenyl)mercapturic acid (NANPC), N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine (mixture; PHEMA), N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine-D_3 + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine-D_3 (mixture; PHEMA-D_3; isotopic purity, 98.7%), N-acetyl-S-benzyl-L-cysteine (SBMA), and N-(acetyl-D_3)-S-benzyl-L-cysteine (SBMA-D_3; isotopic purity, 97.8%) were purchased from Toronto Research Chemicals (Ontario, Canada). N-acetyl-S-phenyl-L-cysteine (SPMA) and N-acetyl-S-phenyl-L-cysteine-D_2 (SPMA-D_2; isotopic purity, 99%) were purchased form Tokyo Chemical Industry (Tokyo, Japan) and CDN Isotope (Pointe-Claire, Quebec, Canada), respectively. The molecular structure of the radical moiety R (Figure 1.1) typical of each mercapturic acid, and the Chemical Abstracts Service registry number of the free carboxylic acid, when available, are shown in Table 1.1.

Analytical grade acetonitrile, formic acid, aqueous ammonia (30%), and methanol were purchased from Sigma-Aldrich (Milan, Italy). Purified water was obtained using a Milli-Q Plus ultrapure water system (Millipore, Milford, MA, USA). Synthetic urine (Surine^{TM}) was purchased from Sigma-Aldrich and used as a negative control.

1.2.2 Standard solution preparation

Each analytical standard (native or isotopically labeled) was weighed and dissolved in methanol to obtain a stock solution at the concentration of 1.0 mg/mL (0.5 mg/mL for CMEMA and CMEMA-D_3, 0.1 mg/mL for SBMA). Stock solutions of isotopically labeled standards were diluted in water to obtain a working solution of

internal standards (concentrations of 0.25 mg/L for 2-HPMA-D_3, EMA-D_5, PHEMA-D_3, SBMA-D_3, and SPMA-D_2; 2.50 mg/L for 3-HPMA-D_3, CEMA-D_3, CMEMA-D_3, DHBMA-D_7, GAMA-D_3, HEMA-D_4, MHBMA-D_6, and MMA-D_3; and 25 mg/L for AAMA-D_3, AMCC-D_3, and HMPMA-D_3). Stock solutions of native standards were diluted in synthetic urine to obtain a working solution of native standards (free acid concentrations of 0.04 mg/L for EMA, MHBMA, MMA, NANPC, PHEMA, SBMA, and SPMA; 1.6 mg/L for 2-HPMA, 3-HPMA, AAMA, CEMA, DHBMA, GAMA, and HEMA; and 3.2 mg/L for AMCC, CMEMA, and HMPMA). This solution was further diluted in synthetic urine to obtain calibration curve and quality control (QC) solutions.

Ten solutions at different concentrations were prepared for the calibration curve, along with an unadulterated (blank) sample of synthetic urine. To cover the levels expected in real samples, analyte concentrations in these solutions ranged from 0.01 to 40 µg/L for EMA, MHBMA, MMA, NANPC, PHEMA, SBMA, and SPMA; from 0.39 to 1600 µg/L for 2-HPMA, 3-HPMA, AAMA, CEMA, DHBMA, GAMA, and HEMA; and from 0.78 to 3200 µg/L for AMCC, CMEMA, and HMPMA. Independent concentrations for QC were 0.08, 0.63, and 5.00 µg/L for EMA, MHBMA, PHEMA, SBMA, and SPMA; 3.1, 25, and 200 µg/L for 2-HPMA, 3-HPMA, CEMA, DHBMA, GAMA, and HEMA; 6.3, 50, and 400 µg/L for AMCC, CMEMA, and HMPMA; 0.63 and 5.00 µg/L for MMA and NANPC; and 25 and 200 µg/L for AAMA.

Stock and working solutions of native and internal standards were stored at –20°C in the dark. Under these conditions, the solutions remained stable for at least 6 months, as determined by LC-MS/MS comparison to freshly prepared solutions.

1.2.3 Sample preparation

Formic acid (0.2 M; 500 µL) and the working solution of internal standards (20 µL) were added to a 500-µL urine sample. This solution was vortexed, filtered with a 0.45-µm regenerated cellulose membrane filter (Agilent Technologies, Cernusco

Sul Naviglio, Italy), collected in an autosampler vial, and submitted to analysis. Calibration curve and QC solutions were prepared using the same process.

1.2.4 LC-MS/MS analysis

A hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500; AB Sciex, Monza, Italy) with an electrospray ionization source interfaced with a high-pressure liquid chromatograph (Agilent 1260; Agilent Technologies) equipped with a Betasil C18 column (150 ×, 2.1 mm, 5 µm ; Thermo Fisher Scientific, Rodano, Italy) and a Betasil C18 pre-column (10 × 2.1 mm, 5 µm; Thermo Fisher Scientific, Rodano, Italy) was used to separate the metabolites of interest. The autosampler temperature was set at 8°C and the injection volume was set at 20 µL. Chromatographic separation was performed using a linear gradient with two mobile phases; the A phase was an aqueous solution of ammonium formate (5 mM) with 0.1% formic acid, and the B phase was acetonitrile. The gradient was programmed as follows: 0–0.5 min, 0.5% B isocratic; 0.5–2 min, from 0.5% to 30% B; 2–7 min, 30% B isocratic; 7–8 min, from 30% to 100% B; 8–12 min, 100% B isocratic; 12–12.1 min, from 100% to 0.5% B; and 12.1–18 min, 0.5% B isocratic. The flow rate was set at 200 µL/min and the column was kept at 40°C.

The mass spectrometer operated in scheduled multiple reaction monitoring (MRM) mode, with a retention time window of 120 s. The target scan time was set at 1 s. The polarity was set to negative and the following conditions were used: gas 1 (air) pressure, 55 psi; gas 2 (air) pressure, 65 psi; curtain gas (N_2) pressure, 35 psi; heater temperature, 550°C; ion spray voltage, –4500 V; and entrance potential, 10 V. The collision energy and precursor ion/product ion pairs were optimized manually through direct infusion of each analyte. The precursor ion was always the deprotonated molecular ion $(M - H)^-$. The two most intense MRM transitions were recorded for each compound (when available); the most intense transition was used for quantitation and the other transition was used for qualification (Table 1.1). For internal standards, we did not choose the transitions with the most intense signals;

rather, we chose those with suitable signals and less interference in chromatographic separation due to matrix components.

The Analist® software (version 1.6.3; AB Sciex) was used to prepare batches for analysis, and the MultiQuant™ software (version 3.0.8664.0; AB Sciex) was used for data integration.

1.2.5 Method validation

The method was validated according to the FDA guidelines and the considerations reported in the review by González and coworkers (FDA, 2013; González et al., 2014).

1.2.5.1 Calibration curve

Two replicates of each calibration concentration were analyzed on the same day. The ranges of concentrations used for the analytes are reported in Section 2.2. Calibration curves were built by plotting the concentration of each analyte on the *x* axis, and the ratio of the chromatographic peak areas of each analyte and the relevant internal standard the *y* axis. For NANPC, the peak area of the relevant native standard was plotted on the *y* axis because no isotopically labeled compound was available. The calibration curves were built with weighted least-squares linear regression, with a weighting factor of 1/x.

1.2.5.2 Sensitivity

The limit of quantitation (LOQ) was calculated using a linear regression curve focused on the lowest concentrations, according to the following expression:

$$LOQ = (3SE_q + q) / m,$$

where q is the intercept, m is the slope of the linear regression, and SE_q is the standard error of q (Miller and Miller, 2005).

1.2.5.3 Precision and accuracy

Precision and accuracy were determined by analyzing low-, medium-, and high-concentration QC solutions. Intraday precision was evaluated by calculating the

coefficient of variation [relative standard deviation (RSD), %] of peak area ratios of five replicates injected on the same day. Interday precision was determined by calculating coefficients of variation on five different days. Accuracy was calculated as the percent ratio between the concentration calculated using the calibration curve and the theoretical value of the prepared solution.

1.2.5.4 Internal standard interference and carryover effect

Internal standard interference was evaluated by analyzing a blank sample consisting of synthetic urine spiked with the internal standard working solution. For this solution, the absence of interfering peaks, i.e., peaks with the same retention times and MRM transitions of the analytes, was evaluated. A sample of water was analyzed immediately after analysis of the highest point of the calibration curve to test the carryover effect.

1.2.5.5 Process efficiency

The considerations raised in the review by González and coworkers (González et al., 2014) were taken into account when setting up experiments for the evaluation of recovery, the matrix effect, and process efficiency (PE). As an initial experiment revealed that recovery was good (91.0–120.7%) for all analytes considered, we assessed only PE, which reflected the matrix effect. Two sets of samples were prepared: water spiked with standards (W) and urine spiked with standards (U). Three concentrations of each compound were evaluated, along with blank samples. Urine samples were donated by five healthy volunteers. Samples were processed as described in Section 2.3. PE was calculated as follows:

PE (%) = (U / W) * 100.

As most analytes were present in unspiked urine, the obtained concentrations were subtracted from those of the analytes in the blank sample.

1.2.5.6 External verification

In autumn 2018, we took part in the German External Quality Assessment Scheme (G-EQUAS) for analyses in biological materials (G-EQUAS, 2019), which is an

interlaboratory comparison. Two urine samples with unknown concentrations of 2-HPMA, 3-HPMA, AAMA, AMCC, CEMA, DHBMA, GAMA, HEMA, MHBMA, and SPMA in the ranges of occupational levels were delivered to our laboratory from the Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg and analyzed as described above.

1.2.5.7 Stability

Short-term stability was tested using three QC solutions. Signals of analytes in fresh samples were compared with those of samples kept in an autosampler at 8°C for 9 days.

Mid-term stability was evaluated as the variability in the slopes of calibration curves (n = 5) prepared from working solutions of native and internal standards over a period of 4 weeks.

Long-term stability was evaluated using four batches of two urine samples delivered to our laboratory in the context of the external verification exercise (see Section 2.5.6) every 6 months for a period of 2 years. The samples were analyzed upon delivery and then stored at –20°C. With each new batch, old samples were re-analyzed together with the new samples.

1.2.6 Analytical sequence

During routine analysis, calibration curve and QC solutions were run along with each set of samples. A typical sequence consisted of the analysis of calibration curve levels, followed by the analysis of unknown samples along with the analysis of low-, medium-, and high-concentration QC solutions and two sample duplicates for every 10 samples, followed by a second analysis of calibration curve levels.

1.2.7 Study subjects

The developed method was applied to the analysis of urinary mercapturic acids in 46 end of shift urine samples form non-smoker workers belonging to seven work

settings with different exposures and available from previous studies. In particular, urine samples were obtained from: seven refinery workers (Fustinoni et al., 2011), six coke oven workers (Campo et al., 2012), seven traffic policemen (Campo et al., 2011), six rotogravure printing workers (Fustinoni et al., 2007), seven gasoline station attendants (Campo et al., 2016b), six asphalt workers (Campo et al., 2006) and seven workers whose job did not include exposure to the investigated toxicants (non-exposed) (Campo et al., 2016b).

1.2.8 Statistical analysis

Statistical analyses were performed using the SPSS package for Windows (version 25; IBM SPSS Statistics). A value corresponding to a half of the LOQ was assigned to each measurement falling below analytical quantitation. The *t*-test was used to evaluate differences in urinary mercapturic acids (data were $\log_{10}$-transformed to ensure normal distribution) between the two groups of workers.

Table 1.1 - List of the acronyms for the analytes investigated, with molecular structures of R, the radical variable for mercapturic acid (Figure 1.1), molecular weights, Chemical Abstracts Service registry numbers, and corresponding parent compounds. For each analyte, the MRM ion transitions used for quantitation (quant) and qualification (qual) are given; moreover, the MRM ion transition of the isotopically labeled internal standard (IS) is given. Finally, the retention time (RT) of each chemical and the collision energy (CE) used to obtain the MRM ion transition are specified.

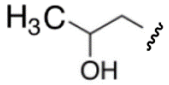
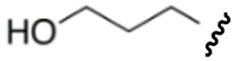
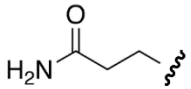
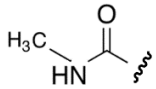
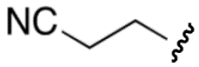
Investigated analytes	Molecular structure of R (see Figure 1.1)	Molecular weight (Dalton)	CAS number	Parent compounds	MRM transitions (precursor ion/product ion) (m/z)		RT (min)	CE (V)
2-HPMA		221	38130-86-8	propylene oxide	Quant.:	220.0/91.0	3.75	-18
					Qual.:	-		
					IS (-D_3 isotope):	223.0/91.2	3.73	-18
3-HPMA		221	NA	acrolein	Quant.:	220.0/91.0	3.45	-18
					Qual.:	220.0/89.0		-26
					IS (-D_3 isotope):	223.0/91.2	3.43	-18
AAMA		234	81690-92-8	acrylamide	Quant.:	233.0/104.0	2.75	-18
					Qual.:	233.0/162.0		-13
					IS (-D_3 isotope):	235.5/104.0	2.75	-18
AMCC		220	103974-29-4	N,N-dimethylformamide	Quant.:	218.9/161.9	3.42	-12
					Qual.:	218.9/84.0		-20
					IS (-D_3 isotope):	221.6/165.0	3.41	-12
CEMA		216	168208-30-8	acrylonitrile	Quant.:	215.0/86.0	4.40	-18
					Qual.:	215.0/162.0		-10
					IS (-D_3 isotope):	217.5/86.0	4.37	-18

Table 1.1 - Continue

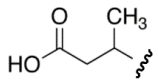
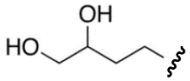
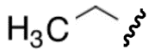
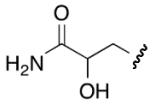
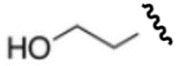
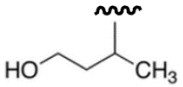
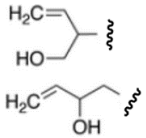
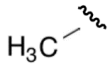
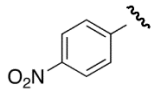
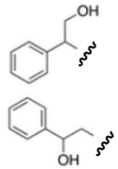
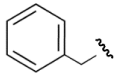
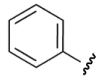
Investigated analytes	Molecular structure of R (see Figure 1.1)	Molecular weight (Dalton)	CAS number	Parent compounds	MRM transitions (precursor ion/product ion) (m/z)		RT (min)	CE (V)
CMEMA		249	1041285-62-4	crotonaldehyde	Quant.:	248.0/161.9	7.15	-16
					Qual.:	248.0/128.0		-14
					IS (-D_3 isotope):	250.8/164.8	7.13	-16
DHBMA		251	144889-50-9	1,3-butadiene	Quant.:	250.0/121.0	2.77	-19
					Qual.:	250.0/75.0		-32
					IS (-D_7 isotope):	257.0/78.0	2.76	-34
EMA		191	31386-36-4	ethylating agents	Quant.:	190.0/61.0	7.65	-23
					Qual.:	190.0/84.0		-14
					IS (-D_5 isotope):	195.0/66.0	7.61	-22
GAMA		250	NA	acrylamide	Quant.:	249.0/120.0	2.75	-18
					Qual.:	249.0/128.0		-15
					IS (-D_3 isotope):	252.0/131.0	2.72	-15
HEMA		207	1331896-18-4	acrylonitrile ethylene oxide	Quant.:	206.0/77.0	2.77	-15
					Qual.:	206.0/75.0		-27
					IS (-D_4 isotope):	210.0/82.2	2.76	-48
HMPMA		235	33164-70-4	crotonaldehyde	Quant.:	234.0/105.0	6.36	-18
					Qual.:	234.0/103.0		-28
					IS (-D_3 isotope):	236.5/105.0	6.32	-18

Table 1.1 - Continue

Investigated analytes	Molecular structure of R (see Figure 1.1)	Molecular weight (Dalton)	CAS number	Parent compounds	MRM transitions (precursor ion/product ion) (m/z)		RT (min)	CE (V)
1-MHBMA 2-MHBMA		233	NA	1,3-butadiene	Quant.: Qual.: IS (-D_6 isotope):	232.0/103.0 232.0/73.0 238.0/109.2	5.09/5.45 5.01/5.27	-16 -37 -16
MMA		177	16637-59-5	methylating agents	Quant.: Qual.: IS (-D_3 isotope):	176.0/84.1 176.0/57.0 178.9/84.1	4.33 4.28	-14 -20 -14
NANPC		284	91088-55-0	4-chloronitrobenze	Quant.: Qual.:	283.0/154.0 283.0/124.0	11.04 	-20 -44
PHEMA1 PHEMA2		283	NA	styrene	Quant.: Qual.: IS (-D_3 isotope):	282.0/153.0 282.0/123.0 284.6/152.8	8.86/8.63 8.80/8.55	-16 -28 -18
SBMA		253	19542-77-9	toluene	Quant.: Qual.: IS (-D_3 isotope):	252.0/123.0 252.0/84.0 255.0/123.0	11.44 11.41	-17 -17 -17
SPMA		239	4775-80-8	benzene	Quant.: Qual.: IS (-D_2 isotope):	238.0/109.0 - 240.0/109.0	10.40 10.37	-18 -16

N.A.: not available

Table 1.2 - Limits of quantification (LOQ), calibration curves (investigated range, mean R^2, and mid-term stability), precision, and accuracy for the analytes investigated.

Investigated analytes	Calibration curve				QC precision and accuracy			
	LOQ (µg/L)	Investigated ranges (µg/L)	R^2 Mean (n=5)	Mid-term stability (%RDS_{slope} n=5)	Spiked Concentrations (µg/L)	Intra-day precision %RSD (Min-Max)	Inter-run precision %RSD	Accuracy %Theoretical (Min-Max)
2-HPMA	0.5	LOQ-1600	0.993	5.0	3.1	6.2 (4.9-7.8)	9.7	98.8 (91.1-110.9)
					25	6.9 (2.8-9.9)	8.0	102.9 (97.7-109.7)
					200	6.1 (2.3-10.4)	7.2	108.0 (102.4-112.2)
3-HPMA	0.2	LOQ-1600	0.992	4.7	3.1	5.8 (3.6-7.5)	6.9	105.3 (96.7-111.5)
					25	4.0 (1.1-6.3)	5.9	107.3 (93.2-115.7)
					200	4.9 (3.5-5.7)	4.7	106.5 (98.5-112.2)
AAMA	3.2	LOQ-1600	0.977	22.7	25	5.6 (3.0-8.9)	19.7	107.1 (99.6-115.4)
					200	5.5 (2.2-7.9)	18.2	108.4 (99.0-115.3)
AMCC	2	LOQ-3200	0.982	6.0	6	4.0 (2.8-6.2)	6.2	101.7 (97.0-107.4)
					50	3.4 (1.6-5.3)	5.4	101.6 (98.4-103.8)
					400	2.8 (2.0-4.1)	4.8	103.2 (98.1-106.2)

Table 1.2 - Continue

Investigated analytes	Calibration curve				QC precision and accuracy			
	LOQ (µg/L)	Investigated ranges (µg/L)	R^2 Mean (n=5)	Mid-term stability (%RDS_{slope} n=5)	Spiked Concentrations (µg/L)	Intra-day precision %RSD (Min-Max)	Inter-run precision %RSD	Accuracy %Theoretical (Min-Max)
CEMA	0.9	LOQ-1600	0.984	21.3	3.1	6.0 (2.2-8.9)	19.6	106.7 (95.2-112.8)
					25	5.6 (1.9-8.6)	20.2	111.6 (107.4-114.3)
					200	5.3 (1.1-9.0)	20.9	112.4 (107.2-118)
CMEMA	2	LOQ-3200	0.995	7.0	6	4.3 (0.8-6.4)	5.1	105.1 (96.5-113.1)
					50	2.2 (0.6-4.1)	3.1	110.2 (101.1-117.5)
					400	2.8 (1.4-4.4)	3.4	110.1 (101.8-116.0)
DHBMA	1.0	LOQ-1600	0.982	3.2	3.1	3.4 (1.7-4.2)	4.3	112.6 (110.6-114.6)
					25	2.7 (0.8-3.6)	3.4	113.6 (111.2-115.9)
					200	3.2 (1.7-5.7)	3.4	114.9 (111.1-117.6)
EMA	0.01	LOQ-40	0.996	2.1	0.08	6.4 (3.3-11.0)	6.9	93.4 (88.8-102.8)
					0.63	4.2 (1.5-7.0)	4.7	95.9 (92.1-98.3)
					5.00	4.9 (3.5-6.8)	5.7	101.7 (97.6-107.1)

Table 1.2 - Continue

Investigated analytes	Calibration curve				QC precision and accuracy			
	LOQ (µg/L)	Investigated ranges (µg/L)	R^2 Mean (n=5)	Mid-term stability (%RDS_{slope} n=5)	Spiked Concentrations (µg/L)	Intra-day precision %RSD (Min-Max)	Inter-run precision %RSD	Accuracy %Theoretical (Min-Max)
GAMA	1.0	LOQ-1600	0.992	3.8	3.1	5.6 (1.8-7.9)	6.9	109.3 (104.6-116.5)
					25	5.4 (3.7-8.5)	5.6	105.5 (102.9-108.5)
					200	5.3 (4.3-6.6)	5.4	110.1 (107.9-113.3)
HEMA	0.3	LOQ-1600	0.990	2.3	3.1	6.1 (5.4-7.7)	5.8	104.5 (100.6-107.9)
					25	5.4 (3.9-7.2)	6.0	102.9 (97.3-107.3)
					200	4.5 (3.7-6.8)	5.3	107.5 (104.7-111.2)
HMPMA	2	LOQ-3200	0.975	26.4	6	4.1 (1.5-7.5)	16.5	103.7 (90.3-115.7)
					50	4.9 (3.1-7.6)	16.2	105.9 (89.0-115.0)
					400	3.7 (2.0-7.0)	16.0	107.9 (89.9-117.2)
MHBMA	0.04	LOQ-40	0.999	2.3	0.08	3.7 (1.5-7.6)	13.2	99.6 (94.1-105.0)
					0.63	8.1 (4.3-10.6)	8.6	99.9 (94.1-106.3)
					5.00	4.1 (1.1-6.6)	4.7	102.8 (101.4-105.2)
MMA	0.09	LOQ-40	0.997	2.1	0.63	6.6 (3.1-10.3)	10.8	96.7 (92.6-99.1)
					5.00	4.4 (2.8-6.6)	5.5	103.1 (98.5-107.6)

Table 1.2 - Continue

Investigated analytes	Calibration curve				QC precision and accuracy			
	LOQ (µg/L)	Investigated ranges (µg/L)	R^2 Mean (n=5)	Mid-term stability (%RDS_{slope} n=5)	Spiked Concentrations (µg/L)	Intra-day precision %RSD (Min-Max)	Inter-run precision %RSD	Accuracy %Theoretical (Min-Max)
NANPC	0.11	LOQ-40	0.979	3.4	0.63	2.9 (1.3-4.2)	10.2	101.7 (89.5-115.2)
					5.00	3.4 (0.9-4.5)	4.6	100.9 (94.9-105.4)
PHEMA	0.01	LOQ-40	0.991	10.0	0.08	4.3 (2.7-6.3)	10.3	93.7 (87.5-100.8)
					0.63	2.9 (1.4-6.8)	7.6	100.6 (91.4-104.6)
					5.00	2.3 (0.8-3.2)	6.8	104.6 (94.2-110.6)
SBMA	0.02	LOQ-40	0.999	0.5	0.08	1.4 (0.8-2.3)	2.3	99.5 (97.4-103.9)
					0.63	0.8 (0.5-1.1)	1.1	100.0 (99.0-101.2)
					5.00	0.6 (0.4-0.8)	0.9	102.8 (102.0-104.0)
SPMA	0.01	LOQ-40	0.989	0.6	0.08	1.9 (1.1-2.5)	4.0	103.0 (99.6-107.8)
					0.63	2.1 (1.7-2.5)	9.3	107.2 (102.8-115.1)
					5.00	1.1 (0.7-1.7)	1.7	106.1 (105.3-106.7)

1.3 Results

1.3.1 Validation

Figure 1.2 shows superimposed extracted ion chromatograms of a standard mixture of mercapturic acids in synthetic urine, and Figure 1.3 (a and b) shows single extracted ion chromatograms for each analyte and its internal standard from an urine sample of a non-smoking male gasoline station attendant. The analytes were eluted within 12 min, although the chromatographic run lasted 18 min due to instrumentation reconditioning. Although some analytes (i.e. HEMA, GAMA, AAMA, and DHBMA) were co-eluted, their peaks were separated in their MRM transitions.

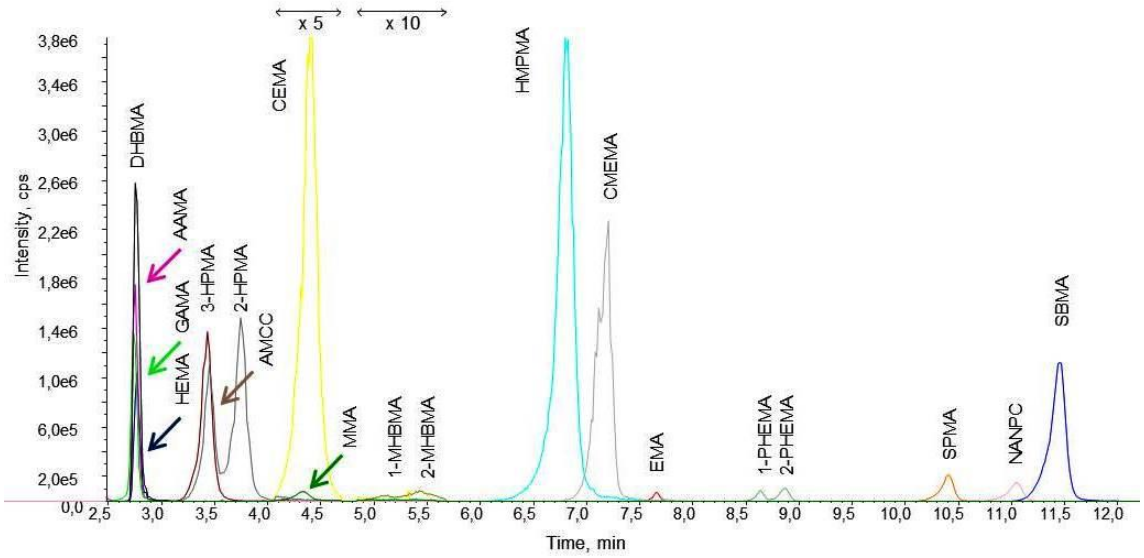


Figure 1.2 - Superimposed extracted ion chromatograms of a standard mixture of mercapturic acids in synthetic urine at concentrations of 10 µg/L for EMA, MHBMA, MMA, NANPC, PHEMA, SBMA and SPMA ; 400 µg/L for 2-HPMA, 3-HPMA, AAMA, CEMA, DHBMA, GAMA, and HEMA ; and 800 µg/L for AMCC, CMEMA, and HMPMA.

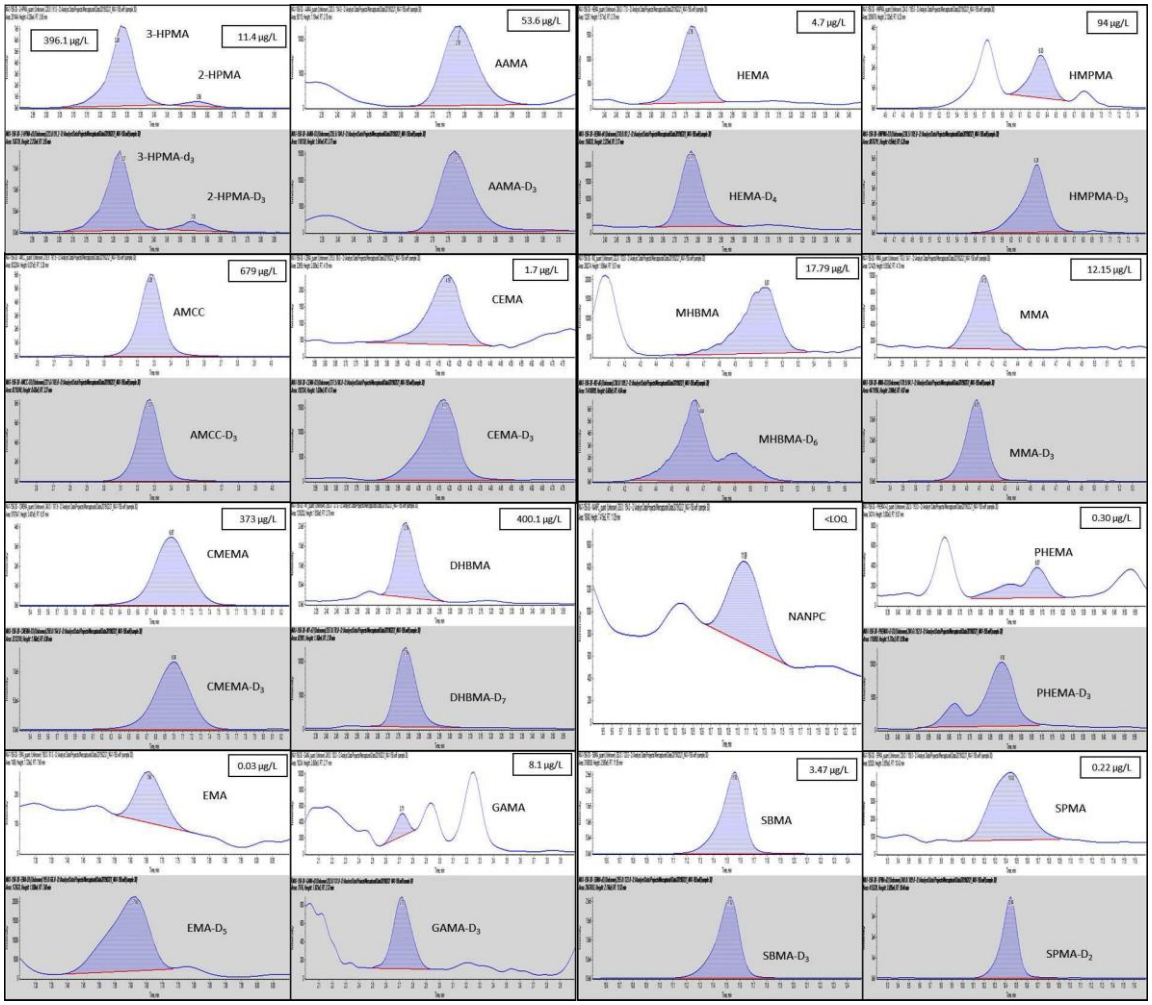


Figure 1.3 - Single extracted ion chromatograms of 2-HPMA, 3-HPMA, AAMA, AMCC, CEMA, CMEMA, DHBMA, EMA, GAMA, and their internal standards (a), and single extracted ion chromatograms of HEMA, HMPMA, MHBMA, MMA, NANPC, PHEMA, SBMA, SPMA, and their internal standards (b), from an authentic urine sample of non-smoking male unexposed worker.

1.3.1.1 Calibration curves

Coefficient of determination (R^2) values were close to 1 for all compounds, demonstrating optimal linearity for the concentration ranges investigated (Table 1.2).

1.3.1.2 Sensitivity

LOQ values ranged from 0.01 to 3.2 µg/L (Table 1.2).

1.3.1.3 Precision and accuracy

Intraday assays yielded optimal values for all compounds, with all RSDs < 10%. Interday assays yielded larger RSD values in a few cases, such as 18.2–19.7% for AAMA, 19.6–20.9% for CEMA, and 16.0–16.5% for HMPMA. All accuracy values were acceptable, ranging from 93.4% to 114.9% (Table 1.2).

1.3.1.4 Internal standard interference and carryover effect

When analyzing a blank sample containing the working solution of internal standards, peaks corresponding to the native chemicals, present as impurities in the labeled internal standards, were found for AAMA, AMCC, CMEMA, DHBMA, GAMA, and HMPMA; however, the amount of this interference was less than the estimated LOQs. No carry-over effect was detected.

1.3.1.5 Process efficiency

Although most PE values were around 100%, they were much lower (8.9–24.9%) for AAMA, DHBMA, GAMA, and HEMA, indicating that matrix reduced the instrument signals for these compounds (Table 1.3). However, the use of isotopically labeled internal standards compensated for these effects, demonstrating the ability of these standards to improve analytical performance. With the use of internal standards, results ranged from 79.6% to 109.1%, with the exception of those for low-concentration HEMA (70.6%) and high-concentration AAMA and DHBMA (133.9% and 147.4%, respectively).

Table 1.3 - Evaluation of the method's process efficiency. Results are given as means ± standard deviations from the urine of five healthy volunteers. Low, medium and high spiked concentrations were 0.75, 2, and 15 µg/L, respectively, for EMA, MHBMA, NANPC, PHEMA, SBMA, and SPMA; 30, 80, and 600 µg/L, respectively, for 2-HPMA, CEMA, GAMA, HEMA, and MMA; and 112.5, 300, and 2250 µg/L, respectively, for 3-HPMA, AAMA, AMCC, CMEMA, DHBMA, and HMPMA.

Investigated analytes	**Process efficiency (PE) for low concentrations (%mean±SD)**		**Process efficiency (PE) for medium concentrations (%mean±SD)**		**Process efficiency (PE) for high concentrations (%mean±SD)**	
	without IS	**with IS**	**without IS**	**with IS**	**without IS**	**with IS**
2-HPMA	72.3 ± 11.8	95.8 ± 14.8	87.4 ± 11.9	109.1 ± 16.7	84.4 ± 9.8	87.6 ± 10.6
3-HPMA	67.9 ± 13.7	82.0 ± 7.5	75.0 ± 14.8	91.5 ± 11.3	85.0 ± 10.5	97.6 ± 4.5
AAMA	8.9 ± 4.9	84.4 ± 5.2	10.6 ± 6.0	98.3 ± 7.5	18.7 ± 10.8	133.9 ± 10.5
AMCC	65.6 ± 13.7	91.5 ± 9.0	72.4 ± 12.2	101.6 ± 3.2	80.4 ± 11.4	105.4 ± 9.4
CEMA	87.4 ± 18.3	96.1 ± 3.1	96.7 ± 16.1	107.8 ± 4.6	98.6 ± 17.3	101.2 ± 5.6
CMEMA	74.9 ± 5.0	89.3 ± 6.8	79.4 ± 8.7	95.2 ± 7.3	83.3 ± 6.1	94.8 ± 4.3
DHBMA	11.6 ± 4.8	94.4 ± 17.6	13.2 ± 6.8	95.2 ± 10.7	24.9 ± 11.6	147.4 ± 11.4
EMA	53.2 ± 17.7	87.0 ± 11.4	60.6 ± 19.2	83.6 ± 12.6	65.3 ± 18.3	85.1 ± 8.2
GAMA	9.8 ± 4.6	105.5 ± 21.4	9.6 ± 5.8	88.0 ± 13.8	10.1 ± 6.5	86.3 ± 21.6
HEMA	10.8 ± 4.6	70.6 ± 16.6	12.9 ± 6.1	79.6 ± 19.8	16.7 ± 7.4	87.5 ± 19.1
HMPMA	77.1 ± 10.9	90.6 ± 4.1	85.6 ± 9.6	95.5 ± 3.0	88.2 ± 7.3	94.9 ± 6.7
MHBMA	93.0 ± 9.6	103.6 ± 11.4	96.4 ± 12.2	102.0 ± 9.0	89.9 ± 12.8	101.6 ± 2.3
MMA	69.2 ± 8.9	87.0 ± 17.8	73.2 ± 12.6	96.6 ± 16.8	72.7 ± 15.1	101.4 ± 13.5
NANPC	87.4 ± 7.6	NA	98.9 ± 8.8	NA	99.6 ± 6.1	NA
PHEMA	76.0 ± 26.9	105.6 ± 23.6	69.3 ± 23.6	102.0 ± 18.9	58.9 ± 22.8	78.3 ± 5.9
SBMA	86.4 ± 5.0	101.6 ± 9.3	92.9 ± 9.3	101.6 ± 4.3	91.8 ± 8.2	98.9 ± 6.5
SPMA	68.5 ± 13.2	86.7 ± 12.6	80.7 ± 12.6	98.1 ± 6.5	86.7 ± 7.4	102.3 ± 6.4

1.3.1.6 External verification

Evaluations from the G-EQUAS were positive for all compounds considered.

1.3.1.7 Stability

Short-term stability tests showed that the areas for mercapturic acids stored at 8°C for 9 days, relative to those of freshly prepared solutions, ranged from 82% to 122%; this variability was in line with the inter-run precision results (Table 1.2), suggesting the absence of analyte degradation. Conversely, loss of area (~50% peak area) was found for isotopically labeled internal standards of AAMA, CEMA, and HMPMA. This loss was probably due to the exchange of deuterium with hydrogen in the presence of formic acid, which was added during sample preparation. No such behavior was noted for the other internal standards.

Mid-term stability (reported as $\%RSD_{slope}$) results ranged from 0.5% to 10.0% for all compounds except AAMA, CEMA, and HMPMA (range, 21.3–26.4%; Table 1.2).

Long-term stability tests showed that the mercapturic acids considered remained stable in urine stored at –20°C for up to 2 years.

1.3.2 Application

The method was applied to the analysis of 46 urine samples from different groups of non-smoking workers. Results are reported in Table 1.4, expressed as median, minimum and maximum. ANOVA test showed statistically significant differences among groups for CEMA, DHBMA, MHBMA, PHEMA and SPMA.

Table 1.4 - Results (median, minimum and maximum) of mercapturic acids in end-shift urine samples of non-smoking male subjects belonging to different working groups. The difference among groups was tested using ANOVA performed on log_{10}-transformed data

Investigated analytes	Statistics	Non-exposed workers	Refinery workers	Coke oven workers	Traffic policemen	Rotogravure printing workers	Gasoline station attendants	Asphalt workers	ANOVA (p-value)
2-HPMA (μg/L)	**Minimum**	17.41	28.31	19.61	11.02	39.33	22.94	11.03	0.380
	Median	43.13	44.26	58.48	28.50	52.00	47.24	108.46	
	Maximum	79.45	60.77	114.52	72.00	343.99	1514.52	148.96	
3-HPMA (μg/L)	**Minimum**	41.96	35.75	111.50	47.82	19.38	60.65	9.72	0.131
	Median	94.11	88.89	175.98	76.03	59.48	146.84	127.52	
	Maximum	262.68	167.79	229.86	180.57	75.30	314.26	639.51	
AAMA (μg/L)	**Minimum**	33.78	24.37	52.33	35.24	33.68	30.73	27.67	0.620
	Median	69.37	73.52	92.24	65.17	50.01	69.42	92.39	
	Maximum	119.93	97.55	210.32	140.85	110.06	111.69	192.62	
AMCC (μg/L)	**Minimum**	160.80	71.34	170.77	163.84	164.10	145.33	133.38	0.253
	Median	373.87	305.83	399.70	221.45	361.75	339.54	401.38	
	Maximum	5240.26	341.43	817.47	598.29	769.05	673.45	1227.00	
CEMA (μg/L)	**Minimum**	1.23	2.29	2.73	1.51	1.22	0.77	1.11	**0.004**
	Median	2.09	4.80	5.42	3.79	1.65	1.69	3.53	
	Maximum	2.83	10.05	25.83	88.54	2.32	2.73	7.19	
CMEMA (μg/L)	**Minimum**	196.86	163.09	244.41	303.82	220.31	271.74	68.36	0.229
	Median	314.00	431.15	439.26	353.20	336.59	691.51	979.23	
	Maximum	543.38	749.85	1903.30	891.11	407.22	2769.26	6584.10	
DHBMA (μg/L)	**Minimum**	68.54	277.01	295.515	213.95	280.45	323.995	68.54	**0.040**
	Median	564.47	558.765	647.315	505.815	358.145	456.86	564.47	
	Maximum	830.2	848.57	789.51	729.2	392.83	905.44	830.2	
EMA (μg/L)	**Minimum**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.02	<LOQ	0.062
	Median	0.03	0.06	0.02	0.02	<LOQ	0.04	0.04	
	Maximum	0.09	0.12	0.37	0.31	<LOQ	0.57	0.62	
GAMA (μg/L)	**Minimum**	2.97	2.09	7.11	0.51	3.79	3.11	3.46	0.924
	Median	6.21	7.44	10.33	9.44	17.63	6.85	11.27	
	Maximum	10.91	20.30	12.84	1516.66	36.00	21.90	21.11	
HEMA (μg/L)	**Minimum**	1.68	1.53	4.09	1.48	1.84	2.57	1.55	0.314
	Median	5.62	7.44	7.39	3.52	4.79	5.74	4.57	
	Maximum	9.87	9.37	16.73	18.77	7.01	91.51	7.56	
HMPMA (μg/L)	**Minimum**	63.63	53.69	172.30	94.32	38.86	115.98	29.12	0.340
	Median	234.02	135.18	225.96	220.47	98.56	297.87	260.60	
	Maximum	669.95	2040.10	312.78	349.89	420.68	3046.94	2299.96	
MHBMA (μg/L)	**Minimum**	<LOQ	1.13	0.42	0.07	0.47	0.61	1.91	**0.013**
	Median	1.07	1.51	3.23	1.05	0.95	6.48	7.13	
	Maximum	4.02	38.02	16.82	4.97	7.17	30.97	32.84	

Table 1.4 - Continue

Investigated analytes	Statistics	Non-exposed workers	Refinery workers	Coke oven workers	Traffic policemen	Rotogravure printing workers	Gasoline station attendants	Asphalt workers	ANOVA (p-value)
MMA (µg/L)	**Minimum**	0.84	2.46	0.92	1.67	0.58	2.04	<LOQ	0.372
	Median	2.88	4.73	4.98	4.59	2.41	4.05	4.84	
	Maximum	4.20	9.70	42.46	11.73	6.28	12.06	21.56	
NANPC (µg/L)	**Minimum**	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	**NA**
	Median	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	
	Maximum	<LOQ	0.26	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	
PHEMA (µg/L)	**Minimum**	<LOQ	0.32	0.16	0.06	<LOQ	0.02	<LOQ	**0.000**
	Median	0.08	0.70	0.29	0.15	0.08	0.05	0.10	
	Maximum	0.27	3.06	0.93	0.79	0.19	0.31	0.42	
SBMA (µg/L)	**Minimum**	0.33	0.33	1.77	0.37	1.18	1.00	0.24	0.167
	Median	1.39	1.88	2.60	1.38	6.95	2.31	5.47	
	Maximum	8.92	8.42	10.62	3.49	10.46	6.73	19.28	
SPMA (µg/L)	**Minimum**	0.03	0.46	0.38	0.19	<LOQ	0.21	0.13	**0.000**
	Median	0.09	0.97	0.63	0.25	0.11	0.47	0.20	
	Maximum	0.11	4.49	1.24	0.45	0.18	0.87	0.33	

NA = not assessed

1.4 Discussion

In this work, we developed a rapid method for the profiling of mercapturic acids, as suitable biomarkers of exposure to occupational and environmental toxicants, in noninvasively collected urine samples. The mercapturic acid considered include metabolites of toxicants such as benzene, toluene, styrene, 1,3-butadiene, acrylamide, acrolein, propylene oxide, N,N-dimethylformamide, acrylonitrile, crotonaldehyde, ethylene oxide, 4-chloronitrobenze, and methylating and ethylating agents.

Mathias and B'hymer (Mathias and B'hymer, 2016) reviewed previously described LC-MS methods for the analysis of mercapturic acids. Most of these methods enable consideration of only small numbers of mercapturates. Pluym and coworkers (Pluym et al., 2015), whose methods cover 18 mercapturic acids, found that two different chromatographic runs were necessary for suitable quantitation of mercapturates with different polarities and expected concentration ranges. Although we encountered such issues during method development, we found strategies to

overcome them and developed a single high-throughput method, which speeds sample preparation and analysis.

The first issue that we faced was the great variability in the polarities of the molecules considered, which included polar compounds such as HEMA and DHBMA and more lipophilic compounds, such as SPMA and SBMA. The use of a single chromatographic run for all of these compounds affected chromatographic separation; in fact, complete separation of polar compounds (e.g., AAMA, DHBMA, GAMA, HEMA) was not possible with the reversed-phase chromatography used. Nevertheless, the high-performance mass spectrometer used allowed the setting of scheduled MRM with a suitable target scan cycle (1 s), which provided high sensitivity and well-defined peak shapes.

Another issue that we faced was the wide range of concentrations at which the different mercapturic acids may be present in samples. Whereas some analytes (e.g., SPMA, SBMA, PHEMA) require very high sensitivity and are typically present at concentrations < 1 μg/L, others (e.g., AMCC, CMEMA, HMPMA) are present at concentrations that are two or three orders of magnitude higher. To obtain suitable LOQs without time-consuming sample preparation, we increased the injection volume to 20 μL. Under this condition, we observed no loss of peak symmetry, but we had to carefully choose the concentrations of native standards for the calibration curve levels and those of isotopically labeled standards to avoid signal saturation at increasing concentrations. Furthermore, increasing the injection volume to 20 μL created significant matrix effects for some analytes, particularly AAMA, DHBMA, GAMA, and HEMA; however, the use of internal standards compensated for this effect, as shown in Table 1.3.

We experienced another issue in the preparation of calibration curves for some analytes, such as AAMA, AMCC, CMEMA, DHBMA, GAMA, and HMPMA; we noticed that the peak areas of the internal standards at the highest levels of the calibration curve were greater than expected. We realized that this effect was attributable to presence of a signal of each analyte superimposed over the signal of

the deuterated internal standard, due to the natural isotopic abundance of atoms in the precursor ion $[M - H]^-$ and the limited number of deuterium atoms in the internal standard. To overcome this issue, we prepared an internal standard mixture with higher concentrations of deuterated standards for the affected analytes, thereby reducing the observed effects to negligible (<10%). The downside of operating with high internal standard concentrations was the increasing relevance of the impurity of native analytes in the deuterated standards; however, as reported in Section 3.1.4, the estimated concentrations of these impurities were always below the estimated LOQs. The final concentrations of the internal standards were chosen carefully to balance these two effects and to obtain sufficiently low LOQs to enable the quantification of mercapturic acids in real samples.

This study included analysis of NANPC, a urinary metabolite of 4-chloronitrobenzene (Yoshida, 1993), which is a chlorinated nitroaromatic intermediate used in the production of drugs, pesticides, dyes, and antioxidants. The scientific literature contains very few studies of NANPC, and Mathias and B'hymer (Mathias and B'hymer, 2016) did not mention this mercapturic acid in their complete review, probably because the only published method for its determination involves the use of LC coupled with UV detection (Jones et al., 2007). To our knowledge, we report here the first LC-MS/MS method that includes this mercapturic acid and analysis of its fragmentation pattern.

1-MHBMA/2-MHBMA and PHEMA1/PHEMA2 are pairs of structural isomers. Although they were chromatographically separated by our method, we decided to quantify each pair of isomers as a single compound because it was included in the same commercial standard and was present at very low concentrations in samples.

The method was validated following the FDA guideline and the considerations raised by González and coworkers (FDA, 2013; González et al., 2014). Despite the slightly high RSD values for AAMA, CEMA, and HMPMA in the interday assays, the method was shown to be suitable for targeted analysis of the mercapturic acids considered in a single analytical framework. Furthermore, the results obtained in the

external verification exercise, although limited to 10 analytes, confirmed the accuracy of the method. In addition, the sensitivity of the assay was satisfactory, as LOQ values were suitable for the detection of analytes in almost all samples from occupationally exposed and unexposed subjects. The LOQ values were comparable to, or often lower than those obtained with previously described methods (Pluym et al., 2015; Zhang et al., 2014; Alwis et al., 2012; Li et al., 2005; Kotapati et al., 2015). Table 1.5 provides an overview of validation parameters reported for other methods developed for the simultaneous determination of mercapturic acids. The main advantage of the method reported here is the ability to quantify a larger number of mercapturic acids with a single chromatographic run. Another advantage, lacking for all previously reported methods, is represented by the success achieved in the external verification exercise.

We applied the method to different groups of workers. Since mercapturic acid concentrations are highly affected by smoking status (Eckert et al., 2011; Zhang et al., 2014; Pluym et al., 2015; Alwis et al., 2012; Maestri et al., 2005), we decided to include only samples from non-smoking workers. The group with the highest amount of mercapturic acids was that of refinery workers; when compared to non-exposed workers, they showed significant higher levels of CEMA, DHBMA, PHEMA, and SPMA, which indicate exposure to acrylonitrile, 1,3-butadiene, styrene and benzene. Although with slightly lower values, also coke oven workers showed a similar profile with significant higher levels of the same mercapturates. On the contrary, rotogravure printing workers showed higher levels (although not statistically significant) of SBMA, a metabolite of toluene, which is a component of inks that evaporates in the workshop during rotogravure printing. This application of the developed assay, although performed on a limited sample size, allowed to demonstrate the suitability of the developed method for profiling a wide range of mercapturic acids with a single, rapid and high-throughput assay. The method can therefore be applied in biological monitoring studies for the simultaneous assessment of occupational and environmental exposure to several toxicants.

Table 1.5 - Comparison of methods for the simultaneous quantification of mercapturic acids according to analytes considered, sample preparation, run time, LOQ, precision, accuracy, stability, and matrix effect.

Compounds	**Sample preparation**	**Run time**	**LOQ**	**Precision**	**Accuracy**	**Stability**	**Matrix effects**	**Reference**
SBMA, SPMA, MBMA	SPE	27 minutes	From 0.60 to 0.80 µg/L	From 1.7 to 14.0%	From 91.1 to 114.9%	< 6%	< 8% (R.S.D. of slopes of calibration curves)	(Sabatini et al., 2008)
2-HPMA, 3-HPMA, AAMA, AMCC, HEMA	SPE	26 minutes	LOQ not reported LOD: from 0.5 to 5 µg/L	From 2.3 to 22.2%	From 70 to 126%	Performed but results not shown	From -44 to +14% (deviation of slope)	(Schettgen et al., 2008)
CEMA, DHBMA, MHBMA	RAM phase column switching	25 minutes	From 1 to 10 µg/L	From 4.9 to 9.9%	From 87 to 121%	Performed but results not shown	From 84 to 128% (accuracy in 5 individual samples)	(Schettgen et al., 2009)
2-HPMA, 3-HPMA, DHBMA, HEMA, MHBMA, DHPMA	SPE	22 minutes	LOQ not reported LOD: from 3.0 to 7.0 µg/L	From 0.9 to 9.2%	From 24 to 136%	Not reported	From 24 to 136% (accuracy in 10 individual samples)	(Eckert et al., 2010)
CEMA, HEMA, MMA	Liquid extraction, SPE, and PFBBr derivitization	15 minutes	From 1.0 to 5.0 µg/L	From 0.9 to 13.9%	From 80.6 to 116.1%	From 90.4 to 112.6%	From 21.3 to 107.9% (peak area ratios between analyte in matrix and solvent)	(Scherer et al., 2010)

Table 1.5 - Continue

Compounds	**Sample preparation**	**Run time**	**LOQ**	**Precision**	**Accuracy**	**Stability**	**Matrix effects**	**Reference**
2-HPMA, 3HPMA, AAMA, AMCC, CEMA, DHBMA, GAMA, HEMA, HMPMA, 1-MHBMA, 2-MHBMA, 3-MHBMA, PHEMA, SBMA, SPMA and others	Protein precipitation	9 minutes	LOQ not reported LOD: from 0.30 to 20.0 µg/L	From 2.5 to 11%	From 83 to 108%	Performed but results not shown	From 82.1 to 120% (process efficiency)	(Alwis et al., 2012)
EMA, MMA, iPMA, NPMA	RAM phase column switching	28 minutes	From 6.6 to 15.5 µg/L	From 4.5 to 12.2%	From 92.3 to 103.0%	Nor reported	From 92.3 to 103.0% (accuracy in 10 individual samples)	(Eckert and Göen, 2014)
3-HPMA, CEMA, DHBMA, HMPMA, SPMA	RAM phase column switching	15 minutes	From 0.050 to 0.177 µg/L	From 0.7% to 15.2%.	From 79.3 to 116.0%	< 20%	< 30% (reduction of peak area ratios between analyte in matrix and solvent)	(Zhang et al., 2014)
CEMA, DHBMA, GAMA, 3-MHBMA, SPMA, 2,4-DPMA, CYMA, tt-MA	SPE column switching	11 minutes	From 0.033 to 2.564 µg/L	From 0.9 to 8.5%	From 90.8 to 108.9%	From 80.5 to 116.8%	From 86.7 to 120.1% (ratio of peak areas between analyte in matrix and solvent)	(Chiang et al., 2015)

Table 1.5 - Continue

Compounds	**Sample preparation**	**Run time**	**LOQ**	**Precision**	**Accuracy**	**Stability**	**Matrix effects**	**Reference**
3-HPMA, CEMA, DHBMA, MHBMA, SPMA, HMPMA, PHEMA	Ionic DLLME	30 minutes	From 0.20 to 8.25 µg/L	From 1.64 to 7.74%	From 85.32 to 127.61%	Not reported	From 85.32 to 127.61% (accuracy in 5 samples)	(Li et al., 2015)
CEMA, CMEMA, EMA, HEMA, HMPMA, MMA, PHEMA1, PHEMA2, SBMA, SPMA	RAM phase column switching	18 minutes	From 0.03 to 5.0 µg/L	From 1.5 to 20.4%	From 94.0 to 119.2%	Performed but results not shown	From 88 to 114% (peak area ratios between analyte in matrix and solvent)	(Pluym et al., 2015)
2-HPMA, 3-HPMA, AAMA, AMCC, DHBMA, GAMA, 1-MHBMA, 2-MHBMA	Concentration and resuspension	21 minutes	From 0.12 to 25.0 µg/L	From 2.0 to 15.3%	From 80.7 to 104.0%	Performed but results not shown	From 88 to 114% (Peak area ratios between analyte in matrix and solvent)	(Pluym et al., 2015)
2-HPMA, 3-HPMA, AAMA, AMCC, CEMA, CMEMA, DHBMA, EMA, GAMA, HEMA, HMPMA, MHBMA, MMA, NANPC, PHEMA, SBMA, SPMA	Dilution and filtration	18 minutes	From 0.01 to 3.2 µg/L	From 0.6 to 20.9%	From 93.4 to 114.9%	From 82 to 122% (short-term)	From 70.6 to 147.4% (Process efficiency)	This work

2 Biomonitoring of coke oven workers by quantitation of urinary mercapturic acids

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2.1 Introduction

Volatile organic compounds (VOCs) are defined, according to the European Union (EU, 2004), as any organic compound having an initial boiling point less than or equal to 250 °C measured at a standard atmospheric pressure. Some VOCs are toxic to humans and some of them have also been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC), including 1,3-butadiene and benzene (known carcinogenic, group 1) (IARC, 2012b; IARC, 2018a), acrylamide and styrene (probable carcinogenic, group 2A) (IARC, 1994; IARC, 2018b), propylene oxide and acrylonitrile (possible carcinogenic, group 2B) (IARC, 1994; IARC, 1999). Moreover, chronic exposure to VOCs is associated with respiratory, neurological, reproductive, and developmental effects (ATSDR, 2007; ATSDR, 2010; ATSDR, 2012; ATSDR, 1990). Exposure to toxic VOCs may derive both from occupational and non-occupational sources (IARC, 2012c; IARC,

2012b), with tobacco smoking as the most important non-occupational source in smokers (IARC, 2004).

Biomonitoring is a useful approach to assess the exposure to VOCs in human subjects. It consists in the analysis of toxicants, or their specific metabolites, in the subjects' biological fluids, such as urine. Its main advantage is the potential to assess the body burden of a given toxicant including all sources and exposure routes. Several mercapturic acids are useful biomarkers of exposure to VOCs. Mercapturic acids are N-acetyl cysteine derivatives of electrophilic compounds initially conjugated with glutathione and then biotransformed to highly hydrosoluble chemicals excreted in urine (De Rooij et al., 1998; Parkinson and Ogilvie, 2010). For the sake of occupational risk assessment, biological limit values and general population reference values have been recommended by committees of experts such as the American Conference of Governmental Industrial Hygienists (ACGIH) (ACGIH, 2017), the MAK-Commission (German Committee for the determination of occupational exposure limits of the Deutsche Forschungsgemeinschaft, German Research Foundation, DFG) (DFG, 2018) and the Risk Assessment Committee of the European Chemicals Agency (ECHA) (ECHA, 2018). In particular, N-acetyl-S-phenyl-L-cysteine (SPMA) has been recommended as a biomarker of exposure to benzene by all these committees. A summary of the biological limit values for the mercapturic acids of VOCs is reported in Table 2.1.

Coke, a porous fuel with a high carbon content and few impurities, is essential for the manufacture of steel. It is produced by heating coal in the absence of oxygen in a process called destructive distillation, in order to remove volatile components. Coke production has been classified as carcinogenic to humans (group 1 according to IARC classification) (IARC, 2012b). The primary chemical exposure in coke oven workers is to polycyclic aromatic hydrocarbons (PAHs) (Zając et al., 2016; Campo et al., 2012; IARC, 2012b). Moreover, coke oven workers might also be exposed to VOCs emitted during destructive distillation. Indeed, a certain number of studies assessed the exposure to VOCs in coke oven workers quantifying their

levels in workplace and breathing zone air, with overall levels ranging from 22.6 μg/m^3 to 2.17 mg/m^3 (Dehghani et al., 2018; Shi et al., 2015; Bieniek and Łusiak, 2012; Chang et al., 2010; Ciaparra et al., 2009; Tsai et al., 2008; Bieniek et al., 2004; Bieniek, 1998; Lovreglio et al., 2018). Only a few studies performed biomonitoring of VOC metabolites in coke oven workers. In particular, the levels of the benzene metabolites trans, trans-muconic acid (Lovreglio et al., 2018; Hotz et al., 1997; Kivistö et al., 1997; Fan et al., 2014) and S-phenyl mercapturic acid (SPMA) (Lovreglio et al., 2018; Colman and Coleman, 2006; Pople et al., 2002; Hotz et al., 1997; Kivistö et al., 1997; Fan et al., 2014) and the toluene metabolite S-benzyl mercapturic acid (SBMA) (Fan et al., 2014) have been measured in the urine of coke oven workers. However, the urinary concentrations of these metabolites have been reported to be highly affected by smoking habit: e.g., Lovreglio and co-workers reported median levels of SPMA equal to 1.35 μg/g creatinine in smoking coke oven workers versus 0.23 μg/g in non-smoking workers (Lovreglio et al., 2018).

The aim of the present study was to assess the exposure to VOCs in coke oven workers, through the determination of seventeen mercapturic acids in the workers' urine. This was achieved by applying a recently developed and validated isotopic dilution liquid chromatography–tandem mass spectrometry (LC-MS/MS) method (chapter 1). The exposure was then compared with that of a matched group of individuals belonging to the general population; further comparisons were performed with occupational limit values and reference values. To avoid the known confounding effect of cigarette smoking, the study was performed enrolling only non-smokers.

Table 2.1 - Biological limit values for mercapturic acids of different volatile organic compounds recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) (ACGIH, 2017), the Deutsche Forschungsgemeinschaft (DFG) (DFG, 2018), and the European Chemicals Agency (ECHA) (ECHA, 2018).

Chemical	CAS Number	Organization	Biomarker	Sampling Time	Biological value	Value	
Acrolein	107-02-8	DFG	3-HPMA	End of shift/for long-term exposures: at the end of the shift after several shifts	BAR (NS)	600 μg/g creatinine	
Acrylamide	79-06-1	DFG	AAMA	End of shift	BAR (NS)	100 μg/g creatinine	
Benzene	71-43-2	ACGIH	SPMA	End of shift	BEI	25 μg/g creatinine	
		ECHA		End of shift, after several shifts	BLV	2 μg/g creatinine	
					BGV	0.5 μg/g creatinine	
		DFG		End of shift	EKA	Air (mg/m³)	Biomarker (μg/g creatinine)
						0.1	1.5 (NS)
						0.2	3 (NS)
						0.5	5
						1.0	12
						2.0	25
						3.3	45
						6.5	90
					BAR (NS)		0.3
1,3-butadiene	106-99-0	DFG	DHBMA	End of shift/for long-term exposures: at the end of the shift after several shifts	EKA	Air (mg/m³)	Biomarker (μg/g creatinine)
						0.45	600
						1.1	1000
						2.3	1600
						4.5	2900
						6.8	4200
					BAR (NS)		400
			MHBMA	End of shift/for long-term exposures: at the end of the shift after several shifts	EKA	Air (mg/m³)	Biomarker (mg/g creatinine)
						0.45	10
						1.1	20
						2.3	40
						4.5	80
						6.8	120
					BAR (NS)		<2
N,N-dimethylformamide	68-12-2	ACGIH	AMCC	End of shift at end of work week	BEI	30 mg/L	
		DFG			BAT	25 μg/g creatinine	

CAS = Chemical Abstracts Service. NS = non-smokers. BEI = biological exposure indices (ACGIH). EKA = exposure equivalents for carcinogenic substances (DFG). BAR = biological reference value (DFG). BAT = biological tolerance value for occupational exposure (DFG). BLV = biological limit values (ECHA). BGV = biological guidance values (ECHA).

2.2 Materials and Methods

2.2.1 Study Design

The enrollment of subjects, investigation design, and sample collection have been described in previous works (Pavanello et al., 2008; Campo et al., 2014). Briefly, the study included 49 coke oven workers and 49 subjects form the general population living in the same area (controls), with mean age 39.3 (20–59) and 39.7 (21–58), respectively. All subjects were males living in Poland. Active tobacco smoking was an exclusion criterion for this enrolment. Informed consent was signed from each participant and the study was approved by the Ethic Committee of the Institute of Occupational Medicine and Environmental Health in Sosnowiec.

A questionnaire was administered to each subject by trained interviewers, including: personal characteristics, residence, industrial and/or heavy traffic exposure in living place, type of heating at and near home, recent food habits, and hobbies at home involving exposure during the last three days. Workers were also asked about the use of protective equipment and whether their skin was dirty at the end of the work-shift. Workers were from three different plants: 17 worked in a plant producing low-phosphor coke and broken coke (plant J), 24 in a plant producing domestic coke (plant D), and 8 in a plant producing foundry and blast furnace coke (plant R). Among all workers, 11 were expert foremen; 13 were engine drivers, operators, or machine workers; while 25 were coke makers or gas workers. Control subjects, living in the same area and matched for age and gender to workers, were enrolled among clerks involved in a health check-up program at the Institute of Occupational Medicine and Environmental Health in Sosnowiec (Poland).

The collection of a urine sample was performed in workers at the end of the work-shift and after at least three consecutive working days, while it was performed in the late afternoon for control subjects. Urine samples were stored at −20 °C. Cotinine concentrations were quantified with a previously published method via LC-MS/MS

(Fustinoni et al., 2013). Urinary creatinine was measured using the Jaffè colorimetric method (Kroll et al., 1986).

Although active tobacco smoking was an exclusion criterion, the analysis of urinary cotinine showed that three control subjects had cotinine levels greater than 50 µg/L (212, 195, 668 µg/L), which was considered as the cutoff to distinguish active smokers from persons exposed to passive smoke (Haufroid and Lison, 1998). Furthermore, most of the other subjects (87%) had levels of cotinine greater than the limit of quantitation (LOQ) (0.1 µg/L), with a median value equal to 2.2 µg/L (<LOQ and 12.5 µg/L, 5th and 95th percentile respectively), showing that most subjects were exposed to passive smoking.

2.2.2 Mercapturic Acid Analysis

The analysis of mercapturic acids, were carried out with the validated LC-MS/MS isotopic dilution method (Chapter 1). The presence of 17 mercapturic acids was investigated (Table 2.2), in particular: N-acetyl-S-(2-hydroxypropyl)cysteine (2-HPMA) (metabolite of propylene oxide), N-acetyl-S-(3-hydroxypropyl)cysteine (3-HPMA) (metabolite of acrolein), N-acetyl-S-(carbamoylethyl)-L-cysteine (AAMA) (metabolite of acrylamide), N-acetyl-S-(N-methylcarbamoyl)-L-cysteine (AMCC) (metabolite of N,N-dimethylformamide), N-acetyl-S-(2-cyanoethyl)-L-cysteine (CEMA) (metabolite of acrylonitrile), N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine (CMEMA) (metabolite of crotonaldehyde), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA) (metabolite of 1,3-butadiene), N-acetyl-S-ethyl-L-cysteine (EMA) (metabolite of ethylating agents), N-acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine (GAMA) (metabolite of acrylamide), N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA) (metabolite of acrylonitrile and ethylene oxide), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HMPMA) (metabolite of crotonaldehyde), (R,S)-N-acetyl-S-[1-(hydroxymethyl)-2-propen-1-yl)-L-cysteine + (R,S)-N-acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine (MHBMA) (metabolites of 1,3-butadiene), N-acetyl-S-methyl-L-cysteine (MMA) (metabolite of methylating agents), S-(4-nitrophenyl)mercapturic acid (NANPC) (metabolite of 4-chloronitrobenze), N-

acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine (PHEMA) (metabolites of styrene), N-acetyl-S-benzyl-L-cysteine (SBMA) (metabolite of toluene), and N-acetyl-S-phenyl-L-cysteine (SPMA) (metabolite of benzene).

2.2.3 Data Elaboration and Statistical Analysis

The MultiQuant™ software (version 3.0.8664.0; Ab Sciex S.r.l, Milano, Italy) was used for data integration. Statistical analysis was performed using R (version 3.6.1, R Foundation, Vienna, Austria) (R-Core-Team, 2019) with the Rstudio interface (Version 1.2.1335, RStudio Inc., Boston, Massachusetts, United States). The package "tidyverse" was used for data elaboration and visualization (Wickham, 2017).

Measurements below the limit of quantitation were replaced with a value equal to half the LOQ before statistical analysis. Values were corrected with creatinine and then descriptive statistics was performed after grouping by workers and controls: in particular, median, 5th, and 95th percentile of the distribution were calculated for each analyte, along with the percentage of samples above LOQ. Data were $\log_{10}$ transformed to ensure normal distribution and Student's t-test was applied to evaluate statistically significant differences in the levels of mercapturic acids between groups.

To assess the determinants of urinary mercapturic acid levels, different multiple linear regression analyses were computed. In each linear model, the dependent variable was the $\log_{10}$-transformed concentration of a specific mercapturic acid, while the independent variables were age, urinary creatinine ($\log_{10}$ transformed), and urinary cotinine ($\log_{10}$ transformed). The other independent variables added one at time and tested as predictors were residence (rural or urban), industrial exposure (no or yes), heavy traffic exposure near home (no or yes), type of heating at home (wood, coal, oil, gas, or other), type of heating near home (wood, coal, oil, gas, or other), heating whole-building (no or yes), individual home heating (no or yes),

consumption of grilled/smoked meat during the last 24 hours (no or yes), consumption of grilled/smoked meat (times/week), and hobbies at home involving exposure to mineral oils, tar, soot, combustion fumes from wood, leaves, other combustible materials, and vehicle exhaust fumes during the last 3 days (no or yes). For workers, the following variables were also tested: plant (J, D, or R), job title (foremen, engine operators, or gas workers), use of individual protective equipment (no or yes), use of mask (hours/day), use of gloves (hours/day), use of overalls (hours/day), use of mask on the day before (hours/day), use of gloves in the day before (hours/day), use of overalls on the day before (hours/day), dirty skin in the last 3 days (a little or moderate/a lot), hand dirty in the last 3 days (a little, moderate, or a lot), and face dirty in the last 3 days (a little, moderate, or a lot).

Most of the considered variables were not significantly associated with the considered mercapturic acids and, therefore, not included in the final models. Then, two different linear models were built. Each model was run separately for each mercapturic acid (µg/L), which was imputed as dependent variable using the $\log_{10}$-transformed data. The first linear model was built using data from all subjects included in the study (n = 94 since four observations were excluded due to missing values) and was aimed to evaluate the differences between workers and controls. The independent variables were: study group (controls or workers), age (years), $\log_{10}$ creatinine (g/L), $\log_{10}$ cotinine (µg/L), presence of hobbies at home involving exposure during the last 3 days (no or yes). The second linear model was built considering only data from the coke oven workers (n = 49), and it was aimed to determine the role played by plant, job title, and dirty skin. The independent variables included in this model were: plant (J = reference, D, or R), job title (foremen = reference, engine operators, or coke markers), dirty skin (no or yes), age (years), $\log_{10}$ creatinine (g/L), and $\log_{10}$ cotinine (µg/L).

For all models, regression slopes were exponentiated in order to obtain the geometric mean ratio (GMR).

2.3 Results

The levels of urinary mercapturic acids in the subjects' urine samples are reported in Table 2.2, in μg/g creatinine, and in Table 2.3, in μg/L. Concentrations of mercapturic acids were greater than the LOQ in all samples for 2-HPMA, 3-HPMA, AAMA, AMCC, CMEMA, DHBMA, GAMA, and SBMA; quantifiable in most samples (from 86% to 99%) for CEMA, EMA, HEMA, HPMPA, MHBMA, MMA, PHEMA, and SPMA; while NANPC was quantified only in 4% of samples. For this reason, NANPC was not included in statistical analyses.

The results of the Student's t-test performed to compare controls and workers revealed significant differences for CEMA ($p < 0.001$), MHBMA ($p = 0.001$), PHEMA ($p < 0.001$), and SPMA ($p < 0.001$).

The linear model computed to estimate the determinants of each mercapturic acid (μg/L) in workers compared to controls, and corrected for age, creatinine, cotinine, and exposure during last 3 days, showed a significant increase in levels of CEMA ($p < 0.001$, GMR = 1.75), MHBMA ($p = 0.010$, GMR = 2.06), PHEMA, ($p < 0.001$, GMR = 2.15), and SPMA ($p < 0.001$, GMR = 9.53). Furthermore, creatinine was a variable associated with a significant increase of all mercapturic acids (GMR from 6.7 for HEMA to 145.01 for MHBMA); age was associated with a significant increase of AMCC, CMEMA, DHBMA, and SPMA; cotinine was associated with a significant increase of AAMA, AMCC, CEMA, and GAMA; finally, the assessment of activities at home involving exposure during the last 3 days was significantly associated with an increase of AMCC, CEMA, and SPMA, showing a possible non-occupational exposure. The adjusted coefficient of determination (R^2) ranged from 0.08 (EMA) to 0.56 (SPMA) and was significantly different from zero for all mercapturic acids (Table 2.4 and Figure 2.1).

The second linear model built considering only the coke oven workers, which was corrected for age, creatinine, and cotinine, showed that both the production plant and the job title were associated with an increase for some mercapturic acids. In

particular, considering the plant J as reference, workers of plant D had significantly higher levels of SPMA (p = 0.008, GMR = 3.00), while workers of plant R had significantly higher levels of AAMA (p = 0.013, GMR = 2.50). Considering values of foremen as reference, engine operators had no significantly different levels of any of the considered mercapturic acids, while coke makers had significantly higher levels of PHEMA (p = 0.023, GMR = 2.04) and SPMA (p = 0.032, GMR = 2.71). Workers declaring that their skin was dirty at the end of the work-shift had significantly higher levels of 3-HPMA (p < 0.001, GMR = 15.48), DHBMA (p = 0.010, GMR = 2.35), GAMA (p = 0.029, GMR = 1.78), HMPMA (p < 0.001, GMR = 6.70), and SBMA (p = 0.018, GMR = 4.13). The adjusted coefficient of determination (R^2) ranged from 0.05 (HEMA) to 0.56 (GAMA) and was significantly different from zero for all mercapturic acids but for AMCC, CMEMA, EMA, HEMA (Table 2.5).

Table 2.2 - Median, 5th, and 95th percentile for the levels of mercapturic acids in subjects' urine samples, expressed as µg/g of creatinine, after grouping by controls and workers. For each compound, the limit of quantitation (LOQ) is also reported, along with the percentage of quantified samples. Finally, the P-value of the Student's T-test performed on log_{10}-transformed values is reported to evaluate differences between the two groups.

Mercapturic acid	Metabolite of	LOQ (µg/L)	Statistics	Controls (*n* = 49) (µg/g creatinine)	Workers (*n* = 49) (µg/g creatinine)	T-test on log_{10}-transformed data (*p*-value)
2-HPMA	propylene oxide	0.5	Median	3.5	4.8	0.293
			5th–95th	0.9–11.6	1.1–11.1	
			%>LOQ	100	100	
3-HPMA	acrolein	0.2	Median	219.1	215.7	0.092
			5th–95th	81.2–1109.1	26.7–841.1	
			%>LOQ	100	100	
AAMA	acrylamide	3.2	Median	21.3	25.8	0.385
			5th–95th	11.5–117.5	8.9–97.5	
			%>LOQ	100	100	
AMCC	N,N-dimethylformamide	2	Median	112	119	0.272
			5th–95th	40–214	34–256	
			%>LOQ	100	100	
CEMA	acrylonitrile	0.9	Median	1.4	3.7	<0.001
			5th–95th	<LOQ–16.6	1.4–9.6	
			%>LOQ	88	98	
CMEMA	crotonaldehyde	2	Median	300	265	0.613
			5th–95th	99–809	97–1080	
			%>LOQ	100	100	
DHBMA	1,3-butadiene	1.0	Median	177.2	212.7	0.222
			5th–95th	109.7–345.4	96.8–413.5	
			%>LOQ	100	100	
EMA	ethylating agents	0.01	Median	0.04	0.03	0.153
			5th–95th	<LOQ–0.32	<LOQ–0.11	
			%>LOQ	82	90	
GAMA	acrylamide	1.0	Median	5.0	6.0	0.092
			5th–95th	2.5–13.3	3.3–11.8	
			%>LOQ	100	100	
HEMA	acrylonitrile ethylene oxide	0.3	Median	0.5	0.6	0.658
			5th–95th	<LOQ–1.6	<LOQ–1.6	
			%>LOQ	86	86	
HMPMA	crotonaldehyde	2	Median	109	101	0.262
			5th–95th	56–270	46–278	
			%>LOQ	100	98	
MHBMA	1,3-butadiene	0.04	Median	0.42	1.10	0.001
			5th–95th	<LOQ–2.47	0.18–3.35	
			%>LOQ	90	96	
MMA	methylating agents	0.09	Median	3.53	2.95	0.175
			5th–95th	0.66–11.28	<LOQ–12.61	
			%>LOQ	100	92	
NANPC	4-chloronitrobenze	0.11	Median	<LOQ	<LOQ	NA
			5th–95th	<LOQ	<LOQ	
			%>LOQ	4	4	
PHEMA	styrene	0.01	Median	0.07	0.15	<0.001
			5th–95th	<LOQ–0.23	0.04–0.4	
			%>LOQ	88	100	
SBMA	toluene	0.02	Median	0.62	0.80	0.316
			5th–95th	0.22–2.00	0.25–3.58	
			%>LOQ	100	100	
SPMA	benzene	0.01	Median	0.02	0.31	<0.001
			5th–95th	<LOQ–0.25	0.04–2.98	
			%>LOQ	71	100	

NA: not assessed.

Table 2.3 - Median, 5th and 95th percentile for the levels of mercapturic acids in subjects' urine samples, expressed as µg/L, after grouping by controls and workers. For each compound, the limit of quantitation (LOQ) is also reported, along with the percentage of quantified samples. Finally, P-value of the student's T-test performed on log10 transformed values is reported to evaluate differences between the two groups.

	LOQ (µg/L)	Statistics	Controls (n=49) (µg/L)	Workers (n=49) (µg/L)	T-test on log_{10} transformed data P-value
2-HPMA	0.5	**Median**	5.5	8.9	0.13
		5th - 95th	1.4 - 27.2	1.5 - 19.6	
		%>LOQ	100	100	
3-HPMA	0.2	**Median**	317.6	440.2	0.283
		5th - 95th	116.8 - 2109.0	35.1 - 1505.9	
		%>LOQ	100	100	
AAMA	3.2	**Median**	35.7	54.6	0.151
		5th - 95th	13.8 - 212.8	11.8 - 204.8	
		%>LOQ	100	100	
AMCC	2	**Median**	174	208	0.081
		5th - 95th	53 - 445	64 - 520	
		%>LOQ	100	100	
CEMA	0.9	**Median**	2.3	6.6	<0.001
		5th - 95th	<LOQ - 19.0	1.7 - 20.9	
		%>LOQ	88	98	
CMEMA	2	**Median**	483	531	0.82
		5th - 95th	114 - 1744	109 - 2057	
		%>LOQ	100	100	
DHBMA	1.0	**Median**	307.5	410.4	0.082
		5th - 95th	125.3 - 769.2	121.4 - 910.0	
		%>LOQ	100	100	
EMA	0.01	**Median**	0.06	0.05	0.393
		5th - 95th	<LOQ - 0.49	<LOQ - 0.22	
		%>LOQ	82	90	
GAMA	1.0	**Median**	7.5	11.5	0.032
		5th - 95th	3.5 - 27.0	4.5 - 27.7	
		%>LOQ	100	100	
HEMA	0.3	**Median**	0.9	1.3	0.28
		5th - 95th	<LOQ - 2.8	<LOQ - 3.0	
		%>LOQ	86	86	
HMPMA	2	**Median**	181	205	0.747
		5th - 95th	71 - 515	59 - 489	
		%>LOQ	100	98	
MHBMA	0.04	**Median**	0.85	1.86	0.001
		5th - 95th	<LOQ - 6.56	0.29 - 6.67	
		%>LOQ	90	96	
MMA	0.09	**Median**	6.12	5.89	0.438
		5th - 95th	0.59 - 22.07	<LOQ - 21.88	
		%>LOQ	100	92	

Table 2.3 - Continue

	LOQ (µg/L)	Statistics	Controls (n=49) (µg/L)	Workers (n=49) (µg/L)	T-test on log_{10} transformed data P-value
NANPC	0.11	**Median**	<LOQ	<LOQ	
		5th - 95th	<LOQ - <LOQ	<LOQ - <LOQ	
		%>LOQ	4	4	NA
PHEMA	0.01	**Median**	0.11	0.27	<0.001
		5th - 95th	<LOQ - 0.41	0.05 - 0.88	
		%>LOQ	88	100	
SBMA	0.02	**Median**	0.98	1.26	0.141
		5th - 95th	0.26 - 4.26	0.32 - 6.21	
		%>LOQ	100	100	
SPMA	0.01	**Median**	0.03	0.48	<0.001
		5th - 95th	<LOQ - 0.33	0.09 - 4.87	
		%>LOQ	71	100	

Table 2.4 - Results of the linear models built to evaluate the differences between controls and workers. The dependent variable of each linear model was the log_{10}-transformed value of a mercapturic acid (µg/L). The independent variables were study group (controls or workers), log_{10} creatinine (g/L), age (years), log_{10} cotinine (µg/L), hobbies at home involving exposure during the last 3 days (no or yes) (n = 94, four observations removed due to missing values).

Mercapturic acids	Group = workers (reference = controls)	Log_{10} creatinine (g/L)		Age (years)		Log_{10} cotinine (µg/L)		Exposure last 3 days = yes (reference = no)	R^2 adj *p*-value
	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	
2-HPMA2	1.05 0.75–1.46 0.77	20.20 7.39–55.17 <0.001	0.54 0.37–0.67 <0.001	1.00 0.98–1.02 0.906	−0.01 −0.21–0.19 0.904	1.12 0.90–1.39 0.305	0.11 −0.10–0.31 0.294	1.36 0.92–1.99 0.117	0.31 <0.001
3-HPMA3	0.63 0.39–1.02 0.06	26.01 6.05–111.85 <0.001	0.43 0.25–0.58 <0.001	1.01 0.99–1.04 0.257	0.12 −0.08–0.32 0.247	1.19 0.87–1.62 0.28	0.12 −0.09–0.31 0.269	1.00 0.57–1.74 0.998	0.16 <0.001
AAMA	0.98 0.72–1.33 0.889	14.22 5.62–35.98 <0.001	0.52 0.35–0.65 <0.001	1.01 1.00–1.03 0.164	0.15 −0.06–0.34 0.154	1.28 1.05–1.57 0.014	0.26 0.06–0.44 0.012	1.08 0.76–1.54 0.677	0.29 <0.001
AMCC	1.00 0.79–1.27 0.989	10.09 4.8820.85 <0.001	0.56 0.40–0.68 <0.001	1.02 1.01–1.03 0.004	0.30 0.11–0.48 0.003	1.20 1.03–1.40 0.022	0.24 0.04–0.42 0.019	1.61 1.22–2.13 <0.001	0.39 <0.001

Table 2.4 - Continue

Mercapturic acids	Group = workers (reference = controls)	Log_{10} creatinine (g/L)		Age (years)		Log_{10} cotinine (µg/L)		Exposure last 3 days = yes (reference = no)	R^2 adj
	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	*p*-value
CEMA	1.75 1.26–2.42 <0.001	7.98 2.97–21.43 <0.001	0.41 0.22–0.56 <0.001	1.01 0.99–1.02 0.455	0.08 -0.12–0.28 0.444	1.88 1.52–2.32 <0.001	0.53 0.37–0.66 <0.001	1.63 1.12–2.38 0.011	0.52 <0.001
CMEMA	0.95 0.68–1.31 0.741	18.73 6.96–50.41 <0.001	0.53 0.37–0.66 <0.001	1.02 1.00–1.03 0.039	0.22 0.02–0.40 0.035	0.94 0.76–1.16 0.563	−0.06 −0.26–0.14 0.555	0.98 0.67–1.43 0.907	0.25 <0.001
DHBMA	1.05 0.86–1.28 0.662	21.62 11.77–39.74 <0.001	0.73 0.62–0.81 <0.001	1.01 1.00–1.02 0.019	0.25 0.05–0.43 0.016	1.13 0.99–1.29 0.061	0.2 0.00–0.39 0.055	1.06 0.84–1.33 0.632	0.54 <0.001
EMA	0.75 0.48–1.19 0.226	9.02 2.23–36.51 0.002	0.32 0.12–0.49 0.002	1.02 0.99–1.04 0.176	0.14 −0.06–0.34 0.167	0.84 0.63–1.14 0.261	−0.12 −0.31–0.08 0.25	1.22 0.71–2.07 0.469	0.08 0.027
GAMA	1.10 0.90–1.34 0.363	14.62 7.95–26.88 <0.001	0.68 0.56–0.78 <0.001	1.01 1.00–1.02 0.136	0.16 −0.05–0.35 0.127	1.19 1.05–1.36 0.009	0.28 0.08–0.45 0.007	0.99 0.78–1.25 0.926	0.49 <0.001
HEMA	1.00 0.73–1.38 0.994	6.7 2.55–17.63 <0.001	0.38 0.20–0.54 <0.001	0.99 0.98–1.01 0.378	−0.09 −0.29–0.11 0.368	0.98 0.79–1.20 0.814	–0.03 −0.23–0.18 0.81	1.33 0.92–1.93 0.128	0.15 0.001
HMPMA	0.85 0.62–1.18 0.341	13.6 5.03–36.79 <0.001	0.49 0.31–0.63 <0.001	1 0.98–1.02 0.922	0.01 −0.19–0.21 0.921	1.11 0.90–1.38 0.315	0.11 −0.10–0.30 0.304	0.99 0.68–1.45 0.96	0.21 <0.001
MHBMA	2.06 1.19–3.57 0.010	145.01 27.31–769.98 <0.001	0.53 0.37–0.66 <0.001	1.02 0.99–1.05 0.181	0.14 −0.06–0.34 0.171	1.2 0.84–1.72 0.313	0.11 −0.10–0.30 0.302	1.01 0.54–1.92 0.966	0.33 <0.001
MMA	0.63 0.38–1.05 0.078	86.08 18.39–402.96 <0.001	0.52 0.36–0.66 <0.001	1.01 0.99–1.04 0.375	0.09 −0.11–0.29 0.364	0.91 0.66–1.27 0.591	−0.06 −0.26–0.15 0.583	1.31 0.73–2.36 0.364	0.24 <0.001
PHEMA	2.15 1.46–3.16 <0.001	36.9 11.38–119.66 <0.001	0.54 0.38–0.67 <0.001	0.99 0.97–1.01 0.38	−0.09 −0.29–0.11 0.37	1.19 0.93–1.53 0.168	0.15 −0.06–0.34 0.159	0.99 0.63–1.55 0.965	0.43 <0.001

Table 2.4 - Continue

Mercapturic acids	Group = workers (reference = controls)	Log_{10} creatinine (g/L)		Age (years)		Log_{10} cotinine (µg/L)		Exposure last 3 days = yes (reference = no)	R^2 adj *p*-value
	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	r (95%CI) *p*-value	GMR (95%CI) *p*-value	
SBMA	1.09 0.78–1.52 0.606	33.41 12.10–92.27 <0.001	0.59 0.44–0.71 <0.001	1.03 1.01–1.04 0.001	0.33 0.14–0.50 0.001	1.01 0.81–1.25 0.962	0.01 −0.20–0.21 0.961	1.37 0.93–2.02 0.108	0.36 <0.001
SPMA	9.53 5.71–15.91 <0.001	9.9 2.09–46.93 0.004	0.3 0.10–0.47 0.004	1.01 0.98–1.03 0.582	0.06 −0.15–0.26 0.574	1.28 0.91–1.78 0.149	0.15 −0.05–0.35 0.14	2.09 1.15–3.79 0.016	0.56 <0.001

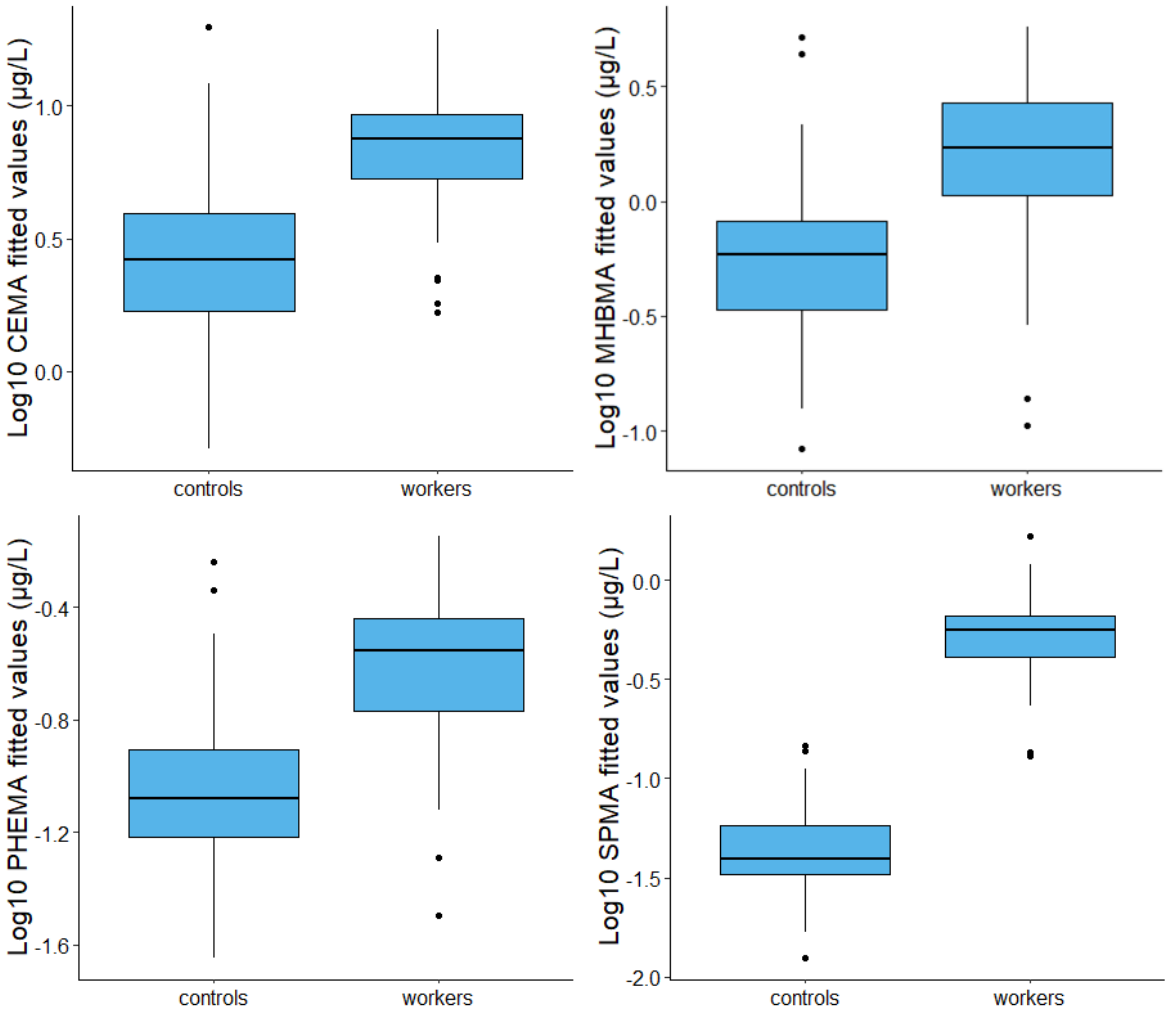

Figure 2.1 - Box plot obtained from fitted values of linear models reported in Table 2.4, showing the distribution of CEMA, MHBMA, PHEMA, and SPMA in controls and coke oven workers. A box plot is a graphical representation of the data distribution. The box contains the 50% of the observations, with the median dividing the box in two areas and the upper and lower hinge representing the 25th and 75th percentile of the distribution. Outside the box, the upper whisker extends from the hinge to the highest value no further than 1.5 × interquartile range (IQR) from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 × IQR of the hinge. Data beyond the whiskers are plotted individually and represented as dots.

Table 2.5 - Results of the linear models built to evaluate the differences in levels of considered analytes among workers. The dependent variable of each linear model was the log_{10}-transformed value of a mercapturic acid (µg/L). The independent variables were: plant (J = reference, D, or R), job title (foremen = reference, engine operators, or coke markers), dirty skin (no or yes), age (years), log_{10} creatinine (g/L), and log_{10} cotinine (µg/L) (*n* = 49).

Mercapturic acids	**Company = D (reference = J)**	**Company = R (reference = J)**	**Job title = engine operators (reference = foremen)**	**Job title = coke makers (reference = foremen)**	**Dirty skin = yes (reference = no)**	
	GMR (95%CI) *p*-value	**GMR (95%CI) *p*-value**	**GMR (95%CI) *p*-value**	**GMR (95%CI) *p*-value**	**GMR (95%CI) *p*-value**	**R^2 adj *p*-value**
2-HPMA	1.18 0.71–1.96 0.504	1.06 0.51–2.19 0.882	1.29 0.62–2.71 0.487	0.70 0.39–1.26 0.230	0.73 0.28–1.90 0.516	0.23 0.015
3-HPMA	1.49 0.67–3.27 0.317	1.15 0.37–3.57 0.807	0.86 0.27–2.71 0.786	1.15 0.46–2.86 0.755	15.48 3.51–68.30 <0.001	0.31 0.002
AAMA	1.04 0.63–1.70 0.888	2.50 1.23–5.11 0.013	1.17 0.57–2.42 0.656	0.85 0.48–1.50 0.562	1.24 0.49–3.15 0.646	0.26 0.007
AMCC	1.10 0.69–1.76 0.673	1.04 0.53–2.04 0.900	0.79 0.40–1.55 0.481	1.06 0.62–1.82 0.821	2.23 0.93–5.35 0.073	0.15 0.065
CEMA	1.55 0.97–2.46 0.066	0.91 0.46–1.77 0.772	0.80 0.40–1.57 0.505	1.30 0.76–2.22 0.331	1.09 0.46–2.63 0.836	0.35 <0.001
CMEMA	1.38 0.78–2.46 0.264	0.87 0.38–1.99 0.730	1.10 0.48–2.56 0.815	1.29 0.67–2.50 0.441	2.67 0.90–7.89 0.074	0.14 0.072
DHBMA	1.39 0.99–1.94 0.058	1.36 0.84–2.20 0.209	1.21 0.74–1.98 0.443	1.32 0.89–1.94 0.161	2.35 1.24–4.42 0.010	0.50 <0.001
EMA	1.27 0.68–2.37 0.452	0.61 0.25–1.49 0.266	1.55 0.62–3.88 0.336	1.00 0.49–2.06 0.997	1.32 0.41–4.29 0.636	0.02 0.362
GAMA	0.88 0.67–1.16 0.362	1.08 0.73–1.60 0.684	1.18 0.79–1.75 0.414	1.27 0.93–1.73 0.134	1.78 1.06–2.96 0.029	0.56 <0.001
HEMA	0.93 0.51–1.68 0.803	0.60 0.25–1.40 0.229	0.92 0.39–2.18 0.841	1.13 0.57–2.25 0.711	0.62 0.20–1.88 0.385	0.05 0.274

Table 2.5 - Continue

Mercapturic acids	Company = D (reference = J)	Company = R (reference = J)	Job title = engine operators (reference = foremen)	Job title = coke makers (reference = foremen)	Dirty skin = yes (reference = no)	
	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	GMR (95%CI) *p*-value	R^2 adj *p*-value
HMPMA	1.62 0.94–2.81 0.082	2.05 0.93–4.51 0.075	0.99 0.44–2.21 0.983	1.26 0.67–2.38 0.461	6.70 2.38–18.81 <0.001	0.34 0.001
MHBMA	1.81 0.88–3.71 0.103	1.98 0.71–5.57 0.187	1.04 0.37–2.97 0.939	1.39 0.61–3.17 0.428	2.76 0.72–10.65 0.136	0.23 0.014
MMA	1.90 0.74–4.91 0.179	1.44 0.37–5.65 0.591	1.07 0.27–4.29 0.919	0.96 0.32–2.86 0.940	2.86 0.48–17.03 0.242	0.22 0.019
PHEMA	0.71 0.41–1.20 0.193	0.75 0.35–1.60 0.444	1.30 0.60–2.82 0.505	2.04 1.11–3.77 0.023	0.76 0.28–2.06 0.577	0.32 0.002
SBMA	0.95 0.51–1.77 0.873	0.57 0.24–1.40 0.216	0.83 0.34–2.05 0.679	1.01 0.49–2.07 0.976	4.13 1.29–13.29 0.018	0.18 0.036
SPMA	3.00 1.36–6.59 0.008	1.21 0.39–3.76 0.732	2.38 0.75–7.50 0.136	2.71 1.10–6.72 0.032	0.92 0.21–4.04 0.909	0.22 0.019

2.4 Discussion

In this work, we assessed the occupational exposure to VOCs in coke oven workers using seventeen urinary mercapturic acids as biomarkers.

Higher concentrations of urinary CEMA, MHBMA, PHEMA, and SPMA were found in coke oven workers than in controls, indicating an occupational exposure to acrylonitrile, 1,3-butadiene, styrene, and benzene. However, several other mercapturic acids were similar in these groups, showing that coke oven workers were not exposed to the majority of the considered VOCs (Table 2.2).

The exposure to VOCs was generally low when compared to occupational limit values. Considering SPMA, metabolite of benzene, the levels in workers were about an order of magnitude lower than the biological exposure indices (BEI) (25 µg/g creatinine) proposed by ACGIH (ACGIH, 2017). It is worth mentioning that lower limit values have been recently proposed for benzene. In particular, ECHA has recently proposed a biological limit value (BLV) equal to 2 µg/g creatinine, corresponding to an occupational limit value of 0.2 mg/m^3 of airborne benzene. Among our study subjects, three workers exceeded this limit. ECHA also suggested a biological guidance value (BGV) equal to 0.5 µg/g creatinine; all subjects in the control group had SPMA levels below than this value, while 15 workers had higher levels (ECHA, 2018). Furthermore, levels of SPMA were comparable with Italian reference values (only non-smokers: 0.18 µg/g creatinine, 95th percentile) (SIVR, 2017) and US reference values (3.03 µg/g creatinine, 95th percentile) (NHANES, 2017). Comparing the concentration of the other mercapturic acids with biological reference values (BARs) (Table 2.1) we note that 3-HPMA (metabolite of acrolein) was higher than the BAR values in seven controls and in five workers (about 12% of the total population in study); AAMA (metabolite of acrylamide) was higher than the BAR values in five controls and two workers (about 7% of the total population in study); DHBMA (metabolite of 1,3-butadiene) was higher than the BAR value in two controls and three workers (about 5% of the total population in study), MHBMA (another metabolite of 1,3-butadiene) was higher than the BAR value in four controls and nine workers (about 13 % of the total population in study). Interestingly, all the subjects (both controls and workers) had levels of AMCC (metabolite of N,N-dimethylformamide) higher than biological tolerance value for occupational exposure (BAT) (DFG, 2018).

The levels of mercapturic acids found in the present study were largely comparable with those previously reported for non-smokers of the general population; differences were noted only for HMPMA and SBMA, with control subjects of this study showing higher levels for HMPMA and lower levels for SBMA than in previous works (Pluym et al., 2015; Schettgen et al., 2009; Schettgen et al., 2008).

Comparing workers and controls, higher concentrations of CEMA, MHBMA, PHEMA, and SPMA were found in workers, but the increased association with occupational exposure was small in comparison to the association with tobacco smoking. Median levels of CEMA were 3.7 and 1.4 µg/g creatinine in controls and in workers, respectively; while the levels reported in smokers were some orders of magnitude higher with mean/median levels ranging from 72.5 to 240 µg/g creatinine (Pluym et al., 2015; Schettgen et al., 2009; Goniewicz et al., 2018; Keith et al., 2019). MHBMA levels were 0.42 and 1.10 µg/g creatinine in controls and in workers, respectively; reported levels of this metabolite for smokers ranged from similar to the levels found in workers (1.08 µg/g creatinine) (Pluym et al., 2015) to an order of magnitude higher (19.5–27.9 µg/g creatinine) (Keith et al., 2019; Goniewicz et al., 2018). PHEMA was significantly higher in workers than in controls with a median level equal to 0.15 versus 0.07 µg/g creatinine. Once again, these results were lower if compared to values reported in smoking subjects, with mean/median values from 0.83 to 2.3 µg/g creatinine (Pluym et al., 2015; Keith et al., 2019). SPMA levels were an order of magnitude higher in workers than in controls (median 0.31 vs. 0.02 µg/g creatinine). Nevertheless, levels were still lower if compared with smoking subjects, for which median/mean values close to 1 µg/g creatinine have been reported (Pluym et al., 2015; Goniewicz et al., 2018).

The possible exposure to benzene in coke oven workers has been reported in previous studies. Dehghani and co-workers determined the levels of benzene, toluene, xylene, and ethylbenzene in the breathing zone air of workers through individual sampling in the coke-making unit of a steel plant and found that benzene levels were higher than the local exposure limits (Dehghani et al., 2018). In another study, toluene and benzene were among the highest VOCs present in the workplace air of the coke-making process; however, the authors evidenced that the concentrations of workplace air pollutants were lower than their local hazardous air pollutant standards for workplace air (Tsai et al., 2008). In another work, personal air samples were analyzed to determine benzene, toluene, and other VOCs, and it was reported that exposure levels of coke workers were relatively low if compared

to the exposure limits (Bieniek et al., 2004). Only a few other studies quantified some mercapturic acids in coke oven workers. In particular, Fan and co-workers determined the concentrations of SPMA and SBMA in coke oven workers and found no significant differences when compared with a control group of farmers. It is noteworthy to mention, though, that the values reported were surprisingly high (median levels of 2.14 and 5.30 µg/g creatinine for SPMA and SBMA, respectively) and this might be due to the presence of tobacco smokers among study subjects, which was not controlled (Fan et al., 2014). Lovreglio and co-workers, assessing SPMA levels, revealed that coke oven workers were exposed to a low level of benzene and reported a median level of SPMA in the end-shift urine equal to 0.50 µg/g creatinine (from 0.10 to 6.89 µg/g creatinine); they also reported higher concentrations in oven standpipe workers than in byproduct workers (Lovreglio et al., 2018). Previous studies reported urinary levels of SPMA from 3.16 to 34.53 µmol/mol creatinine (6.68–72.96 µg/g creatinine) (Colman and Coleman, 2006), from 0.4 to 62.6 µmol/mol creatinine (0.85–132.3 µg/g creatinine) (Pople et al., 2002), from 0.40 to 38.56 µg/g creatinine (Hotz et al., 1997), and from <0.3 to 1020 µg/g creatinine (Kivistö et al., 1997).

Urinary levels of mercapturic acids are influenced by several factors, including environmental exposures. The linear models presented in Table 2.4 aimed to compare workers to controls while correcting for potential confounders. The inclusion of urinary cotinine levels was useful to correct for environmental tobacco smoke: indeed, even though subjects were non-smokers, most of them had quantifiable low levels of cotinine, thus showing an exposure to second-hand tobacco smoke; furthermore, unlike what was declared, three control subjects were probably active smokers due to their very high cotinine levels (>50 µg/L). These linear models highlighted that CEMA, MHBMA, PHEMA, and SPMA were significantly different between controls and workers, with levels about 2-fold (CEMA, MHBMA, and PHEMA) and 10-fold (SPMA) higher in workers, thus showing an exposure to benzene and, with a lower impact, an exposure to acrylonitrile, 1,3-butadiene, and styrene.

Among coke oven workers, the possible determinants of mercapturic acids were studied (Table 2.5). Some significant differences in companies were found, showing the variability across the different plants: indeed, if compared to the plant producing low-phosphor coke and broken coke (J), significant higher levels of SPMA, metabolite of benzene, were found in the plant producing domestic coke (D), and significant higher levels of AAMA, metabolite of acrylamide, were found in the plant producing foundry and blast furnace coke (R). Comparing job titles, engine operators were similar to foremen, while coke markers had significantly higher concentrations (about 2–3 fold) of PHEMA and SPMA than foremen, thus showing that the task of coke markers was associated with higher exposure to styrene and benzene. The most important determinant of exposure among coke oven workers was the dirty skin variable: indeed, during the interview, workers were asked whether they had dirty skin at the end of the work-shift. The subjects who answered affirmatively had about 15-fold higher levels of 3-HPMA (metabolite of acrolein), 1.5-fold higher levels of DHBMA (metabolite of 1,3-butadiene), 1.8-fold higher levels of GAMA (metabolite of acrylamide), 6.7-fold higher levels of HMPMA (metabolite of crotonaldehyde), and 4-fold higher values of SBMA (metabolite of toluene). Not having the skin dirty could be a sign of higher working skill and/or the correct use of personal protective equipment, and both concur to decrease the body burden of VOCs.

Since it has been extensively reported that active tobacco smoke represents the most important non-occupational exposure to VOCs (IARC, 2004), tobacco smoking was an exclusion criterion during the enrollment. This is a strength of this work, further strengthened by the analysis of urinary cotinine, which revealed the presence of three active smokers and an overall exposure to second-hand smoke. To take this exposure into consideration, urinary cotinine was included as independent variable into the linear regressions. Another strength of this study is that several variables were tested for their inclusion in the linear models, since levels of mercapturic acids might be influenced by several other confounding factors, such as diet (Ji et al., 2013; Goerke et al., 2019) and environmental pollution (Borgie et al., 2014;

Manuela et al., 2012). Finally, to our knowledge, this is the first time that such a broad spectrum of mercapturic acids has been determined in urine samples of coke oven workers. A limitation of this study is that the results cannot be representative of all workers of a coke plant since, in a coke refinery, other job titles are present such as byproduct workers. Indeed, a previous study assessing occupational exposure through personal air measurements showed that the exposure to benzene, toluene, and xylene was higher in coke byproduct workers, while coke oven workers are more exposed to PAHs (Bieniek and Łusiak, 2012).

In conclusion, the quantitation of several urinary mercapturic acids showed that coke oven workers were exposed to higher levels of benzene (about 10-fold), acrylonitrile, 1,3-butadiene, and styrene (about 2-fold) than control subjects. However, the impact of working exposure to the body burden of VOCs was within 20% of existing biological limit values for most biomarkers; for SPMA only three subjects exceeded the restrictive biological limit value recently proposed by the ECHA.

3 Biomonitoring of subjects with different smoking habits by quantitation of urinary mercapturic acids

Most of the content of this chapter has been published in *Toxicology Letters*.

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3.1 Introduction

Although tobacco smoking is carcinogenic to humans (group 1 according to the International Agency of Cancer Research, IARC) (IARC, 2004; IARC, 2012c), its prevalence is still considerable since smokers represent 22.5% of the global population (32.0% of males and 7.0% of females) (Gowing et al., 2015). In Italy, the prevalence is very similar to the global one since 22% of Italian people are smokers (28% of males and 16.5% of female) (Pacifici, 2019). Tobacco smoke is a mixture of chemicals containing more than 5000 compounds (Rodgman and Perfetti, 2013), among which over 70 have been classified as carcinogens (IARC, 2004; IARC, 2012c). The list of carcinogenic chemicals (IARC group 1) includes 1,3-butadiene (IARC, 2012b), ethylene oxide (IARC, 2012b) and benzene (IARC, 2018a). Furthermore, probably carcinogenic chemicals (group 2A) such as acrylamide (IARC, 1994) and styrene (IARC, 2018b) and possibly carcinogenic chemicals (group 2B) such as propylene oxide (IARC, 1994) and acrylonitrile (IARC, 1999), are present (Table 3.1).

Besides traditional tobacco smoking, electronic cigarette is a relatively new product that is gaining increasing consideration. While traditional cigarettes use combustion to burn tobacco, electronic cigarette use electricity to heat and aerosolize a liquid containing nicotine and flavourings. The prevalence of electronic cigarettes is increasing among both adolescents and adults (Breland et al., 2017), likely due to the low perceived risk associated with this smoking mode. Among Italian people, 1.7% uses electronic cigarette (Pacifici, 2019). While the presence of nicotine in the aerosol generated from the electronic cigarette is considerable (Goniewicz et al., 2013), the amount of toxicants and carcinogens is lower if compared to traditional tobacco smoke (Goniewicz et al., 2014). Few evidences reported the presence of some toxic compounds in both vapour (Laugesen, 2008; Goniewicz et al., 2014; Uchiyama et al., 2013; Sleiman et al., 2016; Schripp et al., 2013) and liquid (Varlet et al., 2015; Lim and Shin, 2017; Sleiman et al., 2016) of electronic cigarettes (quantified analytes are reported in Table 3.1).

Although the internal dose of carcinogens, assessed quantifying urinary mercapturic acid levels, in traditional tobacco smokers has extensively been reported, only few evidences on electronic cigarette smokers are present, especially quantifying a wide range of mercapturic acids (Goniewicz et al., 2018; Keith et al., 2019).

The aim of this study was to assess exposure to a different toxic chemicals, among which some carcinogens, in subjects with different smoking habits, namely non-smokers (NS), traditional tobacco smokers (S) and electronic cigarette smokers (e-cig), using urinary mercapturic acids as biomarkers, and to perform a comparison between the burden of toxicants associated with different smoking modes.

Table 3.1 - List of toxic compounds, information about their presence in tobacco and electronic cigarette smoke according to the literature, and derived mercapturic acids investigated in this study.

Toxic compounds	**IARC classification of carcinogenicty[a]**	**Presence in tobacco smoke**		**Presence in electronic cigarette**		**Urinary mercapturic acids investigated**
		Mainstream smoke[b]	**Sidestream smoke[b]**	**Vapour**	**Liquid**	
1,3-butadiene	1	20-122.5 µg/cigarette (IARC, 2004)	81.3–250 µg/cigarette (IARC, 2004)	Not detected in e-cig mist (Laugesen, 2008)	Detected in 2% of refill liquids (10 µg/g) (Varlet et al., 2015)	DHBMA MHBMA
4-chloronitrobenzene	2B	NF	NF	NF	NF	NANPC
acrolein	3	51.2–223.4 µg/cigarette (IARC, 2004)	342.1–1000 µg/ cigarette (IARC, 2004)	Not detected in e-cig mist (Laugesen, 2008) N.D. - 41.9 µg/150 puffs (Goniewicz et al., 2014) N.D. - 36 mg/m^3 (Uchiyama et al., 2013) 0.17 – 3.70 µg/cigarette equivalent (Papoušek et al., 2014) 120 - 10060 ng/mg in mainstream vapour (mass per e-liquid consumed) (Sleiman et al., 2016)	Detected in 7% of refill liquids; in those positive: 0.18 - 1.03 µg/g (Varlet et al., 2015)	3-HPMA
acrylamide	2A	Present (IARC, 2004)	NF	No detectable levels (Papoušek et al., 2014)	NF	AAMA GAMA
acrylonitrile	2B	3-39.1 µg/cigarette(IARC, 2004)	24.1–43.9 µg/cigarette (IARC, 2004)	NF	NF	CEMA HEMA
benzene	1	12–105.9 µg/cigarette (IARC, 2004)	70.7–529 µg/cigarette (IARC, 2004)	Not detected in e-cig mist (Laugesen, 2008) <1 µg/m^3 emission test chamber measurement (Schripp et al., 2013) 34 – 440 ng/mg in mainstream vapour (mass per e-liquid consumed) (Sleiman et al., 2016)	Detected in 24% of nicotine liquids; in those positive: 0.008 - 2.28 mg/L (Lim and Shin, 2017)	SPMA

Table 3.1 - Continue

Toxic compounds	IARC classification of carcinogenicty[a]	Presence in tobacco smoke		Presence in electronic cigarette		Urinary mercapturic acids investigated
		Mainstream smoke[b]	Sidestream smoke[b]	Vapour	Liquid	
crotonaldehyde	3	11.6–66.2 µg/cigarette (IARC, 2004)	62.2–121.8 µg/cigarette (IARC, 2004)	N.D - 720 ng/mg in mainstream vapour (mass per e-liquid consumed) (Sleiman et al., 2016)	Detected in 5% of refill liquids; in those positive: 0.067 - 0.084 µg/g) (Varlet et al., 2015)	CMEMA HMPMA
N-nitrosodiethylamine and others ethylating agents	2A	ND–25 ng (IARC, 2004)	NF	NF	NF	EMA
ethylene oxide	1	7 µg/cigarette (IARC, 2004)	NF	NF	Detected in 5% of refill liquids; in those positive: 9 - 13 µg/g) (Varlet et al., 2015)	HEMA EMA
N-nitrosodimethylamine and others methylating agents	2A	0.1–180 ng/cigarette (IARC, 2004)	NF	NF	NF	MMA
N,N-dimethylformamide	2A	Present in tobacco smoke (Talhout et al., 2011)	NF	NF	NF	AMCC
propylene oxide	2B	0–100 ng/cigarette (IARC, 2004)	NF	Not detected in e-cig mist (Laugesen, 2008)	4.2 - 6.7 mg/mL in E-Liquid (Sleiman et al., 2016)	2-HPMA
styrene	2A	4.5–19.3 µg/cigarette (IARC, 2004)	23.2–46.1 µg/cigarette (IARC, 2004)	0.29 ppm in 38 mL sample of e-cigarette mist (Laugesen, 2008)	Detected in 11% of flavoured and nicotine liquids; in those positive: 0.011 - 0.201 mg/L (Lim and Shin, 2017)	PHEMA
toluene	3	48.3–173.7 µg/cigarette (IARC, 2004)	134.9–1060 µg/cigarette (IARC, 2004)	< 1 µg/m^3 emission test chamber measurement (Schripp et al., 2013) ND - 6.3 µg/150 puffs (Goniewicz et al., 2014)	Detected in 17 % of flavoured and nicotine liquids; in those positive: 0.006 - 0.687 mg/L (Lim and Shin, 2017)	SBMA

NF: information not found in the literature

ND: not detected

a IARC classification: group 1, carcinogenic to humans; group 2A, probably carcinogenic to humans; group 2B, possibly carcinogenic to humans; group 3; not classifiable as to its carcinogenicity to humans

b Mainstream smoke is the smoke released at the mouth end of the cigarette during puffing while sidestream smoke is the smoke released from the burning cone and through the cigarette paper (IARC, 2004).

3.2 Materials and methods

3.2.1 Study subjects

The study involved 67 healthy workers, belonging to a plant recycling exhausted oil, among which office workers (n = 9) and plant workers. (n = 58). Job tasks included plant management, exhaust oil receiving, remote and on-site plant control, plant maintenance, regenerated oil quality controls, regenerated oil storage and delivery.

For these workers a survey to assess the exposure to several chemicals, among which volatile organic compounds, was performed in June 2017. The survey protocol included the assessment of personal exposure during the work-shift and the biological monitoring, collecting a spot urine sample at the end of the shift. A questionnaire to collected data on personal characteristics, such as age, height, body weight, and smoking habits, and work activities was administered by the research team. Individuals were classified accordingly to their different smoking habits, in NS, S and e-cig.

The research was conducted in the frame of the risk assessment activity, according to the Italian legislation D.Lgs. 81/08, for the protection of workers' health, under the supervision of the plant occupational health service. Each study subject read, understood and signed the informed consent form.

3.2.2 Urine collection and mercapturic acids analysis

Urine samples were stored on site at 4 °C and delivered to the laboratory within 72 h; once in the laboratory they were stored at -20 °C until analysis.

The concentration of urinary mercapturic acids was assessed as reported in the section 3.

3.2.3 Urinary cotinine, nicotine and creatinine analysis

Cotinine and nicotine concentration was quantified with an LC-MS/MS method (Fustinoni et al., 2013). The limit of quantification was 0.1 µg/L for both cotinine and nicotine. Subjects with urinary cotinine >30 µg/L were classified as active smokers (either electronic or traditional tobacco smokers) (Campo et al., 2016a).

Creatinine in urine was measured using Jaffé reaction colorimetric method (Kroll et al., 1986).

3.2.4 Data processing and statistical analysis

The MultiQuant™ software (version 3.0.8664.0; AB Sciex) was used for chromatographic data integration. Statistical analysis was performed using both SPSS 25.0 for Windows (IBM SPSS Statistics) and RStudio (Version 1.2.1335).

A value corresponding to one-half of the LOQ was assigned to measurements below the analytical quantitation. Values were then adjusted for urinary creatinine and, for the descriptive analysis, median, 5^{th} and 95^{th} percentile values of the distribution were calculated. The variables were transformed into their decimal logarithms and a one-way analysis of variance (ANOVA) was applied to evaluate the difference among groups. A Bonferroni post-hoc test was also applied to evaluate differences between each group pairs.

Data were included in two different multiple linear regression models. The first model (model A) was applied to evaluate the effect of cotinine on the urinary mercapturic acids while the second model (model B) aimed to evaluate the effect of the smoking mode on the concentration of urinary mercapturic acids. The dependent variable in the linear regression model A was the $\log_{10}$ urinary mercapturic acid (µg/L), while independent variables were: $\log_{10}$ cotinine (µg/L), $\log_{10}$ creatinine (g/L), age (years), body mass index (BMI) (kg/m^2), gender (male or female), and occupational exposure (office workers or plant workers). The dependent variable in the linear regression model B was the $\log_{10}$ urinary mercapturic acid (µg/L), while independent variables were: smoking mode (NS or e-cig or S), $\log_{10}$ creatinine (g/L),

age (years), BMI (kg/m^2), gender (male or female), occupational exposure (office workers or plant workers).

The regression slopes were exponentiated to obtain the geometric mean ratio (GMR), representing the fold increase of geometric mean value of a mercapturic acid following a 10-fold increase in cotinine concentration for model A, and changing smoking mode from NS, taken as reference, to e-cig and S, for model B.

3.3 Results

3.3.1 Subjects, cotinine, nicotine and creatinine levels

The main characteristics of study subjects are reported in Table 3.2. Of the 67 subjects, only 4 were females; the age of the subjects ranged from 27 to 62 years and BMI ranged from 19.0 to 37.0 kg/m^2. According to questionnaire, 39 subjects classified themselves as NS, 7 as e-cig, and 21 as S. When the urinary cotinine cut-off ($\geq$ 30 µg/L) was applied, one of the self-declared non-smokers showed a cotinine concentration much higher than the cut-off (1228 µg/L). After a further contact with the health personal of the plant, the subject was reclassified in the S group. The number of cigarettes smoked per day in S ranged between 0 (considering the subject who did not declare smoking) to 25.

Nicotine levels in NS were above LOQ in 19 out of 38 individuals (50%) with values not exceeding 2.34 µg/L. Nicotine levels in e-cig and S had medians of 2003 and 1456 µg/L, respectively. Nicotine levels were significantly higher in e-cig vs NS ($P < 0.001$) and in S vs NS ($P < 0.001$) while similar in e-cig vs S ($P = 1$).

Cotinine levels in NS were above LOQ in 27 out of 38 individuals (71%) with values not exceeding 3.05 µg/L. Cotinine levels in e-cig and S had medians of 1530 and 1772 µg/L, respectively. Cotinine levels were significantly higher in e-cig vs NS ($P < 0.001$) and in S vs NS ($P < 0.001$) while similar in e-cig vs S ($P = 1$).

Creatinine was similar in all groups: overall median was 1.5 g/L and values ranged between 0.3 and 3.1 g/L.

Table 3.2 - Main personal characteristics and information on smoking habits of study subject divided into no-smokers (NS), e-cig smokers (e-cig), and tobacco smokers (S, according to cotinine cut off ≥30 μg/L).

		NS	**smokers**	
			e-cig	**S**
Smoking status	N	38	7	22
Male gender	N	34	7	22
Age (y)	Mean (min-max)	46 (28 - 62)	46 (37 - 55)	45 (27 - 57)
BMI (kg/m^2)	Mean (min-max)	26.2 (19.6 - 37.0)	25.9 (22.8 - 30.8)	26.2 (19.0 - 30.5)
Plant workers	N	32	7	19
Intensity of tobacco smoking (cigarettes/day)	Mean (min-max)	-	-	13 (0 - 25)
nicotine (μg/L)	Median (5^{th} - 95^{th}) %>LOQ	0.11 (<0.10 - 1.63) 50	2003 (537 - 4486) 100	1456 (225 - 5120) 100
cotinine (μg/L)	Median (5^{th} - 95^{th}) %>LOQ	0.35 (<0.10 - 1.93) 71	1530 (1179 - 2772) 100	1772 (601 - 4000) 100
creatinine (g/L)	Median (5^{th} - 95^{th})	1.5 (0.4 - 2.6)	1.5 (0.5 - 2.5)	1.4 (0.6 - 2.8)

3.3.2 Mercapturic acid levels

The results from the analysis of urinary mercapturic acids, adjusted for creatinine, are reported in Table 3.3, along with the p-value of the Anova and Bonferroni post-hoc test. Results, not adjusted for creatinine, are reported in Table 3.4.

Mercapturic acid concentrations were above the LOQ for all samples for 2-HPMA, 3-HPMA, AAMA, AMCC, CMEMA, DHBMA, HMPMA, and SBMA; a few samples showed non-quantifiable levels of CEMA, GAMA, HEMA, MHBMA, MMA, PHEMA and SPMA (from 78% to 99% of samples greater than LOQ); about a half of samples showed non-quantifiable levels of EMA (58% of samples greater than the LOQ) and almost all samples showed non-quantifiable levels of NANPC (only 10% of samples above the LOQ). For this reason, NANPC was excluded from statistical analyses.

Mercapturic acid medians ranged between 0.03 (EMA in NS and e-cig) and 1301 µg/g creatinine (3-HPMA in S). Anova test revealed significant differences among groups for all mercapturic acids, with the exception of MMA and SBMA. After applying the Bonferroni post-hoc test, e-cig showed significant higher levels than NS for CEMA and marginally significant for 3-HPMA (P = 0.069); S showed significant higher levels than e-cig for 2-HPMA, 3-HPMA, AAMA, CEMA, DHBMA, HMPMA, MHBMA, and SPMA; S showed significant higher levels than NS for all analytes but MMA, and SBMA.

Table 3.3 - Concentration of mercapturic acids in urine samples (µg/g creatinine), expressed as median, 5th and 95th percentile, and % of samples >LOQ, in subjects divided according to smoking habit, together with results of Anova test and Bonferroni *post-hoc* test.

Urinary mercapturic acids	**LOQ (µg/L)**	**statistics**	**NS (µg/g creatinine)**	**e-cig (µg/g creatinine)**	**S (µg/g creatinine)**	**P (Anova)**	**P NS vs e-cig**	**P NS vs S**	**P e-cig vs S**
2-HPMA	0.5	Median	8.8	9.8	28.4	< 0.001	1.000	< 0.001	< 0.001
		5th - 95th	4.2 - 16.4	6.7 - 17.4	9.4 - 70.9				
		%>LOQ	100	100	100				
3-HPMA	0.2	Median	160.6	222.1	1301.2	< 0.001	0.069	< 0.001	< 0.001
		5th - 95th	77.9 - 318.5	196.6 - 738.2	328.9 - 3661.1				
		%>LOQ	100	100	100				
AAMA	3.2	Median	47.9	55.8	114.6	< 0.001	1.000	< 0.001	< 0.001
		5th - 95th	24.2 - 95.4	34.4 - 65.5	55.1 - 223.9				
		%>LOQ	100	100	100				
AMCC	2	Median	142	243	405	< 0.001	1.000	<0.001	0.105
		5th - 95th	55 - 434	60 - 519	90 - 844				
		%>LOQ	100	100	100				
CEMA	0.9	Median	0.9	2.7	163.1	< 0.001	<0.001	<0.001	<0.001
		5th - 95th	<LOQ - 2.1	0.9 - 36.5	45.8 - 358.4				
		%>LOQ	63	86	100				

Table 3.3 - Continue

Urinary mercapturic acids	**LOQ (µg/L)**	**statistics**	**NS (µg/g creatinine)**	**e-cig (µg/g creatinine)**	**S (µg/g creatinine)**	**P (Anova)**	**P NS vs e-cig**	**P NS vs S**	**P e-cig vs S**
CMEMA	2	Median	273	233	400	0.018	1.000	0.031	0.100
		5^{th} - 95^{th}	122 - 603	154 - 542	220 - 774				
		%>LOQ	100	100	100				
DHBMA	1.0	Median	247.5	263.8	479.1	< 0.001	1.000	< 0.001	< 0.001
		5^{th} - 95^{th}	163.6 - 348.5	177.3 - 298.7	273.2 - 925.6				
		%>LOQ	100	100	100				
EMA	0.01	Median	0.03	0.03	0.06	0.033	1.000	0.029	0.615
		5^{th} - 95^{th}	<LOQ - 0.11	<LOQ - 0.10	<LOQ - 0.80				
		%>LOQ	55	57	64				
GAMA	1.0	Median	2.5	3.9	5.3	0.002	0.974	0.001	0.501
		5^{th} - 95^{th}	<LOQ - 7.1	1.4 - 6.7	1.7 - 30.4				
		%>LOQ	84	100	95				
HEMA	0.3	Median	1.3	2.0	3.2	0.002	0.872	0.002	0.657
		5^{th} - 95^{th}	0.1 - 4.1	1.3 - 2.2	1.0 - 26.7				
		%>LOQ	95	100	100				
HMPMA	2	Median	48	38	268	< 0.001	1.000	<0.001	<0.001
		5^{th} - 95^{th}	15 - 265	19 - 133	96 - 580				
		%>LOQ	100	100	100				

Table 3.3 - Continue

Urinary mercapturic acids	**LOQ (µg/L)**	**statistics**	**NS (µg/g creatinine)**	**e-cig (µg/g creatinine)**	**S (µg/g creatinine)**	**P (Anova)**	**P NS vs e-cig**	**P NS vs S**	**P e-cig vs S**
MHBMA#	0.04	Median	0.27	0.55	4.07	< 0.001	0.410	< 0.001	0.016
		5th - 95th	<LOQ - 2.47	0.14 - 2.07	0.74 - 11.38				
		%>LOQ	63	100	100				
MMA	0.09	Median	2.57	4.70	2.64	0.662	1.000	1.000	1.000
		5th - 95th	0.36 - 10.52	1.64 - 7.15	0.70 - 17.39				
		%>LOQ	97	100	100				
NANPC	0.11	Median	<LOQ	<LOQ	<LOQ	N.A	N.A	N.A	N.A
		5th - 95th	<LOQ - 0.16	<LOQ - 0.11	<LOQ - <LOQ				
		%>LOQ	13	14	5				
PHEMA#	0.01	Median	0.53	0.68	1.05	0.003	1.000	0.002	0.138
		5th - 95th	0.09 - 1.36	0.17 - 1.29	0.39 - 2.55				
		%>LOQ	100	86	100				
SBMA	0.02	Median	2.22	1.42	1.47	0.096	0.472	0.158	1.000
		5th - 95th	0.55 - 12.74	0.40 - 4.28	0.53 - 2.96				
		%>LOQ	100	100	100				
SPMA	0.01	Median	0.06	0.16	0.48	< 0.001	0.560	< 0.001	0.001
		5th - 95th	<LOQ - 0.23	0.03 - 0.34	0.08 - 1.45				
		%>LOQ	92	86	100				

N.A. Not assessed

#As a sum of isomers

Table 3.4 - Concentration of mercapturic acids in urine samples (µg/L) , expressed as median, 5th and 95th percentile, and % of samples >LOQ, in subjects divided according to smoking habit, together with results of Anova test and Bonferroni *post-hoc* test.

Urinary mercapturic acids	**LOQ (µg/L)**	**statistics**	**NS (µg/L)**	**e-cig (µg/L)**	**S (µg/L)**	**P (Anova)**	**NS vs e-cig**	**NS vs S**	**e-cig vs S**
2-HPMA	0.5	Median	10.3	16.3	33.6	< 0.001	0.947	< 0.001	0.034
		5th - 95th	3.6 - 27.4	5.2 - 41.9	9.1 - 129.8				
		%>LOQ	100	100	100				
3-HPMA	0.2	Median	223.9	414.4	1594.5	< 0.001	0.232	< 0.001	0.002
		5th - 95th	46.5 - 696.3	130.0 - 1862.2	265.3 - 6846.8				
		%>LOQ	100	100	100				
AAMA	3.2	Median	65.4	84.4	112.6	<0.001	1.000	< 0.001	0.088
		5th - 95th	18.0 - 126.8	21.4 - 165.9	43.6 - 450.5				
		%>LOQ	100	100	100				
AMCC	2	Median	220	364	572	0.009	1.000	0.007	0.686
		5th - 95th	44 - 649	38 - 1089	54 - 2257				
		%>LOQ	100	100	100				
CEMA	0.9	Median	1.2	4.1	147.3	<0.001	<0.001	<0.001	<0.001
		5th - 95th	<LOQ - 2.8	1.1 - 96.8	38.1 - 627.5				
		%>LOQ	63	86	100				
CMEMA	2	Median	340	349	578	0.245	1.000	0.315	0.921
		5th - 95th	84 -1243	124 - 898	103 - 1166				
		%>LOQ	100	100	100				
DHBMA	1.0	Median	347.5	404.8	643.7	0.002	1.000	0.002	0.111
		5th - 95th	87.5 - 689.1	112.1 - 702.6	184.5 - 1563.2				
		%>LOQ	100	100	100				
EMA	0.01	Median	0.03	0.02	0.12	0.048	1.000	0.044	0.676
		5th - 95th	<LOQ - 0.17	<LOQ - 0.20	<LOQ - 0.78				
		%>LOQ	55	57	64				
GAMA	1.0	Median	3.5	3.6	7.6	0.011	0.822	0.009	1.000
		5th - 95th	<LOQ - 9.7	2.6 - 9.7	1.1 - 20.9				
		%>LOQ	84	100	95				

Table 3.4 - Continue

Urinary mercapturic acids	**LOQ (µg/L)**	**statistics**	**NS (µg/L)**	**e-cig (µg/L)**	**S (µg/L)**	**P (Anova)**	**NS vs e-cig**	**NS vs S**	**e-cig vs S**
HEMA	0.3	Median	1.4	3.1	2.8	0.001	0.482	<0.001	0.903
		5^{th} - 95^{th}	0.3 - 4.7	1.1 - 4.1	0.9 - 18.8				
		%>LOQ	95	100	100				
HMPMA	2	Median	54	77	326	<0.001	1.000	< 0.001	1.000
		5^{th} - 95^{th}	15 - 312	11 - 337	59 - 1147				
		%>LOQ	100	100	100				
MHBMA#	0.04	Median	0.42	1.16	4.94	< 0.001	0.435	< 0.001	0.061
		5^{th} - 95^{th}	<LOQ - 4.41	0.12 - 5.18	0.92 - 21.06				
		%>LOQ	63	100	100				
MMA	0.09	Median	4.34	5.09	4.20	0.626	1.000	1.000	1.000
		5^{th} - 95^{th}	0.31 - 15.79	2.71 - 8.92	0.42 - 20.68				
		%>LOQ	97	100	100				
NANPC	0.11	Median	<LOQ	<LOQ	<LOQ	N.A	N.A	N.A	N.A
		5^{th} - 95^{th}	<LOQ - 0.14	<LOQ - 0.10	<LOQ - <LOQ				
		%>LOQ	13	14	5				
PHEMA#	0.01	Median	0.60	1.09	1.24	0.013	1.000	0.011	0.355
		5^{th} - 95^{th}	0.08 - 1.84	0.11 - 3.08	0.50 - 5.14				
		%>LOQ	100	86	100				
SBMA	0.02	Median	2.69	2.94	2.18	0.240	1.000	0.336	1.000
		5^{th} - 95^{th}	0.52 - 13.07	0.39 - 8.55	0.51 - 4.67				
		%>LOQ	100	100	100				
SPMA	0.01	Median	0.07	0.17	0.64	< 0.001	0.492	< 0.001	0.014
		5^{th} - 95^{th}	<LOQ - 0.37	0.03 - 0.83	0.13 - 2.82				
		%>LOQ	92	86	100				

N.A. Not assessed

#As a sum of isomers

3.3.3 Multiple regression models

Results of the multiple linear regression analyses are showed in Table 3.5.

In the linear model A, the adjusted coefficient of determination (R^2) was significantly greater from zero for all analytes and ranged from 0.11 (EMA) to 0.72 (CEMA). The GMR for cotinine as determinant of urinary mercapturic acids was significantly greater than one for all compounds with the exception of CMEMA, MMA, and SBMA; where significant, it ranged from 1.13 (DHBMA) to 2.92 (CEMA). The partial correlation coefficients (r) of cotinine were significantly greater than zero for all compounds with the exception of MMA and SBMA; where significant, it ranged from 0.25 (CMEMA) to 0.85 (CEMA).

As regarding the occupational exposure, considering office workers as reference, the GMR associated with plant workers was increased only for SPMA (2.46 , 95%CI: 1.15 - 5.25, P = 0.021) and decreased for GAMA (0.55, 95%CI: 0.31 - 0.99, 0 = 0.046); other mercapturic acids were not affected by occupational exposure.

In the linear model B, the adjusted coefficient of determination (R^2) was significantly greater from zero for all analytes and ranged from 0.13 (EMA) to 0.92 (CEMA). The smoking habits influenced urinary levels of mercapturic acids with notable differences. Considering NS as the reference group, the GMR relative to e-cig was significantly greater than one for 3-HPMA (1.78) and for CEMA (4.85); while the GMR relative to S was significantly greater than one for all analytes with the exception of MMA and SBMA; where significant, it ranged from 1.44 (CMEMA) to 164.97 (CEMA). Distributions of analytes, corrected for all variables included in this linear model, are visually reported in box-plot in Figure 3.2.

As regarding the occupational exposure, considering office workers as reference, the GMR associated with plant workers was increased for SPMA (2.83, 95%CI: 1.42 - 5.62, P = 0.004) and PHEMA (1.89, 95%CI: 0.95 - 3.77, P = 0.069) and decreased for GAMA (0.57, 95%CI: 0.31 – 1.03, P = 0.062); other mercapturic acids were not affected by occupational exposure.

Table 3.5 - Results of multiple linear regression models A and B in which the concentration of each urinary mercapturic acid is the dependent variable. In the linear model A the independent variables are: cotinine (log_{10} transformed, µg/L), creatinine (log_{10} transformed, g/L), age (years), gender (male = reference, female), BMI (kg/m^2), and occupational exposure (office workers = reference, plant workers). In the linear model B urinary cotinine was replaced by smoking mode (NS = reference, e-cig, S). Results are reported as Geometric Mean Ratio (GMR), with respective 95% confidence interval (95% CI), obtained performing exponentiation on the relatives adjusted slopes.

Urinary mercapturic acids	Linear model A			Linear model B		
	Cotinine			e-cig	S	
	GMR (95% CI) P value	r (95% CI) P value	R^2_{adj} P value	GMR (95% CI) P value	GMR (95% CI) P value	R^2_{adj} P value
2-HPMA	1.30 1.20 - 1.42 <0.001	0.62 0.45 - 0.75 <0.001	0.55 <0.001	1.30 0.80 - 2.13 0.289	3.56 2.57 - 4.91 <0.001	0.63 <0.001
3-HPMA	1.54 1.39 - 1.70 <0.001	0.75 0.62 - 0.84 <0.001	0.68 <0.001	1.78 1.09 - 2.90 0.021	8.14 5.91 - 11.22 <0.001	0.81 <0.001
AAMA	1.20 1.13 - 1.28 <0.001	0.60 0.41 - 0.73 <0.001	0.66 <0.001	1.07 0.74 - 1.53 0.720	2.42 1.91 - 3.07 <0.001	0.73 <0.001
AMCC	1.23 1.12 - 1.35 <0.001	0.50 0.30 - 0.66 <0.001	0.61 <0.001	1.25 0.70 - 2.24 0.437	2.62 1.79 - 3.84 <0.001	0.63 <0.001
CEMA	2.92 2.46 - 3.47 <0.001	0.85 0.76 - 0.90 <0.001	0.72 <0.001	4.85 2.68 - 8.77 <0.001	164.97 111.66 - 243.73 <0.001	0.92 <0.001
CMEMA	1.07 1.00 - 1.14 0.051	0.25 0.01 - 0.46 0.042	0.63 <0.001	0.90 0.59 - 1.36 0.605	1.44 1.09 - 1.89 0.010	0.65 <0.001
DHBMA	1.13 1.07 - 1.20 <0.001	0.50 0.29 - 0.66 <0.001	0.67 <0.001	0.93 0.69 - 1.27 0.662	2.02 1.65 - 2.48 <0.001	0.77 <0.001
EMA	1.20 1.01 - 1.42 0.035	0.27 0.03 - 0.48 0.028	0.11 0.044	1.10 0.37 - 3.23 0.866	2.51 1.23 - 5.10 0.012	0.13 0.035
GAMA	1.19 1.08 - 1.32 0.001	0.40 0.18 - 0.59 0.001	0.43 <0.001	1.42 0.73 - 2.77 0.291	2.08 1.34 - 3.22 0.001	0.42 <0.001
HEMA	1.27 1.12 - 1.43 <0.001	0.45 0.23 - 0.62 <0.001	0.18 0.005	1.72 0.79 - 3.75 0.171	2.78 1.66 - 4.65 <0.001	0.18 0.007
HMPMA	1.36 1.21 - 1.53 <0.001	0.55 0.36 - 0.70 <0.001	0.48 <0.001	0.88 0.47 - 1.65 0.691	5.26 3.49 - 7.93 <0.001	0.66 <0.001
MHBMA	1.85 1.51 - 2.28 <0.001	0.61 0.43 - 0.74 <0.001	0.46 <0.001	2.56 0.79 – 8.28 0.114	21.99 10.16 - 47.61 <0.001	0.58 <0.001
MMA	1.08 0.95 - 1.23 0.238	0.15 -0.09 - 0.38 0.220	0.39 <0.001	1.72 0.75 - 3.96 0.198	1.24 0.72 - 2.15 0.435	0.38 <0.001

Table 3.5 - Continue

Urinary mercapturic acids	**Linear model A**			**Linear model B**		
	Cotinine		**R^2_{adj} P value**	**e-cig**	**S**	**R^2_{adj} P value**
	GMR (95% CI) P value	**r (95% CI) P value**		**GMR (95% CI) P value**	**GMR (95% CI) P value**	
PHEMA	1.23 1.09 - 1.39 <0.001	0.41 0.19 - 0.59 <0.001	0.33 <0.001	1.00 0.46 - 2.15 0.997	2.56 1.54 - 4.23 <0.001	0.35 <0.001
SBMA	0.90 0.81 - 1.01 0.073	-0.23 -0.44 - 0.01 0.062	0.32 <0.001	0.61 0.30 - 1.27 0.183	0.65 0.40 - 1.05 0.076	0.32 <0.001
SPMA	1.53 1.34 - 1.75 <0.001	0.64 0.47 - 0.76 <0.001	0.48 <0.001	1.51 0.70 - 3.25 0.285	7.65 4.62 - 12.66 <0.001	0.58 <0.001

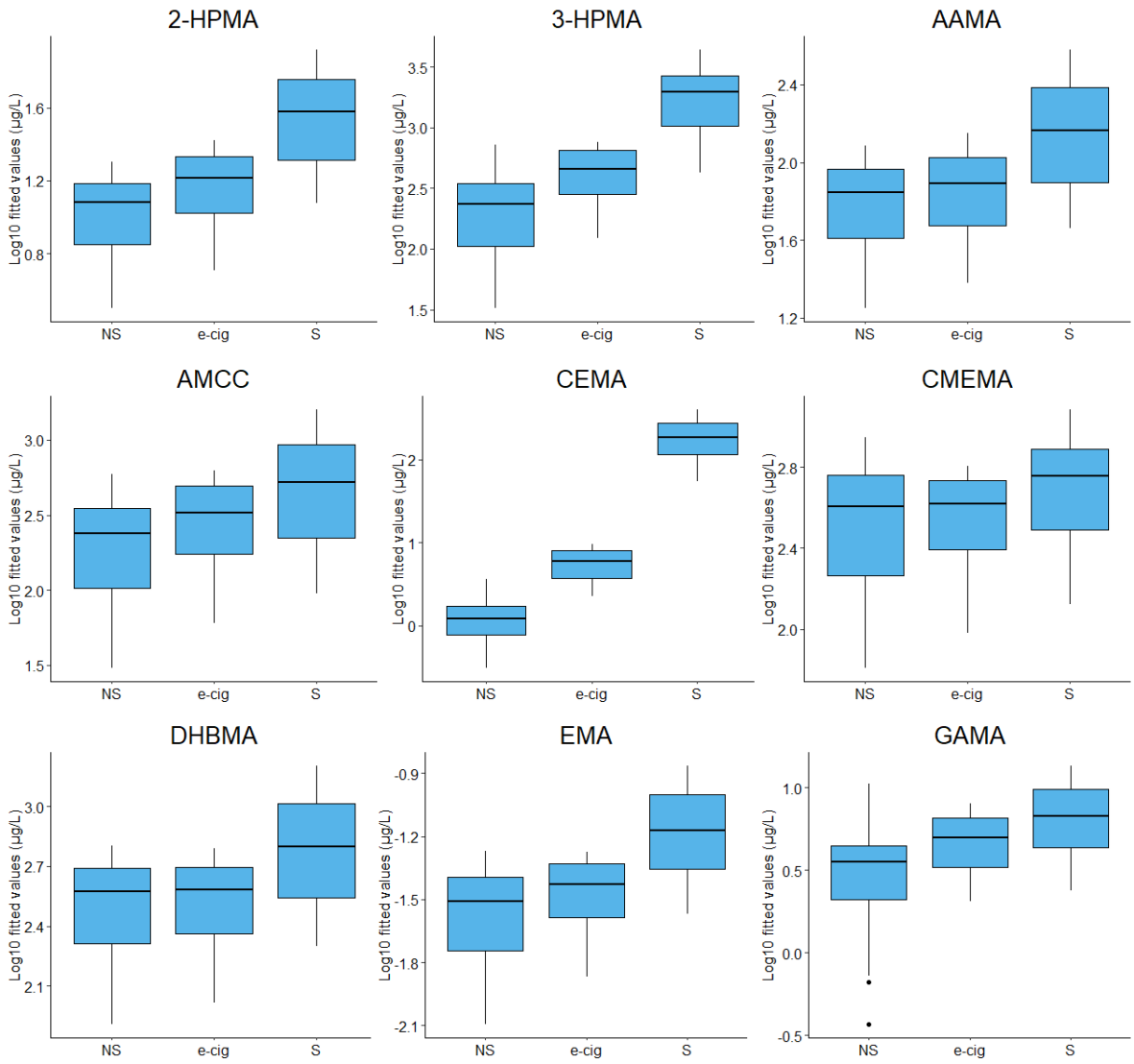

Figure 3.1 - Boxplots representing the distribution of fitted values obtained from the multiple linear model B for all investigated mercapturic acids. Data reported are corrected for creatinine, age, gender, BMI, and occupational exposure.

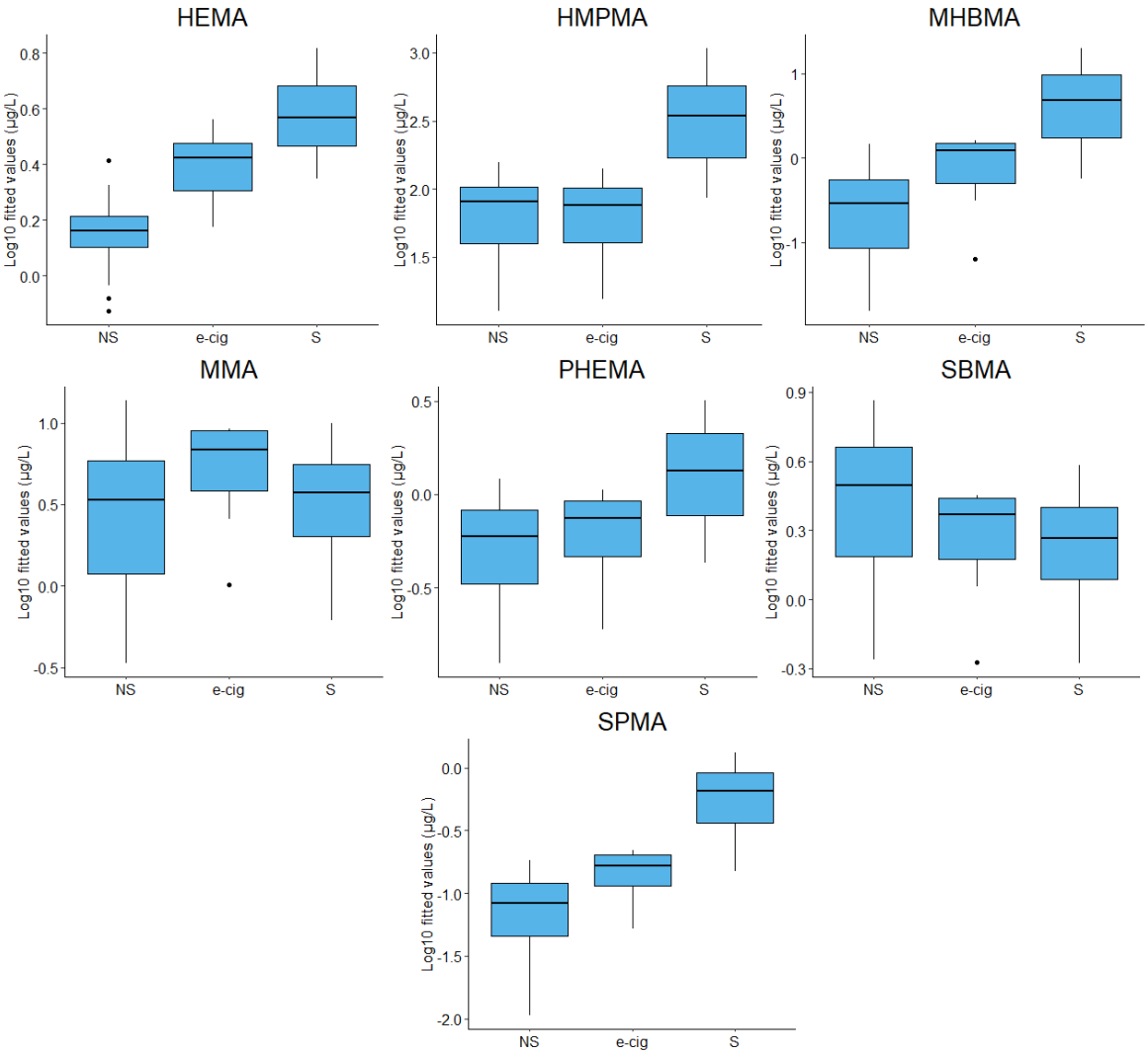

Figure 3.2 -continue

3.4 Discussion

We conducted a biomonitoring study to assess the level of several urinary mercapturic acids associated to carcinogenic and non-carcinogenic chemicals in subjects with different smoking habits. As expected, the levels of most mercapturic acids were higher in S than in both NS and e-cig; however, they were higher also in e-cig compared to NS, showing an impact of this smoking mode on the body burden of toxicants.

The considered mercapturates are the urinary metabolites of different toxicants, among which 1,3-butadiene, which is a recognised carcinogen (IARC, 2012b). While it has been quantified in both main and side stream smoke of traditional cigarettes (IARC, 2004), there are limited evidences of its presence in the vapour or liquid of electronic cigarettes (Laugesen, 2008; Varlet et al., 2015). DHBMA and MHBMA are two metabolites derived from 1,3-butadiene. For both compounds we found significant difference between NS and S, and between e-cig and S, while levels are comparable in e-cig and NS. This finding is in agreement with a previous study (Keith et al., 2019). This is also confirmed in studies of substitution of cigarettes with electronic cigarettes, which observed a significant reduction in MHBMA after the replacement (D'Ruiz et al., 2016; Goniewicz et al., 2017).

NANPC is a metabolite of 4-chloronitrobenzene and, to our knowledge, this is the first time it has been determined in a biomonitoring study in relation to the smoking habits. However, it was below the limit of quantification for most of the samples, showing that the general population is not exposed to 4-chloronitrobenzene and it is not related to the smoking habits. Indeed, 4-chloronitrobenzene has been identified in subjects occupationally exposed (Jones et al., 2007; Sabbioni et al., 2016).

Acrolein is a substance with different toxic effects (Moghe et al., 2015) which has been detected both in tobacco smoke (IARC, 2004) and in vapour of electronic cigarettes (Goniewicz et al., 2014; Uchiyama et al., 2013; Sleiman et al., 2016). 3-HPMA is a urinary metabolite of acrolein and we detected it with levels almost ten time higher in S than NS. Significant higher levels of 3-HPMA in S were found in previous studies with a similar pattern (Alwis et al., 2012; Pluym et al., 2015; Schettgen et al., 2008; Eckert et al., 2011; Li et al., 2015; Zhang et al., 2014). Considering e-cig, levels of 3-HPMA were significantly lower than S and non-significantly higher than NS (P-value of 0.069). However, it is noteworthy to mention that we used the Bonferroni post-hoc test, which is the most conservative post-hoc test, and that the sample size of e-cig was limited to only 7 subjects. Indeed, in the multiple linear regression model B, where correction with additional variables

were performed, we found a significant GMR (P = 0.029) comparing e-cig to NS (see below for further comments on the linear models). Keith et al. found comparable results: higher non-significant level of 3-HPMA in e-cig than NS (Keith et al., 2019); however is worth to mention that they also measured another metabolite of acrolein, N-Acetyl-S-(2-carboxyethyl)-L-cysteine, which was significantly higher in e-cig than NS. Interestingly, the study form Goniewicz at al., quantifying both of the acrolein derived mercapturic acids, did not find any significant differences between e-cig and NS (Goniewicz et al., 2018), while Rubinstein and co-workers, who analysed urine samples from adolescents, found significant higher median levels of 3-HPMA in e-cig than NS (Rubinstein et al., 2018).

Acrilamide is a neurotoxic, genotoxic and probably carcinogenic compound (IARC, 1994) present in the mainstream smoke of tobacco cigarettes (IARC, 2004), while it has not been detected in smoke from electronic cigarettes (Papoušek et al., 2014). We quantified two metabolites of acrylamide, AAMA and GAMA: the first has been estimated to account for 51.7% of the assumed dose of acrylamide, while the second for 4.6% (Hartmann et al., 2009). This is in agreement with the results of our study, since the concentrations of AAMA were about ten time higher than concentrations of GAMA. We found significant higher concentration of AAMA in S than tNS and in e-cig than S. This pattern is similar for GAMA, except for a non-significant difference between e-cig and S. The study of Keith and co-workers reported similar differences (Keith et al., 2019) while Goniewicz and co-workers also found significant difference between levels of GAMA between e-cig and S (Goniewicz et al., 2018). It is noteworthy to mention that there are some conflicting results in the literature: in a study of substitution conducted by Pulvers and co-workers (Pulvers et al., 2018) non-significant differences were found after 4 weeks in smokers who switched to electronic cigarette smokers, while Goniewicz and co-workers found a significant reduction after a period of 2 weeks (Goniewicz et al., 2017); however this could be due to the different rate of people who completely replace cigarettes

with the electronic ones. Moreover, significantly higher levels of AAMA were found in e-cig than NS in a study conducted on adolescents (Rubinstein et al., 2018).

Acrylonitrile is a toxic compound (Woutersen, 1998). It was initially classified as probably carcinogen to human (IARC, 1979) and after it was downgraded to possible carcinogen (IARC, 1999), and this was also confirmed in a more recent review of cohort studies (Cole et al., 2008). While it has been reported to be present in both mainstream and sidestream smoke of traditional tobacco cigarettes (IARC, 2004), we did not find information in literature about its presence in electronic cigarette vapour or liquid. However several studies reported the presence of its metabolite (CEMA) in urine of electronic cigarette smokers (Shahab et al., 2017; Goniewicz et al., 2018; Keith et al., 2019; Rubinstein et al., 2018). In our study, CEMA was the only analyte showing a significant difference between NS and e-cig in the Anova post-hoc test, indicating that, although lower than traditional tobacco smoke, electronic cigarette smoke might represent a considerable source of exposure to acrylonitrile. CEMA showed also significant differences between all other group pairs. These differences were in agreement with other studies (Goniewicz et al., 2018; Rubinstein et al., 2018; Keith et al., 2019).

Benzene is recognised carcinogen (IARC, 2018a), and it is a well-known molecule present in both main and sidestream of cigarettes (IARC, 2004). Very low concentrations of this compound were found in the mainstream vapour (Sleiman et al., 2016) and in some nicotine liquids. In our study, significant higher levels of SPMA, a metabolite of benzene, were found in S compared to NS and in S compared to e-cig, while the median in e-cig was non-significantly higher than NS. Results are similar with other studies which quantified SPMA in S and NS (Pluym et al., 2015; Alwis et al., 2012), and in studies of replacement of cigarettes with electronic cigarettes (O'Connell et al., 2016; D'Ruiz et al., 2016; Goniewicz et al., 2017; Pulvers et al., 2018). Interestingly, the work from Goniewicz and co-workers did not report any significant differences among groups (Goniewicz et al., 2018). Similarly, the study from Keith and co-workers, who measured trans,trans-muconic

acid, instead SPMA, as a metabolite of benzene, did not find significant differences (Keith et al., 2019).

Crotonaldehyde is an unsaturated aldehyde with irritating properties which can be present in food as well as in air (IARC, 1995). It has been detected both in mainstream and in sidestream of tobacco smoke (IARC, 2004). Regarding electronic cigarettes, it was detected only in very a few samples of vapour (Sleiman et al., 2016) and refill liquids (Varlet et al., 2015). The two mercapturic acids derived from crotonaldehyde are HMPMA and CMEMA (Gray and Barnsley, 1971). HMPMA is the major metabolite and it has been reported to be a more suitable biomarker for smoking-related exposure to crotonaldehyde than CMEMA (Scherer et al., 2007). Indeed, in our biomonitoring study, comparing S to NS, we found significant higher median values of HMPMA (268 vs 48 µg/g creatinine, $P < 0.001$); values for CMEMA were also significantly higher but with a minor difference (400 vs 273 µg/g creatinine, $P = 0.031$). Results between NS and e-cig were comparable for both HMPMA and CMEMA, indicating that electronic cigarettes are not a significant source of crotonaldehyde. Other studies which quantified HMPMA showed similar results (Keith et al., 2019; Goniewicz et al., 2018). Replacement studies also showed a decrease in HMPMA levels when switching from cigarettes to electronic cigarettes (O'Connell et al., 2016; Goniewicz et al., 2017), with the exception of one study (Pulvers et al., 2018). Interestingly, in a study conducted on adolescents, significantly higher levels of HMPMA were found in e-cig than NS (Rubinstein et al., 2018).

The mercapturic acids EMA and MMA are metabolites of ethylating and methylating agents, respectively. Although present at very low concentrations, significant higher levels of EMA were found in S if compared to NS, while results in e-cig were similar to NS. This is a unique finding since the study of Pluym et al. did not reveal significant differences between NS and S (Pluym et al., 2015). Results for MMA were comparable in all three groups, indicating that metabolite is not able to discriminate for the smoking habits. Similarly the diet-controlled clinical study

from Pluym et al. and the study of substitution of cigarettes with electronic cigarettes did not report differences among groups (Pluym et al., 2015; Pulvers et al., 2018).

Ethylene oxide is a carcinogenic molecules and tobacco smoke is the main non-occupational source (IARC, 2012b). Varlet and co-workers showed its presence only in a restricted number of refill liquids for e-cigarettes (Varlet et al., 2015). HEMA is the mercapturic acid derived from ethylene oxide (Gérin and Tardif, 1986). A significant difference was found from S to NS, indicating that tobacco smoke may represent an exposure to this compound. No significant differences were found between e-cig and S nor between e-cig and NS. Results are similar with previously studies, which also reported a significant difference between e-cig and S (Keith et al., 2019; Goniewicz et al., 2018).

N,N-dimethylformamide is a probable carcinogen present in tobacco smoke (IARC, 2004). We evaluated its exposure by measuring urinary levels of its metabolite AMCC. S showed significantly higher levels than NS, non-significantly higher levels than e-cig, while results were similar between e-cig and NS. Keith and co-workers (Keith et al., 2019) showed similar results, but with a significant difference between S and e-cig, while Goniewicz and co-workers interestingly also found significant higher values in e-cig than NS (Goniewicz et al., 2018).

Propylene oxide is a possible carcinogen detected in the mainstream of tobacco smoke (IARC, 2004). It has also been detected in some liquid for electronic cigarettes (Sleiman et al., 2016). 2-HPMA is its metabolites which we quantified in the subjects' urine. Levels were very similar in NS and e-cig, and significantly lower if compared to S. These findings are in agreement with other studies (Keith et al., 2019; Goniewicz et al., 2018). Conflicting results are present in the literature in replacement studies since Goniewicz et a. found a significant decrease after the substitution of cigarettes with electronic ones (Goniewicz et al., 2017), while Pulvers et al. did not reveal any changes (Pulvers et al., 2018). Furthermore, in a study conducted on adolescents, significantly higher levels of 2-HPMA were found in e-cig than NS (Rubinstein et al., 2018).

Styrene is another probable carcinogen which has been detected both in mainstream and sidestream tobacco smoke (IARC, 2004). Some studies reported its presence also in electronic cigarette mist (Laugesen, 2008) and in some nicotine liquids (Lim and Shin, 2017). The mercapturic acid derived from styrene is PHEMA (measured as a sum of two isomers). Levels of PHEMA were significantly higher in S than NS, non-significantly higher than e-cig, while levels in e-cig were comparable to NS. The study from Keith and co-workers also reported a significant difference between S and e-cig (Keith et al., 2019), similarly to Goniewicz and co-workers who quantified phenylglyoxylic acid as a metabolite of styrene, instead of PHEMA (Goniewicz et al., 2018).

Toluene is a toxic compound detected both in mainstream and sidestream tobacco smoke (IARC, 2004). It has also been detected in some vapours (Goniewicz et al., 2014) and liquids of electronic cigarettes (Lim and Shin, 2017). However, we did not find any significant difference in levels of SBMA, the mercapturic acid derived from toluene, among groups. These results are confirmed by other studies measuring SBMA levels in urine (Alwis et al., 2012; Pluym et al., 2015; Keith et al., 2019), with the exception of the study of Goniewicz who reported a significant difference between e-cig and NS, although the actual difference in the geometrical mean is low (Goniewicz et al., 2018). Interestingly, toluene levels in blood have been reported to be significant higher in S than NS (Jain, 2016; Chambers et al., 2011). For these reasons, SBMA could not be an ideal biomarker for toluene when considering the smoking habits.

With the multiple linear regression analyses we first tried to understand the role played by cotinine as a determinant for urinary mercapturic acids (model A). Cotinine, a metabolite of nicotine, has a half-life of about 17 hours (Benowitz, 1996), and therefore represents exposure to nicotine over the previous days. Results revealed that cotinine is a significant determinant for most of the considered mercapturic acids (Table 3.5); however, it did not allow to discriminate between

tobacco and electronic cigarette smoking, as both contain nicotine. For these reason another linear model, model B, was computed introducing the categorical variable "smoking mode" (NS, e-cig or S) instead of cotinine. Overall, the adjusted coefficient of determinations improved for all compounds. Indeed, smoking mode allowed us to properly take into account the presence of combustion processes, typical of tobacco smoking. The linear model B highlights that being a tobacco smokers is a significant determinant of exposure for the large majority of studied mercapturic acids, with an increase ranging from + 40% to 165-fold. The highest increases were observed for CEMA, MHBMA, 3-HPMA, and SPMA (165, 22, 8, and 7.7-fold, respectively) (Table 3.5), and among these metabolite we note the presence of those of 1,3-butadiene and benzene, both classified as known carcinogens to humans. On the other hand, e-cig showed increments of urinary mercapturic acids of several toxicants, among which 2-HPMA, 3-HPMA, AMCC, CEMA, GAMA; HEMA, MHBMA, MMA, SPMA (from +25% to 385%) (Table 3.5 and Figure 3.2). Such increments were statistically significant for 3-HPMA and CEMA which were about + 80% and 4.9-fold higher in e-cig than in NS. These results indicate that electronic cigarette smoke is a source of exposure to acrylonitrile and acrolein, and suggest that it is a source also of propylene oxide, N,N-dimethylformamide, ethylene oxide, 1,3-butadiene, methylating agents, and benzene. The ratio of the increment between S and e-cig shows that tobacco smoking contributes to the internal dose of these chemicals from + 60% to 34-fold more than e-cig. When considering benzene and 1,3-butadiene, their internal dose, estimated as urinary mercapturic acids, in e-cig accounts only for 23% and 13% of that observed in S. The multiple linear regression analysis allowed us to take into consideration the role played by occupational exposure on levels of urinary mercapturic acids. It was found that plant workers had higher levels of SPMA and PHEMA, but not of other mercapturic acids, in comparison with office workers; however, for all study subjects, SPMA levels were low and within the reference values (CDC, 2019) and comparable to previous experiences in the Italian

population (Ranzi et al., 2013) and in other countries (Alwis et al., 2012; Chiang et al., 2015; Li et al., 2015; Zhang et al., 2014; Goniewicz et al., 2018).

Generally speaking, comparing the concentration of mercapturic acids with other studies (Table 3.6), we notice some discrepancies, such as GAMA, MHBMA, and SPMA, suggesting critical issues associated with analytical methods applied to the determination of very low levels of mercapturic acids in urine. To tackle with the issue of accuracy, the assay used in the present work was extensively validated (chapter 1) and accuracy was verified by the successful participation in an external verification exercise, in which 10 urinary mercapturic acids, each tested at two different levels of occupational interest, are circulated among participating laboratories (Göen et al., 2012; G-EQUAS, 2019). However, at the moment, reference materials containing these analyses at levels of interest for the general population are lacking, while they would be useful for future applications of these measurements in epidemiological studies.

This study has some limitations, the major of which is the low number of study subjects, especially electronic cigarette smokers. Although we found increases in the concentration of several mercapturic acids in e-cig compared to NS, only for CEMA and 3-HPMA these were statistically significant; it is expected that a greater sample size could have highlighted significant differences also for other mercapturic acids. Another limitation is the lack of information about other lifestyle sources of exposure to chemicals biotransformed to mercapturic acids, such air pollution, and a further correction for other sources could have led to more accurate results in the linear regression models. Diet is another confounding factor (e.g.: presence of acrylamide in food, (Semla et al., 2017)) and, although diet was assessed in the questionnaire, we considered data not suitable for a proper inclusion in the linear model. A further issue is the lack of information about the type of electronic cigarette smoked (e.g. first or more advanced generation electronic cigarettes) and about the time elapsed from the last cigarette.

Among the strengths of this work there are the assessment of a large number of mercapturic acids, which can take into account for the exposure to a mixture of different toxicants and the good quality of the analytical data, as testified by the external verification exercise.

In conclusion, the results of this study allow to estimate the exposure to several toxic compounds, including some carcinogens. The comparative evaluation of the contribute of different smoking modes to the internal dose of chemicals confirms that tobacco smoking is a major source of exposure to carcinogenic and non-carcinogenic chemicals, and it highlights that also electronic cigarette smoking contributes to the internal dose of toxicants, in particular to acrylonitrile and acrolein.

Table 3.6 - Comparison of the results obtained in the present study with a selection of previous studies.

Urinary mercapturic acids		This work	(Alwis et al., 2012)	(Pluym et al., 2015)	(Schettgen et al., 2008; Schettgen et al., 2009)	(Eckert et al., 2011)	(Chiang et al., 2015)	(Li et al., 2015)	(Zhang et al., 2014)	(Goniewicz et al., 2018)	(Rubinstein et al., 2018) subjects: adolescents	(Keith et al., 2019)
		Median µg/g crea	Mean µg/L	Median µg/g crea	Median µg/L (µg/g crea)	Median µg/L (µg/g crea)	Mean µg/L	Mean µg/L	Mean µg/L	Geometric mean ng/mg creatinine	Median ng/mg creatinine	Mean ng/mg creatinine
2-HPMA	NS	8.8	81	3.2	7.1 (4.7)	11.5 (12.1)				33.79	15.2	84.4
	E-cig	9.8								34.45	28.8	34.8
	S	28.4	185	19.0	41.7 (37.5)	49.8 (46.2)				71.10		60.1
	Dual									84.13	40.2	48.6
3-HPMA	NS	160.6	406	62.5	155.0 (112.8)	179 (146)		408.5	607.51	272.4	192.8	223
	E-cig	222.1								314.8	254.3	338.6
	S	1301.2	1546	372	1681 (1630)	1219 (884)		1479	1481.31	1143.5		724.4
	Dual									1317.8	439.7	569.5
AAMA	NS	47.9	82	11.1	52.6 (55.2)					47.28	34.5	67.8
	E-cig	55.8								56.05	67.3	88.5
	S	114.6	196	68.4	242.7 (178.7)					136.4		191.9
	Dual									144.0	235.6	181.8
AMCC	NS	142	122	30.6	113.6 (104.9)							127.1
	E-cig	243										169.7
	S	405	479	146	822 (591)							327.7
	Dual											354
CEMA	NS	0.9	4.6	0.46	2.0		30.5	198.8	3.47	1.315	0	3.0
	E-cig	2.7								3.959	1.3	29.3
	S	163.1	187	72.5	240		68.2	347.6	50.69	123.9		129.8
	Dual									146.2	59.4	97

Table 3.6 - Continue

Urinary mercapturic acids		This work	(Alwis et al., 2012)	(Pluym et al., 2015)	(Schettgen et al., 2008; Schettgen et al., 2009)	(Eckert et al., 2011)	(Chiang et al., 2015)	(Li et al., 2015)	(Zhang et al., 2014)	(Goniewicz et al., 2018)	(Rubinstein et al., 2018) subjects: adolescents	(Keith et al., 2019)
		Median µg/g crea	Mean µg/L	Median µg/g crea	Median µg/L (µg/g crea)	Median µg/L (µg/g crea)	Mean µg/L	Mean µg/L	Mean µg/L	Geometric mean ng/mg creatinine	Median ng/mg creatinine	Mean ng/mg creatinine
CMEMA	NS	273		201								
	E-cig	233										
	S	400		226								
	Dual											
DHBMA	NS	247.5	331	76.2	289	187 (159)	133.8	207.8	184.61			283.2
	E-cig	263.8										262.7
	S	479.1	440	122	398	247 (211)	274.8	384.7	230.47			389.9
	Dual											415.6
EMA	NS	0.03		0.018								
	E-cig	0.03										
	S	0.06		0.028								
	Dual											
GAMA	NS	2.5	28	3.9			29.5			9.022		25.4
	E-cig	3.9								9.924		36.5
	S	5.3	57	12.7			53.6			17.33		43.6
	Dual									18.52		39
HEMA	NS	1.3	0.7	1.1	2.0 (1.7)	<2.0 (1.6)				0.955	1.3	1.7
	E-cig	2.0								1.076	0.5	1.1
	S	3.2	1.9	2.0	5.3 (4.0)	4.3 (4.9)				2.744		4.2
	Dual									3.194	1.0	4.5
HMPMA	NS	48	429	18.9				198.6	191.90	457.7	100.4	138.7
	E-cig	38								442.8	148.7	179.1
	S	268	1992	121.7				1741	1287.83	2359.3		462.4
	Dual									2707.7	185.4	433.5

Table 3.6 - Continue

Urinary mercapturic acids		This work	(Alwis et al., 2012)	(Pluym et al., 2015)	(Schettgen et al., 2008; Schettgen et al., 2009)	(Eckert et al., 2011)	(Chiang et al., 2015)	(Li et al., 2015)	(Zhang et al., 2014)	(Goniewicz et al., 2018)	(Rubinstein et al., 2018) subjects: adolescents	(Keith et al., 2019)
		Median µg/g crea	Mean µg/L	Median µg/g crea	Median µg/L (µg/g crea)	Median µg/L (µg/g crea)	Mean µg/L	Mean µg/L	Mean µg/L	Geometric mean ng/mg creatinine	Median ng/mg creatinine	Mean ng/mg creatinine
MHBMA[#]	NS	0.27	6.4	<LOD (0.12)	<2	<5.0 (<5.0)	52.3	9.733		4.543	0	3.6
	E-cig	0.55								4.308	0	6.8
	S	4.07	37.8	1.08	<2	<5.0 (<5.0)	129.2	40.87		27.90		19.5
	Dual									31.92	0	18.7
MMA	NS	2.57		4.1								
	E-cig	4.70										
	S	2.64		3.4								
	Dual											
NANPC	NS	<LOQ		-								
	E-cig	<LOQ										
	S	<LOQ										
	Dual											
PHEMA[#]	NS	0.53	<LOQ (0.7)	<LOD (0.1)				0.372				0.9
	E-cig	0.68										1.0
	S	1.05	<LOQ (0.7)	0.83				0.734				2.3
	Dual											1.5

Table 3.6 - Continue

Urinary mercapturic acids		This work	(Alwis et al., 2012)	(Pluym et al., 2015)	(Schettgen et al., 2008; Schettgen et al., 2009)	(Eckert et al., 2011)	(Chiang et al., 2015)	(Li et al., 2015)	(Zhang et al., 2014)	(Goniewicz et al., 2018)	(Rubinstein et al., 2018) subjects: adolescents	(Keith et al., 2019)
		Median µg/g crea	Mean µg/L	Median µg/g crea	Median µg/L (µg/g crea)	Median µg/L (µg/g crea)	Mean µg/L	Mean µg/L	Mean µg/L	Geometric mean ng/mg creatinine	Median ng/mg creatinine	Mean ng/mg creatinine
SBMA	NS	2.22	15	3.1								10.6
	E-cig	1.42										5.0
	S	1.47	16	2.3								12.8
	Dual											8.3
SPMA	NS	0.06	0.6	0.018			0.6	0.561	0.36	1.038	0	
	E-cig	0.16								1.007	0	
	S	0.48	0.92	1.1			7.0	0.745	0.20	1.090		
	Dual									1.071	0.2	

[#]results are reported as the sum of different isomers

NS = non smokers

e-cig = electronic cigarette smokers

S = tobacco smokers

Dual = tobacco and electronic cigarette smokers

4 Application of an untargeted metabolomic approach on subjects with different smoking habits and the comparison with the targeted measurement of mercapturic acids

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4.1 Introduction

Tobacco smoke is one of the main preventable causes of diseases for humans (Samet, 2013). Its related health effects, among which lung cancer, cardiovascular, and respiratory diseases, are the causes of millions of premature deaths worldwide each year (West, 2017). Tobacco smoke is composed by a mixture of chemicals and more than 5000 compounds have been identified in tobacco smoke (Rodgman and Perfetti, 2013). Molecules present in tobacco smoke derive from the direct volatilization of compounds present in tobacco or are generated from tobacco constituents through pyrogenesis. Mainstream smoke is composed by a vapour phase (>95%) and a wet particulate matter (<5%). The vapour phase contains water, nitrogen, oxygen, carbon dioxide and monoxide, and other volatile compounds such as hydrocarbons, aldehydes and ketones, nitriles, heterocyclic alcohols, acids, and esters; while the particulate contains, in addition, nicotine, partially combusted

particulate matter knowns as tar, smoke pigments, alkaloid derivatives and phenols (Rodgman and Perfetti, 2013).

Besides traditional tobacco cigarette, electronic cigarette is a relatively new product, which is growing in popularity. Electronic cigarettes aerosolize a solvent, such as propylene glycol and glycerol, containing nicotine and flavourings, and the produced vapour is inhaled by the user (Breland et al., 2017; Cheng, 2014). Evidences regarding the health effects of electronic cigarettes are still limited: long-term health effects have not been documented in humans and potential short-term effects include irritation of respiratory tract and inflammation induction, as well as nicotine-related cardiovascular risks (Callahan-Lyon, 2014; Qasim et al., 2017; Benowitz and Fraiman, 2017). The composition of electronic cigarette liquids, cartridges, and aerosols varies among different products. Other than nicotine, the list of compounds includes tobacco-specific nitrosamines, carbonyl compounds, metals, volatile organic compounds, phenolic compounds, polycyclic aromatic hydrocarbons, flavours, solvent carriers, tobacco alkaloids and drugs (Bekki et al., 2014; Goniewicz et al., 2014; Cheng, 2014).

Inhaled compounds from tobacco smoke and electronic cigarette aerosol, once absorbed, may be metabolized and excreted through different routes, and one of the most important is urinary excretion. Indeed, the assessment of exposure to chemicals can be carried out by determining the specific metabolites of toxicants present in urine (biomonitoring). The metabolites measured with this approach are referred to as biomarkers of exposure. Other than monitoring the exposure to toxicants, biomarkers can also be useful to evaluate the biological responses associated with potential health effects (Mattes et al., 2014).

Among metabolites derived from exogenous compounds, mercapturic acids are the urinary end-products of the metabolism of different toxicants (De Rooij et al., 1998). In a previous work, we evaluated 17 urinary mercapturic acids derived from several volatile organic compounds, in 67 subjects with different smoking habits: traditional

tobacco smokers (TTS), electronic cigarette users (ECU) and non-smokers (NS) (Chapter 3).

Untargeted metabolomics is a relatively new approach, whose development has been made possible thanks to advancements in analytical instrumentation and computational power. It aims to study the metabolome, i.e. the ensemble of small molecules produced from the organism presents in a biological fluid and their modification associated with a specific condition (Dunn et al., 2011). Untargeted metabolomics is a promising approach to characterize metabolites associated with the exposure to environmental xenobiotics, thus allowing us to characterize the "exposome" (Dennis et al., 2017). Previous untargeted metabolomic experiments in smoking subjects were conducted mainly in blood (Hsu et al., 2013; Müller et al., 2014; Gu et al., 2016; Kaluarachchi et al., 2016; Hsu et al., 2017), while only a few were conducted in urine (Garcia-Perez et al., 2014; Ramakrishnan et al., 2016), including some experiences assessing volatile metabolites (Rocha et al., 2012; Wang et al., 2018). Other approaches that are in between the targeted measurements and the untargeted metabolomics are also possible, such as the recently published method for the non-targeted screening of mercapturic acids using neutral loss detection and post-column infusion internal standard correction (Bloch et al., 2019).

The aim of this work was to perform an untargeted high performance liquid chromatography-mass spectrometry metabolomic experiment to investigate different smoking modes. Urine samples of subjects previously investigated for mercapturic acids were used (chapter 3). In this untargeted metabolomic approach, the features allowing the differentiation among groups were identified and annotated. Results of the untargeted approach were compared with those of the previous targeted study; to facilitate this comparison the chromatographic conditions and mass spectrometry ionization mode applied in the analysis of samples were the same.

4.2 Materials and methods

4.2.1 Study subject

The experiments were conducted using the same urine samples obtained in the frame of the study aimed to assess the urinary concentrations of mercapturic acids (see chapter 3, for details). Briefly, 67 healthy subjects with comparable age and body mass index were classified for their smoking habits: 38 non-smokers (NS), 7 electronic cigarette users (ECU) and 22 traditional tobacco smokers (TTS).

4.2.2 Chemicals

Analytical grade acetonitrile, aqueous ammonia (30%), formic acid, and methanol were purchased from Sigma-Aldrich (Milan, Italy). Purified water was obtained using a Milli-Q Plus ultrapure water system (Millipore, Milford, MA, USA). Authentic chemical standards of the mercapturic acids were purchased from Toronto Research Chemicals (Ontario, Canada), with exception of N-acetyl-S-phenyl-L-cysteine (SPMA), which was purchased form Tokyo Chemical Industry (Tokyo, Japan), and N-acetyl-S-phenyl-L-cysteine-D2 (SPMA-D2), which was purchased from CDN Isotope (Pointe-Claire, Quebec, Canada). O-, m-, and p- methylhippuric acid were purchased from Tokio Casei (Nihonbashi, Chūō, Tokyo, Japan) and creatinine was purchased from Merck KGaA (Darmstadt, Germany).

4.2.3 Sample preparation

500 µL of each urine samples was added to 500 µL of an aqueous solution of 0.2 M formic acid and 20 µL of a mixture containing deuterated internal standards of mercapturic acids (chapter 1). This solution was mixed through vortex, filtered using a regenerated cellulose membrane filter (0.45 µm) (Agilent Technologies, Cernusco Sul Naviglio, Italy), and transferred to an autosampler vial.

4.2.4 Analytical experiment

Analytical experiments were carried out using a liquid chromatograph system coupled with tandem mass spectrometry (LC-MS/MS). In particular, the LC part consisted of a UHPLC Exion LC (AB Sciex, Monza, Italy). The autosampler temperature was set at 8 °C and the injection volume was set at 2 µL. The column used was a Betasil C_{18} column (150 ×, 2.1 mm, 5 µm; Thermo Fisher Scientific, Rodano, Italy) along with the Betasil C_{18} pre-column (10 × 2.1 mm, 5 µm; Thermo Fisher Scientific, Rodano, Italy). The column oven was set at 40 °C. A linear gradient with two mobile phases was applied; the A phase was an aqueous solution of ammonium formate (5 mM) with 0.1% formic acid, and the B phase was acetonitrile. The gradient was programmed as follows: 0–0.5 min, 0.5% B isocratic; 0.5–2 min, from 0.5% to 30% B; 2–7 min, 30% B isocratic; 7–8 min, from 30% to 100% B; 8–12 min, 100% B isocratic; 12–12.1 min, from 100% to 0.5% B; and 12.1–18 min, 0.5% B isocratic. The flow rate was set at 200 µL/min. The mass spectrometer part consisted of a time of flight Triple-TOF 6600 (AB Sciex, Monza, Italy). The instrument was set in data-dependent mode: a full mass experiment with range 100-600 m/z was carried out, while the MS/MS experiments triggered when the signal exceeded 1000 cps, for the 10 most intense signal, and excluding the same parent ion for 10 seconds after two consecutive triggers. The acquisition time for the full mass experiment was 250 ms; while, for each data dependent experiment, it was 75 ms. Polarity was set in negative and the following parameters were used: curtain gas (N_2), 30 psi; ion spray voltage, -4500 V; temperature, 350°C; ion source gas 1 (air), 50 psi; ion source gas 2 (air), 45 psi; declustering potential, -50 V; collision energy, -15 V; collision energy spread, 30 V. During each analytical sequence, an external calibration was performed every three analysis, according to the manufacturer's instructions, to calibrate mass accuracy of the mass spectrometer. The Analist® software (version 1.7.1; Ab Sciex S.r.l, Milano, Italy) was used to prepare batches for analysis. Data were acquired in profiling mode.

4.2.5 Data integration, analysis and metabolite annotation

Data obtained with the analyses were converted from "wiff" files to "mzML" files using ProteoWizard MSConverter 3.0.19248 (Chambers et al., 2012) using the peak piking algorithm on all MS levels. These files were then processed using the IPO algorithm (Libiseller et al., 2015), which was run using R (version 3.6.1, R Foundation, Vienna, Austria) (R-Core-Team, 2019) with the Rstudio interface (Version 1.2.1335, RStudio Inc., Boston, Massachusetts, United States) in order to obtain the optimal parameters for XCMS (*centWave* algorithm). The dataset containing the detected features was obtained using the on-line version of XCMS (Tautenhahn et al., 2012; Tautenhahn et al., 2008), using the following parameters: minimum peak width, 3; maximum peak width, 95; ppm, 28.45; mzdiff, -0.0175; signal to noise threshold, 3; noise, 0; prefilter, 3; value of prefilter, 100; integration method, 1; bw, 0.88; minfrac, 0.5; mzwid, 1×10^{-4}.

The obtained dataset was investigated using MetaboAnalyst (Chong et al., 2018) to perform both multivariate and univariate analysis. Principal Components Analysis (PCA) was performed for dimensional reduction and data visualization. Features with more than 50% missing values were removed, missing value imputation was performed using the *k*-nearest neighbour (KNN) algorithm, data were normalised by sum and log transformed, and a pareto scaling was applied. One-way ANOVA was performed, with data normalised by sum and log-transformed. A Fisher's LSD post-hoc test was also applied for inter-group comparison.

The software BEAMS (Birmingham mEtabolite Annotation for Mass Spectrometry), developed at the University of Birmingham (unpublished), was then implemented for grouping adducts and isotopes, and to perform annotation of metabolite features. The following parameters were used for grouping features: maximum RT difference (sec), 5; coefficient threshold, 0.70; grouping method, Pearson correlation; P-value threshold, 0.01; cpus, 3; block size, 5000; annotation of peak patterns was performed considering adducts, isotopes and multiple charged ions, with a mass tolerance of 6 ppm. Then, features which were statistically

significantly different among groups in the one-way ANOVA test were further investigated, scrutinizing each feature, and merging entries with close masses or retention times and other possible adducts. Metabolite annotation was completed by manually comparing the fragmentation pattern obtained (where available) from each $[M-H]^-$ parent ion with data stored in the on-line databases of Metlin (Guijas et al., 2018) and HMDB (Wishart et al., 2018), checking data from all the levels of collision energies available, along with comparison with fragmentation patterns obtained in-silico using MS-Finder (Tsugawa et al., 2016) and Met-frag (Ruttkies et al., 2016). Annotated metabolites were grouped as confidently identified compounds (level 1), putatively annotated compounds (level 2), putatively annotated compound classes (level 3), or unknown compounds (level 4) according to the proposed minimum reporting standards as suggested by the Metabolomics Standards Initiative (Sumner et al., 2007).

4.3 Results

An overview of the workflow and a summary of the results are given in Figure 4.1.

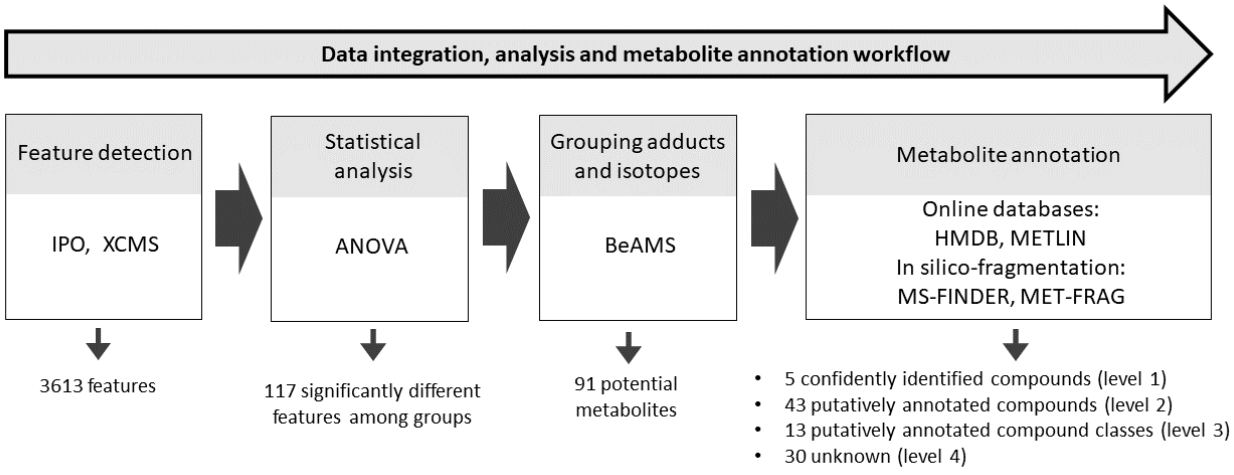

Figure 4.1 - Workflow implemented for the data integration, analysis and metabolite annotation.

4.3.1 Statistical analyses and metabolite annotation

Signals from the analyses of all urine samples elaborated with XCMS were summarised in to 3613 features. A clear separation of groups was not achieved with the PCA (Figure 4.2 and Figure 4.3).

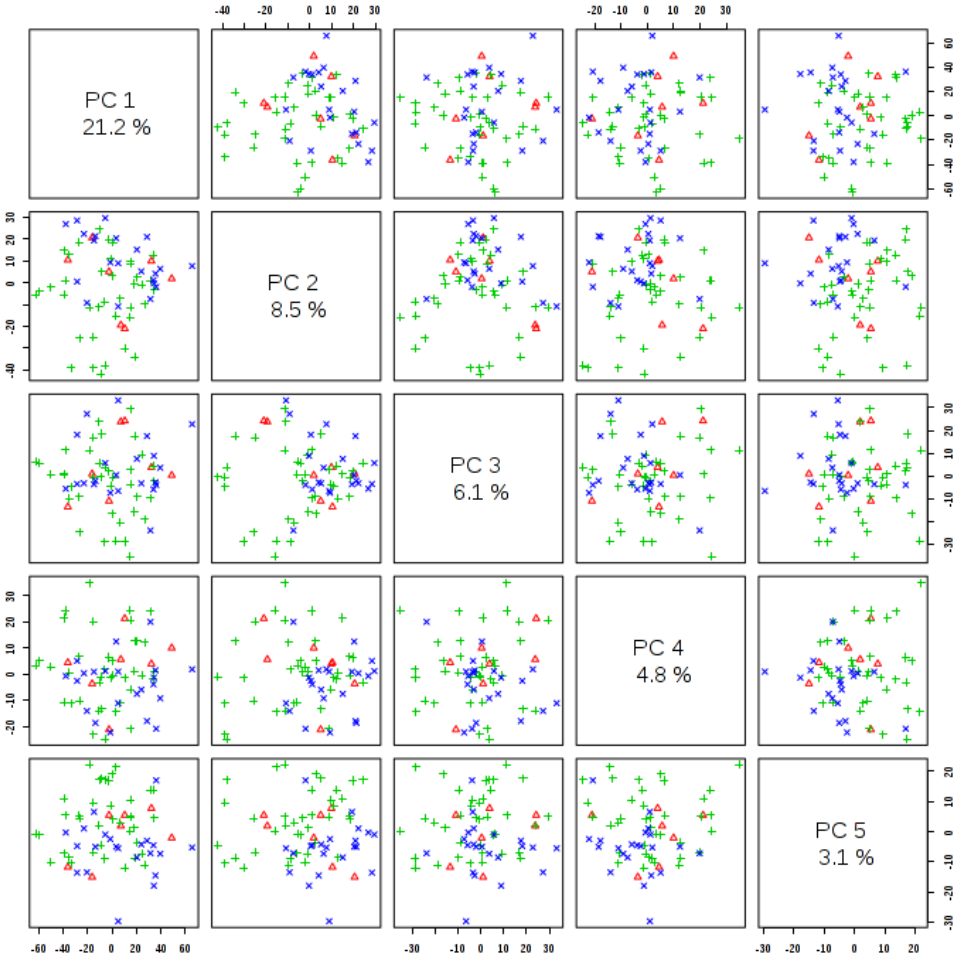

Figure 4.2 - Results from PCA, all pairs from Principal Component 1 to Principal Component 5

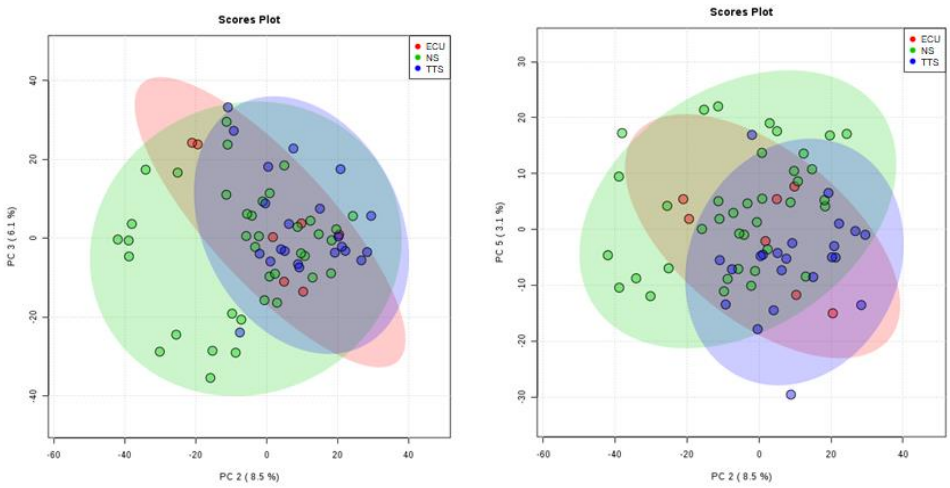

Figure 4.3 - Results of PCA, scores plot Principal Component 2 vs Principal Component 3 (left) and scores plot Principal Component 2 vs Principal Component 5 (right). Each point represents a subject (red for ECU, green for NS and blue for TTS), while the percentage of variance of each component is given in brackets

From the 3613 features, the application of the one-way ANOVA test identified 117 features with significant differences among groups. Using the software BEAMS for grouping adducts and manually inspecting each feature, 91 potential metabolites were defined. After manually inspecting mass fragmentation spectra, 5 metabolites were confidently identified through comparison (retention time and MS/MS mass spectrum) with purchased external chemical standards analysed under the same analytical conditions (level 1), 43 were putatively annotated (level 2) by matching m/z and MS/MS fragments with those reported in different databases, 13 compounds were putatively annotated only for their compound class (level 3), and 30 were considered unknown compounds (level 4). The list of compounds annotated with level 1, 2, and 3 is reported in Table 4.1, while some examples of confidently identified compounds and putatively annotated compounds are reported in Figure 4.4 and Figure 4.5.

Table 4.1 - List of confidently identified compounds (level 1 according to Sumner et al., 2007), putatively annotated compounds (level 2) and putatively annotated compound classes (level 3). For each compound, significance differences in the Fisher's LSD post-hoc test are given, along with molecular formula, ppm error, compound name, an identification code, and the supposed parent compound (for most compounds this information was obtained from HMDB).

Level 1 – confidently identified compounds						
ANOVA P-value	**Fisher's LSD**	**Molecular formula**	**ppm error**	**Annotated compounds name**	**Code**	**Metabolite of**
3.56×10^{-12}	TTS > ECU; TTS > NS	C9H17NO4S	+3.19	N-Acetyl-S-(3-hydroxy-1-methylpropyl)-L-cysteine (HMPMA)	PubChem CID: 46780019	crotonaldehyde
7.69×10^{-12}	TTS > ECU; TTS > NS	C8H15NO4S	-0.86	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA)	PubChem CID: 119083	acrolein
4.03×10^{-4}	TTS > ECU; TTS > NS	C9H17NO5S	-1.65	N-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA)	PubChem CID: 44151464	butadiene
3.57×10^{-12}	TTS > ECU; TTS > NS	C10H11NO3	-1.13	m- or p-methylhippuric acid	HMDB0013245 / HMDB0013292	m- or p-xylene
9.90×10^{-4}	NS > TTS	C4H7N3O	-4.78	Creatinine	HMDB0000562	-
Level 2 – putatively annotated compounds						
ANOVA P-value	**Fisher's LSD**	**Supposed molecular formula**	**ppm error**	**Annotated compounds name**	**Code**	**Metabolite of**
1.19×10^{-21}	ECU > NS; TTS > NS	C16H20N2O8	-7.05	trans-3-Hydroxycotinine glucuronide	HMDB0001204	nicotine
8.86×10^{-21}	TTS > ECU; TTS > NS	C8H10O4S	+1.47	3-Ethylphenyl sulfate / 4-Ethylphenylsulfate	HMDB0062721 / HMDB0062551	3-ethylphenol / 4-ethylphenol
1.61×10^{-9}	TTS > ECU; TTS > NS					
3.06×10^{-5}	ECU > NS; TTS > NS	C7H8O5S	-0.83	O-methoxycatechol-O-sulphate	HMDB0060013	Guaiacol (o-Methoxyphenol)
3.69×10^{-18}	TTS > ECU; TTS > NS	C7H5NO4S	-3.8	5-Mercapto-2-Nitro-Benzoic Acid	DrugBank=DB02763	-
1.80×10^{-16}	TTS > ECU; TTS > NS	C11H9N3O3	-4.84	1-(2-hydroxyethyl)-4-[1]benzopyrano[3,4-d]triazolone	CHEBI:114849	-
5.07×10^{-16}	ECU > NS; TTS > ECU; TTS > NS	C8H8O5S	-4.04	(5-ethenyl-2-hydroxyphenyl)oxidanesulfonic acid	HMDB0124978	4-ethenylbenzene-1,2-diol
8.12×10^{-16}	TTS > ECU; TTS > NS	C13H10O4	+2.91	7-hydroxy-6-(3-oxobut-1-en-1-yl)-2H-chromen-2-one / Coriandrin	HMDB0128952 / HMDB33329	7-methoxy-6-(3-oxobut-1-en-1-yl)-2h-chromen-2-one
8.48×10^{-16}	TTS > ECU; TTS > NS	C9H12O4S	-1.18	(3-phenylpropoxy)sulfonic acid	HMDB0135313	3-phenylpropan-1-ol
1.33×10^{-15}	TTS > ECU; TTS > NS	C26H40O8	-2.39	xylarenone E	CHEBI:69734	-

Table 4.1 - Continue

ANOVA P-value	Fisher's LSD	Supposed molecular formula	ppm error	Annotated compounds name	Code	Metabolite of
3.39×10^{-15}	TTS > ECU; TTS > NS	C11H12O5S	-5.76	[(3-methyl-2-oxo-4-phenylbut-3-en-1-yl)oxy]sulfonic acid / [4-(2-methyl-3-oxobut-1-en-1-yl)phenyl]oxidanesulfonic acid / [3-oxo-2-(phenylmethylidene)butoxy]sulfonic acid / [3-oxo-2-(phenylmethylidene)butoxy]sulfonic acid	HMDB0133689 / HMDB0133695 / HMDB0133691 / HMDB0133693	1-hydroxy-3-methyl-4-phenylbut-3-en-2-one / 4-(4-hydroxyphenyl)-3-methylbut-3-en-2-one / 3-(hydroxymethyl)-4-phenylbut-3-en-2-one / 4-(3-hydroxyphenyl)-3-methylbut-3-en-2-one
1.01×10^{-10}	ECU > NS; TTS > NS					
5.31×10^{-7}	ECU > NS; TTS > ECU; TTS > NS					
9.82×10^{-15}	TTS > ECU; TTS > NS	C10H12O4S	-2.44	[(2E)-2-methyl-3-phenylprop-2-en-1-yl]oxy]sulfonic acid / [((3E)-4-phenylbut-3-en-2-yl)oxy]sulfonic acid	HMDB0133620 / HMDB0133731	(2e)-2-methyl-3-phenylprop-2-en-1-ol / 4-phenylbut-3-en-2-ol
2.49×10^{-12}	TTS > ECU; TTS > NS	C21H30O7	-2.99	Pteroside Z / Secoeremopetasitolide B	HMDB32587 / HMDB41363	-
8.84×10^{-10}	TTS > ECU; TTS > NS	C5H9NO3S	-2.09	Tiopronin	CHEBI:32229	-
1.12×10^{-9}	ECU > NS; TTS > ECU; TTS > NS	C8H10O5S	-1.93	Tyrosol 4-sulfate / (5-ethyl-2-hydroxyphenyl)oxidanesulfonic acid	HMDB0041785 / HMDB0124986	polyphenol metabolite / 4-ethylbenzene-1,2-diol
7.59×10^{-7}	TTS > ECU; TTS > NS					
2.43×10^{-9}	TTS > ECU; TTS > NS	C12H22O4	-2.76	Dodecanedioic acid	HMDB0000623	-
5.06×10^{-9}	TTS > ECU; TTS > NS	C8H7NO4S	+0.93	Indoxyl sulfate	HMDB0000682	tryptophan
6.61×10^{-9}	ECU > NS; TTS > NS	C10H10O6S	-2.07	[2-hydroxy-5-(3-oxobut-1-en-1-yl)phenyl]oxidanesulfonic acid / (2E)-2-methyl-3-[4-(sulfooxy)phenyl]prop-2-enoic acid / (2E)-2-methyl-3-[3-(sulfooxy)phenyl]prop-2-enoic acid / [4-((1E)-3-methoxy-3-oxoprop-1-en-1-yl)phenyl]oxidanesulfonic acid	HMDB0135681 / HMDB0133667 / HMDB0133664 / HMDB0131180	4-(3,4-dihydroxyphenyl)but-3-en-2-one / (2e)-3-(4-hydroxyphenyl)-2-methylprop-2-enoic acid / (2e)-3-(3-hydroxyphenyl)-2-methylprop-2-enoic acid / (2e)-3-(4-hydroxyphenyl)prop-2-enoate
2.56×10^{-4}	ECU > NS; TTS > NS					
2.59×10^{-7}	TTS > ECU; TTS > NS	C16H20O8	+0.76	trans-isoeugenol-O-glucuronide	HMDB0060021	trans-isoeugenol
1.53×10^{-6}	TTS > ECU; TTS > NS	C8H8O4S	-1.78	4-Vinylphenol sulfate	HMDB0062775	4-Hydroxystyrene
2.82×10^{-6}	TTS > ECU; TTS > NS	C9H12O5S	+1.87	(4-ethyl-2-methoxyphenyl)oxidanesulfonic acid	HMDB0127988	4-Ethyl-2-methoxyphenol
3.40×10^{-6}	TTS > ECU; TTS > NS	C21H32O8	+0.63	5-(2,3-Dihydroxy-3-methylbutyl)-4-(3,4-epoxy-4-methylpentanoyl)-3,4-dihydroxy-2-isopentanoyl-2-cyclopenten-1-one / Abscisic alcohol 11-glucoside	HMDB0030082 / HMDB39636	-
3.61×10^{-6}	ECU > NS; TTS > NS	C7H9NO4S	-0.26	Cystathionine ketamine	HMDB0002015	-
1.05×10^{-5}	ECU > TTS; NS > TTS	C11H15NO3S	-2.87	methiocarb-sulfoxide / 2-(Ethylsulfinylmethyl)phenyl methylcarbamate	CHEBI:83542 / HMDB0040289	Methiocarb / Ethiofencarb
1.10×10^{-05}	TTS > NS	C10H8O6S	-7.78	4-Methylumbelliferyl sulfate / [(1-oxo-1H-isochromen-3-yl)methoxy]sulfonic acid	CHEBI:1905 / HMDB0128627	4-methylumbelliferone / 3-(hydroxymethyl)-1h-isochromen-1-one

Table 4.1 - Continue

ANOVA P-value	Fisher's LSD	Supposed molecular formula	ppm error	Annotated compounds name	Code	Metabolite of
1.46×10^{-5}	ECU > NS; TTS > NS	C12H22O11	-0.4	disaccharide (Lactose, Maltose, Melibiose, Sucrose, Trehalose)	HMDB0000186 / HMDB0000163 / HMDB0000048 / HMDB0000258 / HMDB0000975	-
4.96×10^{-4}	ECU > NS; TTS > NS					
2.79×10^{-5}	TTS > ECU; TTS > NS	C20H30O7	-2.56	12-Oxo-20-trihydroxy-leukotriene B4	HMDB0012553	leukotriene B4 (LTB4)
7.03×10^{-5}	TTS > NS	C8H11NO4S	+3.69	Tyramine-O-sulfate	HMDB0006409	Tyramide
1.06×10^{-4}	ECU > NS; TTS > NS	C12H16N2O5	+0.95	Tyrosyl-Serine / Serinyl-Tyrosine	HMDB29114 / HMDB29051	-
2.59×10^{-4}	ECU > TTS; NS > TTS	C10H12N2O5	-4.79	(±)-2-(1-Methylpropyl)-4,6-dinitrophenol / Dinoterb	HMDB0032559 / CHEBI:81883	-
3.19×10^{-4}	ECU > NS; TTS > NS	C26H32O10	-2.53	Myricatomentoside I	HMDB0031536	-
5.71×10^{-4}	ECU > NS; TTS > NS	C13H10O5S	+5.82	MINEs-120960 / MINEs-120956	MINEs-120960 / MINEs-120956	
7.44×10^{-4}	ECU > NS; TTS > NS	C16H20O10	-3.16	Dihydroferulic acid 4-O-glucuronide	HMDB0041723	-
8.45×10^{-4}	TTS > ECU; TTS > NS	C7H6O8S	+8.99	2,4-dihydroxy-3-(sulfooxy)benzoic acid / 3,5-dihydroxy-4-(sulfooxy)benzoic acid	HMDB0130471 / HMDB0126639	trihydroxybenzoic acid
1.14×10^{-3}	ECU > NS; TTS > NS	C6H9N3O2	+2.59	L-Histidine	HMDB0000177	-
1.27×10^{-3}	ECU > NS; TTS > NS	C18H22O11	-0.57	5-(3",4",5"-trihydroxyphenyl)-gamma-valerolactone-O-methyl-4"-O-glucuronide / 5-(3",4",5"-trihydroxyphenyl)-gamma-valerolactone-O-methyl-5"-O-glucuronide	HMDB0060027 / HMDB0060028	5-(3',4',5'-trihydroxyphenyl)-gamma-valerolactone-O-methyl / 5-(3',4',5'-trihydroxyphenyl)-gamma-valerolactone-O-methyl
1.32×10^{-3}	ECU > NS; TTS > NS	C8H9NO4	+0.1	4-Pyridoxic acid	HMDB0000017	vitamin B6
1.53×10^{-3}	ECU > TTS; NS > TTS	C10H16N2O4	-0.14	Prolylhydroxyproline	HMDB0006695	-

Level 3 – putatively annotated compound classes

ANOVA P-value	Fisher's LSD	Supposed molecular formula	ppm error	Annotated compound class
1.04×10^{-25}	ECU > NS; TTS > ECU; TTS > NS	C11H12O4S	+1.05	Sulfonic acid
2.87×10^{-20}	TTS > ECU; TTS > NS	C12H12O4S	-0.99	Sulfonic acid
1.82×10^{-17}	TTS > ECU; TTS > NS	C12H14O4S	-5.94	Sulfonic acid
6.48×10^{-4}	TTS > NS	C8H8O5S	+0.15	Sulfonic acid

Table 4.1 - Continue

ANOVA P-value	Fisher's LSD	Supposed molecular formula	ppm error	Annotated compounds name	Code	Metabolite of
5.55×10^{-5}	TTS > NS	C8H12O2	-6.14	Methyl-branched fatty acids / Cyclic ketones / Fatty acid esters / Medium-chain fatty acids		
5.57×10^{-5}	TTS > ECU; TTS > NS	C10H12O6S	-4.57	Sulfonic acid / Phenylsulfates		
8.96×10^{-5}	TTS > ECU; TTS > NS	C15H22O9	-3.5	conjugated polyphenol		
1.91×10^{-4}	TTS > NS	C7H10O2	-2.43	Cyclic ketones		
5.75×10^{-4}	TTS > NS	C12H16O8	-0.49	Phenolic glycosides		
6.00×10^{-4}	NS > ECU; TTS > ECU	C9H10O4	+2.03	Phenols		
8.54×10^{-4}	TTS > NS	C9H10O3	-5.56	Benzenoids		
1.06×10^{-3}	TTS > ECU; TTS > NS	C5H8O3	-4.07	Short-chain keto acids and derivatives		
1.39×10^{-4}	ECU > NS; TTS > NS	C15H16O8S	+0.81	sulfonic acid		

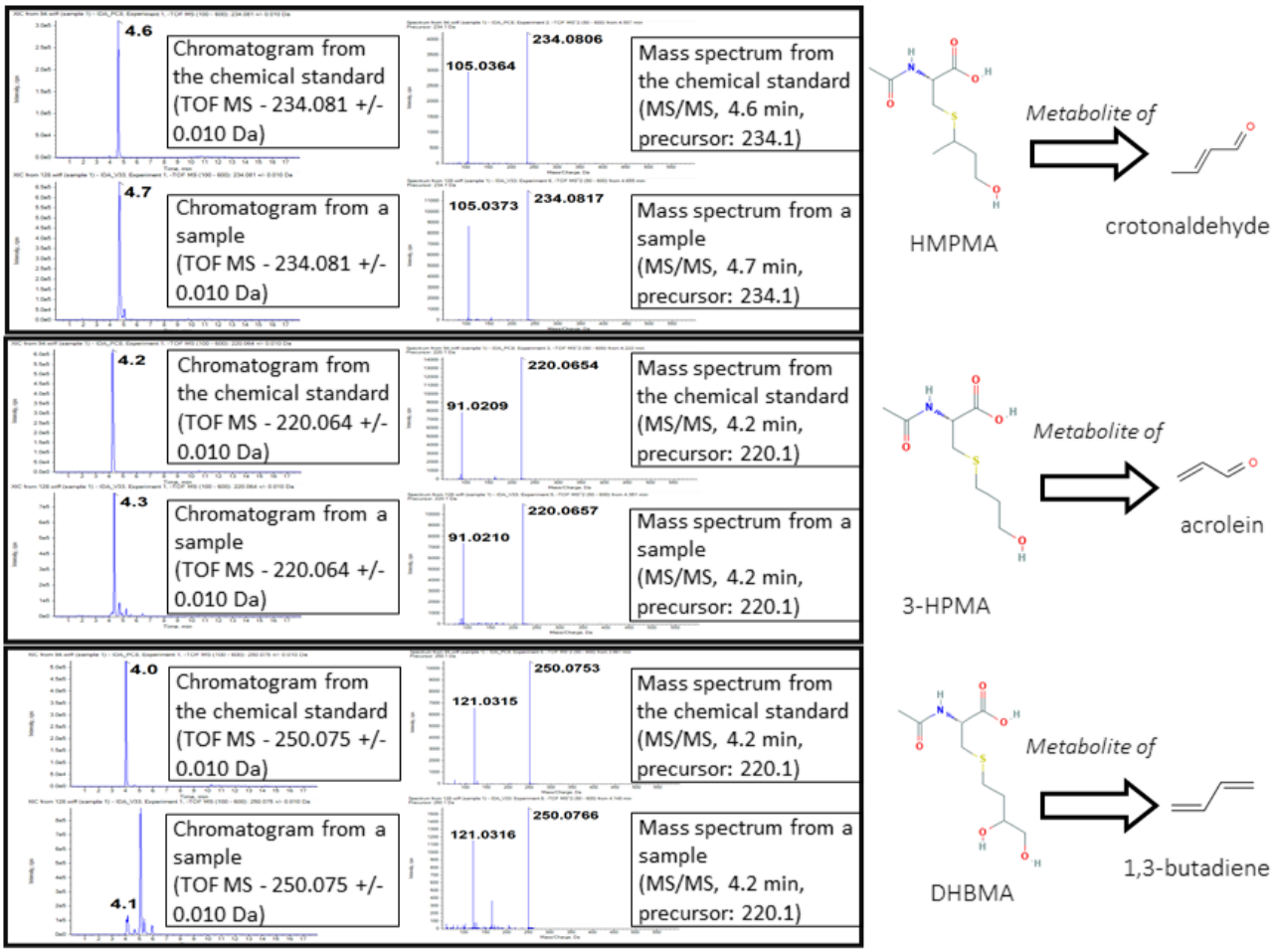

Figure 4.4 - Examples of confidently identified compounds (level 1)

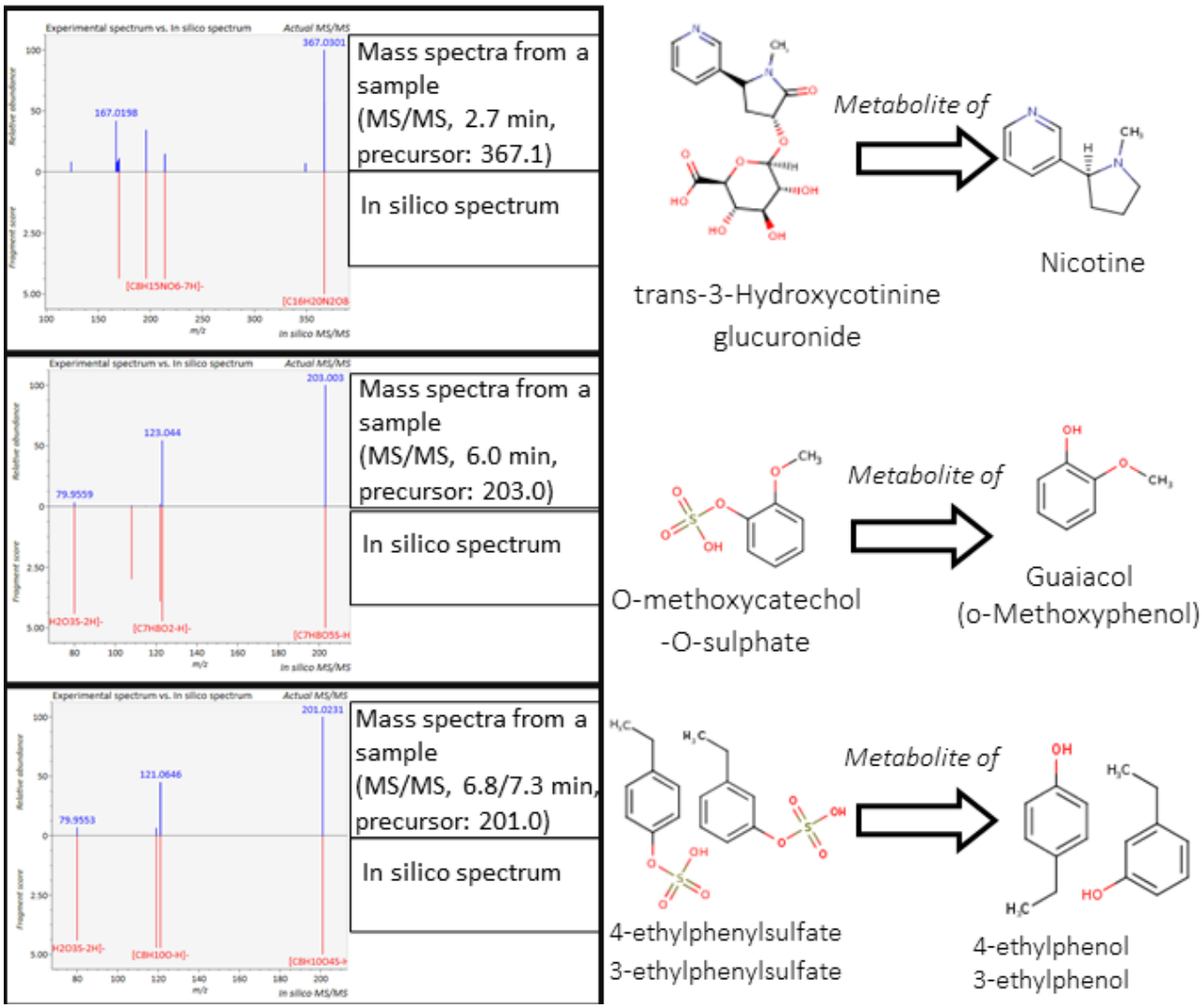

Figure 4.5 - Examples of putatively annotated compounds (level 2)

4.3.2 Comparison between the untargeted metabolomic approach and the targeted approach

The results related to mercapturic acids obtained in the present study were compared with those obtained quantifying urinary mercapturic acid concentrations with a targeted approach (chapter 3). As reported in Table 4.2, only three mercapturic acids (3-HPMA, DHBMA, and HMPMA) were identified as a feature by the XCMS algorithm and their intensities were significantly different among groups. CMEMA and AMCC were also identified as features, but their intensities were not different among groups; this is consistent with results from the targeted approach for

CMEMA, while it is not for AMCC. Several molecules (2-HPMA, AAMA, CEMA, GAMA, HEMA, MHBMA, and SBMA) were not identified as a feature by the XCMS algorithm, but their presence was verified after inspecting the chromatograms. Finally, some of the compounds present at the lowest concentrations (EMA, MMA, PHEMA, and SPMA) were not detected neither using the algorithms nor inspecting the chromatograms.

Table 4.2 - Comparison between the targeted and the untargeted approach in the detection of urinary mercapturic acids.

	Targeted approach				**Untargeted approach**			**Coherence of targeted vs untargeted approach**
Urinary mercapturic acids	**Median concentrations in NS (µg/L)**	**Median concentrations in ECU (µg/L)**	**Median concentrations in TTS (µg/L)**	**P-value of ANOVA**	**Part of the 91 different features**	**Part of the 3613 total features**	**Identified after inspecting the chromatogram**	
3-HPMA	224	414	1594	< 0.001	✓	✓	✓	✓
DHBMA	348	405	644	0.002	✓	✓	✓	✓
HMPMA	54	77	326	< 0.001	✓	✓	✓	✓
AMCC	220	364	572	0.009		✓	✓	
CMEMA	340	349	578	0.245		✓	✓	✓
2-HPMA	10.3	16.3	33.6	< 0.001			✓	
AAMA	65	84	113	< 0.001			✓	
CEMA	1.2	4.1	147	< 0.001			✓	
GAMA	3.5	3.6	7.6	0.011			✓	
HEMA	1.4	3.1	2.8	0.001			✓	
MHBMA	0.42	1.16	4.94	< 0.001			✓	
SBMA	2.69	2.94	2.18	0.240			✓	
EMA	0.03	0.02	0.12	0.048				
MMA	4.34	5.09	4.20	0.626				
NANPC	< LOQ	< LOQ	< LOQ	N.A				✓
PHEMA	0.60	1.09	1.24	0.013				
SPMA	0.07	0.17	0.64	< 0.001				

4.4 Discussion

In this work, we presented the results of an untargeted metabolomic experiment carried out on urine samples from a pilot cross-sectional study involving subjects with different smoking habits.

Multivariate analyses were used only for dimensional reduction and data visualization of features, but no clear separation was achieved with these approaches (Figure 4.2 and Figure 4.3). Univariate one-way ANOVA was implemented to find significantly different features among groups and metabolite annotation was performed only for these features. After grouping possible adducts, the annotation was performed on 91 potential metabolites. We were able to confidently identify with an external chemical standard (level 1 according to the proposed minimum reporting standard) (Sumner et al., 2007) only 5 compounds (Table 4.1). Three of the five identified compounds are mercapturic acids: N-acetyl-S-(3-hydroxy-1-methylpropyl)-L-cysteine (HMPMA), N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3-HPMA), and N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA). These compounds were significantly higher in TTS when compared to both NS and ECU. Indeed they are the metabolites of crotonaldehyde, acrolein, and 1,3-butadiene, respectively, and these compounds have been reported to be present in tobacco smoke (IARC, 2004). The other identified compounds (level 1) were firstly annotated by comparison of mass spectra with on-line databases, and afterwards by analysis, comparing their signal with an external chemical standard. Methylhippuric acid is a metabolite of xylene (Kira, 1977), which is a known compound present in tobacco smoke (IARC, 2004). Indeed, the feature identified as methylhippuric acid was significantly higher in TTS than ECU/NS. We tested the three possible isomers with three different standards: o-methylhippuric acid, m-methylhippuric acid, and p-methylhippuric acid. While o-methylhippuric acid eluted earlier (5.61 min), m- and p-methylhippuric acid had the same retention time (6.18 min). The peak in samples had the same retention time of m- and p-methylhippuric acid, so it can be one of them or a mixture of both. The last identified compound was creatinine,

which was higher in NS than TTS; this was surprising as in the previous study (chapter 3) creatinine was not different among the groups.

Several compounds were annotated at level 2, for most of which the annotation was based on the predicted fragmentation spectra produced by MS-Finder and the information present in HMDB. Among putatively annotated compounds, 3-hydroxycotinine glucuronide (Table 4.1), a metabolite of nicotine, showed the most significant difference in the ANOVA test ($P = 1.19 \times 10^{-21}$) and it was significantly higher in ECU and TTS than in NS. This result is supported by similar differences of urinary cotinine in TTS and ECU (chapter 3) and also by previously reported data showing that the concentration of nicotine in electronic and traditional cigarette smoke is comparable (Marsot and Simon, 2016). It is worth mentioning that we managed to find only one metabolite of nicotine (3-hydroxycotinine glucuronide), while several other nicotine metabolites have been reported to be present in urine; the discrepancy is due to the fact that our experiment was performed in the negative ion mode, while the large majority of nicotine metabolites is detected in the positive ion mode (Ramakrishnan et al., 2016).

Among the other annotated features at level 2, we proposed the presence of 3-ethylphenyl sulfate, 4-ethylphenylsulfate, and methoxycatechol-sulphate, which are metabolites of 3-ethylphenol, 4-ethylphenol, and guaiacol, respectively. All of them are compounds reported to be present in tobacco smoke (Clark and Bunch, 1996; Rodgman and Perfetti, 2013). Interestingly, the two isomers of ethylphenyl sulfate were significantly higher in TTS than ECU/NS, while methoxycatechol-sulphate was significantly higher in ECU/TTS than NS, indicating that both electronic cigarette users and traditional tobacco smokers might be equally exposed to this compound. We annotated other metabolites of toxicants with a sulfate group. For all of them we identified a characteristic product ion of *m/z* 79.96. With this information, we managed to annotate the compound class (sulfonic acid) of other features (level 3). Some of the other compounds annotated are endogenous metabolites, which can be related to the alteration of metabolism induced by the

smoking habits. As an example, TTS showed higher levels of dodecanedioic acid (an indicator of hepatic carnitine palmitoyltransferase I deficiency) and higher levels of indoxyl sulfate, a metabolite of the amino acid tryptophan, that may point to alterations in the tryptophan metabolism in smokers, as previously reported in a smoking cessation study (Goettel et al., 2017). Furthermore, TTS had higher levels of 12-oxo-20-trihydroxy-leukotriene B4, which is linked to inflammation. It is important to mention, however, that results obtained with annotated compounds (level 2) should be interpreted with caution, since some of them may derive from a false positive annotation. Different compounds deriving from confounding factors may have been annotated; for example some of the polyphenol derivatives might derive from tobacco smoking and/or the consumption of coffee; in fact the association of smoking with coffee consumption has been reported (Bjørngaard et al., 2017).

Other untargeted metabolomic experiments carried out on urine samples of subjects with different smoking habits reported metabolites of nicotine as the main discriminant compounds between TTS and NS (Garcia-Perez et al., 2014; Ramakrishnan et al., 2016). Furthermore, the study by Garcia-Perez and co-workers reported the presence of other discriminating endogenous metabolites, which are different from the ones annotated in this work. This may be attributable to the different polarity mode applied (negative in this study, positive in the one by Garcia-Perez and co-workers).

Comparing the results relative to mercapturic acids to those obtained with the targeted approach (chapter 3), HMPMA, 3-HPMA, and DHBMA (with median levels ranging from 54 to 1595 µg/L) were the only mercapturic acids identified as a features and significantly different among groups with the approach reported in this study. Interestingly, these mercapturic acids are not currently present in the HMDB database, although they are well-known metabolites of xenobiotics. This highlights that the limited coverage of metabolites in on-line databases is still a shortcoming of untargeted metabolomics, especially when trying to characterize

metabolites of xenobiotics or environmental exposures. Another major limitation of untargeted metabolomics highlighted in this pilot experiment was the lack of sensitivity of such an untargeted approach. Indeed, in our previous work we used a targeted approach to determine a total of seventeen mercapturic acids in the same urine samples (chapter 3). Besides HMPMA, 3-HPMA, and DHBMA, others urinary mercapturic acids were significantly higher in TTS, as SPMA (metabolite of benzene, median concentration from 0.07 to 0.64 µg/L) and CEMA (metabolite of acrylonitrile, median concentration from 1.2 to 147.3 µg/L), the latter being also different between ECU and NS. In that previous work, we used a low-resolution triple quadrupole mass spectrometer and each mercapturic acid was quantified with its own mass transition. Moreover, the parent/fragment ion pair and the collision energy were optimised for each analyte to obtain the highest signal sensitivity. With such a targeted approach, it was possible to accurately quantify even compounds present in concentrations lower than one µg/L (as for SPMA). In the non-targeted approach presented in this work, using a data-dependent mode and applying a non-specific ramp of collision energies, we did not manage to detect molecules present at median concentrations ranging from 0.03 to 4.20 µg/L (EMA, MMA, PHEMA, and SPMA). The missing detection of SPMA has been similarly reported in other non-targeted approaches (Bloch et al., 2019; Wagner et al., 2007). Furthermore, despite being detected after inspecting the chromatograms, others mercapturic acids, with median concentration from 0.42 to 147.3 µg/L were not present in the list of 3613 features identified by the XCMS algorithm (2-HPMA, AAMA, CEMA, GAMA, HEMA, MHBMA, and SBMA). This could derive both from a poor chromatographic separation of peaks and to a non-ideal choice of XCMS parameters, as the “minfrac” parameter (which is the minimum fraction, for each feature, of positive presence among all samples to keep that feature in the final result table). These limitations of untargeted approaches should be considered in every study aimed to assess the exposure to exogenous compounds in a certain condition (e.g. environmental or occupational). A possible solution to overcome these limitations could be the use of methods focused on a particular class of metabolites,

such as the one recently proposed for mercapturic acids, using a neutral loss detection (Bloch et al., 2019).

Some limitations may be identified in this study. The most important is the low number of subjects involved in the study, in particular for the ECU class. Another limitation is the use of negative polarity in the mass spectrometer, therefore excluding metabolites forming positive ions. This strategy was meant to directly compare the results of this study with those of the previous study on mercapturic acids and to focus on metabolites of exogenous chemicals, known to be mostly eliminated as carboxylic acids and phenols, following oxidative metabolism. Another weakness of this experiment was the lack of suitable quality controls, such as pooled quality controls, which could have improved the quality of the dataset (Broadhurst et al., 2018).

A strength of the work is the annotation of several different compounds; the effort was supported by the targeted study on urinary mercapturic acids previously performed and on the large literature on tobacco smoking. Furthermore, with this approach, we described the use of a combination of open-source tools, which can be useful for the interpretation of data from any untargeted metabolomic experiment. Finally, to the best of our knowledge this is the first untargeted metabolic experiment conducted in electronic cigarette users.

In conclusion, this untargeted metabolomic approach allowed to investigate metabolic changes in subjects with different smoking habits, showing several differences in both metabolites associated with exposure to toxicants and with precocious effects. The comparison with the results obtained with the targeted study quantifying mercapturic acids (chapter 3) highlights the limited power of the untargeted approach to identify differences between exposure groups when the compounds are present at concentration of tens of µg/L or lower. Another strength of this work is this attempt to quantify the range of applicability of an untargeted approach.

5 Biomonitoring of persistent organic compounds: development of a method to quantify bisphenol A and metabolites of phthalates and terephthalates in urine samples

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5.1 Introduction

During the last century, the production of industrial chemicals has greatly increased and their use has spread in general population. However, in recent years, some concerns have been raised regarding their possible adverse effects. In particular, certain pollutants have been defined as endocrine disrupter chemicals (EDCs) due to their ability to interfere with the physiological function of hormones (Kabir et al., 2015; Monneret, 2017; Darbre, 2018).

Phthalates are among compounds which have showed to act as EDCs, with estrogenic and androgenic disrupting properties (Darbre, 2018). They are used in several consumer products, and their low cost and attractive properties are likely the reasons of their success and extensive use. Among phthalates, di-2-ethylhexyl

phthalate (DEHP) have been used in a huge variety of products including food packaging and medical products (Benjamin et al., 2017). However, because of concerns regarding its potential adverse effects, the use of DEHP has been restricted, in particular in toys and childcare articles (EU, 2005). Di-2-ethylhexyl terephthalate (DEHTP) is chemically related to DEHP and it has been used as a substitute to DEHP as a safer alternative (Silva et al., 2017) and, for this reason, the human exposure to this compound may be on the rise (Silva et al., 2017; Silva et al., 2019; Lessmann et al., 2017). Others phthalates, recognized to be ECDs, used in food packaging, personal care products, and medication excipients are butylbenzyl phthalate (BBzP), diethyl phthalate (DEP), di-n/i-butyl phthalate (DnBP/DiBP) (Braun, 2017). Generally, phthalates are predominately excreted from the body after hydroxylation to their respective monoester metabolites and sometimes after a further glucuronide or sulphate conjugation (Frederiksen et al., 2007). For this reason, phthalate exposure can be assessed using urine biospecimens (Silva et al., 2007). In particular, the two major DEHP oxidative metabolites are mono-2-ethyl-5-carboxypentyl phthalate (MECPP) and mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) (Frederiksen et al., 2007) while the analogous, recently identified, DEHTP metabolites are mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP) and mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP) (Silva et al., 2015; Lessmann et al., 2016b). The searchable metabolites in urine of BBzP, DEP and DnBP/DiBP are monobenzyl phthalate (MBzP), monoethyl phthalate (MEP) and mono-n/i-butyl phthalates (MnBP/MiBP), respectively (Frederiksen et al., 2007).

Another recognised EDC is Bisphenol A (BPA), which is widely produced for the manufacture of polycarbonate plastic and other products, among which food containers, bottles, medical equipment, electronic devices, and toys (Mikołajewska et al., 2015; Vandenberg et al., 2007). Due to its extensive use, BPA is ubiquitous in the environment and can reach the human body mainly through food-contamination, drinking water and dust (Michałowicz, 2014). Once in the organism, the main metabolic reaction undergone by BPA is glucuronidation (Völkel et al., 2002). For these reason, the most common method to evaluate the human exposure

to BPA is to measure urinary concentrations of both free and conjugated BPA (Frederiksen et al., 2013).

Human biomonitoring, conducted by quantifying the specific biomarkers of EDCs in human non-invasive biological fluids, is a useful approach to evaluate the exposure to these compounds. Several methods have been developed in order to quantify the most common phthalates and bisphenol A (Silva et al., 2007; Myridakis et al., 2015; Wang et al., 2013; Heffernan et al., 2016; Chen et al., 2012; Tranfo et al., 2013). Only recently, a few new methods have been set up for the determination of alternatives phthalates such as DEHTP (Lessmann et al., 2016a; Nayebare et al., 2018; Been et al., 2019). The aim of this work was to develop and validate a suitable analytical method for the simultaneous determination of exposure biomarkers of bisphenol A and phthalates recognised to be ECDs, along with the novel alternative phthalate DEHTP.

5.2 Material and method

5.2.1 Chemicals

For the preparation of standard solutions, bisphenol A (BPA), bisphenol A-d8 (BPA-d8), monobenzyl phtalate (MBzP), monobenzyl phtalate-d4 (MBzP-d4), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), monoethyl phthalate (MEP), monoethyl phthalate-d4 (MEP-d4), monoisobutyl phthalate (MiBP), monoisobutyl phthalate-d4 (MiBP-d4), mono-n-butyl phthalate (MnBP), and mono-n-butyl phthalate-d4 (MnBP-d4) were purchased from Toronto Research Chemicals (Ontario, Canada) while mono-2-ethyl-5-carboxypentyl phthalate-d4 (MECPP-d4), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP), mono-2-ethyl-5-carboxypentyl terephthalate-d4 (MECPTP-d4), mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP), and mono-2-ethyl-5-hydroxyhexyl terephthalate-d4 (MEHHTP-d4), were purchased form Cansyn Chem Corp (Toronto, Canada).

Molecular structures of the considered compounds are shown in Table 5.1. Analytical grade acetonitrile, acetic acid, aqueous ammonia (30%), methanol, and the enzyme β-Glucuronidase from E. coli K 12 were purchased from Sigma-Aldrich (Milan, Italy). Purified water was obtained using a Milli-Q Plus ultrapure water system (Millipore, Milford, MA, USA).

5.2.2 Standard solution preparation

Each analytical standard (both the native and the isotopic labelled ones) were weighted and dissolved in methanol in order to obtain stock solutions at the concentration of 1 mg/mL.

Isotopically labelled standards were diluted in water in order to obtain a mixture at the final concentration of 1 mg/L for each compound (internal standards working solution).

Stock solutions of native standards were diluted in water in order to obtain a working solution of native standards at the final concertation of 0.5 mg/mL for BPA, 1 mg/mL for MBzP, MECPTP, MECPTP, and MEHHTP, and 5 mg/mL for MEP, MiBP, and MnBP. This solution was further diluted in water in order to obtain calibration curve and quality control (QC) solutions. In particular, ten solutions at different concentrations were prepared for the calibration curve, along with an unadulterated (blank) sample of water. Analyte concentrations in these solutions ranged from 0.01 to 50 μg/L for BPA; from 0.02 to 100 μg/L for MBzP, MECPTP, MECPTP, and MEHHTP, and from 0.1 to 500 for MEP, MiBP, and MnBP.

Independent concentrations for QC were 1.56 and 12.5 μg/L for BPA; 3.13 and 25 μg/L for MBzP, MECPTP, MECPTP, and MEHHTP; 15.63 and 125 μg/L for MEP, MiBP, and MnBP.

Stock and aliquoted working solutions of native and internal standards were stored at -20°C in the dark.

5.2.3 Sample preparation

1 mL of each sample (or blank, calibration curve levels, and QCs) was added to 500 µL of ammonium acetate buffer (1 M, pH 6.5) and to 5 µL of the internal standards working solution. 30 µL of β-glucuronidase from *E. coli* K 12 (diluted 1:1 with the buffer) was then added, the solution was vortexed incubated at 37°C for 2.5 hours. After incubation, 50 µL of pure acetic acid was added and the solution was vortexed again. The resulting solution was directly loaded on an Oasis® Prime HLB 3cc (60 mg) extraction cartridge (Waters S.p.A., Sesto San Giovanni, Milan, Italy) connected to a vacuum chamber. The cartridge was first washed with 2 mL of an aqueous solution containing 30% of methanol and 1% of acetic acid, and then the analytes were eluted with 1.5 mL of pure methanol. The obtained eluate was evaporated to dryness under nitrogen flow while kept at 40°C. Finally, 100 µL of water was added, the resulting solution was vortexed thoroughly and placed in the Autosampler for the analysis.

5.2.4 LC-MS/MS analysis

The instrumentation used for achieve the separation and the determination of considered compounds consisted of a high-pressure liquid chromatograph Agilent 1260 (Agilent Technologies, Cernusco Sul Naviglio, Italy) coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500; AB Sciex, Monza, Italy) with an electrospray ionization source. The autosampler temperature was set at 10 °C and the injection volume was set at 2 µL. The column used was an Accucore Phenyl-X (150 x 2.1 mm, 2.6µm) (Thermo Fisher Scientific, Rodano, Italy) along with a pre-column Accucore Phenyl-X (10 x 2.1 mm, 2.6µm) (Thermo Fisher Scientific, Rodano, Italy). The mobile phase was composed by a linear gradient with two mobile phases; the A phase was composed by water with 0.02% acetic acid, while the B phase was composed by acetonitrile with 0.02% acetic acid. The gradient was programmed as follows: 0-0.5 min, 20% B isocratic; 0.5-4 min,

from 20% to 40% B; 4–7 min, 40% B isocratic; 7-11 min, from 40% to 100% B; 11-16 min, 100% B isocratic; 16–16.1 min, from 100% to 20% B; and 16.1–22 min, 20% B isocratic. The flow rate was set at 300 μL/min and the column was kept at 40 °C.

The mass spectrometer operated in scheduled multiple reaction monitoring (MRM) mode, with a retention time window of 240 s. The target scan time was set at 1 s. The polarity was set to negative and the following conditions were used: gas 1 (air) pressure, 40 psi; gas 2 (air) pressure, 65 psi; curtain gas (N_2) pressure, 35 psi; heater temperature, 550 °C; ion spray voltage, −4500 V; and entrance potential, 10 V. For each analyte, the precursor ion/product ion pair and the collision energy were manually optimized through direct infusion. The precursor ion was always the pseudo molecular ion $[M - H]^-$. The two most intense MRM transitions were recorded for each native analyte; the most intense transition was used for quantitation and the other transition was used for qualification (Table 5.1). The most intense transition was reordered for each isotopically labelled standard.

The Analist® software (version 1.6.3; AB Sciex) was used to prepare the method and the batches for analysis, while the MultiQuant™ software (version 3.0.8664.0; AB Sciex) was used for quantification.

Table 5.1 - List of the acronymus of investigated analytes, with molecular structures, molecular weight, Cas Numbers and relative parent compounds. For each analyte, the MRM ion transitions used for quantitation (quant) and qualification (qual), are showed; moreover, the MRM ion transition of the isotopic labelled internal standard (IS) is showed. Finally, the retention time (RT) of each chemical and the collision energy (CE) used to obtain the MRM ion transition are provided.

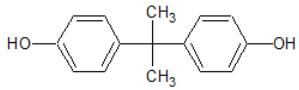
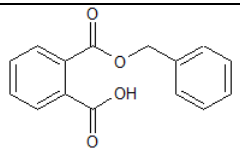
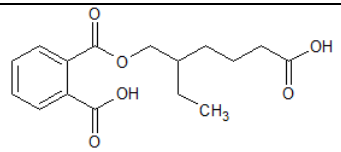
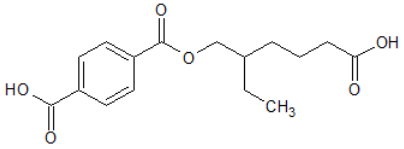
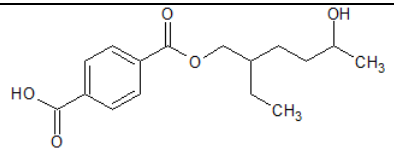
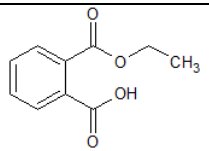
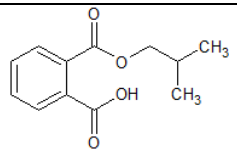
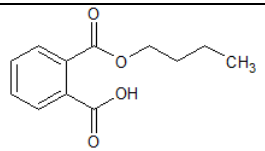
Investigated analytes	Molecular structure	Mole-cular weight (Dalton)	Cas number	Parent compounds	MRM transitions (precursor ion/product ion) (m/z)		RT (min)	CE (V)
BPA		228	80-05-7	BPA	Quant.: Qual.: IS (-d8 isotope):	227.0/212.0 227.0/133.0 235.0/220.0	10.13 10.01	-25 -32 -26
MBzP		256	2528-16-7	butylbenzyl phthalate (BBzP)	Quant.: Qual.: IS (-d4 isotope):	255.0/77.0 255.0/106.9 259.0/77.0	14.59 14.52	-26 -18 -28
MECPP		308	40809-41-4	di-2-ethylhexyl phthalate (DEHP)	Quant.: Qual.: IS (-d4 isotope):	307.3/158.8 307.3/113.0 311.3/159.0	14.00 13.93	-17 -37 -17
MECPTP		308	1684398-42-2	di-2-ethylhexyl terephthalate (DEHTP)	Quant.: Qual.: IS (-d4 isotope):	307.2/164.8 307.2/121.0 311.3/168.7	14.40 14.36	-20 -31 -20
MEHHTP		294	1684398-38-6	di-2-ethylhexyl terephthalate (DEHTP)	Quant.: Qual.: IS (-d4 isotope):	293.2/120.9 293.2/76.9 297.2/125.0	13.82 13.77	-23 -40 -23

Table 5.1 - Continue

Investigated analytes	**Molecular structure**	**Mole-cular weight (Dalton)**	**Cas number**	**Parent compounds**	**MRM transitions (precursor ion/product ion) (m/z)**		**RT (min)**	**CE (V)**
MEP		194	2306-33-4	diethyl phthalate (DEP)	Quant.: Qual.: IS (-d4 isotope):	193.0/77.0 193.0/121.0 197.0/81.0	10.38 10.22	-22 -14 -22
MiBP		222	30833-53-5	diisobutyl phthalate (DiBP)	Quant.: Qual.: IS (-d4 isotope):	221.0/77.0 221.0/133.8 225.0/81.0	13.37 13.29	-22 -18 -23
MnBP		222	131-70-4	di-n-butyl phthalate (DnBP)	Quant.: Qual.: IS (-d4 isotope):	221.0/77.0 221.0/71.0 225.0/81.0	13.57 13.50	-22 -18 -23

5.2.5 Method validation

A complete validation of the method was performed according to FDA guidelines (FDA, 2013). Furthermore, the consideration raised by González et al. were taken into account (González et al., 2014).

5.2.5.1 Calibration curve

The ranges of concentrations used to build the calibration curve are reported in "Standard solution preparation" section, and they were analysed at least twice in each sequence. For each analyte, the calibration curve was built by plotting on the x axis the concentrations, and on the y axis the ratios between the chromatographic peak areas of native compound and those of the relative internal standard. A weighted least-squares linear regression, with a weighting factor of 1/x, was then interpolated.

5.2.5.2 Sensitivity

For each analyte, a calibration curve built with low concentration levels were used to estimate the limit of quantitation (LOQ). In particular, the following formula was used:

$$LOQ = (3SEq + q)/m,$$

Where SEq is the standard error of the intercept, q is the intercept, and m is the slope of the linear calibration curve (Miller and Miller, 2005).

5.2.5.3 Precision and accuracy

Precision and accuracy were determined by analysing QC solutions. Intraday precision was evaluated by calculating the relative standard deviation (RSD) of peak area ratios of five replicates injected on the same day. Interday precision was determined by calculating RDS on five different days. Accuracy was calculated as the percent ratio between the concentration calculated using the calibration curve and the actual value of the prepared solution.

5.2.5.4 Accuracy with standard reference material

Accuracy of the method was also verified analysing two Standard Reference Materials (SRM) for organic contaminants in urine, developed by the National Institute of Standards and Technology: SRM 3672 and SRM 3673 (Schantz et al., 2015). These samples reported reference values for BPA, MBzP, MECPP, MEP, MiBP, and MnBP. Reference values were converted from µg/kg to µg/L using the density of urine (1.019 kg/L), and then accuracy was calculated as described above.

5.2.5.5 Selectivity and carryover effect

Selectivity was evaluated analysing the blank sample, while carryover effect was tested analysing the same blank sample after an analysis of the highest concentration level of the calibration curve. The presence of interfering peaks with the same transitions and retention times of the considered compounds was investigated.

5.2.5.6 Matric effect, recovery, and process efficiency

The experiments for the evaluation of matrix effect, recovery, and process efficiency were set up taking into account guidelines reported in the literature (González et al., 2014; Matuszewski et al., 2003). Using urine donated from five different healthy volunteers, three different sets of samples were prepared: water spiked with standards before the sample preparation (W), urine spiked with standard before the sample preparation (U-pre) and urine spiked with standard after the sample preparation (U-post). The three different validation parameters were calculated as follows:

Matrix effect (ME) (%) = (U-post / W) * 100

Recovery (REC) (%) = (U-pre / U-post) * 100

Process efficiency (PE) (%) = (U-pre / W) * 100

Two different concentrations, corresponding to QCs, were evaluated for each parameter and the peak area ratio values in spiked urine were subtracted with values found in healthy volunteers' urine before calculation.

5.2.5.7 Stability

The stability of considered compounds was evaluated as short-term and long-term stability. Short-term stability was tested comparing signals of analytes of QC solutions kept in the autosampler (10 °C) for 2 weeks with those of the same fresh-prepared solutions. Analogously, long-term stability was tested keeping the solutions at -20°C for more than 6 months and after four freeze-thaw cycle.

5.2.5.8 External verification

We took part to the ICI-EQUAS study, which is an inter-laboratory verification in the frame of the European Human Biomonitoring Initiative (HBM4EU) (Bopp et al., 2018). Participating in the third round of the ICI-EQUAS study, two urine samples containing unknown levels (low and high) of MBzP, MECPP, MEP, MiBP, MnBP and two urine samples containing unknown levels (low and high) of BPA were shipped to our laboratory. We analysed the samples and sent the results, which were compared to the results obtained from reference laboratories. Z-scores were calculated according to the following formula:

$$Z = (x - C) / \sigma_T$$

Where x is the result submitted by the laboratory, C is the expert-assigned value, and σ_T is 0.25*C (a fit-for-purpose target standard deviation). $|Z| \leq 2$ was considered satisfactory, $2 < |Z| < 3$ was considered questionable, and $|Z| \geq 3$ was considered unsatisfactory.

5.2.6 Analytical sequence

A typical sequence consisted of the analysis of calibration curve levels, followed by the analysis of unknown samples along with the analysis of QC solutions and two sample duplicates for every 10 samples, followed by a second analysis of calibration curve levels.

5.2.7 Study subjects

With the purpose of a pilot biomonitoring study, the method was applied to urine from 36 healthy subjects living in Milan. The subjects were monitored in the period November 2014-March 2015 and recruited in the frame of the Sphere project (http://users.unimi.it/sphere) (Bonzini et al., 2017). The Ethical Committee of the University of Milan approved the project. Written informed consent was collected from each participant at the study. All subjects were not occupationally exposed to phthalates or BPA. Among subjects 14 males and 22 females were present, the median age was 45 years (from 38 to 73), and the median body mass index was 24.7 kg/m^2 (from 18.6 to 29.6). All subjects were non-smokers.

5.2.8 Statistical analysis

Descriptive statistical analyses of the pilot study were performed using the software Rstudio (version 1.2.1335). For each analyte, a value corresponding to half of the LOQ was attributed when the concentration was not quantifiable. Median, 5^{th} percentile, and 95^{th} percentile were then calculated. A correlation between MECPTP and MEHHTP (both metabolites of DEHTP) were carried out calculating the Pearson correlation coefficient on log10-transformed values.

5.3 Results

5.3.1 Validation

Figure 5.1 shows the overlapped extracted ion chromatograms of a mixture of the chemical standards.

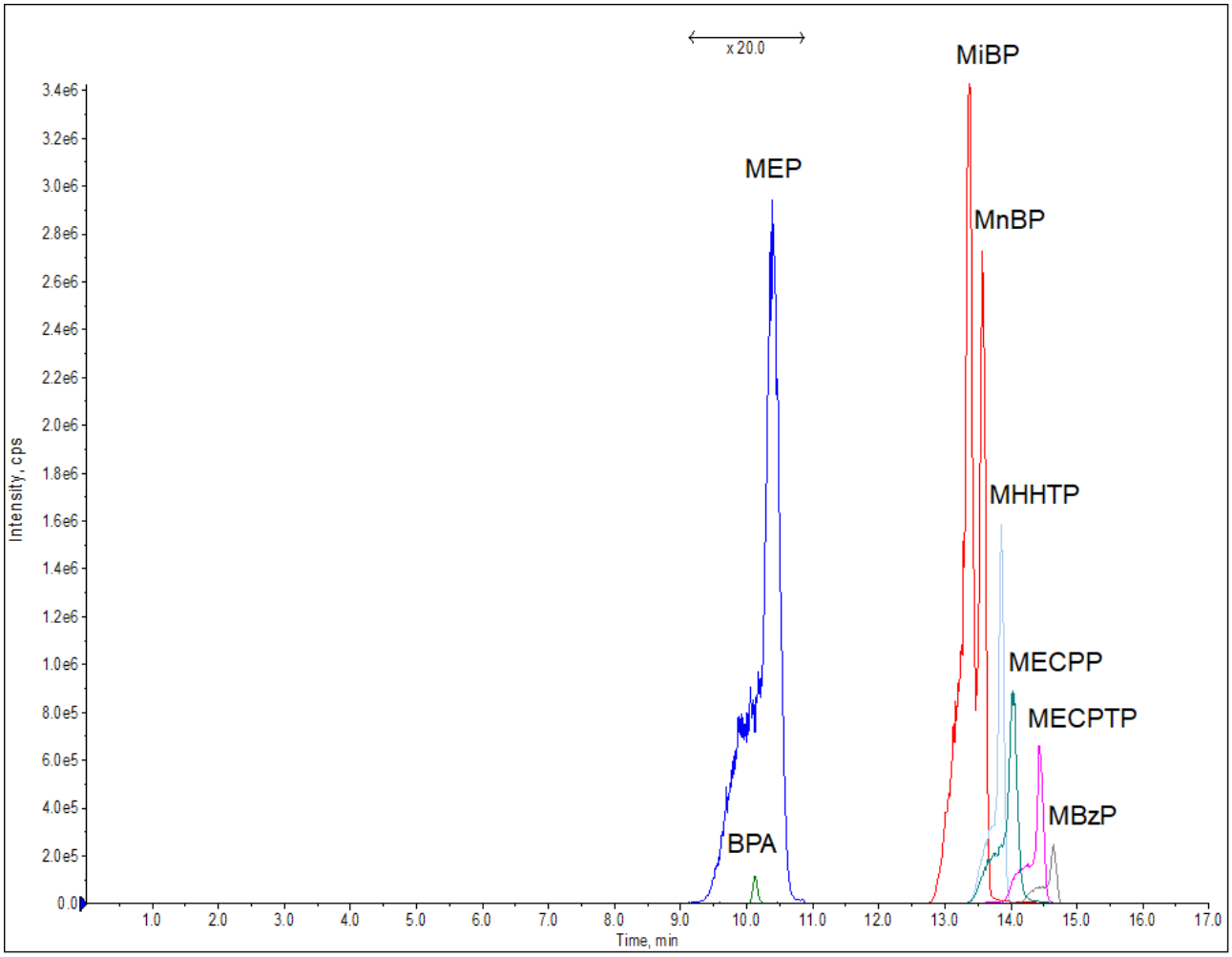


Figure 5.1 - Superimposed extracted ion chromatograms of a standard mixture in water at concentrations of 12.5 µg/L for BPA; 25 µg/L for MBzP, MECPP, MECPTP, and MEHHTP; and 125 µg/L for MEP, MiBP, and MnBP.

5.3.1.1 Calibration curve

For each linear regression, the coefficient of determination (R^2) was calculated. The values ranged from 0.9803 (MiBP) to 0.9970 (MEP), demonstrating optimal linearity for the concentration ranges investigated (Table 5.2).

5.3.1.2 Sensitivity

LOQ values are reported in Table 5.2 and ranged from 0.02 (MECPP) to 1 µg/L (MiBP and MnBP). The background contamination led to higher LOQ values for BPA, MiBP, and MnBP (see "3.1.5. Selectivity and carryover effect" section).

5.3.1.3 Precision and accuracy

Results from accuracy and precision tests are reported in Table 5.2. The RDS mean values for the intra-day precision ranged from 1.3% (QC low of MECPTP) to 7.3% (QC low of BPA), while the inter-day precision ranged from 2.1% (QC low of MECPTP) to 9.4% (QC high of MnBP). The mean percent ratios between the calculated concentration and the theoretical values ranged from 92.1% (QC low of BPA) and 109.2% (QC low of MiBP).

5.3.1.4 Accuracy with standard reference material

The results from standard reference material (with relative accuracy) are the following: BPA, SRM3672: 2.52 µg/L (81.1%), SRM3673: 2.30µg/L (114.9%); MBzP, SRM3672: 7.33 µg/L (85.9%), SRM3673: 4.91 µg/L (84.7%); MECPP, SRM3672: 30.52 µg/L (85.1%), SRM3673: 22.78 µg/L (74.3%); MEP, SRM3672: 113.14 µg/L (117.5%), SRM3673: 90.17 µg/L (44244.4%); MiBP, SRM3672: 5.33 µg/L (81.7%), SRM3673: 4.99 µg/L (94.5%); MnBP, SRM3672: 8.92 µg/L (82.5%), SRM3673: 9.55 µg/L (83.7%). Overall the results are acceptable since accuracy ranged from 74.3% to 117.5%, except for SRM3673 which gives an exceeding result. However, the reference value for this compound is 0.2±2.1 µg/kg, which is surprisingly well below the levels commonly found in samples.

Table 5.2 - Results from the method validation: limits of quantification (LOQ), calibration curves (investigated range and mean R^2), precision, accuracy, short- and long-term stability.

Investigated analytes	Calibration curve			QC precision and accuracy				Stability	
	LOQ (µg/L)	Investigated ranges (µg/L)	R^2 Mean (n=5)	Spiked Concentrations (µg/L)	Intra-day precision %RSD (Min-Max)	Inter-day precision %RSD	Accuracy %Theoretical (Min-Max)	Short term stability (ratio% with fresh solution)	Long term stability (ratio% with fresh solution)
BPA	0.50	LOQ - 50	0.9872	1.56	7.3 (1.0-12.2)	8.3	92.1 (87.2-95.8)	101.2	97.9
				12.5	4.9 (2.3-6.5)	5.3	94.2 (92.3-95.8)	106.3	105.1
MBzP	0.03	LOQ - 100	0.9956	3.13	2.5 (1.6-3.5)	4.1	100.4 (98.0-103.0)	109.6	89.5
				25	2.6 (0.7-4.5)	5.0	100.6 (96.4-103.6)	111.7	98.4
MECPP	0.02	LOQ - 100	0.9966	3.13	2.0 (1.6-2.5)	4.0	104.4 (97.8-114.0)	100.4	109.8
				25	1.8 (1.0-2.6)	2.4	100.1 (96.3-107.6)	105.9	103.6
MECPTP	0.11	LOQ - 100	0.9925	3.13	1.3 (0.4-2.3)	2.1	96.7 (91.2-100.7)	101.3	80.3
				25	1.4 (0.7-1.7)	2.8	98.7 (93.1-103.9)	101.1	84.8
MEHHTP	0.03	LOQ - 100	0.9953	3.13	1.6 (1.2-2.1)	4.5	98.6 (87.8-110.0)	98.2	116.7
				25	1.7 (0.8-3.0)	3.2	100.7 (95.5-109.0)	100.7	105.8

Table 5.2 - Continue

Investigated analytes	Calibration curve			QC precision and accuracy				Stability	
	LOQ (µg/L)	**Investigated ranges (µg/L)**	**R^2 Mean (n=5)**	**Spiked Concentrations (µg/L)**	**Intra-day precision %RSD (Min-Max)**	**Inter-day precision %RSD**	**Accuracy %Theoretical (Min-Max)**	**Short term stability (ratio% with fresh solution)**	**Long term stability (ratio% with fresh solution)**
MEP	0.28	LOQ - 500	0.9970	15.63	2.7 (0.7-4.5)	4.5	101.9 (95.8-108.7)	102.3	107.7
				125	2.2 (0.7-4.0)	3.3	98.0 (94.6-101.5)	102.0	103.8
MiBP	1	LOQ - 500	0.9803	15.63	2.7 (1.7-3.6)	3.9	109.2 (102.7-117.6)	100.2	110.1
				125	3.1 (1.9-4.4)	7.9	95.0 (77.3-103.7)	101.7	101.0
MnBP	1	LOQ - 500	0.9906	15.63	4.3 (3.4-5.4)	5.0	105.4 (91.1-110.5)	101.0	108.1
				125	4.1 (3.0-5.0)	9.4	100.6 (81.8-110.5)	105.7	98.7

5.3.1.5 Selectivity and carryover effect

There were no interfering signals, except for BPA, MiBP, and MnBP. For these analytes, a background level was present in every analysis of the black sample. These contaminations impacted the LOQ values, as reported in the "sensitivity" section. No carry-over effect was observed.

5.3.1.6 Matrix effect, recovery, and process efficiency

Table 5.3 shows the results from matrix effect, recovery and process efficiency. ME, calculated without considering the internal standards, were below 80% for most of the considered analytes (with a minimum of 42.5% for QC low of BPA) indicating that the matrix might be able to reduce the signal for these molecules. However, after the correction with the internal standards, ME ranged from 74.0% (QC low of BPA) to 144% (QC low of MEP), indicating that ISs are able to properly adjust the results. The recovery of the method ranged from 66.5% (QC low of MECPTP) to 126.0 (EC low of BPA) without considering the ISs and from 82.0% (QC low of MiBP) to 123.8% (QC low of BPA) after the correction with the ISs. Process efficiency, which measures the overall contribution of both matrix effect and recovery, ranged from 48.7% (QC low of MiBP) to 108.9 (QC low of MEP) without considering the ISs and from 84.5% (QC high of BPA) to 116.0% (QC low of MEP) after the correction with the ISs. These results confirm the suitability of the method.

Table 5.3 - Results from the method validation: Evaluation of the matrix effect, recovery and process efficiency. Results are given as means ± standard deviations from the urine of five healthy volunteers.

Investigated analytes	**Spiked Concentrations**	**Matrix effect (ME) (%mean±SD)**		**Recovery (REC) (%mean±SD)**		**Process efficiency (PE) (%mean±SD)**	
		without IS	**with IS**	**without IS**	**with IS**	**without IS**	**with IS**
BPA	1.56	42.5±9.6	74.0±13.3	126.0±29.4	123.8±26.3	55.5±17.2	100.1±12.9
	12.5	54.1±17.3	84.4±3.4	113.2±51.7	100.2±9.2	54.3±7.9	84.5±7.2
MBzP	3.13	63.2±14.8	91.8±21.6	86.0±10.3	105.8±18.1	54.0±13.1	94.1±8.2
	25	74.8±21.6	100.7±13.5	75.1±22.7	93.9±5.7	52.8±10.1	94.1±7.3
MECPP	3.13	76.7±31.3	83.8±18.2	87.2±27.6	114.1±19.7	62.4±22.7	93.4±13.8
	25	88.6±40.8	89.0±25.8	83.8±30.3	102.1±13.5	70.4±27.4	89.1±18.2
MECPTP	3.13	72.4±54.4	115.8±37.6	66.5±34.6	89.4±30.2	55.9±43.8	94.8±6.6
	25	97.8±46.8	104.9±26.1	75.4±40.7	96.6±14.8	65.7±17.5	98.3±7.4
MEHHTP	3.13	66.9±11.8	98.9±14.2	88.2±8.6	101.0±12.5	58.4±7.4	98.6±4.8
	25	76.3±12.0	104.8±13.0	78.5±24.0	99.3±7.7	57.8±11.0	103.3±6.5
MEP	15.63	111.6±54.6	144.7±31.9	76.5±14.4	82.0±15.1	108.9±62.4	116.0±9.7
	125	76.3±30.9	92.6±29.1	108.0±87.4	111.3±41.5	55.2±25.9	97.0±2.5
MiBP	15.63	53.9±3.3	82.6±24.0	82.3±19.3	107.4±49.7	48.7±17.8	99.2±31.3
	125	73.4±12.5	97.8±19.7	80.5±20.6	102.9±4.9	54.0±10.6	108.3±21.4
MnBP	15.63	60.5±6.4	97.1±19.5	92.3±8.0	110.4±25.4	55.5±4.5	103.4±4.7
	125	78.6±8.7	107.4±11.6	79.2±17.7	103.5±4.1	61.2±8.6	110.8±7.8

5.3.1.7 Stability

The results of the short-term stability tests (expressed as ratio with the fresh-prepared solution) ranged from to 98.2% (QC low of MEHHTP) to 111.7% (QC high of MBzP), indicating the absence of degradation in the autosampler up to two weeks. Long-term stability results ranged from 80.3% (QC low of MECPTP) to 116.7% (QC low of MEHHTP), indicating a good reproducibility over 6 months and after freeze-thaw cycles.

5.3.1.8 External verification

The results from the ICI-Equas study were satisfactory for all considered compounds. In particular, z-scores were -1.9 and -1.6 (low and high sample for BPA), -0.1 (high sample for MBzP; concentration was below LOQ of expert labs

for the low sample and no Z-scores were calculated), -0.9 and -0.7 (low and high sample for MECPP), 0.5 and 0.6 (low and high sample for MEP), 0.0 and 0.2 (low and high sample for MiBP), 0.3 and -0.1 (low and high sample for MnBP).

5.3.2 Application of the method

The results of the application of the method to urine samples from healthy subjects are reported in Table 5.4. All samples were quantifiable for their levels of MBzP, MECPP, MECPTP, and MEP. 30 out of 36 (83%) samples were >LOQ for BPA, 33 (92%) for MHHTP, and 34 (94%) for both MiBP and MnBP. Median values ranged from 2.07 (BPA) to 117.08 µg/L (MEP), 5th percentile ranged from <LOQ (BPA) to 18.99 µg/L (MEP), 95th percentile ranged from 3.39 (MEHHTP) to 651.93 µg/L (MEP). Figure 5.2 shows the correlation between MECPTP and MEHHTP log10-transformed levels. The Pearson correlation coefficient was 0.861 (CI95%: 0.742 - 0.927, $p < 0.001$).

Table 5.4 - Results of the application of the method to a pilot biomonitoring study on 36 adult subjects.

	Median (µg/L)	**5th percentile (µg/L)**	**95th percentile (µg/L)**	**Number of samples >LOQ (%)**
BPA	2.07	<LOQ	6.67	30 (83%)
MBzP	4.00	1.26	20.90	36 (100%)
MECPP	12.75	4.56	36.24	36 (100%)
MECPTP	3.73	0.85	35.25	36 (100%)
MEHHTP	0.27	0.02	3.39	33 (92%)
MEP	117.08	18.99	651.63	36 (100%)
MiBP	22.15	4.70	42.04	34 (94%)
MnBP	16.02	2.35	43.61	34 (94%)

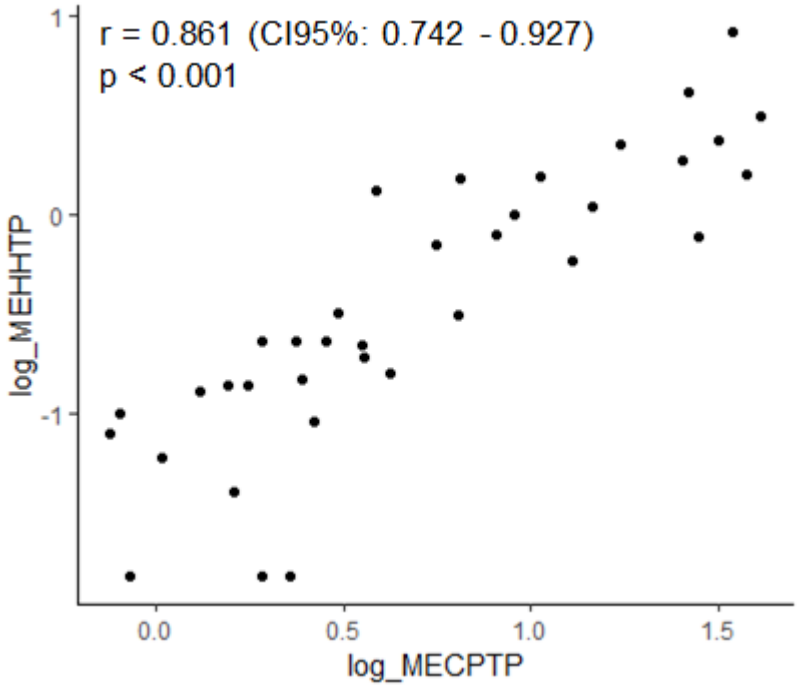


Figure 5.2 - Correlation between log_{10}-transformed levels of MECPTP and MEHHTP (metabolites of DEHTP) in investigated urine samples.

5.4 Discussion

In this work, we developed an analytical method for the simultaneous determination of both BPA and the metabolites MBzP, MECPP, MECPTP, MEHHTP, MEP, MiBP, and MnBP, which are exposure biomarkers of BBzP, DEHP, DEHTP, DEP, DiBP, and DnBP.

The main advantage of the method is its ability to simultaneously quantify the urinary biomarkers of both well-recognised EDCs (BPA, BBzP, DEHP, DEP, DiBP, and DnBP) as well as a novel phthalate used as alternative (DEHTP) which exposure is reported to be on the raise (Silva et al., 2017; Silva et al., 2019). To choice of the metabolites to be used as biomarker was carefully made: indeed, the hydrolytic monoesters (MBzP, MEP, MiBP, and MnBP) are reported to be the most abundant metabolites of phthalates with a short alkyl chain (BBzP, DEP, DiBP, and DnBP), while long-chain phthalates (DEHP and DEHTP) are excreted mainly as oxidized

monoesters (Koch and Calafat, 2009). Indeed, the toxicokinetics of DEHTP has been recently studied and revealed that it is eliminated as mono(2-ethylhexyl) terephthalate (MEHTP) only with a magnitude <0.1% of the oral dose, while the oxidized MECPTP and MEHHTP represent about 13 and 6% of the oral dose (Lessmann et al., 2016b). We decided to include both oxidized compounds in our method in order to be able to eventually further study the behaviour of these two novel metabolites: indeed, Silva and co-worker reported interesting differences in the ratio of molar concentrations of MEHHTP and MECPTP in urine samples collected in different time of the day or from subjects with different ages (Silva et al., 2019). The choice of oxidised monoesters was also useful for the contamination issue: indeed, we detected a considerably low background level for MECPP, MECPTP, and MEHHTP, therefore obtaining a low LOQ for these compounds, while we observed a high background level especially for MiBP, and MnBP, besides BPA. We managed to reduce the entity of these contaminations applying additional short C18 columns, as suggested form previous report (Ye et al., 2013): one was applied after the HPLC pump A, in order to delay contamination present in the aqueous phase, and one just before the Autosampler. Nevertheless, we still had a background level for BPA, MiBP, and MnBP, which we were not able to avoid, and this affected the LOQ values.

An advantage of this analytical assay is its ability to quantify both phthalates and BPA in a single chromatographic run. Only a few methods able to simultaneously separate these chemically different compounds have been reported (Myridakis et al., 2015; Chen et al., 2012; Heffernan et al., 2016). However, some considerations need to be addressed when developing such an approach. Because of the very low expected levels of BPA in the general population and its low signal intensity, (especially if compared with phthalates), we decided to focus our efforts in the optimization of the chromatographic method for this molecule. Indeed, we tested different acids and at different concentrations in the mobile phases: although a stronger acid (e.g. formic acid) and a relative higher concentration is required for a better resolution of phthalates, we observed a loss in signal for BPA under these

conditions. This finding was in agreement with previous works (Völkel et al., 2005; Myridakis et al., 2015; Chen et al., 2011), where it has been reported that the best sensitivity for BPA is obtained avoiding the organic acids in the mobile phase at all. In the end, we set up mobile phase with a relatively weak acid (acetic acid) and at a very low concentration (0.02%), in order to optimize the response of BPA and still retain the phthalates. Even though peak shape of considered phthalates is not ideal (Figure 5.1), this did not affect neither quantitation nor accuracy of the method.

The choice of the enzyme was made according to the considerations reported by Koch and co-workers (Koch et al., 2018): indeed, the use of β-glucuronidase from *E. coli* K 12 allowed a specific cleavage of glucuronide conjugates, since it has been reported that the use of enzymes with other enzymatic activities (e.g.: lipase or arylsulfatase) could further cleave the interested metabolites (Lessmann et al., 2016a). Furthermore, the glucuronide moiety is the only conjugate detected for phthalates (Albro et al., 1982; Lessmann et al., 2018). However, although the use of the specific β-glucuronidase was ideal for phthalates, it was not for BPA: indeed, other than the glucuronide conjugate, BPA is also excreted as monosulfate (Provencher et al., 2014). However, the percentage of BPA excreted as sulphate is reported to be about 3% (Thayer et al., 2015). For this reason, we considered the amount of BPA sulphates as negligible and chose to use an enzyme with only glucoronidase activity, in order to obtain the best condition for phthalates. The suitability of our decision was also confirmed by the results obtained in the external verification, which were acceptable, although lower if compared to the results obtained from reference laboratories.

A limitation of this method for its applicability on large epidemiologic studies is the relatively long time required for the sample preparation, due to the several steps involved (incubation, SPE cartridges, evaporation). However, we partially managed to speed it up using the novel SPE cartridges “Oasis PRiME HLB”, which do not require neither conditioning nor equilibrations, therefore allowing to load the sample directly on the cartridge.

Overall, the method underwent a complete validation which demonstrated the suitability of the method. Furthermore a strength of our work is the validation using Standard Reference Materials (SRM) (Schantz et al., 2015), along with the successful participation to an external verification exercised (ICI-Equas studies), although this approach was not possible for MECPTP and MEHHTP, since this novel compounds were still not included either in the SRMs or in the ICI-EQUAS.

The method was applied to a pilot biomonitoring study and the results obtained are in agreement with those previously reported (Ye et al., 2015; Silva et al., 2017; Tranfo et al., 2013). Regarding the metabolites of DEHTP, we confirm that MECPTP is the most abundant metabolite as their levels were higher than MEHHTP. This was also confirmed by the significant positive correlation between the two metabolites, which is similar with previous study (Silva et al., 2017; Lessmann et al., 2017; Silva et al., 2019).

In conclusion, the reported analytical assay can be considered a useful tool for the simultaneous biomonitoring of bisphenol A and phthalates, including DEHTP, which is increasingly used as substitutes for DEHP. Since exposure data to terephthalates are limited, an assay like the one presented in this work may be used to fill this void since it can be used to monitor both the potential increasing exposure to DEHTP along with the exposure to other phthalates and BPA which use has been restricted.

Conclusions

The assessment of the environmental or occupational exposure to pollutants can be performed through biological monitoring, i.e., the indirect measurement of the compounds, or their specific metabolites, in the subjects' biological samples, such as biological fluids. The aim of this PhD project was to develop specific analytical assays, both targeted and untargeted, for the measurement of mercapturic acids and endocrine disrupter chemicals in urine samples.

Overview of the results obtained

Mercapturic acids are urinary metabolites of occupational and environmental toxicants. The first assay developed was a liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) method for the sensitive and rapid determination of 17 mercapturic acids in urine samples. Considered mercapturic acids included metabolites derived from several compounds, among which: benzene, toluene, 1,3-butadiene, styrene, acrylonitrile, 4-chloronitrobenzene, acrylamide, acrolein, propylene oxide, N,N-dimethylformamide, crotonaldehyde, ethylene oxide, and methylating and ethylating agents. The chromatographic run lasted 18 min. Limits of quantitation ranged from 0.01 to 3.2 µg/L. A complete validation was carried out: precision, expressed as relative standard deviation, ranged from 0.6 to 20.9%; accuracy ranged from 93.4 to 114.9% of theoretical values; and the use of deuterated internal standards was suitable for control of the matrix effect. The assay allowed the simultaneous quantitation of urinary mercapturic acids at different concentration ranges. The external verification exercise produced good results.

This method was applied to a group of 49 coke-oven workers and 49 individuals living in the same area. Active tobacco smoking was an exclusion criterion for both groups. Linear models were built to correct for different confounding variables.

Urinary levels of N-acetyl-S-phenyl-L-cysteine (SPMA) (metabolite of benzene), N-acetyl-S-(2-hydroxy-1/2-phenylethyl)-L-cysteine (PHEMA) (metabolite of styrene), N-acetyl-S-(2-cyanoethyl)-L-cysteine (CEMA) (metabolite of acrylonitrile), N-acetyl-S-[1-(hydroxymethyl)-2-propen-1-yl)-L-cysteine and N-acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine (MHBMA) (metabolites of 1,3-butadiene) were 2–10 fold higher in workers than in controls ($p < 0.05$). For SPMA, in particular, median levels were 0.02 and 0.31 µg/g creatinine in workers and controls, respectively. Among workers, coke makers were more exposed to PHEMA and SPMA than foremen and engine operators. The comparison with biological limit values shows that the exposure of workers was within 20% of the limit values for all biomarkers, moreover three subjects exceeded the restrictive occupational limit value recently proposed by the European Chemicals Agency (ECHA) for SPMA.

The method was also applied to a group of subjects with different smoking habits, in particular: 38 non-smokers (NS), 7 electronic cigarette users (ECU), and 22 traditional tobacco smokers (TTS). Most mercapturic acids were 2 - 165 fold-higher in TTS compared to NS, with CEMA, MHBMA, N-acetyl-S-(3-hydroxypropylcysteine (3-HPMA) (metabolite of acrolein), and SPMA showing the most relevant increases. Furthermore, some mercapturic acids were higher in ECU than NS; CEMA and 3-HPMA, in particular, showed significant increases and were 1.8 and 4.9 fold-higher, respectively. These results confirm that tobacco smoking is a major source of carcinogenic chemicals such as benzene and 1,3-butadiene; electronic cigarette use is a minor source, mostly associated with exposure to chemicals with less carcinogenic potential such as acrylonitrile and acrolein.

An untargeted metabolomic approach was applied to the same population of subjects with different smoking habits. Samples were analysed by liquid chromatography/time-of flight mass spectrometry. Data were processed using the R-packages IPO and XCMS to perform feature detection, retention time correction and alignment. One-way ANOVA test was used to identify different features among groups. One hundred and seventeen features, out of 3613, were different among

groups. They corresponded to 91 potential metabolites, 5 of which were identified vs authentic standards, 43 were putatively annotated and 13 were attributed to chemical classes. Among identified compounds there were the mercapturic acids of acrolein, 1,3-butadiene, and crotonaldehyde; among putatively annotated compounds there were the glucuronide conjugated of 3-hydroxycotinine and the sulfate conjugate of methoxyphenol; with the lowest degree of confidence several sulfate conjugates of small molecules were annotated. Considering mercapturic acids, the coherence between the targeted and untargeted approach was found for a limited number of chemicals, typically the most abundant.

Finally, several phthalates and bisphenol A are endocrine-disrupting chemicals (EDCs). Recently, their use has been partially restricted and less toxic compounds, such as di-2-ethylhexyl terephthalate (DEHTP), have been placed on the market. An isotopic dilution high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC-MS/MS) method for the determination of bisphenol A (BPA), monobenzyl phthalate (MBzP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP), mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP), monoethyl phthalate (MEP), and mono-n/i-butyl phthalates (MnBP/MiBP) in human urine was developed. A complete validation was carried out. Limits of quantitation ranged from 0.02 (MECPP) to 1 μg/L (MnBP and MiBP). Relative standard deviations below 10% indicated a suitable precision; accuracy, evaluated using a standard reference material, ranged from 74.3% to 117.5%; isotopically labelled internal standards were suitable for correcting the matrix effect. The method was applied to 36 non-occupationally exposed adults and levels of the investigated chemicals in subjects were in line with those previously reported.

Concluding remarks

Overall, the methods presented in this thesis were suitable for the biomonitoring of the considered compounds. The main advantages of the developed assays are a high-

throughput and a great sensitivity. The determination of specific biomarkers allows an accurate assessment of exposure to selected parent compounds. We, as organisms living in the environment, are continuously exposed to complex mixtures of compounds and the analysis of a wide number of mercapturic acids in a single chromatographic run allows the assessment of exposure to several volatile organic compounds. Furthermore, the analysis of bisphenol A and metabolites of phthalates allows to measure both persistent compounds which are currently under restrictions and emergent compounds (terephthalates) which exposure could be on the rise.

The biomonitoring studies carried out in this thesis indicated that the biological monitoring is a useful tool for the chemical risk assessment since they have been suitable to evaluate occupational and environmental chemical exposure. Indeed, the application of the method for the mercapturic acids determination to the two different populations (coke oven workers and subjects with different smoking habits) has shown interesting results: comparing the two different groups (each paired with their independent control group of subjects), smokers were exposed to a greater number of volatile organic compounds (14 mercapturic acids were significantly higher than their related control subjects while only 4 mercapturic acids were significantly higher in coke-oven workers than their controls). Furthermore, smokers had higher absolute levels of mercapturic acids than coke oven workers (median level of CEMA were 3.7 and 163.1 µg/g creatinine, median levels of MHBMA were 1.10 and 4.07 µg/g creatinine, median levels of PHEMA were 0.15 and 1.05 µg/g creatinine, and median levels of SPMA were 0.31 and 0.48 µg/g creatinine in coke oven workers and smokers, respectively). These results further highlight how tobacco smoking is a harmful habit due to its related exposure to elevated amounts of hazardous volatile compounds, including carcinogenic ones.

These applications, performed both on individuals occupationally and environmentally exposed to toxic substances, demonstrated the suitability of biomonitoring as a tool for measuring the exposure to dangerous compounds in different scenarios. The usefulness of biomonitoring for the chemical risk

assessment is related to its ability to measure the internal exposure to given chemicals and aggregating the exposure from different routes. This is both an advantage, since it can provide an estimate of the total body burden, and also a disadvantage, since it cannot unbundle all the possible sources: as example, in the study of the coke-oven workers, urinary concentration of SPMA, despite indicating specifically an exposure to benzene, cannot distinguish between exposure caused by the occupational environment (which evaluation was the aim of the work) and other possible sources, thus specific cares in controlling all possible confounding factor should be made (as we did excluding smoking subjects). Taking into account these observations, the studies reported in this thesis confirmed biomonitoring as a powerful methodology for the chemical risk assessment.

Finally, the innovative untargeted approaches are both an opportunity and a challenge in the field of biological monitoring. Their potential to detect any differences in the metabolome of groups of individuals subjected to various exposures (both occupational and environmental) might allow to detect new metabolites of exogenous compounds as well as alteration in endogenous metabolites related to the intake of the dangerous agents; thus allowing the identification of new biomarkers of exposure and effects. The work conducted in this thesis, though, highlighted that these results could not be achieved for those compounds present at low concentrations, whose presence can be detected only with more specific and targeted approaches. All these considered, I believe untargeted metabolic approaches are very powerful tools, with their own advantages and disadvantages, which are not going to replace the target approaches in the field of the biological monitoring, rather to integrate them as useful hypothesis generators, to which complementary targeted assays must follow as hypothesis testing and validation.

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