Date: Mar 24, 2020
To: "Silvia Fustinoni" silvia.fustinoni@unimi.it
cc: kate.jones@hse.gov.uk;radu_duca@yahoo.com;radu.duca@kuleuven.be
From: Wolfgang Dekant eesserver@eesmail.elsevier.com
Reply To: Wolfgang Dekant editor.TL@toxi.uni-wuerzburg.de
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Ms. Ref. No.: TOXLET-D-19-01064R1
Title: Urinary biomonitoring of subjects with different smoking habits. Part II: an untargeted metabolomic approach and the comparison with the targeted measurement of mercapturic acids
Toxicology Letters

Dear Professor Silvia Fustinoni,

I am pleased to confirm that your paper "Urinary biomonitoring of subjects with different smoking habits. Part II: an untargeted metabolomic approach and the comparison with the targeted measurement of mercapturic acids" has been accepted for publication in Toxicology Letters.

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Wolfgang Dekant
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Article Type: VSI: Biomonitoring: ISBM-11

Keywords: smoking habits; electronic cigarette; traditional tobacco smoking; untargeted metabolomics; exposomics

Corresponding Author: Professor Silvia Fustinoni, PhD

Corresponding Author's Institution: University of Milan

First Author: Gianfranco Frigerio, M.D.

Order of Authors: Gianfranco Frigerio, M.D.; Rosa Mercadante; Laura Campo; Elisa Polledri; Luca Boniardi; Luca Olgiati; Pasquale Missineo; William J Nash; Warwick B Dunn; Silvia Fustinoni

Abstract: Background. Although thousands of different chemicals have been identified in cigarette smoke, the characterization of their urinary metabolites still requires significant research. The aim of this work was to perform an untargeted metabolomic approach to a pilot cross-sectional study conducted on subjects with different smoking habits and to compare the results with those of the targeted measurement of mercapturic acids.
Methods. Urine samples from 67 adults, including 38 non-smokers, 7 electronic cigarette users, and 22 traditional tobacco smokers were collected. 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. Quantitative determination of 17 mercapturic acids was available from a previous study.
Results. 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.
Conclusions. Differences in the urinary levels of several compounds were associated to the different smoking habits, suggesting that the proposed approach is useful for the investigation of the metabolite patterns related to the exposure to toxicants. However, limitations were highlighted, in particular regarding the identification of low concentration compounds.

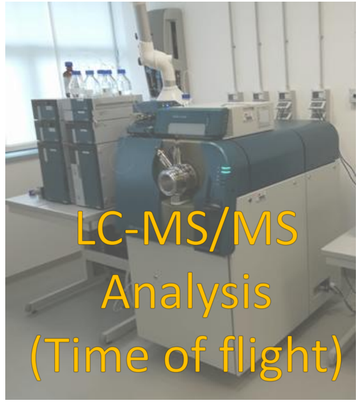
Graphical Abstract

Urine samples from:

- Non smokers

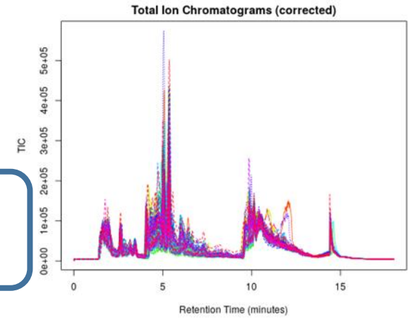
- Electronic cigarette users

- Traditional tobacco smokers



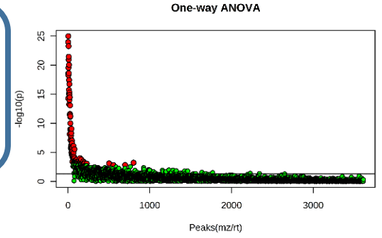
IPO

XCMS



MetaboAnalyst 4.0

One-way ANOVA



BEAMS

hmdb The Human Metabolome Database

METLIN

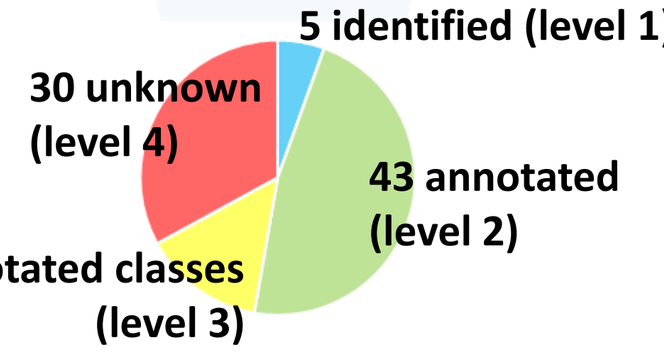
MetFrag

MS Finder

3613 features

117 significantly different features among groups

91 potential metabolites



Highlights

- LC-MS/MS untargeted metabolomics applied to subjects with different smoking habits
- 91 potential urinary metabolites out of 3613 features were different among groups
- 61 potential metabolites were annotated with various degree of confidence
- Annotated metabolites derived from smoke pollutants and metabolism modifications
- Among different features, 3 corresponded to mercapturic acids previously measured

Urinary biomonitoring of subjects with different smoking habits. Part II: an untargeted metabolomic approach and the comparison with the targeted measurement of mercapturic acids

Gianfranco Frigerio[1], Rosa Mercadante[1], Laura Campo[2], Elisa Polledri[1], Luca Boniardi[1], Luca Olgiati[2], Pasquale Missineo[2], William J. Nash[3], Warwick B. Dunn[3,4,5] and Silvia Fustinoni[1,2*]

[1] Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy

[2] Environmental and Industrial Toxicology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[3] School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

[4] Phenome Centre Birmingham, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

[5] Institute of Metabolism and Systems Research, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

[*] Corresponding author

Silvia Fustinoni

Via S. Barnaba, 8 - 20122 Milan, Italy

e-mail: silvia.fustinoni@unimi.it

phone: +39 02 503 20158

Abstract

Background. Although thousands of different chemicals have been identified in cigarette smoke, the characterization of their urinary metabolites still requires significant research. The aim of this work was to perform an untargeted metabolomic approach to a pilot cross-sectional study conducted on subjects with different smoking habits and to compare the results with those of the targeted measurement of mercapturic acids.

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Abbreviation

2-HPMA, N-acetyl-S-(2-hydroxypropyl)cysteine; 3-HPMA, N-acetyl-S-(3-hydroxypropyl)cysteine; AAMA, N-acetyl-S-(carbamoylethyl)-L-cysteine; AMCC, N-acetyl-S-(N-methylcarbamoyl)-L-cysteine; ANOVA, analysis of variance; CEMA, N-acetyl-S-(2-cyanoethyl)-L-cysteine; CMEMA, N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine; DHBMA, N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine; ECU, electronic cigarette users; EMA, N-acetyl-S-ethyl-L-cysteine; GAMA, N-acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine; HEMA, N-acetyl-S-(2-hydroxyethyl)-L-cysteine; HMPMA, N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MHBMA, (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; MMA, N-acetyl-S-methyl-L-cysteine; NANPC, S-(4-nitrophenyl)mercapturic acid; NS, non-smokers; PCA, Principal Components Analysis; PHEMA, N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine; SBMA, N-acetyl-S-benzyl-L-cysteine; SPMA, N-acetyl-S-phenyl-L-cysteine; TTS, traditional tobacco smokers.

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 (Benowitz and Fraiman, 2017; Callahan-Lyon, 2014; Qasim et al., 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; Cheng, 2014; Goniewicz et al., 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) (Frigerio et al., 2020; this issue).

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 (Gu et al., 2016; Hsu et al., 2017; Hsu et al., 2013; Kaluarachchi et al., 2016; Müller et al., 2014), 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 (Frigerio et al., 2020; this issue). 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.

Materials and methods

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 Frigerio et al., 2020, this issue, 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).

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).

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 (Frigerio et al., 2019). 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.

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.

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., 2008; Tautenhahn et al., 2012), 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).

Results

An overview of the workflow and a summary of the results are given in Fig. 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 (supplementary Figures S1 and S2).

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 1, while some examples of confidently identified compounds and putatively annotated compounds are reported in Fig. 2A and 2B.

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 (Frigerio et al., 2020; this issue). As reported in Table 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.

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 (supplementary Figures S1 and S2). 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 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 (Frigerio et al., 2020; this issue) 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 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 (Frigerio et al., 2020; this issue) 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 (Frigerio et al., 2020; this issue), 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 (Frigerio et al., 2020; this issue). 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 (Frigerio et al., 2020; this issue) 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.

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Declarations of interest

None.

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Tables

Table 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	$C_{13}H_{10}O_4$	+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	$C_9H_{12}O_4S$	-1.18	(3-phenylpropoxy)sulfonic acid	HMDB0135313	3-phenylpropan-1-ol
1.33×10^{-15}	TTS > ECU; TTS > NS	$C_{26}H_{40}O_8$	-2.39	xylarenone E	CHEBI:69734	-
3.39×10^{-15}	TTS > ECU; TTS > NS	$C_{11}H_{12}O_5S$	-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	$C_{10}H_{12}O_4S$	-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	$C_{21}H_{30}O_7$	-2.99	Pteroside Z / Secoeremopetasitolide B	HMDB32587 / HMDB41363	-
8.84×10^{-10}	TTS > ECU; TTS > NS	$C_5H_9NO_3S$	-2.09	Tiopronin	CHEBI:32229	-
1.12×10^{-9}	ECU > NS; TTS > ECU; TTS > NS	$C_8H_{10}O_5S$	-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	$C_{12}H_{22}O_4$	-2.76	Dodecanedioic acid	HMDB0000623	-
5.06×10^{-9}	TTS > ECU; TTS > NS	$C_8H_7NO_4S$	+0.93	Indoxyl sulfate	HMDB0000682	tryptophan
6.61×10^{-9}	ECU > NS; TTS > NS	$C_{10}H_{10}O_6S$	-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	$C_{16}H_{20}O_8$	+0.76	trans-isoeugenol-O-glucuronide	HMDB0060021	trans-isoeugenol
1.53×10^{-6}	TTS > ECU; TTS > NS	$C_8H_8O_4S$	-1.78	4-Vinylphenol sulfate	HMDB0062775	4-Hydroxystyrene
2.82×10^{-6}	TTS > ECU; TTS > NS	$C_9H_{12}O_5S$	+1.87	(4-ethyl-2-methoxyphenyl)oxidanesulfonic acid	HMDB0127988	4-Ethyl-2-methoxyphenol
3.40×10^{-6}	TTS > ECU; TTS > NS	$C_{21}H_{32}O_8$	+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	$C_7H_9NO_4S$	-0.26	Cystathionine ketamine	HMDB0002015	-
1.05×10^{-5}	ECU > TTS; NS > TTS	$C_{11}H_{15}NO_3S$	-2.87	methiocarb-sulfoxide / 2-(Ethylsulfinylmethyl)phenyl methylcarbamate	CHEBI:83542 / HMDB0040289	Methiocarb / Ethiofencarb
1.10×10^{-05}	TTS > NS	$C_{10}H_8O_6S$	-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

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
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

Table 2

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

	Targeted approach				**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**	**Coherence of targeted vs untargeted approach**
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				

Figure legends

Figure 1. Workflow implemented for the data integration, analysis and metabolite annotation.

Figure 2-A. Examples of confidently identified compounds (level 1)

Figure 2-B. Examples of putatively annotated compounds (level 2)

Figure1
Click here to download Figure: 2020-03-17_TOXLET-D-19-01064_Fig.1.pptx

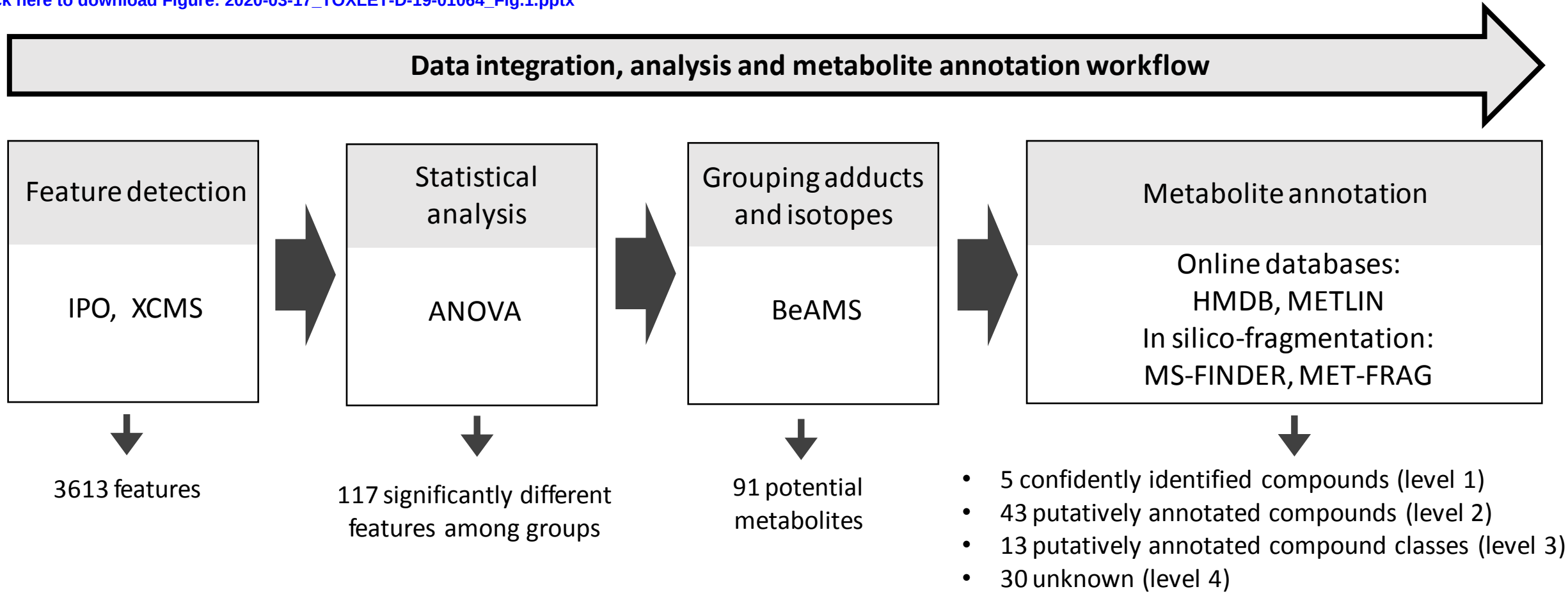
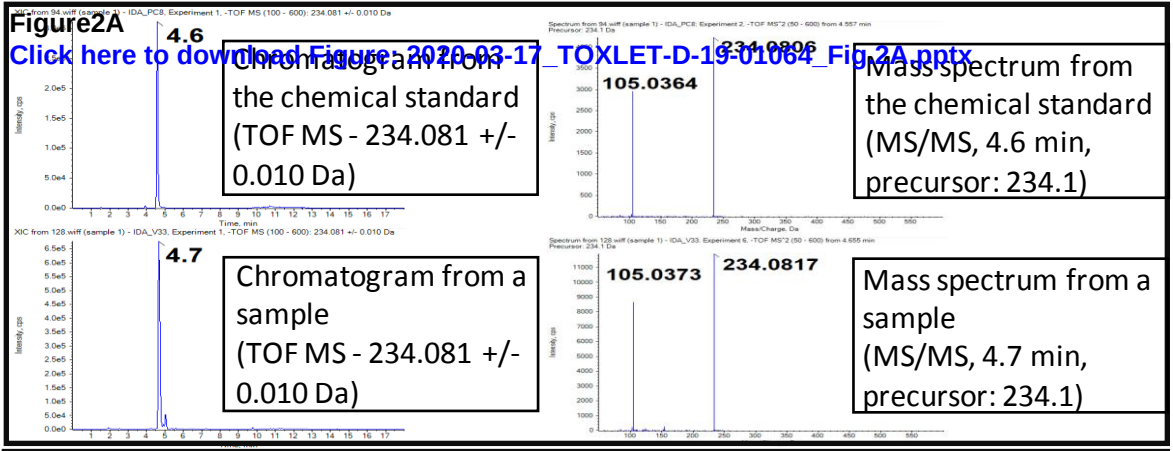


Figure2A

Click here to download Figure: 2020-03-17_TOXLET-D-19-01064_Fig 2A.pptx

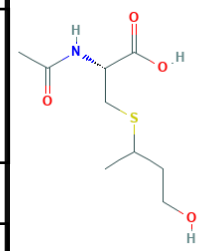


Chromatogram from the chemical standard (TOF MS - 234.081 +/- 0.010 Da)

Mass spectrum from the chemical standard (MS/MS, 4.6 min, precursor: 234.1)

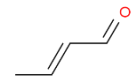
Chromatogram from a sample (TOF MS - 234.081 +/- 0.010 Da)

Mass spectrum from a sample (MS/MS, 4.7 min, precursor: 234.1)

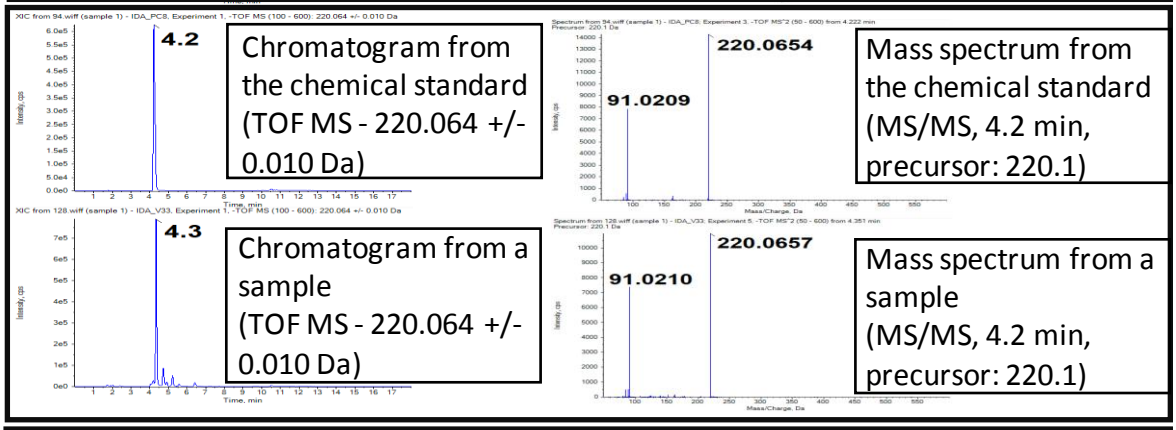


HMPMA

Metabolite of



crotonaldehyde

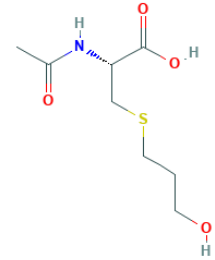


Chromatogram from the chemical standard (TOF MS - 220.064 +/- 0.010 Da)

Mass spectrum from the chemical standard (MS/MS, 4.2 min, precursor: 220.1)

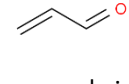
Chromatogram from a sample (TOF MS - 220.064 +/- 0.010 Da)

Mass spectrum from a sample (MS/MS, 4.2 min, precursor: 220.1)

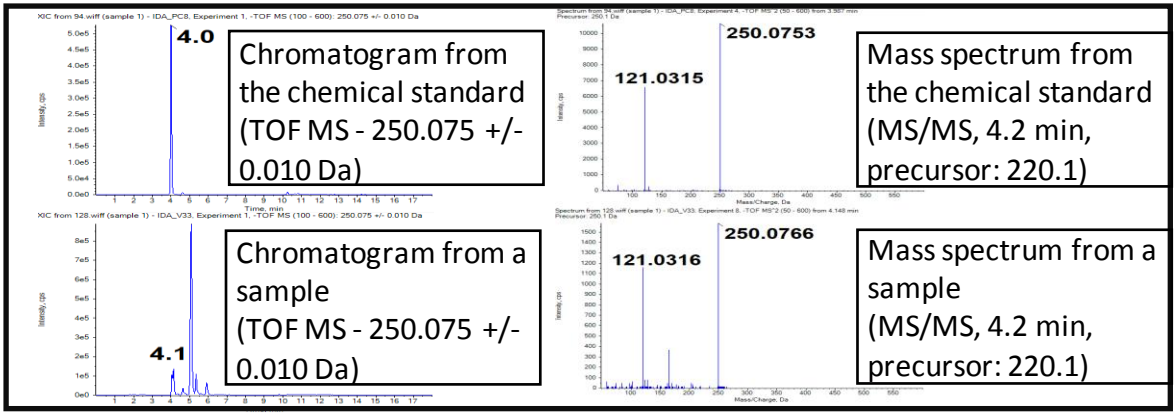


3-HPMA

Metabolite of



acrolein

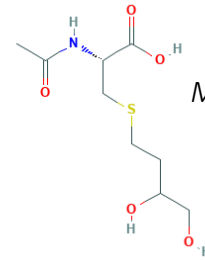


Chromatogram from the chemical standard (TOF MS - 250.075 +/- 0.010 Da)

Mass spectrum from the chemical standard (MS/MS, 4.2 min, precursor: 220.1)

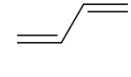
Chromatogram from a sample (TOF MS - 250.075 +/- 0.010 Da)

Mass spectrum from a sample (MS/MS, 4.2 min, precursor: 220.1)



DHBMA

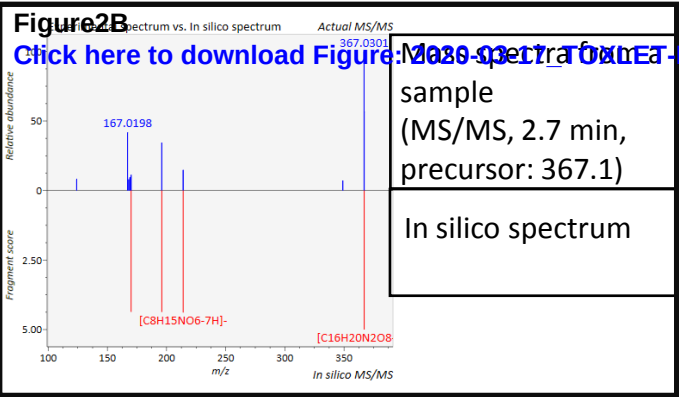
Metabolite of



1,3-butadiene

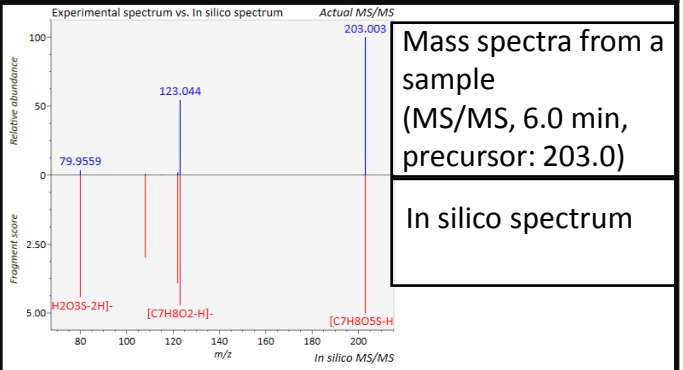
Figure2B

Click here to download Figure: 2020-0217_TOXLET-D-19-01064_Fig.2B.pptx



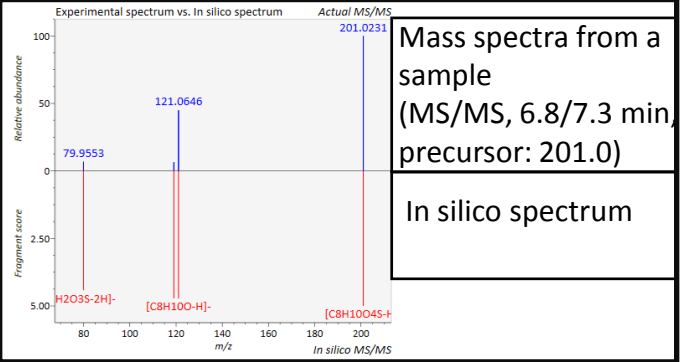
Mass spectra from a sample (MS/MS, 2.7 min, precursor: 367.1)

In silico spectrum



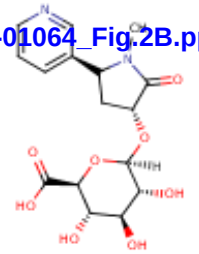
Mass spectra from a sample (MS/MS, 6.0 min, precursor: 203.0)

In silico spectrum



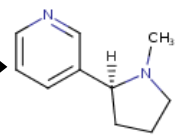
Mass spectra from a sample (MS/MS, 6.8/7.3 min, precursor: 201.0)

In silico spectrum

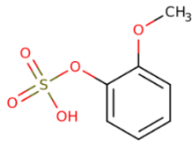


trans-3-Hydroxycotinine glucuronide

Metabolite of

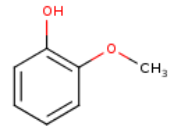


Nicotine

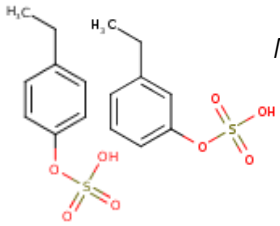


O-methoxycatechol -O-sulphate

Metabolite of

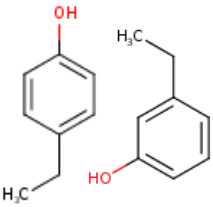


Guaiacol (o-Methoxyphenol)



4-ethylphenylsulfate
3-ethylphenylsulfate

Metabolite of



4-ethylphenol
3-ethylphenol