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Human Biomonitoring of "new" contaminants: Sub-Project 2 - Screening of target and non-target contaminants in human blood and urine

by

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Abstract

The development of screening methods has increased over the last years due to the possibility to search for multiple targets and suspected and so far unknown compounds. Non-target screening has mostly been restricted to water and food samples, only a few studies have shown an application to biological samples. Thus the objective of this project was to develop and apply a non-target screening method to human urine and blood samples. The method development was done by using a broad range of target analytes from various chemical groups, like aromatic amines, industrial chemicals, perfluorinated alkyl acids and UV filters. For sample preparation the QuEChERS (quick, easy, cheap, effective, rugged and safe) method was tested, samples were extracted using acetonitrile and salts for phase separation, followed by a sample clean-up using dispersive solid phase extraction. Urine samples were also directly injected into the LC-HRMS. Using these two methods absolute recoveries between 30 and 150% for 38 of the 40 urine target analytes were achieved. Blood samples were extracted by QuEChERS, resulting in absolute recoveries between 70 and 150% for 43 of the 53 blood target analytes. Using these preparation methods, 16 urine and blood samples from the German Environmental Specimen Bank were extracted for subsequent non-target analysis. After the data processing using MZmine for peak deconvolution and alignment 11 of the 15 internal standards could be detected in all samples. After blank subtraction several targets, suspects and non-targets could be (tentatively) identified. Among these were UV-filters like benzophenone-3 and several benzophenone metabolites, organophosphate flame retardants like triethylphosphate, 4-hydroxy-chlorothalonil and a bromo-quinolinole.

Kurzbeschreibung

Die Anzahl an publizierten Screening-Methoden hat in den letzten Jahren stark zugenommen, da hierbei nach einer großen Zahl von Ziel oder verdächtigen Analyten („Suspects“) gesucht und auch Unbekannte („Non-targets“) detektiert werden können. Bisherige Studien zu Non-target-Methoden sind weitgehend auf Wasserproben und Lebensmittel beschränkt, die Analyse von biologischen Proben wurde bisher kaum durchgeführt. Das Ziel dieses Projektes war es, Non-target-Methoden für humane Urin- und Blutproben zu entwickeln. Die Ziel-Analyten, welche für die Methodenentwicklung herangezogen wurden, repräsentieren unterschiedlichste chemische Gruppen, z.B. aromatische Amine, Industriechemikalien, perfluorierte Alkylsäuren und UV-Filter. Für die Analyse der Urinproben wurde neben einer Direktinjektion in das LC-HRMS System auch die QuEChERS Aufarbeitung getestet. Hierbei wurden die Proben zuerst mittels Acetonitril und Salzen zur Phasentrennung extrahiert und anschließend mit dispersiver Festphasenextraktion aufgereinigt. Mit einer Kombination von Direktinjektion und QuEChERS konnten für 38 von 40 Urin-Ziel-Analyten absolute Wiederfindungen von 30-150% erzielt werden. Die Blutproben wurden nur mit Acetonitril extrahiert, wobei für 43 von 53 Blut-Ziel-Analyten absolute Wiederfindungen von 70-150% erreicht wurden. Mit den optimierten Aufarbeitungsmethoden wurden 16 Urin- und Blutproben aus der Umweltprobenbank des Bundes extrahiert. Für die Non-target Auswertung wurde die Software MZmine herangezogen, welche eine Peakerkennung und ein Alignment durchführt. 11 der 15 zur Überprüfung der Auswertung dotierten internen Standards wurden in allen Proben detektiert. Nach Blindwertsubtraktion konnten mehrere Targets, Suspects und Non-targets vorläufig identifiziert werden. Dies waren unter anderem UV-Filter wie Benzophenon-3 und Benzophenon-Metabolite, Organophosphat Flammschutzmittel wie Triethylphosphat, 4-Hydroxy-Chlorothalonil und ein Brom-Chinolinol.

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List of Abbreviations

ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photon ionization
BMUB	Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety
CID	collision induced dissociation
DI	direct injection
dSPE	dispersive solid phase extraction
ECNI	electron capture negative ionization
EI	electron ionization
ESI	electrospray ionization
GC	gas chromatography
HCD	higher-induced collision dissociation
HMDB	Human Metabolite Database
HpB	heparin blood
HRMS	high resolution mass spectrometry
IS	internal standard
K_{OW}	distribution coefficient between octanol and water
LC	liquid chromatography
LDC	lowest detectable concentrations
LLE	liquid liquid extraction
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
NCI	negative chemical ionization
PAHs	polyaromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCI	positive chemical ionization
PFASs	polyfluorinated alkyl substances
PSA	primary-secondary amine
QqQMS	triple quadrupole MS
QuEChERS	quick easy cheap rugged and safe (extraction method)
RT	retention time
SIM	single ion monitoring

SRM	selected reaction monitoring
TIC	total ion chromatogram
TOF	time-of-flight
VCI	German Chemical Industry Association
VOCs	volatile substances
WB	whole blood

1 Summary

During the last decades an increasing number of chemicals has been used in various applications and consequently been released to the environment. Some chemicals can reach human body tissues via direct usage in consumer products or uptake via the environment and food. Human biomonitoring programs in several countries monitor human body fluids such as urine and blood for a limited number of compounds and biomarkers. This project was set up to support the German human biomonitoring program with a method to allow the detection and identification of so far unknown compounds in human samples. Therefore a non-target method for the analysis of human urine and blood samples was developed.

In general target, suspect and non-target screening methods can be distinguished. Target screening is a (semi-) quantitative or qualitative screening for known compounds with reference standards. The aim is often to obtain a fast overview of a large number of (regulated) contaminants. Such methods are often used in food monitoring and residue analysis to quickly distinguish positive from negative findings below a certain detection limit. Afterwards the concentration of the detected contaminants can be quantified. Suspect screening aims to confirm suspected compounds ("suspects"), for example known or predicted degradation products or compounds for which no reference standards are available. There is no analytical information on the substances (retention time, mass spectrum), but the structures and often other properties are known. From the list of the compounds relevant properties can be calculated (octanol-water partition coefficient, pK_a values, mono-isotopic mass, isotope ratios, etc.). On the basis of this information the chromatograms are searched for the "suspects" which are verified if detected peaks and mass spectra coincide with the calculated properties and the structure. During a non-target screening, also called unknown screening, no information on the pollutants present in a sample is available. The information about the substances is derived solely from the chromatograms and mass spectra. Therefore, the first step is a manual or automatic peak search, which results in a list of detected ions. For each detected ion, lists of possible candidate structures are created based on the mass spectra. As for the suspect screening, inappropriate candidates are filtered by comparison of properties predicted from the structure and properties derived from the chromatogram. A final confirmation of tentatively identified substances is only possible with other spectroscopic techniques or a comparison with a reference standard.

A literature review was conducted regarding non-target methods. Existing methods are mostly focusing on the analysis of water and sediment samples. Only a few studies analyse human tissues. Strategies regarding method development and data evaluation were often not well documented; in most cases simply the highest peaks found in the chromatograms were identified. Some studies describe the usage of software for peak deconvolution, alignment and blank subtraction. We developed a concept for data evaluation, which was to be tested using internal standards (IS) and target analytes.

For the development of sample extraction, target analytes from diverse chemical groups were used. For their selection existing target methods for human samples were reviewed and essentially 12 chemical groups were extracted, being:

- Aromatic amines (blood and urine),
- Fragrances (blood),
- Flame retardants (blood, novel and phosphate-esters in urine),
- Parabens (blood and urine),
- Pesticides (blood and urine),
- Phenols (blood and urine),
- Phthalates (as metabolites in urine),

- Polyaromatic hydrocarbons - PAHs (blood, metabolites in urine),
- Polychlorinated biphenyls – PCBs (blood, metabolites in urine),
- Polyfluorinated alkyl substances - PFASs (blood, short-chain compounds also in urine),
- UV filters (blood and urine),
- Volatile substances - VOC (blood and urine).

A suspect list of 1500 chemicals was set up using information about these already detected compounds in human matrices, but also those that could be relevant due to high production volumes, their occurrence in household products, food and environmental samples, as well as due to their persistency and bioaccumulation potential. The suspect list served as the basis for the suspect screening of blood and urine samples and for the selection of relevant and representative target analytes for the method development.

Target analytes for method development selected from the suspect list were chosen to cover a wide range of functional groups and physicochemical properties ("substance domain"), representing both gas chromatography (GC) as well as liquid chromatography (LC) amenable substances, and represent both new as well as methodically well-established substances to compare the method with already published (standard) methods. 47 analytes were chosen for urine and 56 for blood extraction.

Both LC and GC coupled to mass spectrometry (MS) were to be applied during instrumental analysis of the sample extracts. Urine samples were only analysed by LC-MS, as these contained more polar compounds than blood, for which both LC-MS and GC-MS were applied.

For LC-MS, extracts were injected onto a Kinetex C18 column (100 mm x 3 mm, 2.6 µm, Phenomenex) controlled by an Agilent 1200 LC system. A gradient with a flow rate of 0.2 mL/min was run using water and methanol, both containing 0.1% of formic acid. The LC-system was coupled to an ion trap-Orbitrap hybrid instrument (LTQ Orbitrap XL, Thermo), and separate runs were conducted for positive and negative mode using electrospray ionization (ESI). Detection was conducted with the Orbitrap operating in high resolution (HR) MS full scan mode (m/z 100-1000) using a nominal resolving power of 100,000. For the non-target screening an additional run was conducted where data-dependent HRMS/MS spectra with a resolving power of 30,000 were recorded using five different collision energies for the masses of detected suspects and non-targets showing isotopic patterns in the full scan spectra.

A GC-MS (Agilent Technologies) with electron ionization (EI) was operated in single ion monitoring (SIM) mode for the detection of 27 of the blood targets. A HP-5MS capillary column (30m x 0.25 mm I.D., 0.25 µm film thickness, Agilent Technologies) run with a temperature gradient was used for separation.

Target analytes were optimized using the LC and GC methods. Some targets were not ionisable with the ionization methods used (ESI and EI) or did not show any retention on the selected columns. Thus the number of targets had to be reduced to 40 for urine and 53 for blood.

For method development a pooled urine sample collected from members of the department was used. Pig blood supplied by a local slaughterhouse was used due to its similarity to human blood. Whole blood, heparin blood and plasma were tested during method development.

For the sample preparation the QuEChERS (quick, easy, cheap, effective, rugged and safe) method was tested. This method had been reported to obtain good recoveries for a broad range of pesticides in different food matrices, as well as for pharmaceuticals in whole blood samples. Thus it seemed promising for the extraction of compounds with a broad range of physico-chemical properties. The method uses in a first step a liquid liquid extraction (LLE) with acetonitrile and addition of salts for phase separation. In a second step the supernatant is cleaned-up with dispersive solid phase extraction (dSPE) using primary secondary amine. This procedure was tested both for the extraction of urine and blood samples. For urine

additionally a direct injection (DI) into the LC-HRMS system was tested. Applying both the DI and the QuEChERS extraction 38 of the 40 urine target analytes showed absolute recoveries between 30-150%. Although this meant that the target analytes were prone to matrix effects, the absolute recoveries were acceptable for the intended use, as during a non-target screening no quantification but rather a qualitative detection was intended. The QuEChERS method applied to blood samples resulted in good recoveries, which were similar for all three blood types tested. Differences between LLE and the subsequent dSPE step were only discovered for the perfluorinated carboxylic acids and tetrabromobisphenol A, which were retained by the dSPE material. As the background signal in LC and GC-MS analysis was not substantially higher in the LLE fraction, for the non-target screening a simple LLE extraction of the blood samples was conducted. The recoveries for 43 of 53 analytes were between 70 to 150% for spiked heparin blood. Thus the QuEChERS method is a very promising extraction method for the analysis of diverse chemical groups from blood samples.

For the non-target analysis the developed extraction methods were applied to 16 human urine and blood samples. These samples were supplied by the German Environmental Specimen Bank and were taken in 2013 from 8 female and 8 male students in Greifswald. All samples were spiked with 15 internal standards (IS) used for evaluation of the data processing and for retention time normalization. Following a deconjugation step using β-glucuronidase, urine samples were analysed by direct injection as well as after QuEChERS extraction with LC-HRMS, while blood samples were extracted with LLE and then both LC-HRMS and GC-MS measurements were conducted.

For LC analysis the raw data from 16 samples, 3 method blanks and 2 external standards were imported into the open access program MZmine 2.10. Peaks were deconvoluted and aligned, and six aligned peak lists were obtained, for DI-urine, QuEChERS-urine and blood, each for positive and negative ion mode. On average the lists contained about 33 000 peaks. These lists were compared with the internal standard list, the target list, the suspect list and a HMDB suspect list (all small molecule metabolites detected in urine or blood extracted from the Human Metabolite Database www.hmdb.ca/). These final lists, however, contained many peaks that resulted from the integration of background noise. To eliminate these peaks and to filter out the peaks which are also present in the method blanks an R script was written. In a first step peaks with bad peak shapes defined as having an area to height ratio of > 100 were deleted from the lists. In a second step all peaks in the samples being < 2 times the peak intensity and/or area of the method blank were deleted. In a final step all masses with unreasonable mass defects were filtered out. On average about 10 000 peaks were deleted from the peak lists, leaving about 23 000 peaks in the aligned lists.

By application of the data processing method, all IS were detected in all analysed external standards. Only 4 of the 15 IS were not detected in all analysed samples. Also all target analytes were found in the external standards. Some of the target analytes were additionally detected in a few samples. Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) were detected in all blood samples. Other targets detected in only a few samples were trichloroethylphosphate, perfluorotetradecanoic acid (PFTDA) and tetrachlorosalicylanilide in blood and 2-ethoxyethyl acetate, hydroxyethyl-mercapturic acid, diphenyl phosphate, mono-ethyl-phthalate, mono-benzyl-phthalate, triclosan, ethyl paraben, butyl paraben and benzophenone-3 in urine samples. These results show that the data processing method could successfully be used to detect target analytes. Therefore it should also be possible to detect other compounds present in the samples.

First a suspect search was conducted. The suspects identified by MZmine were visually inspected and those with good peak shapes and present in at least 5 of the 16 samples were selected for further processing. For these MS/MS product ion spectra were recorded. The program MetFrag was used for in-silico fragmentation of the detected suspect and comparison with the recorded MS/MS spectra. If the main fragment ions could be explained by the in-silico fragmentation, the suspect was reported as tentatively

identified. For final identification reference standards were purchased and analysed together with the samples.

Several compounds could be tentatively identified. Compounds such as cotinine, aminophenol and dihydroxychlorobenzene were detected besides several endogenous compounds like isoflavones in many samples. Additionally four compound groups seemed to be interesting. The first being parabens, where ethyl- and butyl-paraben were detected during target analysis, while methyl- and propyl-paraben were additionally detected during suspect analysis and could be verified based on RTs. Parabens are widely used in personal care products and have been analysed in human samples in several studies. The second group, which is also present in personal care products and where at least a few compounds have been detected in human samples before are the UV-filters. Next to benzophenone-3 that has been detected as target analyte, also di-, tri- and tetrahydroxybenzophenone were detected in several urine samples. They could only be tentatively identified by matching their RTs with each other. A third compound group already being analysed for in several human biomonitoring studies are the perfluorinated alkyl acids. As mentioned before, PFOA and PFOS were detected in all blood samples, while PFTDA was detected in only one. The suspect search could additionally detect perfluorinated carboxylic acids with chain lengths of 9 to 13 carbons and perfluorinated sulfonic acids with 6 and 7 carbon chain lengths in some samples. The last compound group are the organophosphate flame retardants (OP-FR). Triethylphosphate, diethylhexylphosphate and dicresylphosphate were the ones detected in many or all blood samples. Due to the phase out of brominated flame retardants the OP-FR are being used in larger quantities and thus it is not surprising for them to be present in human samples.

For the detection of unknowns, the aligned peak lists were separated into individual lists for each sample. Lists for two samples were then imported into the R script “nontarget”, where a pattern search was conducted. The resulting lists contain information about peaks showing isotopic patterns for Cl, Br, N and S. For m/z values of peaks containing isotopes and showing intensities >100 000 a data-dependent HR-MS/MS scan was conducted. The peaks were looked up in the raw files again, where molecular formulas were calculated from the exact mass with a mass tolerance of 10 ppm. For masses with Cl or Br isotope pattern this resulted in 2 up to 400 generated molecular formulas. These were checked for plausibility by application of the Seven-Golden-Rules software, reducing the number to mostly 1-2. The remaining molecular formulas were then searched for in ChemSpider. For most of the compounds found in ChemSpider less than 5 references were listed. One compound detected in blood samples having 19 references in ChemSpider was 4-hydroxy chlorothalonil, a metabolite of the fungicide chlorothalonil. This metabolite could be identified by comparison to a reference standard. Only one peak with a bromine pattern was detected in all urine and all blood samples, both in positive and negative mode. The only possible molecular formula for the detected mass and the bromine pattern was C_9H_6NOBr. A search for this formula in the compound database ChemSpider resulted in 123 hits, which could be reduced to 34 possible ones being analysable both in positive and negative ion mode. 31 of these are bromo-quinolinoles, which are used amongst others for the production of dyes for textiles. This usage could make the presence of these chemicals in human samples likely; however, a final identification using a reference standard is necessary.

By GC-MS analysis of blood sample extracts the target analytes dibutylhydroxytoluene and naphthalene were detected in 13 and 3 samples with approximate concentrations of 10 and 2-3 ng/mL, respectively. Using AMDIS for the deconvolution of the chromatograms and a search in the mass spectra databases NIST and Wiley several non-target compounds could be tentatively identified. Besides some compounds present in food items like caffeine and theobromine, niacidamine (vitamin B3) could be tentatively identified being widely used in personal care products. Other substances of interest were the two phthalates dibutyl- and bis-(2-ethylhexyl) phthalate. Phthalates are known to be present in human samples, however, they are commonly analysed in urine as their mono-phthalate metabolites. Additionally to dibutylhydroxytoluene, which had been detected already during the target analysis, di-tert-butylbenzene,

di-tert-butylphenol and 7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione could tentatively be identified. These substances are used as antioxidation compounds in plastic material, for example in tubes for drinking water supply. Di-tert-butylphenol could be verified using a reference standard and has been calculated to be present at approximately 170 ng/mL. Except for dibutylhydroxytoluene, none of the others has previously been analysed in human blood samples from the general population. The presence of these compounds, however, might need some further investigation.

The detected targets, suspects and non-targets showed the applicability of the developed non-target screening method to human samples. Some suspects and non-targets could tentatively be identified and a few could finally be identified by reference standards. Thus with the application of a non-target screening method new emerging contaminants can be detected, as long as they are present in databases like ChemSpider or PubChem or local databases generated for suspect search. As the suspect search is less time consuming than the search for non-targets, the suspect lists should include all relevant compounds and should be continuously updated. It is also helpful if the information on each suspect is very detailed in regards of additional information such as detections in different matrices, production volumes and usages.

The general data processing during non-target screening is quite laborious and requires manual work. There is thus a lot of potential for software development that could help with the huge amount of data that needs to be handled. The suspect and non-target compounds found are only a small portion of the peaks detected in the human blood and urine samples. Thus with additional time and work there is a potential to detect even more contaminants.

An aspect that has to be taken into account during non-target screening of biological samples is the occurrence of natural substances and metabolites. To the best of our knowledge there is no way to generally distinguish between endogenic and exogenic substances. We tried to take this aspect into account by referring to the Human Metabolome Database, which contains data about small molecule metabolites found in the human body, thus also many naturally occurring substances.

For future non-target screening in human tissues, one should regard different ways to handle the large amount of detected peaks in the samples. One idea could be the examination of time trends using samples from the German Environmental Specimen Bank taken over a time span of several years. By only identifying peaks showing a trend (increasing, decreasing or both), especially compounds with increasing levels becoming interesting for inclusion into human biomonitoring studies could be identified. This would thus reduce the amount of peaks for identification and at the same time filter out the compounds becoming interesting for future studies. Another way to reduce the amount of peaks would be the comparison of different cohorts, for example with and without a certain disease or specific exposure groups such as occupational exposure. Using statistical tools one could determine peaks which occur predominantly in one of the cohorts and try to identify solely these ones.

A third way to reduce the number of relevant peaks is to determine the compounds being of toxicological relevance. Here an effect directed analysis of human blood and urine samples could be developed. A fractionation with a subsequent toxicological test like for example on endocrine disruption could be applied, where the fractions showing effects are filtered out. In these fractions a non-target analysis can then try to identify the compounds responsible for the detected effects. This way the several hundred to thousand peaks found in a sample can be reduced to the relevant ones regarding effects on human health.

2 Zusammenfassung

Während der letzten Jahrzehnte wurde eine zunehmende Anzahl an Chemikalien produziert, welche durch verschiedenste Anwendungen in die Umwelt gelangen können. Einige dieser Chemikalien können entweder durch direkte Verwendung in verbrauchernahen Produkten oder durch die Umwelt bzw. Lebensmittel in den menschlichen Körper gelangen. In einigen Ländern existieren Humanbiomonitoring Programme, die Konzentrationen einer begrenzten Anzahl an Chemikalien und Biomarkern in Körperflüssigkeiten wie Urin und Blut überwachen. Dieses Projekt hat zum Ziel das deutsche Humanbiomonitoring-Programm mit einer Methode zur Bestimmung von relevanten und bisher noch nicht analysierten Chemikalien in menschlichen Proben zu unterstützen. Hierzu wurde eine Non-target Screening Methode für die Analyse von menschlichen Urin- und Blutproben entwickelt.

Generell können Screening Methoden in Target, Suspect und Non-target Methoden unterteilt werden. Das Target Screening ist eine qualitative bzw. (semi)quantitative Screening Methode für bekannte Verbindungen mit Referenzstandards. Ziel ist es hierbei einen schnellen Überblick über eine große Zahl an (regulierten) Kontaminanten zu erhalten. Häufig werden solche Methoden in der Lebensmittelüberwachung und Rückstandsanalytik eingesetzt, um schnell positive von den häufig überwiegend negativen Befunden unterhalb eines bekannten Detektionslimits zu unterscheiden. Die Konzentration der gefundenen Kontaminanten kann anschließend selektiv quantifiziert werden. Ziel des Suspect Screenings ist es, vermutete Verbindungen („Suspects“) zu bestätigen, z.B. vorhergesagte Abbauprodukte oder Substanzen, für die kein Referenzstandard erhältlich ist. Hierbei gibt es zwar keine analytischen Informationen über die Verbindungen (Retentionszeit, Massenspektrum), jedoch sind die Strukturen und eventuell weitere Eigenschaften bekannt. Aus der Liste der Substanzen selbst können für die Analytik relevante Eigenschaften abgeschätzt oder mit Hilfe von Programmen berechnet werden (Oktanol-Wasser-Verteilungskoeffizient, pK_a-Werte, monoisotopische Massen, Isotopenverhältnisse, usw.). Anhand dieser Angaben können die Chromatogramme nach den „Suspects“ durchsucht werden und es kann geprüft werden, ob die gefundenen Peaks und Massenspektren mit den aus der Struktur abgeleiteten Eigenschaften in Einklang stehen. Beim Non-target oder unknown Screening liegen keine Informationen über die vorhandenen oder erwarteten Spurenschadstoffe in einer Probe vor. Die Information über die Substanzen wird allein aus dem Chromatogramm abgeleitet. Folglich ist der erste Schritt eine manuelle oder automatische Peak Suche, die zu einer Liste gefundener Ionen führt. Zu jedem Ion dieser Liste werden basierend auf den Massenspektren Listen mit möglichen Kandidatenstrukturen erstellt. Ein schrittweises Herausfiltern unpassender Kandidaten erfolgt wie beim Suspect-Screening aus dem Vergleich von aus der Struktur vorhergesagten und den aus dem Chromatogramm abgeleiteten Eigenschaften. Wie auch beim Suspect-Screening ist eine endgültige Bestätigung der vorläufig identifizierten Substanzen jedoch nur mit weiteren spektroskopischen Verfahren oder letztlich einem authentischen Referenzstandard möglich.

Zu Beginn des Projektes wurde eine Literaturrecherche in Bezug auf Non-target Screening-Methoden durchgeführt. Vorhandene Methoden konzentrieren sich bisher meist auf die Analyse von Wasser, Sediment und Lebensmitteln. Es gibt nur wenige Studien zur Analyse menschlicher Proben. Die Strategien hinsichtlich Methodenentwicklung und Auswertung der Daten waren oft nicht nachvollziehbar dokumentiert, in den meisten Fällen wurde lediglich eine Auswertung der größten oder gut chromatographisch getrennten Peaks vorgenommen. Einige Studien beschreiben die Verwendung von Software für Peak Erkennung, Alignement und Blindwert Korrektur. Somit wurde in diesem Projekt ein Konzept für die Datenauswertung entwickelt, was anhand von internen Standards (IS) und Target Analyten getestet wurde.

Für eine Selektion der Target Analyten wurden bestehende Analyse-Methoden von menschlichen Proben nach Analyten und ihren Substanzgruppen durchsucht. Resultierend wurden vor allem folgende 12 Substanzklassen gemessen:

- Aromatische Amine (Blut und Urin),
- Duftstoffe (Blut),
- Flammschutzmittel (Blut, Neuartige und Phosphat-Ester in Urin),
- Parabene (Blut und Urin),
- Pestizide (Blut und Urin),
- Phenole (Blut und Urin),
- Phthalate (Metabolite in Urin),
- Polyaromatische Kohlenwasserstoffe - PAKs (Blut, Metabolite in Urin),
- Polychlorierte Biphenyle – PCBs (Blut, Metabolite in Urin),
- Polyfluorierte Alkyl Verbindungen - PFASs (Blut, Kurzkettige auch in Urin),
- UV Filter (Blut und Urin),
- Volatile Verbindungen - VOC (Blut und Urin).

Eine Suspect Liste mit über 1500 Chemikalien wurde aus diesen schon in Humanproben gemessenen Analyten, aber auch aus sonstigen relevanten (auf Grund hoher Produktionsmengen, Vorkommen in Haushaltsprodukten, Lebensmitteln und Umweltproben, als auch Persistenz und Bioakkumulierungs Potential) Verbindungen zusammengestellt. Diese Liste wurde als Basis eines Suspect Screenings für die Urin- und Blutproben verwendet und aus ihr wurden auch repräsentative Target-Analyten für die Methodenentwicklung ausgewählt.

Die ausgewählten Target-Analyten sollten ein großes Substanzspektrum erfassen, sowohl Gaschromatographie (GC)- als auch Flüssigchromatographie (LC)-gängige Analyten beinhalten und sowohl neuartige als auch schon methodisch etablierte Substanzen enthalten. Für die Methodenentwicklung von Urinproben wurden 47 und für Blutproben 56 Analyten ausgewählt.

Für die instrumentelle Analyse wurden sowohl LC als auch GC gekoppelt mit Massenspektrometern (MS) verwendet. Urinproben wurden nur mittels LC-MS analysiert, da sie eher polare Verbindungen enthalten, während Blutproben mit beiden Methoden gemessen wurden. Die instrumentellen Methoden wurden aus schon bestehenden Methoden entwickelt.

Während der LC-MS Analyse wurden Proben auf eine Kinetex C18 Säule (100 mm x 3 mm, 2,6 µm, Phenomenex) injiziert. Ein Gradient aus Wasser und Methanol (beides mit 0,1% Ameisensäure versetzt) wurde mit einer Flussrate von 0,2 mL/min gefahren. Das LC-System war mit einem Iontrap-Orbitrap Instrument (LTQ Orbitrap XL, Thermo) gekoppelt, welches mittels separaten Messungen in positiv und negativ Modus im Elektrospray (ESI) ionisierte. Zur Detektion wurde eine Full Scan Messung (m/z 100-1000) mit einer nominalen Auflösung von 100 000 durchgeführt. Für die Non-target Analyse wurden für detektierte Suspects und Non-targets mit Isotopenmustern zusätzlich MS/MS Spektren mittels fünf verschiedener Kollisionsenergien und einer nominalen Auflösung von 30 000 aufgenommen.

Ein GC-MS (Model 6890 N, MSD 5973, Agilent Technologies) mit Elektronenionisation (EI) wurde im Single Ion Monitoring (SIM) Modus für die Detektion der GC-Target Analyten verwendet. Ein µL Probe wurde auf eine HP-5MS Kapillarsäule (30m x 0,25 mm ID, 0,25 µm Filmdicke, Agilent Technologies) injiziert und mittels eines Temperaturprogramms aufgetrennt.

Mittels dieser LC und GC Methoden wurden die Target-Analyten optimiert. Einige der Targets waren mit den gewählten Ionisierungsmethoden (ESI und EI) nicht ionisierbar oder wurden auf den gewählten Säulen nicht zurückgehalten. Somit reduzierte sich die Anzahl der Target Analyten auf 40 für die Urinproben und auf 53 für die Blutproben.

Für die Methodenentwicklung wurden Urinproben von Mitarbeitern des Departments gesammelt und homogenisiert. Für die Blutproben wurde Schweineblut von einem lokalen Schlachter verwendet, da Schweineblut dem menschlichen sehr ähnlich ist. Hier wurden sowohl Vollblut, Heparin-Blut als auch Plasma getestet.

Für die Probenaufarbeitung sollte die QuEChERS (quick, easy, cheap, effective, rugged and safe) Methode herangezogen werden, welche bisher vor allem zur Analyse von Pestiziden in Lebensmitteln zum Einsatz kommt, aber auch schon zur Analyse von Arzneimitteln in Vollblutproben getestet wurde. Somit schien diese Methode gut geeignet zu sein, um ein breites Substanzspektrum aus diversen Probenarten zu extrahieren. In einem ersten Schritt wird eine Flüssig-Flüssig-Extraktion (LLE) mittels Acetonitril-Zugabe und Salzen zur Phasentrennung durchgeführt, gefolgt von einer Aufreinigung mittels dispersiver Festphasenextraktion (dSPE). Diese Herangehensweise wurde sowohl für Urin- als auch für Blutproben getestet. Für Urinproben wurde zusätzlich eine Direktinjektion (DI) in das LC-MS System getestet. Bei einer Kombination von QuEChERS und DI konnten für 38 der 40 Target Analyten absolute Wiederfindungen von 30-150% erreicht werden. Trotz z.T. hoher Matrixeffekte, sind diese absoluten Wiederfindungen ausreichend für ein Non-target Screening, weil es hier vor allem um die Detektion, nicht aber die Quantifizierung der Substanzen geht. Bei der Anwendung der QuEChERS Methode zur Extraktion der Blutproben konnten für alle drei Blutarten ähnliche Wiederfindungen erzielt werden. Gemessene LLE und dSPE Extrakte unterschieden sich nur bei wenigen Analyten, z.B. werden bei der dSPE Aufreinigung die Perfluorierten Carbonsäuren und Tetrabromobisphenol A zurückgehalten und zeigen somit geringe Wiederfindungsraten. Da der Hintergrund weder bei LC noch GC Messungen wesentlich höher für die LLE Extrakte im Vergleich zu den dSPE Extrakten war, wurde für das Non-target Screening nur eine Extraktion mittels LLE durchgeführt. Bei der LLE Extraktion von dotierten Heparin-Blutproben lagen die Wiederfindungen von 43 der 53 Target Analyten zwischen 70-150%. Somit zeigte sich die QuEChERS Extraktionsmethode als sehr vielversprechend für die Analyse diverser Substanzgruppen vor allem in Blutproben.

Zur Non-target Analyse wurden die entwickelten Extraktionsmethoden auf 16 humane Urin- und Blutproben angewandt. Die Proben wurden von der Umweltprobenbank bereitgestellt und stammten aus dem Jahr 2013 von acht weiblichen und acht männlichen Studenten aus Greifswald. Alle Proben wurden mit internen Standards dotiert, welche zur Evaluierung der Datenauswertung und Retentionszeit-Normierung herangezogen wurden. Nach einer Dekonjugation der Metabolite in Urinproben mittels β-Glucuronidase wurden die Urinproben sowohl mittels QuEChERS extrahiert und analysiert als auch mittels DI gemessen. Die Blutproben wurden mittels LLE extrahiert und anschließend sowohl mit LC-HRMS als auch GC-MS analysiert.

Zur Datenauswertung wurden die Rohdaten der LC Analyse der 16 Proben, drei Methoden-Blindwerten und zwei externen Standards in das open-access-Programm MZmine 2.10 importiert. Hier wurden die Peaks herausgefiltert und anschließend zu einer großen Liste zusammengestellt. Daraus resultierten 6 Peak Listen: für DI-Urin, QuEChERS-Urin und Blut, jeweils im positiven und negativen Modus. Die Listen enthielten im Durchschnitt ca. 33 000 Peaks. Diese Peak Listen wurden mit Listen der internen Standards, Target Analyten, Suspects und Suspects aus der HMDB Liste (alle niedermolekularen Metabolite, welche in Urin- oder Blutproben detektiert wurden, aufgelistet in der Human Metabolite Database www.hmdb.ca/) verglichen und die Ergebnisse wurden den Peak Listen hinzugefügt. Die resultierenden Listen enthalten jedoch viele Peaks, die sich aus der Integration von Hintergrundrauschen ergeben. Um diese Peaks zu beseitigen und Peaks herauszufiltern, die auch in den Methoden-Blindwerten vorhanden sind, wurde ein R-Skript geschrieben. In diesem wurden in einem ersten Schritt Peaks mit schlechten Peak Formen, definiert durch ein Fläche zu Höhenverhältnis > 100, aus den Listen gelöscht. In einem zweiten Schritt wurden alle Peaks aussortiert, deren Intensitäten < 2x der Intensität im Methodenblindwert bzw. deren Flächen < 2x der Fläche im Methodenblindwert waren. Und in einem letzten Schritt wurden alle Massen mit

unangemessenem Massendefekt herausgefiltert. Durchschnittlich wurden so ca. 10 000 Peaks aus den Listen herausgelöscht, so dass ca. 23 000 Peaks übrig blieben.

Durch die Anwendung von MZmine und dem R Skript wurden 11 der 15 internen Standards in allen Proben detektiert. In den externen Standards wurden außerdem alle Target Analyten und internen Standards detektiert. Zusätzlich wurden einige der Target Analyten in ein paar der Proben entdeckt. Perfluoroktansäure (PFOA) und Perfluoroktansulfonsäure (PFOS) wurden in allen Blutproben gefunden. Andere detektierte Target Analyten waren Trichlorethylphosphat, Perfluortetradecansäure (PFTDA) und Tetrachlorosalicylanilide in Blut und 2-Ethoxyethyl-Acetat, Hydroxyethyl-Merkaptursäure, Diphenyl-Phosphat, Mono-Ethyl-Phthalat, Mono-Benzyl-Phthalat, Triclosan, Ethylparaben, Butylparaben und Benzophenon-3 in Urinproben. Diese Ergebnisse zeigen, dass die Datenauswertungs-Methode erfolgreich dazu eingesetzt werden konnte, um Target-Analyten zu detektieren. Daher war es auch möglich, andere vorhandene Verbindungen in den Proben zu identifizieren.

Hierzu wurde zunächst eine Suspect-Suche durchgeführt. Die von MZmine erkannten Suspects wurden bei guten Peak Formen und Detektionen in mindestens 5 der 16 Proben weiter bearbeitet. Für diese wurde eine Fragmentierung mittels datenabhängigem MS/MS-Scan durchgeführt. Das Programm MetFrag wurde anschließend für eine in-silico-Fragmentierung der Suspects und Abgleich mit den aufgezeichneten MS/MS-Spektren verwendet. Wenn die wichtigsten Fragment-Ionen durch die in-silico-Fragmentierung erklärt werden konnten, wurden die Suspects als vorläufig identifiziert betrachtet. Außerdem konnte für einige Substanzen eine endgültige Identifizierung mittels Referenzstandards durchgeführt werden.

Auf diesem Weg konnten einige Suspects vorläufig identifiziert werden. Substanzen wie Cotinin, Aminophenol und Dihydroxychlorbenzol wurden neben mehr endogenen Substanzen wie Isoflavonen in vielen Proben detektiert. Zusätzlich konnten vier interessante Substanzklassen herausgefiltert werden. Die erste Klasse waren die Parabene, von denen Ethyl- und Butylparaben schon während der Target Analyse in Urinproben identifiziert werden konnten. Während der Suspect Suche wurden zusätzlich Methyl- und Propylparaben gefunden, deren Retentionszeiten in homologer Reihe mit den anderen Parabenen waren. Parabene werden weitläufig in Körperpflegeprodukten benutzt und wurden schon in vielen Studien in Urinproben analysiert. Die zweite Klasse sind die UV-Filter, welche auch vorwiegend in Körperpflegeprodukten verwendet werden und von denen einige bereits in anderen Studien analysiert worden sind. Neben Benzophenon-3, welches als Ziel Analyt detektiert wurde, konnten Di-, Tri- und Tetrahydroxybenzophenon in mehreren Urinproben durch ihre zueinander passenden Retentionszeiten vorläufig identifiziert werden. Die dritte Substanzklasse sind die Perfluorierten Alkylsäuren, von denen schon mehrere in diversen Humanbiomonitoring Programmen routinemäßig analysiert werden. Neben PFOA, PFOS und PFTDA, die während der Target Analyse detektiert wurden, konnten aufgrund passender Retentionszeiten auch die Perfluorierten Carbonsäuren mit 9 bis 13 Kohlenstoffatomen und die Perfluorierten Sulfonsäuren mit 6 und 7 Kohlenstoffatomen vorläufig identifiziert werden. Die letzte und vielleicht interessanteste Substanzklasse sind die Organophosphor-Flammschutzmittel. Hier wurden Triethylphosphat, Diethylhexylphosphat und Dicresylphosphat in vielen oder allen Blutproben detektiert. Durch das Phase Out der bromierten Flammschutzmittel, werden zunehmend Flammschutzmittel auf Organophosphat Basis verwendet. Es ist also nicht verwunderlich, diese in menschlichen Proben zu finden.

Für den Nachweis von Non-targets wurden die Peak Listen in einzelne Peak Listen pro Probe unterteilt. Die Listen von zwei Proben wurden dann in das R Skript "nontarget" importiert, wo eine Isotopensuche durchgeführt wurde. Die Ergebnislisten enthalten Informationen über Peaks mit Isotopenmustern für Cl, Br, N und S. Für m/z-Werte der Peaks, die Isotope enthalten und Intensitäten > 100 000 aufweisen wurde ein datenabhängiger MS/MS-Scan durchgeführt. Die Peaks wurden in den Rohdateien überprüft und Molekülformeln wurden für die genaue Masse mit einer Massentoleranz von 10 ppm berechnet. Dies führte für Massen mit Cl oder Br Isotopen zu zwei bis 400 generierten Molekülformeln. Diese wurden durch die Anwendung einer Plausibilitätsprüfung mittels der Software Seven-Golden-Rules auf meist 1-2 reduziert. Nach den verbleibenden Molekülformeln wurde anschließend in der Chemikaliendatenbank ChemSpider

gesucht. Für den Großteil der Molekülformeln waren in ChemSpider nur Verbindungen gelistet, die weniger als fünf Referenzen aufwiesen. Eine Substanz, welche 19 Referenzen aufwies, war 4-Hydroxy-Chlorthalonil, ein Metabolit des Fungizides Chlorthalonil. Dieses wurde mittels Referenzstandards und Abgleich von Retentionszeit und MS/MS-Spektrum identifiziert. Nur ein Peak mit einem Br-Isotopenmuster wurde in allen Blut- und Urinproben detektiert, wobei die Substanz sowohl im positiven als auch im negativen Modus messbar war. Für die exakte Masse und das Isotopenmuster gab es nur eine passende Molekülformel: C_9H_6NOBr. Bei der Suche nach dieser Molekülformel in ChemSpider resultierte eine Liste von 123 passenden Strukturen. 34 von diesen waren sowohl im positiven als auch negativen Modus messbar. 31 von diesen Strukturen waren Bromo-Chinolinole, welche unter anderem in der Produktion von Textilfarbstoffen zur Anwendung kommen. Diese Verwendung würde das Vorkommen dieser Substanzen in menschlichen Proben erklären, jedoch ist eine finale Identifizierung mittels Referenzstandard notwendig.

Mittels der GC-MS Target Analyse der Blutprobenextrakte konnten Dibutylhydroxytoluene (BHT) in 13 und Naphthalen in 3 Proben detektiert werden. Ungefähre Konzentrationen lagen bei 10 ng/mL für BHT und bei 2-3 ng/mL für Naphthalen. Mit dem Programm AMDIS wurden die Peaks mit den zugehörigen Massenspektren aus den Chromatogrammen herausgefiltert und anschließend mit den Spektren der Datenbanken NIST und Wiley abgeglichen. Auf diesem Wege konnten diverse Non-targets vorläufig identifiziert werden. Neben Substanzen, welche von Lebensmitteln stammen wie Kaffein und Theobromine, wurde auch Niacidamine (Vitamin B3) detektiert, welches breite Anwendung in Körperpflegeprodukten findet. Andere interessante Substanzen waren die zwei Phthalate Dibutyl- und Bis-(2-ethylhexyl) Phthalat. Das verbreitete Vorkommen von Phthalaten in menschlichen Proben ist bekannt, jedoch werden meist die Mono-Phthalat Metabolite in Urinproben gemessen. Zusätzlich zu dem Target BHT wurden Di-tert-butylbenzol, Di-tert-butylphenol und 7,9-di-tertbutyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione vorläufig identifiziert. Diese Substanzen werden als Antioxidationsmittel in Plastikmaterialien, wie z.B. Rohren für Trinkwasser verwendet. Di-tert-butylphenol wurde außerdem mittels Referenzstandards verifiziert und die ungefähr berechnete Konzentration in zwei der Proben betrug 170 ng/mL. Außer BHT wurde von diesen Antioxidantien noch keins in menschlichen Proben der generellen Bevölkerung detektiert.

Die detektierten Targets, Suspects und Non-targets zeigen die Anwendbarkeit der entwickelten Non-target Screening Methode auf menschliche Proben. Einige Suspects und Non-targets konnten vorläufig identifiziert und einige konnten sogar abschließend durch Referenzstandards identifiziert werden. So können durch die Anwendung eines Non-target Screenings neue Schadstoffe nachgewiesen werden, solange sie in Datenbanken wie ChemSpider oder PubChem oder lokalen Suspect-Datenbanken vorhanden sind. Da die Suspect Suche weniger zeitaufwendig ist als die Suche nach Non-targets, sollten die Suspect Listen alle relevanten Verbindungen enthalten und kontinuierlich aktualisiert werden. Hierbei ist es sinnvoll, möglichst viele vorhandene Informationen zu den Suspects zu sammeln, wie z.B. Zusatzinformationen wie Detektionen in diversen Proben, Produktionsvolumen und Anwendungsgebiete.

Die allgemeine Datenverarbeitung während einer Non-target Suche ist mühsam und erfordert manuelle Arbeit. Neue Software Entwicklungen könnten es erleichtern, mit den großen Datenmengen zu arbeiten. Die hier gefundenen Suspect und Non-target-Verbindungen sind nur ein kleiner Teil der Peaks, die in den menschlichen Urin- und Blutproben entdeckt wurden. Durch zusätzliche Zeit und Arbeit könnten hier potenziell weitere Verbindungen identifiziert werden.

Ein Aspekt, der beim Non-target Screening von biologischen Proben berücksichtigt werden muss, ist das Auftreten von körpereigenen Stoffen und dessen Metaboliten. Momentan gibt es keine generelle Vorgehensweise, wie diese von den Umweltschadstoffen unterschieden werden können. Wir haben versucht, diesen Aspekt zu integrieren, indem auch nach Verbindungen gesucht wurde, die in der Human Metabolite Database (HMDB) vorhanden sind.

Für zukünftige Non-target Screening Studien in menschlichen Proben sollten verschiedene Möglichkeiten betrachtet werden, um die große Menge an vorhandenen Peaks in den Proben zu bearbeiten. Eine Idee wäre die Suche nach zeitlichen Konzentrationsverläufen anhand von Proben aus der Umweltprobenbank, welche sich über mehrere Jahre erstrecken. Durch die Identifizierung von Substanzen, welche einen ansteigenden Trend zeigen, könnten vor allem Verbindungen welche für zukünftige Humanbiomonitoring Studien interessant wären, herausgesucht werden. Eine weitere Möglichkeit, die zu identifizierenden Peaks zu reduzieren wäre der Vergleich von verschiedenen Kohorten, z. B. mit und ohne Vorkommen einer bestimmten Krankheit oder mit einer bestimmten Exposition. Mithilfe von statistischen Methoden könnten diejenigen Peaks identifiziert werden, welche überwiegend in nur einer der Kohorten auftreten.

Ein Dritter Weg zur Reduzierung der zu identifizierenden Peaks wäre eine Bestimmung der toxikologisch Relevanten. Hierzu könnte eine wirkungsorientierte Analyse von menschlichen Urin- und Blutproben entwickelt werden. Eine Fraktionierung mit einem nachfolgenden toxikologischen Test wie zum Beispiel auf hormonelle Wirksamkeit, würde die Fraktionen herausfiltern, in denen Effekte auftreten. In diesen Fraktionen kann dann eine Non-target Analyse versuchen, die Verbindungen, die für die gefundenen Effekte verantwortlich sind, zu identifizieren. Auf diese Weise könnten mehrere hundert bis tausend in einer Probe gefunden Peaks auf die für die menschliche Gesundheit relevanten reduziert werden.

3 Introduction

Over the last decades an increasing number of chemicals have been produced. Via usage in a wide range of products some of them will reach the environment as contaminants. By an uptake through skin, food, water and air either through direct usage of consumer products or through the environment these chemicals can reach human tissues. After the uptake of chemicals they can be stored in tissues such as fat or blood, or they can be metabolized and excreted via the urine. Today only a small part of the chemicals produced are being analysed in human biomonitoring studies or other smaller scientific projects. Beside the American and Canadian human biomonitoring projects, the German biomonitoring program is one of the most extensive worldwide. Currently the fifth German Environmental Survey is being conducted, in which next to other factors blood and urine samples are analysed for selected contaminants like bisphenol A, phthalates and perfluoroalkyl acids.

A joint initiative of the Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the German Chemical Industry Association (VCI) was started in 2010 to develop further target methods for possible contaminants of interest regarding human biomonitoring (www.vci.de/Presse/Pressemitteilungen/). The goal is to develop target methods for 50 new substances until 2020. A group of experts from industry, academia and government is deciding on which chemicals to include in these 50.

In this context the German Environmental Agency decided to go beyond target analysis with the development of non-target screening methods for human blood and urine samples. The objective of this project was hence to support the German human biomonitoring program with additional methods and substances of interest, which might in the future be included into human biomonitoring programs. To achieve this, non-target screening methods which are currently focusing on the analysis of environmental samples like water and sediment were adapted to the analysis of human blood and urine samples. The sample preparation and analysis methods were first tested with a set of target chemicals. The developed methods were subsequently used for the non-target analysis of 16 human blood and urine samples from the German Environmental Specimen Bank.

After a review of non-target screening methods for environmental samples and target methods for the analysis of human blood and urine samples, this report describes the method development by usage of target analytes. Following the description and validation of the data evaluation process, the identification of both target and non-target chemicals is reported.

4 State of the art in non-target screening

To detect and quantify contaminants in environmental and human samples usually target methods are applied. A relatively small number of target analytes is quantified in the samples using reference standards. This type of analysis is necessary when determining low concentrations in samples, however, this way only a small part of the contaminants present in a sample can be detected.

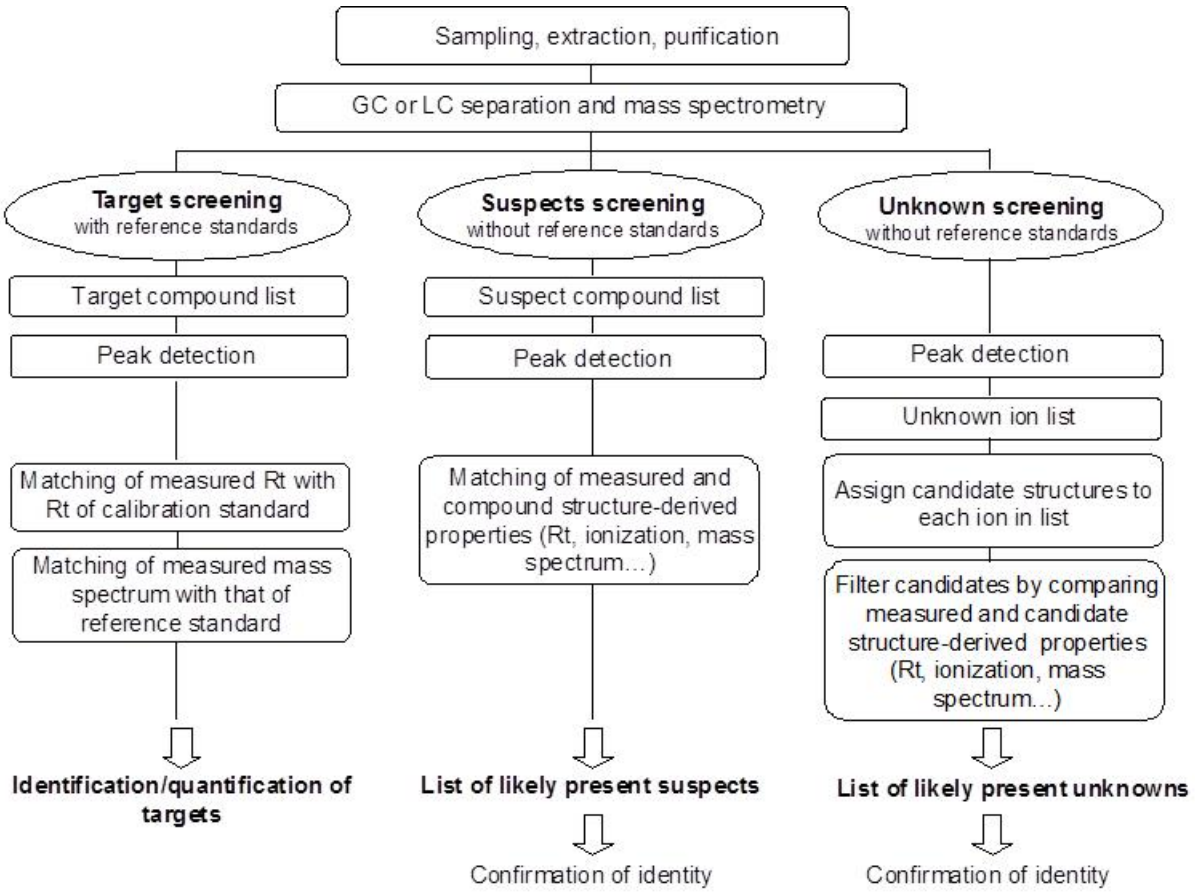
In the literature, there is no generally applicable definition for screening methods, their categorization is based on different criteria [1-4]. We will apply the definitions by Krauss et al. [3], where screening is divided into target, suspect and unknown screening, see Figure 1.

Target screening: (Semi-) quantitative or qualitative screening methods for known compounds with reference standards. The aim is often to obtain a fast overview of a large number of (regulated) contaminants [5, 6]. Such methods are often used in food monitoring and residue analysis, to quickly distinguish positive from negative findings below a certain detection limit. Afterwards the concentration of the detected contaminants can be quantified, which can be done using the same measurement or in a subsequent measurement, if necessary with an additional instrument [7].

Suspect screening: Aims to confirm suspected compounds ("suspects"), for example known or predicted degradation products or compounds for which no reference standards are available. There is no analytical information on the substances (retention time, mass spectrum), but the structures and often other properties are known. From the list of the compounds relevant properties can be calculated (octanol-water partition coefficient, pKa values, mono-isotopic mass, isotope ratios, etc.). On the basis of this information the chromatograms can be searched by the "suspects" and it can be checked if detected peaks and mass spectra coincide with the properties derived from the structure. A similar approach is defined by García-Reyes et al. [2] as "non-target screening", where samples are screened for known substances, which are not analysed in the routine target method, and positive findings are being confirmed with an existing reference standard.

Non-target screening: In this approach, also called "unknown screening", no information on the pollutants present in a sample is available. The information about the substances is derived solely from the chromatograms and mass spectra. Therefore, the first step is a manual or automatic peak search, which leads to a list of detected ions. For each detected ion, lists of possible candidate structures are created based on the mass spectra. As also for the suspect screening, inappropriate candidates are filtered by comparison of properties predicted from the structure and properties derived from the chromatogram. A final confirmation of tentatively identified substances is only possible with other spectroscopic techniques or a comparison with a reference standard. Sancho et al. [4] refer to this approach as "elucidation methods". A structure elucidation of unknowns based on gas chromatography-mass spectrometry (GC-MS) or liquid chromatography (LC)-MS is also carried out in the context of controlled degradation experiments (in vivo, in vitro, or in environmental media). However, in contrast to a real unknown screening, a number of preliminary information such as structural similarity to the initial substance and fundamental degradation mechanisms exist. Also, the detection of substances is facilitated by the existence of a time series or control samples [8]. These studies can therefore conceptually be placed between unknown and suspect screening.

Figure 1: Comparison of systematic workflows for (i) quantitative target analysis with reference standards, (ii) suspect screening without reference standards, and (iii) non-target screening of unknowns in environmental samples by using LC–high resolution (tandem) mass spectrometry. According to [3].

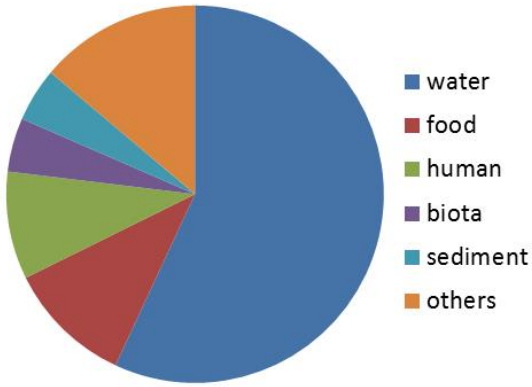


For both target and screening methods instruments like GC and LC coupled to MS are being used, as they possess the required selectivity and sensitivity. For non-target screening methods it is an advantage to use high resolution mass spectrometers, especially when coupled to LC, as there are no large mass spectra libraries available, as it is the case for GC-MS.

4.1 Sample preparation for non-target screening

The literature was searched for existing suspect and non-target screening methods. A significant focus of the reviewed literature was on aquatic ecosystems, with about 60% of the studies analysing water samples (groundwater, surface water, sewage, seawater) and 10% sediments (Figure 2). However, so far almost no methods for air samples (gas phase, aerosols) or soil samples exist. The number of publications on biota and human samples with a share of 5% each is also low, which stands in contrast to the large number of target methods developed for these matrices. Thus, there is a considerable need for the development of non-target screening methods in human samples, which is in agreement with the overall objective of this project. Food is present with a share of approximately 10%, while the remaining studies are on different matrices such as cosmetics or house dust.

Figure 2: Distribution of sample matrices investigated by the reviewed suspect and non-target screening methods, showing the highest percentage (>50%) for water analysis.



To capture a wide range of substances and to avoid any loss of substances in the samples analysed, the sample preparation has to be as broad as possible. But enrichment as well as a purification of the enriched samples to remove disturbing matrix components is often necessary for the analysis of trace amounts in complex environmental samples. In contrary, controlled metabolism studies are often performed at higher concentrations and samples are thus analysed without an enrichment step [9-12].

For a target screening a full validation of the method using reference standards is possible, similar to a target analysis. For a suspect screening a direct validation is not feasible, therefore false negative findings are possible due to analyte losses. To minimize these, it is necessary to define the 'chemical domain' of detectable substances by the simultaneous analysis of target analytes with similar physic-chemical properties. Although often such a method validation is not done with a suspect screening, generic sample preparations are used and discriminatory clean-up steps are avoided.

When analysing water samples with a GC-MS based screening, typically liquid liquid extraction (LLE) with MTBE, pentane, and/or dichloromethane is used (e.g. [13, 14]) or a solid-phase extraction (SPE) with C18-modified silica gel (e.g. [15, 16]) are applied. These methods largely capture a non-polar substance range. For LC-MS based screening methods SPE with various polymer sorbents (often OASIS HLB, but also Lichrolut EN, OASIS MCX, strata X and PRP-1) is applied, which often only cover a small polarity spectrum. For this reason Singer et al. and Kern et al. [5, 17] used a combination of four different neutral and ion-exchange sorbents (OASIS HLB, cation- and anion-exchange resin, Isolute ENV+) for the extraction of water samples.

For the extraction of sediment, accelerated solvent extraction (pressurized liquid extraction, PLE) or shaking extraction techniques using a combination of polar and non-polar solvents are applied (e.g. [18-20]). In most cases, the extracts are purified or fractionated by column chromatography.

For the analysis of food samples a growing number of studies make use of the QuEChERS approach ("quick, easy, cheap, effective, rugged and safe"), which includes a LLE with acetonitrile (ACN) followed by a dispersive SPE [21-25]. The suitability of this extraction method for a wide range of analytes (pesticides) has already been shown by the development of multi target methods [26, 27].

The preparation of human samples is usually carried out by a LLE of blood or tissues for non-polar compounds, where sometimes a protein precipitation and deconjugation step is included. Urine samples are sometimes not cleaned-up and concentrated, but diluted before analysis [28-30].

4.2 Instrumental analysis

For screening methods it is most common to couple GC or LC with MS, as they have the required selectivity and sensitivity for complex matrices and at the same time provide information about the chemical composition and structure. Table 1 gives an overview of the types of mass spectrometers. In

some individual studies also detectors with low selectivity are used to capture all halogenated compounds in a sample, e.g. GC-ECD or AED [13, 14, 31-33] or LC-UV [34]. An application of NMR or the direct coupling of LC and NMR is possible only in a few cases with high concentrations of the analytes at contaminated sites [35, 36]. For analytes in the ng to µg/kg or ng/L range the sensitivity of NMR is not sufficient, or no sufficient purity of the analyte can be achieved despite previous fractionation and chromatographic separation. An interesting combination of elemental analysis using LC-ICP-AES (inductive coupled plasma – atomic emission spectroscopy) and organic mass spectrometry using LC-MS/MS is described by de Brabandere et al. [37] for the identification of organic phosphorus substances, which can in principle also be applied to organometallic compounds.

Table 1: Comparison of commercial mass spectrometers; values usually refer to a mass range of 300-400, special equipment or configurations can have better values. See [3].

Mass spectrometer	Ion trap	Single quadrupole	Triple quadrupole	Double-focussing magnetic sector	(Quadrupol)-Time-of-flight (Q)TOF	Orbitrap	Fourier transform ion cyclotron resonance (FTICR)
resolving power[a]	unit[a]	unit[a]	unit[a]	60 000	20 000 (40 000)	100 000 (250 000)	400 000 (1 000 000)
precision (ppm)	50	50	50	5	3	2	<1
linear range	10^3	10^4	10^4	>10^4	10^2-10^3	10^3-10^4	10^4
sensitivity full scan (absolute mass)[b]	fg-pg (SRM, full scan)	fg-pg (SIM) ng (full scan)	ag-pg (SRM) ng (full scan)	fg-pg	fg-pg (full scan)	fg-pg (full scan)	pg (full scan)
scan rate	fast	average	average	average	very fast	slow-fast	slow
frequency of application in screening-methods	rarely	common	rarely	rarely	common	common	rarely
price	low	low	average	high	high	high	very high
coupled to	GC, LC	GC	GC, LC	GC	GC, LC	LC	LC

[a] The resolution is dependent on mass range and scan rate; standard quadrupol- and ion trap-instruments work with unit resolution, but specific configurations can reach resolutions of 5 000 with a precision < 10 ppm.

[b] The sensitivity is strongly dependent on the ionization efficiency of each substance.

4.2.1 GC-MS

For GC substances most methods use a quadrupole MS, less often an ion trap MS, applying electron ionization (EI), a technology established since over 40 years. EI is a widely standardized, reproducible ionization method that is little prone to matrix effects. A "harmonized" standard ionization energy of 70 eV is commonly used, which results in a good fragmentation and thus delivers often a good structural information. Thus mass spectra are relatively comparable across devices from various manufacturers, which has led to the development of large spectrum databases, intensively used for comparison with unknown peaks in a sample. However, the strong fragmentation can also be a disadvantage of EI as the intensity of the molecular ion (M^+) often is low or missing completely, making it impossible to identify the substance. Softer ionization techniques can provide complementary information; here the positive chemical ionization (PCI) and negative chemical ionization (NCI) are the most widespread. They generate a lower internal energy of the molecules, resulting in less fragmentation and thus a higher intensity of

molecular ions (typically $[M+H]^+$ in PCI and $[M]^-$ in NCI). With PCI adducts are generated depending on the reaction gas used, while with NCI especially halogenated compounds are ionized, which can form a stable anion by thermal electron capture. Despite the fact that PCI and NCI have been established for many years in commercial devices, they so far have been little used in screening methods. Only the study by Portoles et al. [15] shows how a combination of EI, PCI and NCI can lead to complementary information from EI and CI spectra, which contribute to the structure elucidation of unknown compounds.

For unstable or non-volatile compounds derivatization has been applied with target methods and within metabolomics prior to the development of LC-MS, but is still used today [38]. For non-target substances a review of the derivatization efficiency and optimization of conditions is not possible, making it unclear whether all functional groups possibly prone to derivatization are actually collected. In several screening methods of polar unknowns [14, 32, 33, 39] silylation or methylation with diazomethane were used as relatively non-specific reactions covering a large number of functional groups.

Recent developments in the area of GC-MS techniques aim at improving the selectivity both for detection and separation. In the former case, this means the application of high-resolution mass spectrometers (HRMS). Some older studies already used sector field mass spectrometers [40-42], while more recent studies use time-of-flight (TOF)-instruments (e.g. [15, 29, 43-45]). A better chromatographic separation of the peaks in complex mixtures is possible by two-dimensional GC (comprehensive GCxGC). As a faster detector is required to achieve a good resolution for the narrow peaks, GCxGC is coupled to a fast quadrupole MS or more frequently to a (low resolution) TOF [45-49].

4.2.2 LC-MS

For non-GC-amendable, more polar substances analysed in screening methods LC is coupled with different mass spectrometers using atmospheric pressure ionization (API) techniques. The most common ionization technique is electrospray ionization (ESI), followed by atmospheric pressure chemical ionization (APCI). With both techniques, a large number of polar molecules can be ionized. Through the relatively soft ESI resulting in a low amount of fragmentation usually protonated ($[M+H]^+$ in positive mode) or deprotonated ($[M-H]^-$ in negative mode) molecular ions are generated, thus the molecular weight can easily be determined. The harder APCI often results in a stronger fragmentation, and through charge transfer reactions also positively or negatively charged molecular ions can occur (M^+ or M^-). This way also molecules showing a low tendency to (de)protonate can be ionized, e.g. S- and O-heterocycles, nitriles and pyrroles. Matrix effects often pose problems during LC ionization, which can lead to ion suppression [50, 51] and the formation of adducts with alkali metals or other small ions (ammonium, formiate). This makes it difficult to identify the molecular ions in the mass spectrum. A further ionization technique is atmospheric pressure photon ionization (APPI; [52]), with which also highly non-polar, "typical" GC-MS analytes such as polycyclic aromatic hydrocarbons [53] can be detected. In contrast to ESI and APCI, which provide good ionization efficiencies for a wide range of substances, the ionization by APPI is more selective for individual substance groups and more dependent on the ionization conditions such as flow rate, type and quantity of the non-polar dopant (e.g. toluene, acetone) responsible for the charge or proton transfer. Therefore, APPI seems currently less well suited than ESI or APCI for a wide substance screening and has previously only been used for target analysis.

While for most target methods triple quadrupole MS (QqQMS) in selected reaction monitoring (SRM) mode is used, its use for screening methods is possible only to a limited extent. For a target screening in the sensitive SRM mode, the number of identifiable substances with sufficient dwell time for the individual ions is limited, and the sensitivity in full scan mode is several orders of magnitude lower [3]. For ion trap instruments these two disadvantages are not true, since the entire mass range can be analysed with sufficient sensitivity using full scan. However, full scan-LC-MS spectra of complex matrices often show a high ion background and a large number of peaks, which complicates the peak detection. Therefore, the

vast majority of the LC-MS screening methods uses HRMS in the form of TOF, quadrupole TOF (QTOF), quadrupole Orbitrap or ion trap Orbitrap to achieve the required selectivity (see Table 1 and [3, 7]). The use of very expensive Fourier transform ion cyclotron resonance-MS (FTICRMS), which achieve the highest mass resolution, is limited to a few studies. Next to the higher selectivity, a second advantage of HRMS is the opportunity to derive the empirical formula of the molecular ions from the exact mass, which in addition to the resolution also requires a high mass accuracy. Because the API itself results in no or only low fragmentation further structural information has to be gained by in-source fragmentation or real tandem mass spectrometry. Only with the latter a clear assignment of fragment and precursor ions is possible. HRMS such as ion trap Orbitrap, quadrupole Orbitrap or QTOF also allow determining accurate mass-MS/MS spectra and currently represent the most commonly used instruments for screening methods. In particular a data-dependent MS/MS, where the recording of an MS/MS spectrum is triggered by occurrence of specific ions in the full scan, allows for an efficient extraction of MS/MS spectra for a large number of substances in a chromatographic run. The resolution and accuracy required for a unique assignment of molecular formulas and a separation of ions depends on the complexity of the matrix and the mass. The number of meaningful sum formula is greatly increasing with increasing mass. Previous experiences show that for a suspect and non-target screening of pollutants in environmental and food samples a resolution of 20,000 to 60,000 in full scan and a mass accuracy of 2 ppm is sufficient in most cases [3, 54].

4.3 Data evaluation

In addition to the analysis itself, the data evaluation for screening methods is an important and usually the most time consuming step in the identification of substances. While in older studies often a manual evaluation of the data was carried out, the large amount of data obtained from GCxGC-MS, GC-HRMS or LC-HRMS makes an automatic data evaluation necessary. Chromatograms from complex sample matrices typically contain between 1000 and 10,000 peaks. Therefore, many steps of the data analysis have been automated in recent studies, and a large amount of different commercial and freely accessible software has been developed (overviews in references [55, 56]).

The steps of data analysis in target screening largely correspond to those of the target analysis (peak search and integration on the basis of known mass and retention time, confirmation by well-known fragmentation and isotopic peaks if necessary, quantification of calibration standards; see Figure 1) and will not be described in detail. The approaches and procedures for suspect and non-target screening are described in the following sections.

4.3.1 From peak detection to the compound list

In a suspect screening, similar to target screening, chromatograms can be searched directly for a peak with a known mass from a suspect list (e.g. [17]), however, due to an unknown retention time in the entire chromatogram. Subsequently one (or several) peaks found for each suspect must be confirmed. In some studies automatic peak detection is carried out as for a non-target screening, and the detected peaks are compared to the masses in a suspect list (e.g. [25]).

In a non-target screening ideally all peaks in a chromatogram are detected and identified. In older GC-EI-MS methods, but also recent studies, a visual peak search in the total ion chromatogram and manual extraction of the mass spectra was carried out [39, 57]. Thus, only an analysis of the largest or chromatographically well resolved peaks is possible. In most of the studies, however, only insufficient information is given about the peak detection and criteria on which peaks are picked for further identification or on how many peaks were found in total.

Different algorithms that are implemented in a number of software tools are available for automated peak detection in chromatograms and subsequent data processing (Table 2, as described in [55, 58]). The peak

detection is facilitated by a baseline correction in the chromatogram and filtering of background noise, which are implemented in some of the tools. A distinction of real substance peaks from those resulting from contamination of reagents or instruments requires the simultaneous analysis of blank and control samples. Blank and control chromatograms can then be "subtracted" from those of the samples. The chromatographically separated substances always comprise a number of individual masses resulting (i) from isotopic distribution, (ii) from the fragmentation of molecules, and (iii) from formation of adducts in LC-MS analysis (see section 4.3.3). To assign each of these mass peaks to one compound, further data processing steps are necessary: for GC-EI-MS data, typically showing pronounced fragmentation, "clean" mass spectra are extracted from the chromatograms by spectra deconvolution, which are used for the identification usually by comparison with mass spectra databases [59]. For LC-HRMS data, however, the isotope peaks as well as the adduct peaks can due to the exact mass difference, relative signal intensities and matching peak forms be recognized by algorithms and associated with the substances ("de-isotoping").

Table 2: Software for peak detection and data processing.

software	data type	filte-ring	peak-detection	align-ment	comparison to reference samples	de-isotoping	adduct search	availability
MetAlign	MS, HRMS	yes	yes	yes	yes	-	-	open source www.metalign.wur.nl/UK/
XCMS	MS, HRMS	yes	yes	yes	yes	-	yes	open source metlin.scripps.edu/xcms/
Decon2LS	HRMS	yes	yes	-	-	yes	-	open source omics.pnl.gov/software/Decon2LS.php
MZmine2	MS, HRMS	yes	yes	yes	yes	yes	yes	open source mzmine.sourceforge.net/
ACD/ IntelliXtract	MS, HRMS	-	yes	-	-	yes	yes	commercial (ACDLabs)
AMDIS	GC-EI-MS	yes	yes	yes	yes	-	-	open source chemdata.nist.gov/mass-spc/amdis/

4.3.2 From compound list to molecular structure: GC-EI-MS

The methods described in section 4.3.1 results in a list of (unknown or suspected) substances detected in the sample with their retention time and the corresponding mass spectra. In most studies, a search in spectrum databases is done (see Table 3), where the measured spectra are compared with those in the database and a match value is calculated. Databases most often used are the NIST Mass Spectral Library and the Wiley Registry of Mass Spectral Data, which together contain about 870,000 spectra of 736,000 substances. In addition, there are some smaller databases, specialized on individual substance groups such as pesticides or drugs, containing from 100 to more than 10,000 spectra. For a large number of substances in the databases also GC retention time indices (Kovats RI) exist, which can easily be determined by comparing the retention time to RI calibration standards (homologues series of n-alkanes). These criteria can without much additional effort be implemented into a screening method [46, 60]. A good correlation of measured and database-spectra does not necessarily mean the actual occurrence of a substance, as many structural isomers result in similar spectra. A confirmation of mass spectrum and retention time with the help of a reference standard is therefore always necessary. Despite the large number of stored spectra, often only a portion of the existing unknowns in a sample can be identified, although in most

studies no further details are given on the numbers of detected peaks and percentage of identified substances.

An alternative approach to capture all theoretically possible molecular structures of a substance, and thus not to exclude any possible structures is in-silico structure generation. Here the molecular formula of the substance has to be known. Schymanski et al. presented an approach that combines a structure generation and the use of sub structure information from EI-MS spectra by means of the MOLGEN-MS software [61]. For many substances, this method results in a large number of possible candidate structures, which can be restricted by using more information such as predicted vs. measured RIs, and predicted steric energy of candidates [62]. Based on this method, it was possible to identify a set of ground water contaminants [63].

Table 3: Mass spectra databases, partly covering the range of organic pollutants.

Name	type	substance groups	spectra	substances	availability
Wiley 10th edition	EI-MS	all	719.000	638.000	commercial
NIST `11	EI-MS	all	243.893	212.961	commercial
	EI-MS/MS (2012)	all	121.586	6.999	
			346.757 Kovats RI	70.835 Kovats RI	
Massbank	all kind of MS und MS^n spectra	metabolomics and environmental pollutants	40.064	no information	open source www.massbank.jp
ReSpect (RIKEN MS^n spectral database for phytochemicals)	MS^n	metabolomics in plants	8.649	3.595	open source spectra.psc.riken.jp
METLIN	LC-MS/MS high resolution	metabolomics	26.640	no information	open source metlin.scripps.edu
Mass Spectra of Physiologically Active Substances	GC-MS	drugs, steroids, endocrine substances	4.182	no information	commercial (Wiley)
Mass Spectra of Designer Drugs	GC-EI-MS	drugs	>14.000 >6.000 Kovats RI	>12.000	commercial (Wiley)
	LC-ESI-MS/MS		>10.000	750	
Mass Spectra of Pesticides		pesticides	1238	no information	commercial (Wiley)
FFNSC 1.3	GC-EI-MS	essences and fragrances	1831 (+ Linear RI)	1831	commercial (Wiley)
Mass Spectra of Androgens, Estrogens and other Steroids	EI-MS	androgens, estrogens and other steroids	3.722	no information	commercial (Wiley)
Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants and their Metabolites	EI-MS	drugs, poisons, pesticides, pollutants and their metabolites	13.640	7.500	commercial (Wiley)
Mass Spectra of Pharmaceuticals and Agrochemicals 2006	EI-MS	pharmaceuticals and agrochemicals	4.563	no information	commercial (Wiley)
Mass Spectra of Organic Compounds 2004	EI-MS	all	37.055	no information	commercial (Wiley)
Golm Metabolome Database	GC-EI-TOFMS (plus RT index)	metabolomics	6.205	no information	open source gmd.mpimp-golm.mpg.de
ESI-MS/MS (QqITMS) library for forensic and clinical toxicology	ESI-MS/MS	toxicology	5.600	1.253	open source (pdf) www.chemicalsoft.de
ESI-MS^n spectra library	ESI-MS^n		3.766	1.743	[64]
Mass Spectra of Geochemicals, Petrochemicals and Biomarkers	EI-MS	geochemicals, petrochemicals and biomarkers	1.100	no information	commercial (Wiley)

4.3.3 From compound list to molecular structure: LC-MS

Compared to EI-MS spectra databases, API-MS/MS spectra databases are small (see Table 4). This is due to the shorter usage time of API-MS techniques as well as the fact that the MS/MS fragmentation is difficult to standardize and differs significantly between fragmentation techniques, collision gases used and

instrument types. Therefore in many studies only in-house databases are used [1, 24, 30] or EI-MS databases are consulted despite the limited comparability due to different fragmentation mechanisms [18, 65, 66]. In larger, commercial or freely available LC-MS/MS databases, however, spectra at various collision energies are recorded, which improves the comparability between MS/MS techniques and devices [67, 68]. The currently most comprehensive LC-MS/MS databases exist in the field of metabolomics with METLIN (Scripps Research Institute in San Diego, California, United States), as well as MassBank, which is operated by a worldwide consortium and is open to LC-MS and GC-MS spectra [69]. Despite a significant progress in the establishment of LC-MS/MS databases, their application for suspect and non-target screening of trace contaminants is currently still fairly limited.

Therefore the most widely used approach in a non-target screening is to determine the exact mass with HRMS devices and to generate molecular formula for the detected substances. For low molecular weight compounds (< 200 Da) the accurate measurement by itself is often sufficient to determine the empirical formula, however, with increasing mass the number of possible sum formula is increasing significantly. Even with a resolution >100,000, sum formula for masses > 400 containing only C, H, and O cannot be clearly determined [70]. Therefore, taking into account the relative intensities of isotopic peaks is essential for the determination of the sum formula, in most cases the relatively common isotopes ^{13}C, ^{34}S and especially ^{37}Cl and ^{81}Br are applicable. Thus, also the accuracy of the measurement of the isotope distribution ("spectral accuracy") plays an important role. For intense peaks also ^{15}N, ^{18}O, ^{33}S-isotope peaks may be visible. The number of probable sum formula can also be limited by more heuristic filtering rules ("Seven Golden Rules [71]"), which allow only "meaningful" or probable element ratios for organic molecules, for example a H/C ratio < 3, N/C < 2 or Cl/C < 1.

After determination of the sum formula possible structural formulas can be derived. A search in large online substance databases such as SciFinder, PubChem, ChemSpider or smaller databases (see Table 4) can be processed. The number of hits can vary between several thousands and zero. For example, several well-known transformation products of pesticides that are relevant to a screening of food or environmental samples currently cannot be found among the millions of substances. An alternative is the structure generation as described above (see section 4.3.2), but so far no substructure classifiers are available for LC-MS/MS data, so that a reduction in the candidate list is not possible.

Table 4: Commercial and freely available substance databases.

name	substances	availability
ChemSpider	>30.000.000	open source www.chemspider.com
PubChem	>48.800.000	open source pubchem.ncbi.nlm.nih.gov
SciFinder / CAS	>61.000.000	commercial (Chemical Abstracts Services)
Reaxys (before: Beilstein/Crossfire DB)	>19.500.000	commercial (Elsevier)
ChemBioFinder	>2.000.000	commercial (Cambridgesoft/PerkinElmer)
Chemindex	ca. 75.000	commercial (Cambridgesoft)
Kyoto Encyclopedia of Genes and Genomes (KEGG)	16.181 (small molecules)	open source www.genome.jp/kegg
Merck Index	>10.000	commercial
Human Metabolome Database (HMDB)	7.900	open source www.hmdb.ca
Chemical Entities of Biological Interest (ChEBI)	26.091	open source www.ebi.ac.uk/chebi
DrugBank	6.796	open source www.drugbank.ca
Chemical structure lookup service	>74.000.000 from >100 dababases (metasearch)	open source cactus.nci.nih.gov/cgi-bin/lookup/search
ChemIDplus	380.000	open source sis.nlm.nih.gov/chemical.html
Combined Chemical Dictionary	>500.000	commercial (Taylor & Francis Group)
ChemExper	>200.000	open source www.chemexper.com

To further limit the resulting candidate list for a substance peak, manual data processing and expert knowledge as well as automated procedures and software tools are needed. This has to be done not only for a non-target but also for a suspect screening, to check whether a peak found first exclusively due to its mass could actually match the suspect. This is commonly done by (i) MS fragmentation prediction and (ii) prediction of retention time or retention time indices.

Most published studies conduct a manual interpretation of the measured MS/MS data (e.g. [36]). The prediction of mass spectra from the candidate structures followed by a comparison with the measured spectra is so far rarely used [17, 72]. Table 5 contains software programs, which are based on different methodological approaches. While the commercial MassFrontier and ACD/MS Fragmenter programmes are based on fragmentation rules derived from the literature and databases, the programs FiD [73] and MetFrag [74] predict the likely bond dissociation, which excludes possible rearrangement reactions. MetFrag combines the fragmentation prediction directly with a substance database search. Based on exact masses and molecular formulas, candidates are extracted from substance databases, fragmented in silico, and sorted according to their similarity with the appropriate MS/MS spectrum. However, both for MS/MS spectra as well as for EI-MS spectra, case studies often showed no good agreement between simulated and measured spectra [75, 76], requiring additional methods regarding candidate selection.

Table 5: Commercial and freely available software for the prediction of EI-MS and MS/MS spectra.

Software	mass spectra	method	availability
ACD/MS Fragmenter	EI-MS, API-MS/MS	rules	commercial (ACD/Labs)
MassFrontier	EI-MS, API-MS/MS	rules, databases	commercial (Hichem/Thermo)
MetFrag	API-MS/MS EI-MS	bond-dissociation	open source msbi.ipb-halle.de/MetFrag
Fragment Identificator (FiD)	API-MS/MS	bond-dissociation	open source www.cs.helsinki.fi/group/sysfys/software/fragid

A number of models have been developed for the prediction of retention times or retention indices from chemical structures (quantitative structure-retention relationships QSRR; overview in [77]). These can be used for further selection of candidate structures. Such approaches, however, have so far rarely been used. While for the GC-MS Kovats and Lee retention indices can be predicted quite well with existing models [78], the retention index forecast for LC-MS is more difficult. For the latter there is no universal index system and existing QSRRs can only be applied to a relatively narrow substance spectrum due to the more complex interactions. However, also simple $\log K_{OW}$-retention time models can be used to reduce candidate lists [17].

More information about the limitation of candidate structures can be drawn from the preceding analysis. Meinert et al. [79] defines K_{OW} ranges for RP-HPLC fractions derived from standard runs, which are then used for reduction of candidate lists from groundwater analysis. In the studies by Kern et al. [17] and Hug et al. [80] candidate structures are confirmed by a plausibility check of their ionization in positive or negative mode.

4.4 Summary

Non-target screening methods based on GC-EI-MS represent an approved method for non-polar compounds. Developments on the analytical side as GC-EI-TOF devices offer improved resolution of co-eluting peaks and determination of the sum formula. In terms of data analysis the (manual or automated) peak detection dominates. So far data evaluation includes manual search in spectrum databases or spectra interpretation, sometimes also retention indices are used for the preliminary identification of unknown compounds.

For LC-MS-based methods, tandem-HRMS devices like QTOF or ion trap Orbitrap with a data-dependent MS^n spectrum acquisition have become the standard technique. The small size of existing MS/MS spectra libraries and larger device variations so far only lead to circumstantial hits in the identification of peaks. Thus methodological approaches primarily go via the determination of the empirical formula and the search for appropriate candidate structures in substance databases. In most cases only the MS/MS spectrum is used for the restriction of the surviving candidates.

Further methods to limit the candidate structures or to confirm suspects (in silico prediction of fragmentation, retention time forecasts) have been used very little so far. Other approaches such as prediction of ionization behaviour in different modes/sources or adduct formations in API sources are used only as expert knowledge; however, there are no quantitative methods to predict them from the structure for a large number of candidates.

For a comprehensive screening it appears appropriate to combine GC-MS and LC-MS/HRMS methods, because this way a wide range of substances can be covered, and the identification can benefit from complementary information of both techniques.

5 Target methods for human samples

As a basis for a strategic method development, an inventory of target methods for the analysis of trace pollutants in human samples was conducted. Additionally, a list of chemicals which are or might be relevant for human exposure was created, so called suspect chemicals. Based on this a selection of sample preparation steps and representative target substances for the development of a non-target screening method for human urine and blood was made.

5.1 Substances analysed in human matrices

Whether and how trace pollutants can be measured in any matrix, depends on their metabolism and toxicokinetics in the human body. A determination in urine is meaningful for polar and ionic substances, as the renal clearance is high. An analysis of more lipophilic substances is meaningful in blood samples, because these bind stronger to plasma proteins and are hardly renally excreted. For substances that are biologically transformed to a large extent, it is more meaningful to analyse metabolites in urine samples, due to their usually high polarity.

For the analysis of metabolites it should be distinguished between phase I and phase II metabolites. Phase I metabolites are particularly formed from non-polar compounds, which have only few functional groups. Metabolism takes places by oxidation, reduction and hydrolysis, primarily through cytochrome P450 monooxygenases. These metabolites are mostly analysed directly using LC-MS or more seldom GC-MS. Phase II metabolites result from conjugation of trace pollutants or their phase I metabolites with glucuronic acid, sulfate, acetyl moieties, amino acids, methyl groups or glutathione. This results in increase of water solubility and thus renal excretion. Phase II metabolites are analysed only in a few cases directly by LC-MS (for example [81-83]), mostly the conjugates in the sample are cleaved, thus only the deconjugated substance is being analysed. In addition to the application in urine samples, a deconjugation step is included for example in the analysis of phenols and phthalates in blood and breast milk samples [84-86]. If both free and total (including the conjugated) fractions of a substance are of interest, two analyses are necessary, one without and one with a deconjugation step. The deconjugation takes place either by adding enzymes or acid. The latter required that the substance to be analysed is acid resistant.

These fundamental considerations are reflected in the target methods previously developed for trace pollutants and their metabolites in various human matrices. Substance classes such as the classical POPs are only found in blood (or adipose tissue and breast milk) due to their hydrophobic properties. Other substance classes, e.g. different pesticides and UV filters, can be detected in blood as well as urine, whereas e.g. PAHs are analysed in blood as the parent compound and in urine as metabolites.

For following substance classes a number of target methods has been previously published, the approximate number of methods is specified:

- Aromatic amines (blood and urine), > 10 methods
- Fragrances (blood), > 10 methods
- Polybrominated flame retardants (blood, novel and phosphate-esters in urine), > 10 methods
- Parabens (blood and urine) > 5 methods
- Pesticides (blood and urine), > 30 methods
- Phenols (blood and urine), > 10 methods
- Phthalates (as metabolites in urine), > 5 methods
- Polyaromatic hydrocarbons - PAHs (blood, metabolites in urine), > 30 methods

- Polychlorinated biphenyls – PCBs (blood, metabolites in urine), > 10 methods
- Polyfluorinated alkyl substances - PFASs (blood, shorter chains also in urine), > 10 methods
- UV filters (blood and urine), > 10 methods
- Volatile substances - VOC (blood and urine), > 30 methods

Most of the published methods are specialized target methods for a certain substance class. There are only a few publications describing the simultaneous analysis of various – mostly quite similar - substance classes (e.g. polybrominated flame retardants, organochlorine pesticides and PCBs [29, 87]).

5.2 Sample preparation and analysis

Blood: Analyses of blood samples can be carried out in whole blood, plasma or serum. Whole blood samples are instable at room temperature due to coagulation, but can be stored deep frozen for a long period of time. However, hemolysis of the cellular constituents in whole blood takes place upon freezing. Alternatively, anticoagulants (usually EDTA or heparin) are added, however, this could possibly lead to interferences in the analysis of pollutants due to the relatively high concentrations of the anticoagulants. A sorption of trace pollutants to cellular components or the clotted blood can occur both during plasma and serum production. This way substances such as aromatic amines which are bound to the cellular components are removed from the sample [88].

Different amounts of blood samples are taken for analysis, depending on the required concentration factor and expected matrix effects. The quantities used in the reviewed methods range from 50 µL of plasma for the analysis of warfarin [89] and 10 g of serum for the analysis of PCBs and dioxins [90]. Concentration factors are often between 10 to 40, only in a few methods factors as high as 250 to 400 are reached [91-93].

Before the extraction of analytes, plasma proteins are precipitated. For persistent chemicals like PCBs, PFCs and some pesticides this is done by the addition of acids, while for acid-labile substances it is done by the addition of solvents such as acetonitrile and ethanol [94, 95]. Extraction is then usually done by LLE with non-polar solvents or SPE with modified silica gel (usually C18) or polymers such as ENV + and OASIS HLB. If the extracts after LLE or SPE require further purification, in many cases silica gel is used for lipophilic analytes to remove polar constituents.

Urine: For the analysis of trace pollutants in urine samples varying amounts are extracted, 10 µl for the analysis of bisphenol A [85] and up to 80 mL for the analysis of UV filters [96]. The concentration factors vary between 0.1 (i.e. dilution) and 250 [97, 98].

In most methods, the first sample preparation step is the hydrolysis of conjugates, as discussed above. This step is not carried out, if the target analytes are not conjugated or only the free fraction is of interest. Mercapturic acids of the precursor targets were analysed directly in only two studies [81, 83]. For the subsequent extraction also for urine samples SPE or LLE are preferred, only two methods described the analysis of organophosphates and bisphenol A where the samples were simply diluted, filtered and directly injected into the LC-MS [85, 99]. Rarely a clean-up is performed following SPE or LLE using silica gel or florisil [100-102].

6 Establishment of a suspect compound list

The suspect compound list serves as the basis for the suspect screening of blood and urine samples and for the selection of relevant and representative target analytes for the method development. Thus, this list should contain as many trace pollutants as possible, which already have been measured in human blood

and urine samples, or could be relevant due to high production volumes, their occurrence in household products, food and environmental samples, as well as due to persistent and bioaccumulative properties.

The list includes:

1. Substances, which have been detected or for which target methods are described in (i) the monitoring program of the “centers for disease control and prevention” (CDC), United States [103], (ii) the method collection "Analytical test methods for hazardous materials - analysis in biological materials" [104] and (iii) the homepage of the “Institut für Arbeits-, Sozial- und Umweltmedizin” of the University of Erlangen (www.arbeitsmedizin.uni-erlangen.de/biomonitoring/).
2. Substances that have been identified by the BfR in collaboration with the UBA and the BAuA as priority substances for human biomonitoring [105].
3. Substances from the report "Carcinogenic, mutagenic, (CMR toxic for reproduction) and other problematic substances in products" by the UBA [106].
4. Approved substances in cosmetics regulation 2010 (www.gesetze-im-internet.de/bundesrecht/kosmetikv/gesamt.pdf).
5. Due to their approved applications (e.g. disinfectants, biocides for human hygiene, wood preservatives) for human exposure-relevant substances, old biocides as they are listed in the European chemical substances information system (ESIS) (esis.jrc.ec.europa.eu/index.php?PGM=bpd).
6. Substances from various publications:
    a) probably persistent and bio-accumulating substances, which were derived on the basis of predicted properties from a list of 22,263 trading chemicals by Howard & Muir [107], based among others on the Canadian Domestic Substance List and the Toxic Substances Control Act Inventory update rule database by the U.S. EPA;
    b) list of per- and polyfluorinated substances which have been detected in the environment, or their homologues and structurally similar per- and poly-fluorinated substances [108];
    c) new trace pollutants, which have been measured in environmental samples for the first time during the years 2008-2011 [109, 110].

This resulted in a list of 1510 chemicals. For 357 of these chemicals methods for the analysis of blood are described, and for 332 methods in urine. In addition, 222 of these substances were detected in human blood samples and 120 in human urine samples, 39 in both matrices.

For all substances SMILES codes (simplified molecular input line entry system) were retrieved based on their CAS numbers and/or names using the program chemical translation service (cts.fiehnlab.ucdavis.edu/) or the substance databases ChemSpider (www.chemspider.com/Search.aspx) and PubChem (pubchem.ncbi.nlm.nih.gov/). For the chlorinated paraffins, it seemed unreasonable to generate unique SMILES codes because this substance class is a mixture of congeners with different chain lengths, degree of branching and degree and position of chlorination. This results in a large number of chlorinated paraffins with the same mass and structural formula, which complicates the analysis by MS. Generally this substance group is however quite relevant to the human biomonitoring, because until now only a few data exist for breast milk [111]. For other substances that have branched chains such as nonylphenol or perfluorinated sulfonic acids, only the unbranched chains were considered for the generation of SMILES. Using these generated SMILES the molecular structures were imported into a database in the program InstantJChem (ChemAxon, Budapest, www.chemaxon.com). Here, using the ChemAxon calculator plugins, IUPAC names, mono-isotopic masses and substance properties were

calculated ($logK_{OW}$, logD at pH 7.4, pK_a- and pK_b values and topological polar surface area). The EPI SuiteTM v4.10 by the US EPA (www.epa.gov/oppt/exposure/pubs/episuitedl.htm) was applied as a second program to predict $logK_{OA}$, $logK_{OW}$, bio-concentration factors (BCF) and Henry constants.

6.1 Target analytes for method development

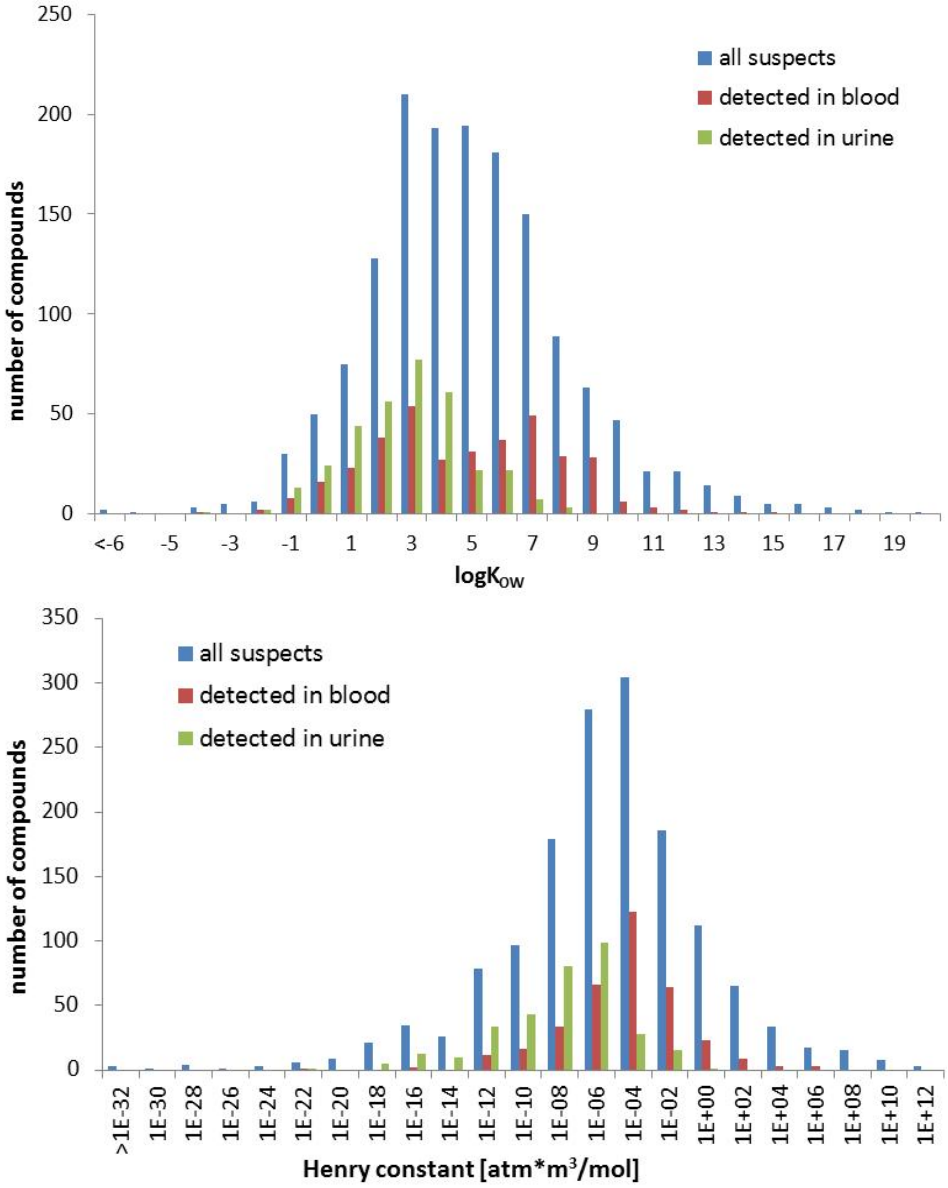
Target analytes for method development were selected from the suspect list of 1510 substances. The group of target analytes was supposed to:

- cover of a wide range of functional groups and physicochemical properties ("substance domain"),
- represent both GC as well as LC amenable substances,
- represent both new as well as methodically well-established substances to compare the method with already published (standard) methods. The substance selection of “new” analytes does not consider whether they would be detectable as the analyte in its original form or as a metabolite in blood and urine.

In a first step substances beyond the mass range of Orbitrap MS and GC-MS (60-2000 u) were removed.

The substance domain was derived by the collection of calculated $logK_{OW}$ and Henry constants of the compounds detected in blood or urine, which can be found in Figure 3. In blood samples the majority (> 90%) of the detected substances have $logK_{OW}$ values between 0 and 10 and Henry constants of 10^{-12} to 100 atm · m^3/mol, in urine > 90% are between $logK_{OW}$ 1 and 6, and Henry constants 10^{-16} to 0.01 atm · m^3/mol. For the substances measured in urine, only the unconjugated analytes are included, as they were mostly measured in this form. In general, conjugates would show lower $logK_{OW}$ values. Compared to the substance domain of all 1510 suspects the largest part is covered by the substances that so far have been detected in human samples.

Figure 3: Histograms of $logK_{OW}$ values and Henry constants (atm*m³/mol) of substances detected in blood, urine and all suspects in the suspect list; predicted using EpiSuite.

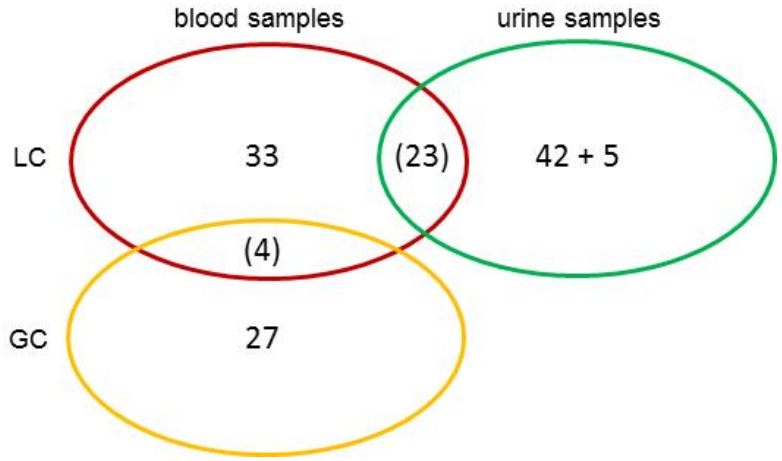


In a second step, representative substances were selected from the different compound classes; mostly one or two substances per class, sometimes more if the classes contained substances with very different functional groups or properties (e.g. in the case of flame retardants). The selected target substances for blood and urine samples are listed in Table 6 and Table 7. Table A 1 and Table A 2 in the Annex also include the calculated properties of the chosen target analytes. Not all substances are within the $logK_{OW}$ and Henry constant range covered by > 90% of the substances detected so far in blood or urine. They therefore represent extreme properties that will help to determine the substance domain of the method. Not considered were siloxanes, as these are relatively volatile and thus purge and trap - headspace extraction methods [112] have been used so far. Siloxanes additionally show high background levels due to their usage in most cosmetics such as hand soaps and deodorants.

In total 65 substances for the determination in blood and 42 for the determination in urine were chosen. Three target compounds had to be removed from the list, as they were not commercially available. For method development in urine also four selected commercially available phase II metabolites (glucuronide and sulfate of methylumbelliferone and estrone, respectively) were chosen to test a deconjugation step

during sample preparation and/or to check whether also conjugates can be detected. Additionally creatinine was selected, which is used frequently for the standardization of the detected concentrations or as a measure of the dilution of the urine. In total, 92 target analytes were selected, where all substances for urine method development should be analysable by LC-MS, see Figure 4.

Figure 4: Number of target substances for method development in the two matrices and their method of analysis.



These substances cover a wide range of suspect substances with respect to their physico-chemical properties, as can be seen for $logK_{OW}$ and Henry constants in Figure 5.

Figure 5: Chemical space plot; distribution of $logK_{OW}$ and Henry constants of the selected target substances for blood and urine compared to all 1500 suspects.

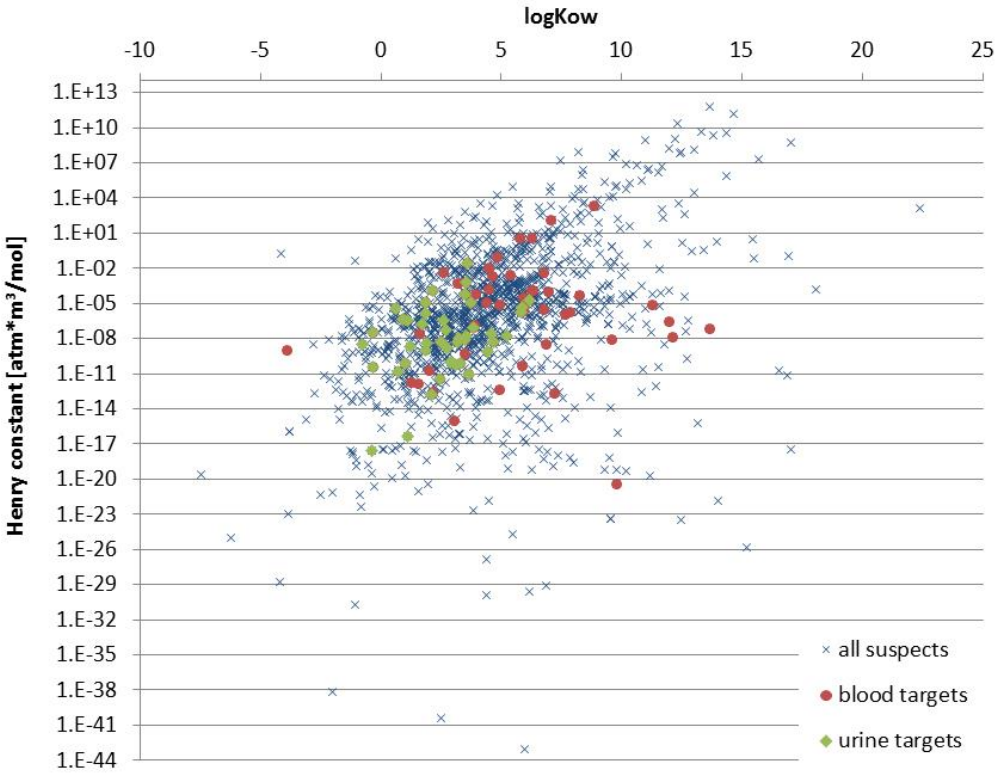


Table 6: Target analytes for the method development of urine samples.

Compound name	CAS No	compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
4-methyl-m-phenylene diisocyanate (2,4-TDI)	26471-62-5, 1321-38-6, 26006-20-2, 584-84-9	allergenic substance			BfR	174.16	$C_9H_6N_2O_2$		LC
5-chloro-2-methyl-4-isothiazolin-3-one (CMIT)	26172-55-4	allergenic substance			BfR, KosmVer	149.60	C_4H_4ClNOS	present in household and cosmetic products	LC
citronellal	106-23-0	allergenic substance			BfR	154.25	$C_{10}H_{18}O$		LC
geraniol	624-15-7, 68311-14-8, 106-24-1, 106-25-2	allergenic substance			BfR, KosmVer	154.25	$C_{10}H_{18}O$	contact allergen, fragrance	LC
resorcinol	108-46-3	allergenic substance			BfR	110.11	$C_6H_6O_2$		LC
2,4-diaminoanisole	615-05-4	aromatic amine			BfR	138.17	$C_7H_{10}N_2O$		LC
4,4'-thiodianiline	139-65-1	aromatic amine			BfR	216.30	$C_{12}H_{12}N_2S$	release from azodyes, which are used for dying clothing and paper, other aromatic amines are being analysed as Hb adduct in blood, BfR suggests detection in urine	LC
4,4'-methylendi-o-toluidine	838-88-0	aromatic amine			BfR	226.32	$C_{15}H_{18}N_2$		LC
4-aminoazobenzene (solvent yellow)	60-09-3	aromatic amine, azodye			BfR, UBA-WF, UBA-T	197.24	$C_{12}H_{11}N_3$		LC
o-dianisidine	20325-40-0, 119-90-4	aromatic amine			BfR	244.29	$C_{14}H_{16}N_2O_2$		LC
mercaptobenzothiazole	149-30-4	industrial chemical			BfR	167.25	$C_7H_5NS_2$	benzothiazoles, used as anticorrosives, likely ubiquitous environmental contaminants, vulcanization accelerators	LC
2-(methylthio)benzothiazole	615-22-5	industrial chemical			BfR	181.28	$C_8H_7NS_2$		LC
8-octyl-4-isothiazolin-3-one (octhilinone)	26530-20-1	industrial chemical			BfR, BPD	213.34	$C_{11}H_{19}NOS$		LC

Compound name	CAS No	compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
4-hydroxybenzotriazole	26725-51-9	industrial chemical			BfR	135.12	$C_6H_5N_3O$	metabolite of benzotriazole, used as anticorrosive, likely ubiquitous environmental contaminant	LC
p-toluene-sulfonamide	70-55-3	industrial chemical, benzosulfon-amide			BfR	171.22	$C_7H_9NO_2S$	used as anticorrosive, likely ubiquitous environmental contaminant	LC
5-methylbenzotriazole	136-85-6	industrial chemical, benzotriazole			BfR	133.15	$C_7H_7N_3$	used as anticorrosives, likely ubiquitous environmental contaminants	LC
ethyltosylamide	80-39-7	industrial chemical, benzene-sulfonamide			BfR	199.27	$C_9H_{13}NO_2S$		LC
dibutylhydroxytoluene	128-37-0	industrial chemical	u		AMBM, UBA-T	164.24	$C_{11}H_{16}O$	widely used as antioxidant in food, cosmetics, rubber etc.	LC
2-ethoxyethyl acetate	111-15-9	industrial chemical			UBA-T	132.16	$C_6H_{12}O_3$	HPV, solvent in production of other chemicals, monitoring data indicate that the general population may be exposed via inhalation of ambient air, but degradation might be fast	LC
hydroxyethyl mercapturic acid (HEMA)	15060-26-1, 19179-72-7, 97170-09-7	industrial chemical	u	urine [113]	detect	207.25	$C_7H_{13}NO_4S$	metabolite (of glycidol, 1,3-butadiene, acrolein, ethylene oxide and propylene oxide)	LC
2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD)	126-86-3	industrial chemical				226.36	$C_{14}H_{26}O_2$	surfactant, antifoaming agent	LC
3-aminomethyl-3,5,5-trimethylcyclohexyl-amine (isophorone diamine)	2855-13-2	industrial chemical				170.30	$C_{10}H_{22}N_2$		LC
1-hydroxypyrene	5315-79-7, 63021-84-1	OH-PAH	u	urine [114, 115]	TM,CDC detect, AMBM	218.25	$C_{16}H_{10}O$	metabolite of pyrene	LC

Compound name	CAS No	compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
1-naphthol	90-15-3	OH-PAH	p,u,hair	urine [115]	detect, CDC, AMBM	144.17	$C_{10}H_8O$	metabolite of naphthalene	LC
3-OH-4,4'-dichloro-biphenyl (3-OH-PCB)	53459-39-5	OH-PCB	u	[116]	TM	239.10	$C_{12}H_8Cl_2O$	metabolite of PCB	LC
4-OH-3,4',5-trichloro-biphenyl (4-OH-PCB)	4400-06-0	OH-PCB	u	[116]	TM	273.54	$C_{12}H_7Cl_3O$	metabolite of PCB	LC
2-isopropyl-6-methyl-pyrimidin-4-ol (IMPY)	2814-20-2	pesticide	u	urine [117]	detect, CDC	152.19	$C_8H_{12}N_2O$	OP, metabolite of diazinon	LC
diazinon	333-41-5	pesticide	s,b	[118]	TM, AMBM	304.35	$C_{12}H_{21}N_2O_3PS$	OP, insecticide	LC
dimethylthiophosphate (DMTP)	1112-38-5, (salts: 23754-87-2, 40633-14-5)	pesticide	u	urine [119]	detect, CDC, AMBM	142.11	$C_2H_7O_3PS$	OP metabolite (unspecific)	LC
diphenyl phosphate	53396-64-8, 838-85-7	pesticide, flame retardant	u	urine [120, 121]	detect	250.19	$C_{12}H_{11}O_4P$	OP metabolite (unspecific)	LC
perfluorobutanoic acid (PFBA)	375-22-4	PFC				214.04	$C_4HF_7O_2$	short chain carboxylic acid, replacement for longer chains	LC
bisphenol A	27100-33-0, 80-05-7	plasticizer	s, u	serum [122], urine [82, 123, 124]	detect, CDC, AMBM, UBA-T	228.29	$C_{15}H_{16}O_2$		LC
monoethyl-phthalate (MEP)	2306-33-4	plasticizer	u	urine [125]	detect, CDC	194.18	$C_{10}H_{10}O_4$	phthalate metabolite	LC
mono-iso-butyl-phthalate (MBP)	30833-53-5	plasticizer	u, breast milk	urine [125], breast milk [86]	detect, CDC	222.24	$C_{12}H_{14}O_4$	phthalate metabolite	LC
monobenzyl-phthalate (MBzP)	2528-16-7	plasticizer	u	urine [125]	detect, CDC	256.25	$C_{15}H_{12}O_4$	phthalate metabolite	LC
triethylcitrate	77-93-0	plasticizer				273.26	$C_{12}H_{17}O_7$		LC

Compound name	CAS No	compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
triclosan	3380-34-5	preservative	s, p, u, breast milk	serum [126], breast milk [127], plasma and milk [84], urine [128]	detect, CDC, UBA-WF, KosmVer, BPD	289.54	$C_{12}H_7Cl_3O_2$	bactericide	LC
ethyl paraben	120-47-8	preservative, paraben	s, u	serum [126], urine [129], [130]	detect, BfR	166.17	$C_9H_{10}O_3$	in cosmetics	LC
butyl paraben	94-26-8	preservative, paraben	u	urine [130]	detect, BfR	194.23	$C_{11}H_{14}O_3$	in cosmetics	LC
triethylenglycol dimethylether (tetraoxadodecan)	112-49-2	SVHC			BfR	178.23	$C_8H_{18}O_4$		LC
2-ethylhexyl-p-methoxycinnamate (cinnamate)	131-57-7	UV filter	s,p, u, epidermis	[95]	TM, BfR, EU authorized	290.40	$C_{18}H_{26}O_3$		LC
salicylic acid 3,3,5-trimethcyclohexyl ester (homosalate)	5466-77-3	UV filter	s,p, u, epidermis	[95]	TM, BfR, KosmVer, EU authorized	262.34	$C_{16}H_{22}O_3$		LC
benzophenone-3	118-56-9	UV filter	u	urine [82, 131]	detect, CDC, UBA-T, KosmVer, EU authorized	228.24	$C_{14}H_{12}O_3$		LC
4-methylumbelliferyl glucuronide (4-MeUmb-gluc)	881005-91-0	conjugate				352.29	$C_{16}H_{16}O_9$	test compounds conjugates	LC
4-methylumbelliferyl sulfate (4-MeUmb-sul)	15220-11-8	conjugate				256.23	$C_{10}H_8O_6S$		LC
estrone 3-(β-D-glucuronide) (estrone-gluc)	15087-01-1	conjugate				446.49	$C_{24}H_{30}O_8$		LC
estrone 3-sulfate (estrone-sul)	1240-04-6	conjugate				350.43	$C_{18}H_{22}O_5S$		LC
creatinine	60-27-5	marker		method in AMBM	AMBM	113.12	$C_4H_7N_3O$		LC

Table 7: Target analytes for the method development of blood samples.

Compound name	CAS No	Compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
4-methyl-m-phenylene diisocyanate (2,4-TDI)	584-84-9	allergenic substance			BfR	174.16	$C_9H_6N_2O_2$		LC
5-chloro-2-methyl-4-isothiazolin-3-one (CMIT)	26172-55-4	allergenic substance, biocide			BfR, KosmVer	149.60	C_4H_4ClNOS	present in household and consumer products	LC
citronellal	106-23-0	allergenic substance			BfR	154.25	$C_{10}H_{18}O$		LC
geraniol	624-15-7, 106-24-1, 106-25-2	allergenic substance			BfR, KosmVer	154.25	$C_{10}H_{18}O$	contact allergen, fragrance	GC
resorcinol	108-46-3	allergenic substance			BfR	110.11	$C_6H_6O_2$		LC
2,4-diaminoanisol	615-05-4	aromatic amine			BfR	138.17	$C_7H_{10}N_2O$		LC
4,4'-thiodianiline	139-65-1	aromatic amine			BfR	216.30	$C_{12}H_{12}N_2S$	release from azodyes, which are used for dying clothing and paper. Other aromatic amines are being analysed as Hb adduct in blood, BfR suggests detection in urine	LC
4,4'-methylendi-o-toluidine	838-88-0	aromatic amine			BfR	226.32	$C_{15}H_{18}N_2$		LC
4-aminoazobenzene (solvent yellow)	60-09-3	aromatic amine, azodye			BfR, UBA-WF, UBA-T	197.24	$C_{12}H_{11}N_3$		LC
o-dianisidine	20325-40-0, 119-90-4	aromatic amine			BfR	244.29	$C_{14}H_{16}N_2O_2$		LC
2,2',4,4'-tetrabromodiphenyl ether (BDE 47)	5436-43-1	flame retardant	s,p, breast milk, hair, adipose tissue	serum, milk [132, 133], adipose tissue [134, 135], breast milk [136, 137]	detect, CDC	485.79	$C_{12}H_6Br_4O$	commonly analysed PBDE	GC

Compound name	CAS No	Compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
2,2',4,4',5-pentabromodiphenyl ether (BDE 99)	32534-81-9	flame retardant	s,p, breast milk, hair, adipose tissue	serum, milk [132, 133], adipose tissue [134, 135], breast milk [136, 137]	detect, CDC	564.69	$C_{12}H_5Br_5O$	commonly analysed PBDE	GC
2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE 209)	1163-19-5	flame retardant	s, breast milk, hair	serum [138], breast milk [136, 137]	detect, BfR, UBA-WF,E	959.17	$C_{12}Br_{10}O$	commonly analysed PBDE, still used	GC
hexabromocyclodo-decane (HBCDD)	3194-55-6, 25637-99-4	flame retardant			UBA-T	641.70	$C_{12}H_{18}Br_6$	commonly analysed brominated flame retardant	LC/GC
syn and anti dechlorane plus (DP)	13560-89-9	flame retardant	s, hair	serum [93], hair [139]	detect	653.72	$C_{18}H_{12}Cl_{12}$	chlorinated flame retardant	GC
tris(2-chloroethyl)phosphate (TCEP)	29716-44-7, 68411-66-5, 115-96-8	flame retardant			BfR, UBA-T,WF	285.49	$C_6H_{12}Cl_3O_4P$	phosphate flame retardant	LC
tetrabromobisphenol A (TBBPA)	79-94-7	flame retardant			UBA-E	543.87	$C_{15}H_{12}Br_4O_2$		GC
decabromodiphenyl ethane (DBDPE)	84852-53-9	flame retardant			[107, 109]	971.22	$C_{14}H_4Br_{10}$		GC
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)	105650-23-5	food -grill product, heterocyclic aromatic amine	u	[140-142]	TM	224.26	$C_{13}H_{12}N_4$		LC
mercaptobenzothiazole	149-30-4	industrial chemical, benzothia-zole			BfR	167.25	$C_7H_5NS_2$	used as anticorrosives, likely ubiquitous environmental contaminant, high water solubility; vulcanization accelerators	LC
2-(methylthio)benzothiazole	64036-43-7, 31621-01-9, 615-22-5	industrial chemical, benzothia-zole			BfR	181.28	$C_8H_7NS_2$	used as anticorrosives, likely ubiquitous environmental contaminant, high water solubility; vulcanization accelerators	LC

Compound name	CAS No	Compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
p-toluene-sulfonamide	70-55-3	industrial chemical, benzosul-fonamide			BfR	171.22	$C_7H_9NO_2S$	used as anticorrosives, likely ubiquitous environmental contaminant, high water solubility	LC
dibutylhydroxytoluene	128-37-0	industrial chemical, antioxidant	u		AMBM, UBA-T	164.24	$C_{11}H_{16}O$	widely used as antioxidant in cosmetics, rubber etc.	GC
2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD)	126-86-3	industrial chemical				226.36	$C_{14}H_{26}O_2$	surfactant, antifoaming agent	LC
4-methylanisole	104-93-8	fragrance			BfR	122.16	$C_8H_{10}O$		GC
ambrettolide	123-69-3	musk - fragrance (macrocyclic)			BfR	252.39	$C_{16}H_{28}O_2$		GC
cashmeran	33704-61-9	musk – fragrance (polycyclic)	b	[143, 144]	TM, BfR	206.32	$C_{14}H_{22}O$		GC
celestolide	88401-65-4	musk – fragrance (polycyclic)	b, u	[127, 143, 144]	TM, BfR, AMBM	244.37	$C_{17}H_{24}O$		GC
galaxolide	1222-05-5	musk – fragrance (polycyclic)	b, u	blood [144]	TM, BfR, AMBM	258.40	$C_{18}H_{26}O$		GC
1,2-bis(2-ethylhexyl) 3,4,5,6-tetrabromobenzene-1,2-dicarboxylate (pyronil 45)	26040-51-7	P&B chemical			[107]	706.14	$C_{24}H_{34}Br_4O_4$	likely penta- and octa-BDE replacement, listed as P&B substance by the European Union, high logK_{OW}	LC
N,N-ethylene-bis(tetrabromophthalimide) (saytex BT 93)	32588-76-4	P&B chemical			[107]	951.47	$C_{18}H_4Br_8N_2O_4$	likely penta- and octa-BDE replacement, listed as P&B substance by the European Union, high logKow	GC

Compound name	CAS No	Compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
hexachlorocyclopenta-1,3-diene (HCCPD)	77-47-4	P&B chemical			[107]	272.77	C_5Cl_6	used as intermediate in production of chlorinated cyclodiene pesticides, also DP, detected in atmosphere above the Great Lakes	GC
naphthalene	91-20-3	PAH	p	plasma [145]	detect, UBA-WF	128.17	$C_{10}H_8$		GC
acenaphthylene	208-96-8	PAH	p	plasma [145]	detect	152.19	$C_{12}H_8$		GC
pyrene	129-00-0	PAH	p	plasma [145]	detect	202.25	$C_{16}H_{10}$		GC
2,2',5,5'-tetrachlorobiphenyl (PCB 52)	35693-99-3	PCB	p,s, adipose tissue	adipose tissue [134]	detect, CDC, AMBM	291.99	$C_{12}H_6Cl_4$	PCB routinely analysed	GC
2,2',4,5,5'-pentachlorobiphenyl (PCB 101)	37680-73-2	PCB	p,s, adipose tissue	adipose tissue [134]	detect, CDC, AMBM	326.43	$C_{12}H_5Cl_5$	PCB routinely analysed	GC
2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180)	35065-29-3	PCB	p, adipose tissue	adipose tissue [134]	detect, CDC, AMBM	395.32	$C_{12}H_3Cl_7$	PCB routinely analysed	GC
carbendazim	37953-07-4, 10605-21-7	pesticide, carbamate	s	[146]	TM, BPD	191.19	$C_9H_9N_3O_2$	biocide/fungicide, some usage has been banned by the EU	LC
diazinon	333-41-5	pesticide, OP	s,b, u	[118]	TM, AMBM	304.35	$C_{12}H_{21}N_2O_3PS$	biocide/insecticide	LC/GC
thiabendazole	148-79-8	pesticide			BPD	201.25	$C_{10}H_7N_3S$	fungicide, used as food preservative (on banana peel and citrus fruits)	LC
perfluorobutanoic acid (PFBA)	375-22-4	PFC				214.04	$C_4HF_7O_2$	short chain carboxylic acid, replacement for longer chains	LC
perfluorooctanoic acid (PFOA)	335-67-1	PFC	s,b, breast milk	serum [147], whole blood [148], serum and milk [132]	detect, AMBM	414.07	$C_8HF_{15}O_2$		LC
perfluorotetradecanoic acid (PFTDA)	376-06-7	PFC	b	whole blood [148]	detect, BfR	714.11	$C_{14}HF_{27}O_2$	long chain carboxylic acid, bioaccumulative	LC

Compound name	CAS No	Compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
perfluorooctane sulfonic acid (PFOS)	1763-23-1	PFC	s,b, breast milk	serum [147], whole blood [148], serum and milk [132]	detect, AMBM	500.13	$C_8HF_{17}O_3S$		LC
2-N-methylperfluoro-octanesulfonamide (MeFOSA)	31506-32-8	PFC	s	serum [147]	detect, BfR	513.17	$C_9H_4F_{17}NO_2S$	precursor of PFOS	LC
perfluorooctyl phosphonic acid (C8-PFPA)	40143-78-0	PFC			[108]	500.05	$C_8H_2F_{17}O_3P$	used in food package material	LC
8:2 fluorotelomer alcohol (8:2 FTOH)	678-39-7	PFC			BfR	464.12	$C_{10}H_5F_{17}O$	precursor of perfluoro carboxylic acids	(GC)
10:2 fluorotelomer alcohol (10:2 FTOH)	865-86-1	PFC			BfR	564.13	$C_{12}H_5F_{21}O$	precursor of perfluoro carboxylic acids	(GC)
4-nonylphenol (4-NP)	25154-52-3, 104-40-5, 68081-86-7	phenol			BfR, UBA-T,WF,E	220.35	$C_{15}H_{24}O$		GC
bisphenol A	80-05-7	plasticiser	s, u	serum [122], urine [82, 123, 124]	detect, CDC, AMBM (urine), UBA-T	228.29	$C_{15}H_{16}O_2$		LC
bis(4-chlorophenyl) sulfone	80-07-9	plasticiser			[107]	287.16	$C_{12}H_8Cl_2O_2S$		GC
triclosan	3380-34-5	preservative, bactericide	s, p, u, breast milk	serum [126], breast milk [127], plasma and milk [84], urine [128]	detect, CDC, UBA-WF, KosmVer, BPD	289.54	$C_{12}H_7Cl_3O_2$	bactericide	LC
ethyl paraben	120-47-8	preservative, paraben	s, u	serum [126], urine [129],[130]	detect, BfR	166.17	$C_9H_{10}O_3$	in cosmetics	LC
butyl paraben	94-26-8	preservative, paraben	u	urine [130]	detect, BfR	194.23	$C_{11}H_{14}O_3$	in cosmetics	LC

Compound name	CAS No	Compound group	Matrix[1]	Method source	Detected, listed[2]	Molecular weight	Formula	Comment	LC/GC
3,5-dichloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide (tetrachlorosalicylanilide)	1154-59-2	preservative			[107]	351.01	$C_{13}H_7Cl_4NO_2$		LC
quaternium 15	4080-31-3	QUAT, biocide, allergenic substance			BfR, BPD	251.16	$C_9H_{16}Cl_2N_4$	strongly allergenic, used in household- and consumer products	LC
benzyldimethyldodecyl-ammonium chloride (benzylQUAT)	139-07-1	QUAT				304.53	$C_{21}H_{38}N$	biocide, surfactant	LC
trimethyloctyl-ammonium bromide (trimethylQUAT)	2083-68-3	QUAT				172.33	$C_{11}H_{26}N$	biocide, surfactant	LC
michlers ketone	90-94-8	SVHC			BfR	268.35	$C_{17}H_{20}N_2O$		LC
phenolphthalein	77-09-8	SVHC			BfR	318.32	$C_{20}H_{14}O_4$		LC
2-ethylhexyl-p-methoxycinnamate (cinnamate)	5466-77-3	UV filter	s,p, u, epidermis	[95]	TM, BfR, EU authorized	290.40	$C_{18}H_{26}O_3$	in sunscreens, cosmetics	LC/GC
salicylic acid 3,3,5-trimethcyclohexyl ester (homosalate)	118-56-9, 52253-93-7	UV filter	s,p, u, epidermis	[95]	TM, BfR, KosmVer, EU authorized	262.34	$C_{16}H_{22}O_3$	in sunscreens, cosmetics	LC/GC
4-methyl-benzylidene camphor (4-MBC)	36861-47-9	UV filter			BfR, KosmVer, EU authorized	254.37	$C_{18}H_{22}O$	in sunscreens, cosmetics	GC
octocrylene (OC)	6197-30-4	UV filter			BfR, EU authorized	361.48	$C_{24}H_{27}NO_2$	in sunscreens, cosmetics	GC

[1] s=serum, p=plasma, b=whole blood, u=urine; [2] TM (target method exists), AMBM (methods described in [104]), BDP (listed in Biocidal Products Directive), CDC (detected in American human biomonitoring surveys), detect (detect reported in any literature), BfR (recommended to look for in human biomonitoring studies by the BfR), UBA-WF/T/E (listed in [106]), EU-authorized (listed in [149])

7 Method development

7.1 Concept for method development

7.1.1 Urine

For a comprehensive screening of trace contaminants, phase I and phase II metabolites can both be analysed directly or as the deconjugated substances after cleavage. To enable a targeted search for conjugates, some samples were to be analysed with and without a deconjugation step, enabling the search for relevant peaks based on the comparison of these two analyses. Therefore, during method development the deconjugation step was to be tested by spiking two sulfate and two glucuronide conjugates listed in Table 6. The enzymatic hydrolysis was preferred over an acidic one to avoid a transformation of acid-labile compounds.

7.1.2 Blood

The extraction procedure was tested particularly for whole blood, as compounds absorbed to cellular components were not a priori excluded. To prevent clotting often anticoagulants such as EDTA or heparin are added to whole blood directly after withdrawal [94, 143, 150, 151]. However, there is also the possibility to freeze the blood at -20°C after sampling and to homogenize after thawing [148, 152-154]. For a non-target screening, it would be generally preferable to freeze the blood without the addition of an anticoagulant, as the addition introduces a risk of contamination of the sample, and an increase of matrix effects. However, the samples from the environmental specimen bank are processed by the addition of heparin (approx. 1 drop of heparin/1% benzyl alcohol per 15 mL of sample) [155], before being stored at -150°C. As the final non-target screening of these samples was an objective of this project, both the analysis of whole blood with and without the addition of heparin was tested regarding recoveries for the target analytes. Additionally also the analysis of plasma samples was tested.

Pig blood was used for the method development, as its matrix is similar to human blood and it could easily be obtained from a local slaughterhouse.

7.1.3 Extraction method

As a general extraction method applicable for a wide range of substances the QuEChERS approach (quick, easy, cheap, effective, robust and save) developed in 2003 for the extraction of pesticides in foods appeared interesting [88, 89]. This approach combines a liquid-liquid-extraction of analytes in aqueous (homogenized) samples with a fairly polar solvent (mostly acetonitrile, occasionally acetone). Phase separation is induced by the addition of desiccant (Na_2SO_4, anhydrous $MgSO_4$) and salt (NaCl). At the same time, the addition of acetone and acetonitrile results in a precipitation of proteins that remain in the aqueous phase and can thus be separated. Removal of matrix components from the solvent phase is subsequently possible with dispersive SPE (dSPE) using different sorbents before the extract is dried and evaporated or a change of solvent for the GC-MS and LC-MS analysis is performed. This method uses low amounts of solvents and materials, is not very time consuming and can thus be viewed as an "environmentally friendly" extraction method. The work on pesticides shows that a very wide range of substances can be extracted (very good recoveries for substances with a $\log K_{OW}$ between 0.6 and 4.9 [89]). This method was used so far mainly for the analysis of pesticides in food, but also the extraction of a wide range of drugs in human whole blood samples and the application to forensic cases have been described [156-159].

Therefore the QuEChERS extraction method was tested both for urine and blood samples. For urine samples additionally the direct injection into the LC-MS system was tested, which is not possible for the analysis of blood samples.

In the context of method development, the recoveries of target substances for the QuEChERS method were optimized regarding salt concentrations for phase separation and composition of the dSPE sorbents, based on previously published methods [90-94]. For the dSPE step in food analysis mainly PSA (primary-secondary amines) has been used for the removal of fatty acids and sugars, C18-modified silica gel for the removal of lipids and GBC (graphitized black carbon) for the removal of carotenoids and chlorophyll [1, 90, 93, 94]. Since all sorbents also remove some of the target analytes, it is necessary to balance between matrix removal and loss of analytes. In this project only PSA was tested, as C18 and GBC were expected to remove too many environmental pollutants and the matrix constituents removed were not relevant for urine and blood.

After method development internal standards (IS) were used for the non-target analysis of human samples. The IS were not used to correct for matrix effects and losses during sample preparation as done during quantitative analysis, but were used as a quality control of the data evaluation procedure. Additionally during the data evaluation they were taken for a retention time normalization of the standard and sample measurements.

7.2 Materials and methods

7.2.1 Chemicals

Stock solutions of each target analyte (1 mg/mL) were prepared in MeOH, ACN or toluene, depending on the solubility of the compound. Spike standards, one for urine and one for blood, were prepared by mixing of stock solutions. Compounds were contained at different concentrations in the standard mixtures, depending on their sensitivity at the MS instruments concentrations of each analyte are listed in Table 8 and Table 9.

7.2.2 Sample material

A pooled urine sample was collected from members in the department. Morning urine was mixed in a 5 L aluminium bottle and subsequently portioned into 5, 10 and 20 mL aliquots in 50 mL PP tubes. These were frozen at -20°C until usage.

Pig blood samples were obtained from organically grown up pigs from the Vorwerk Podemus slaughterhouse in Dresden (www.vorwerkpodemus.de). Whole blood was collected in 125 mL Nalgene bottles. A portion of the blood was directly mixed with a heparin solution (about 7300 units heparin dissolved in 5 mL of bidistilled water for 100 mL of blood) and shaken. The sampled blood was put on ice and transported to the laboratory. The whole blood samples were homogenized using an Ultra Turrax homogenizer. To obtain plasma samples part of the whole blood was centrifuged (15 min at 4000 x g). All samples were thoroughly mixed and 5 mL aliquots were collected into 15 mL PP tubes, followed by deep freezing at -80°C.

7.2.3 Instrumental analysis

7.2.3.1 LC-MS

To determine the detectability and ionization behaviour the target analytes listed in Table 6 and 7 were injected separately into the Orbitrap for determination of their ionization behaviour. 40 µL of solutions with 5 ng/µL in MeOH were injected directly into the ion trap-Orbitrap hybrid instrument (LTQ-Orbitrap XL, Thermo Scientific) by an autosampler, using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in positive and negative mode. The masses and MS/MS ions (if present) were extracted manually from the resulting spectra. Afterwards a standard mixture containing all target analytes was injected into the LC-Orbitrap system to check whether the compounds also elute from the chosen column and if they are still ionisable under the conditions of the LC eluent.

The LC-Orbitrap method was adopted from an established method for the screening of water samples [80]. Extracts were injected onto a Kinetex C18 column (100 mm x 3 mm, 2.6 µm, Phenomenex) controlled by an Agilent 1200 LC system. A gradient with a flow rate of 0.2 mL/min was run using LC-MS grade water (A) and LC-MS grade methanol (B), both containing 0.1% of formic acid. The initial content of 95% A was held for 3.2 minutes and increased to 95% B during 17.8 min. After 20 min at 95% B the column was equilibrated for 9 min to the initial composition. The LC-system was coupled to the ion trap-Orbitrap hybrid instrument, and separate runs were conducted for positive and negative mode using ESI. Detection was conducted with the Orbitrap operating in HRMS full scan mode (m/z 100-1000) using a nominal resolving power of 100 000. For the non-target screening an additional run was conducted where data-dependent HRMS/MS spectra with a resolving power of 30 000 were recorded for selected masses. Dissociation was achieved using collision induced dissociation (CID) at normalized collision energies of 35 and 50% and higher-energy collisional dissociation (HCD) at 50, 90 and 120%.

Table 8 shows the results for the compound optimization of the urine target analytes. Geraniol was not detected; it is probably too volatile and was thus deleted from the list. Resorcinol, dibutylhydroxytoluene and 1-naphthol were not detectable with ESI, only using APCI. For a non-target screening ESI would be the ionization method to choose, as here mostly molecular ions are formed and can thus be identified. APCI is a “harder” ionization method and thus often no molecular ion is found in the spectra, making the identification more difficult. Therefore ESI was the method of choice for the target analytes, reducing their number to 43. Two more compounds posed problems. Bisphenol A is only detectable at very high concentrations with the Orbitrap system and 2,4-diaminoanisole proved to be instable in solution. Creatinine was only monitored as a marker compound and was not used for the recovery experiments during method development. Thus these targets were excluded from the urine target list, reducing the number further to 40 urine targets for method development.

Table 8: Ionization methods and m/z values for the urine target analytes analysed by LC-HRMS.

compound name	monoisotopic mass	formula	ESI	APCI	final method	target m/z	urine spike std [µg/mL]
2,4-TDI	174.0429	$C_9H_6N_2O_2$	+	+/-	ESI+	175.0502	10
CMIT	148.9702	C_4H_4ClNOS	+/(-)	+	ESI+	149.9775	5
citronellal	154.1358	$C_{10}H_{18}O$	+	+	ESI+	155.1430	10
(geraniol)	154.1358	$C_{10}H_{18}O$					-
(resorcinol)	110.0368	$C_6H_6O_2$		+/-	APCI+		50
(2,4-diaminoanisol)	138.0793	$C_7H_{10}N_2O$	+	+	ESI+	139.0866	10
4,4'-thiodianiline	216.0721	$C_{12}H_{12}N_2S$	+	+/-	ESI+	217.0794	5
4,4'-methylendi-o-toluidine	226.1470	$C_{15}H_{18}N_2$	+	+	ESI+	227.1543	5
solvent yellow	197.0953	$C_{12}H_{11}N_3$	+/(-)	+/-	ESI+	198.1026	5
o-dianisidine	244.1212	$C_{14}H_{16}N_2O_2$	+	+	ESI+	245.1285	50
mercaptobenzothiazole	166.9863	$C_7H_5NS_2$	+/-	+/-	ESI+	167.9936	5
2-(methylthio)benzothiazole	181.0020	$C_8H_7NS_2$	+/-	+/(-)	ESI+	182.0093	5
octhilinone	213.1187	$C_{11}H_{19}NOS$	+	+/-	ESI+	214.1260	5
4-hydroxybenzotriazole	135.0433	$C_6H_5N_3O$	+/-	+/-	ESI-	134.0360	5
p-toluene-sulfonamide	171.0354	$C_7H_9NO_2S$	+/-	+/-	ESI+	172.0427	50
5-methylbenzotriazole	133.0640	$C_7H_7N_3$	-	+/-	ESI+	134.0713	5
ethyltosylamide	199.0667	$C_9H_{13}NO_2S$	+	+/-	ESI+	200.0740	5
(dibutylhydroxytoluene)	220.1827	$C_{11}H_{16}O$		-	APCI-		5
2-ethoxyethyl acetate	132.0786	$C_6H_{12}O_3$	+/(-)		ESI+	133.0859	50
HEMA	207.0565	$C_7H_{13}NO_4S$	+/-	+/(-)	ESI-	206.0493	50
TMDD	226.1933	$C_{14}H_{26}O_2$	+	+	ESI+	227.2006	10
isophorone diamine	170.1783	$C_{10}H_{22}N_2$	+	+	ESI+	171.1856	5
1-hydroxy-pyrene	218.0732	$C_{16}H_{10}O$	-	+/-	ESI-	217.0659	5
(1-naphthol)	144.0575	$C_{10}H_8O$	(-)	+/-	APCI+		5
3-OH-PCB	237.9952	$C_{12}H_8Cl_2O$	-	-	ESI-	236.9879	5
4-OH-PCB	271.9562	$C_{12}H_7Cl_3O$	-	-	ESI-	270.9490	5
IMPY	152.0950	$C_8H_{12}N_2O$	+/-	+/-	ESI+	153.1022	5
diazinon	304.1010	$C_{12}H_{21}N_2O_3PS$	+	+/(-)	ESI+	305.1083	5
diphenyl phosphate	250.0395	$C_{12}H_{11}O_4P$	+/-	+/-	ESI-	249.0322	5
PFBA	213.9865	$C_4HF_7O_2$	-	-	ESI-	212.9792	5
(bisphenol A)	228.1150	$C_{15}H_{16}O_2$	-	-	ESI-	227.1078	250
MEP	194.0579	$C_{10}H_{10}O_4$	+/-	+/-	ESI+	195.0652	5
MBP	222.0892	$C_{12}H_{14}O_4$	+/-	+/-	ESI+	223.0965	5
MBzP	256.0736	$C_{15}H_{12}O_4$	+/-	-	ESI+	257.0808	5
triethylcitrate	276.1209	$C_{12}H_{17}O_7$	+	(+)	ESI+	277.1282	5
triclosan	287.9512	$C_{12}H_7Cl_3O_2$	-		ESI-	286.9439	5
ethyl paraben	166.0630	$C_9H_{10}O_3$	+/-	+/-	ESI-	167.0703	5
butyl paraben	194.0943	$C_{11}H_{14}O_3$	+/-	(+)/-	ESI-	193.0870	5
tetraoxadodecan	178.1205	$C_8H_{18}O_4$	+	(+)	ESI+	179.1278	5
cinnamate	290.1882	$C_{18}H_{26}O_3$	+	(+)	ESI+	291.1955	5
homosalate	262.1569	$C_{16}H_{22}O_3$	+	-	ESI+	263.1642	100
benzophenone-3	228.0786	$C_{14}H_{12}O_3$	+/-	+/-	ESI+	229.0859	5
4-MeUmb-gluc	352.0794	$C_{16}H_{16}O_9$	+/-	+/-	ESI-	351.0722	5

compound name	monoisotopic mass	formula	ESI	APCI	final method	target m/z	urine spike std [µg/mL]
4-MeUmb-sul	256.0042	$C_{10}H_8O_6S$	-	+/-	ESI-	254.9969	5
estrone-gluc	446.1941	$C_{24}H_{30}O_8$	-	+/-	ESI-	445.1868	10
estrone-sul	350.1188	$C_{18}H_{22}O_5S$	-	+/-	ESI-	349.1115	5
(creatinine)	113.0589	$C_4H_7N_3O$	+/-	+/-	ESI+	114.0662	-

Table 9 shows the results for the blood target compounds. Substances which were not detectable with LC-MS were tested on the GC-MS, see chapter 7.2.3.2. As mentioned above, bisphenol A and resorcinol were only analysable at very high concentrations with the instrument used and 2,4-diaminoanisole was unstable when in contact with water. They thus had to be excluded. 36 blood targets were suitable for analysis by LC-LTQ-Orbitrap.

Table 9: Ionization methods and m/z values for the blood target analytes analysed by LC-HRMS.

compound name	monoisotopic mass	formula	ESI	APCI	final method	target m/z	QTrap	blood spike std [µg/mL]
2,4-TDI	174.0429	$C_9H_6N_2O_2$	+	+/-	ESI+	175.0502	x	5
CMIT	148.9702	C_4H_4ClNOS	+/(-)	+	ESI+	149.9775	x	5
citronellal	154.1358	$C_{10}H_{18}O$	+	+	ESI+	155.1430	(x)	5
geraniol	154.1358	$C_{10}H_{18}O$			GC			50
(resorcinol)	110.0368	$C_6H_6O_2$		+/-	-		(x)	5
(2,4-diaminoanisol)	138.0793	$C_7H_{10}N_2O$	+	+	ESI+	139.0866	x	5
4,4'-thiodianiline	216.0721	$C_{12}H_{12}N_2S$	+	+/-	ESI+	217.0794	x	5
4,4'-methylendi-o-toluidine	226.1470	$C_{15}H_{18}N_2$	+	+	ESI+	227.1543	x	5
solvent yellow	197.0953	$C_{12}H_{11}N_3$	+/(-)	+/-	ESI+	198.1026	x	5
o-dianisidine	244.1212	$C_{14}H_{16}N_2O_2$	+	+	ESI+	245.1285	x	10
BDE 47	481.7152	$C_{12}H_6Br_4O$		(+)	GC			-
BDE 99	559.6257	$C_{12}H_5Br_5O$		(+)	GC			-
(BDE 209)	949.1783	$C_{12}Br_{10}O$		+	-			5
HBCDD	635.6509	$C_{12}H_{18}Br_6$	-		ESI-/GC	634.6436		10
DP	647.7201	$C_{18}H_{12}Cl_{12}$			GC			5
TCEP	283.9539	$C_6H_{12}Cl_3O_4P$	+	+	ESI+	284.9612	x	5
TBBPA	539.7571	$C_{15}H_{12}Br_4O_2$	-	-	GC		x	50
(DBDPE)	961.2147	$C_{14}H_4Br_{10}$		(-)	-			5
PhIP	224.1062	$C_{13}H_{12}N_4$	+/(-)	+/-	ESI+	225.1135	x	5
mercaptobenzothiazole	166.9863	$C_7H_5NS_2$	+/-	+/-	ESI+	167.9936	(x)	5
2-(methylthio)benzothiazole	181.0020	$C_8H_7NS_2$	+/(-)	+/-	ESI+	182.0093	x	5
p-toluene-sulfonamide	171.0354	$C_7H_9NO_2S$	+/-	+/-	ESI+	172.0427	x	50
dibutylhydroxytoluene	220.1827	$C_{11}H_{16}O$		-	GC			5
TMDD	226.1933	$C_{14}H_{26}O_2$	+	+	ESI+	227.2006	x	5
4-methylanisole	122.0732	$C_8H_{10}O$		+	GC			5
ambrettolide	252.2089	$C_{16}H_{28}O_2$			GC			5
cashmeran	206.1671	$C_{14}H_{22}O$			GC			5
celestolide	244.1827	$C_{17}H_{24}O$			GC			5
galaxolide	258.1984	$C_{18}H_{26}O$			GC			5
pyronil 45	701.9191	$C_{24}H_{34}Br_4O_4$	+	+/-	ESI+	702.9263		50

compound name	monoisotopic mass	formula	ESI	APCI	final method	target m/z	QTrap	blood spike std [µg/mL]
saytex BT 93	943.3638	$C_{18}H_4Br_8N_2O_4$		-	GC			50
HCCPD	269.8131	C_5Cl_6			GC			10
naphthalene	128.0626	$C_{10}H_8$			GC			5
acenaphthylene	152.0626	$C_{12}H_8$			GC			5
pyrene	202.0783	$C_{16}H_{10}$			GC			5
PCB 52	289.9224	$C_{12}H_6Cl_4$			GC			5
PCB 101	323.8834	$C_{12}H_5Cl_5$			GC			5
PCB 180	391.8054	$C_{12}H_3Cl_7$			GC			5
carbendazim	191.0695	$C_9H_9N_3O_2$	+/-	+	ESI+	192.0768	x	5
diazinon	304.1010	$C_{12}H_{21}N_2O_3PS$	+	+/-	ESI+/GC	305.1083	x	5
thiabendazole	201.0361	$C_{10}H_7N_3S$	+/-	+/-	ESI+	202.0433	x	5
PFBA	213.9865	$C_4HF_7O_2$	-	-	ESI-	212.9792	x	5
PFOA	413.9737	$C_8HF_{15}O_2$	-	(-)	ESI-	412.9664	x	5
PFTDA	713.9545	$C_{14}HF_{27}O_2$	-	(-)	ESI-	712.9473	x	50
PFOS	499.9375	$C_8HF_{17}O_3S$	-	(-)	ESI-	498.9302	x	5
MeFOSA	512.9691	$C_9H_4F_{17}NO_2S$	-	-	ESI-	511.9619	x	-
C8-PFPA	499.9470	$C_8H_2F_{17}O_3P$			ESI-	498.9328	(x)	-
(8:2 FTOH)	464.0069	$C_{10}H_5F_{17}O$		-	-			5
(10:2 FTOH)	564.0005	$C_{12}H_5F_{21}O$		-	-			5
4-NP	220.1827	$C_{15}H_{24}O$	-	-	GC			5
(bisphenol A)	228.1150	$C_{15}H_{16}O_2$	-	-	-		x	50
bis(4-chlorophenyl) sulfone	285.9622	$C_{12}H_8Cl_2O_2S$			GC			5
triclosan	287.9512	$C_{12}H_7Cl_3O_2$	-	(-)	ESI-	286.9439	x	5
ethyl paraben	166.0630	$C_9H_{10}O_3$	+/-	+/-	ESI-	167.0703	x	5
butyl paraben	194.0943	$C_{11}H_{14}O_3$	+/-	+/-	ESI-	193.0870	x	5
tetrachlorosalicylanilide	348.9231	$C_{13}H_7Cl_4NO_2$	-	+/-	ESI-	347.9158	x	5
quaternium 15	215.1058	$C_9H_{16}Cl_2N_4$	+		ESI+	215.1058	x	50
benzylQUAT	304.2999	$C_{21}H_{38}N$	+	+	ESI+	304.2999	x	5
trimethylQUAT	172.2060	$C_{11}H_{26}N$	+	+	ESI+	172.2060	x	5
michlers ketone	268.1576	$C_{17}H_{20}N_2O$	+	+	ESI+	269.1648	x	5
phenolphthalein	318.0892	$C_{20}H_{14}O_4$	+/-	+/-	ESI+	319.0965	x	5
cinnamate	290.1882	$C_{18}H_{26}O_3$	+	(+/-)	ESI+/GC	291.1955	x	10
homosalate	262.1569	$C_{16}H_{22}O_3$	+	(+)/-	ESI+/GC	263.1642	x	50
4-MBC	254.1671	$C_{18}H_{22}O$			GC			10
octocrylene	361.2042	$C_{24}H_{27}NO_2$			GC			10

Table 10 shows the results for IS which were analysed by LC-MS. They were spiked both into the urine and blood samples for the non-target analysis. They were, however, not used during method development.

Table 10: Ionization mode and m/z values for the internal standards used during non-target screening.

compound name	monoisotopic mass	ESI	mass analysed	IS spike std conc [µg/mL]
PFOS-$^{13}C_4$	502.9434	-	501.9371	5
PFBA-$^{13}C_3$	216.9964	-	215.9901	5
triclosan-d_3	290.9701	-	289.9638	27
MBP-d_4	226.1144	+	227.1207	10
chlormequat-d_9	131.1298	+	132.1361	10
p-toluene-sulfonamide-d_4	175.0605	+	176.0668	100
carbendazim-d_4	195.0947	+	196.1010	7
creatinine-d_3	116.0778	+	117.0841	10
benzophenone-3-d_5	233.1101	+	234.1164	10
PhIP-d_3	227.1251	+	228.1314	10
cotinine-d_3	179.1139	+	180.1202	10
benzotriazole-d_4	123.0735	+	124.0798	10
tri-butyl-d_{27}-phosphate	293.3348	+	294.3411	10
tonalide-d_3	261.2173	+	262.2236	8
diazinon-d_{10}	314.1640	+	315.1703	10
atrazine-$^{13}C_3$	218.1037	+	219.1100	10

An additional LC-MS instrument was used during method development for the analysis of blood samples. A liquid chromatograph (Agilent 1260 Infinity) was coupled to a quadrupole-iontrap system (QTrap 6500 from ABSciex). This was used due to faster data processing procedures and time limitations at the LC-Orbitrap. The same Kinetex C18 LC column as mentioned above was used with a slightly shortened LC gradient program of 35 min. A flow rate of 0.2 mL/min was run using LC-MS grade water (A) and LC-MS grade methanol (B), both containing 2 mM ammonium acetate. The initial content of 95% A was held for 5 min and increased to 95% B over 5 min. After 15 min with 95% B, it was equilibrated for 10 min to the initial composition. The QTrap instrument was run in scheduled MRM mode. The advantage was that the QTrap could analyse positive and negative ionization in one run, while with the Orbitrap one run each for negative and positive mode was necessary. However, the QTrap could only be run in low resolution, making the instrument unsuitable for the non-target screening. Therefore, during method development also a few extracts were injected into the Orbitrap to check for matrix effects and to test the data evaluation, see chapter 8.1. Table A 3 in the Annex shows the MRM transitions for the blood LC target analytes and the IS.

In total 36 blood targets were detectable with the LC-QTrap. These targets included two analytes not detectable with the LC-Orbitrap system: BPA and TBBPA were analysable probably due to a different ESI source and application of ammonium-containing LC-solvents. On the other hand, HBCDD and pyronil 45, which were analysable with the Orbitrap were not analysable with the LC-QTrap. Citronellal, resorcinol and mercaptobenzothiazole posed another problem as they were analysable by direct injection into the MS, but not when using the LC system. Tetrachlorosalicylanilide and PFOPA were ionisable, but their calibration curves were not useable. Therefore the total LC blood targets were reduced to 31.

7.2.3.2 GC-MS

A gas chromatograph coupled to a mass spectrometer (Model 6890 N, MSD 5973, Agilent Technologies) was used in single ion monitoring (SIM) mode for the detection of blood targets. One µL was injected in splitless mode at 250°C. A HP-5MS capillary column (30m x 0.25 mm I.D., 0.25 µm film thickness, Agilent Technologies) was used for separation with a temperature program starting at 60°C, held for 1 min, ramped with 30°C/min until 90°C, held for 3 min, ramped with 6°C/min to 280°C and held for 16.5 min. The auxiliary line was kept at 280°C and the ion source temperature at 230°C. For the non-target screening the temperature program was started at 60°C, held for 3 min and then ramped at 3°C/min to 300°C (held for 20 min). During method development while analysing extracts in SIM mode, a GC with hydrogen as carrier gas was applied. For the screening helium was used as carrier gas, as hydrogen might result in different fragmentation compared to the EI spectra present in the databases used for identification and might react with unsaturated compounds.

Target analytes from Table 7 that were considered ionisable by GC-EI-MS were injected at concentrations of about 5 ng/µL into the GC-MS system. Scans (m/z 50-600) were conducted and qualifier and quantifier ions were taken to establish a final SIM method for further analysis during method development. LDCs in the standard runs (analytes solved in ACN, with addition of analyte protectant, according to [160]) and in spiked samples were determined for each substance. This information is summarized in Table 11. A few target analytes could not be covered by the GC-EI-MS method. One group were the highly brominated flame retardants BDE 209 and DBDPE. These would be ionisable either by injection on column or by electron capture negative ionization (ECNI), where the Br^- isotopes 79 and 81 would be analysed [161]. Thus highly brominated flame retardants fall outside the range for a non-target screening with GC-EI-MS. Also FTOHs fall outside the range, which commonly are analysed with positive or negative chemical ionization and due to their high volatility are injected at lower temperatures [162, 163]. Integrating lower injection temperatures into the method would however result in bad peak shapes for later eluting compounds. Thus they also needed to be excluded from the final target list, leaving 27 GC-blood-targets for method development, 4 of which are also analysed by LC-QTrap. 56 blood targets remained for method development analysable with LC and GC-MS.

Table 11: Qualifier, quantifier and retention times of target analytes and IS analysed by GC-EI-MS (in SIM mode).

compound name	LC/GC	qualifier	quantifier	RT [min]	instr. LDC [ng/mL extract]	method LDC [ng/mL blood]
geraniol	GC	69	41 / 123	9.2	20.5	10
BDE 47	GC	486	326 / 484	31.2	10	10
BDE 99	GC	566	404 / 406	34.5	10	10
BDE 209	(GC)	-				
HBCDD	LC/GC	239	319 / 401	32.5	51.2	blank
DP	GC	272		49.6	20.5	10
TBBPA	GC	529	544 / 527	36.4	20.5	blank
DBDPE	(GC)	-				
dibutylhydroxytoluene	GC	205	220	14.8	<8.2	blank
4-methylanisole	GC	112	77 / 107	3.9	20.5	10
ambrettolide	GC	67	82 / 252	22.6	51.2	10
cashmeran	GC	191	206 / 135	14.7	20.5	2
celestolide	GC	229	244 / 173	18.9	<8.2	blank
galaxolide	GC	243	258 / 213	21.2	8.2	blank
saytex BT 93	GC	463	420 / 232	46.0	51.2	10
HCCPD	GC	237	272 / 203	10.9	128	-
naphthalene	GC	128	102 / 64	7.6	<8.2	<2
acenaphthylene	GC	152	76	13.8	20.5	<2
pyrene	GC	202	101	25.9	20.5	<2
PCB 52	GC	292	220 / 255	22.9	<8.2	blank
PCB 101	GC	356	254 / 291	25.6	<8.2	blank
PCB 180	GC	394	324 / 252	30.9	<8.2	blank
diazinon	LC/GC	179	137 / 304	20.4	20.5	2
8:2 FTOH	(GC)	-				
10:2 FTOH	(GC)	-				
4-NP	GC	107	220 / 77	21.7	128	blank
bis(4-chlorophenyl) sulfone	GC	159	286 / 111	27.5	20.5	2
cinnamate	LC/GC	178	161 / 290	28.7	<8.2	2
homosalate	LC/GC	138	109 / 262	21.5 + 22.0	8.2	blank
4-MBC	GC	254	221 / 128	24.7	20.5	blank
octocrylene	GC	249	360 / 204	32.9	8.2	blank
Internal standards						
tonalide-d_3	GC	261.1	246.2	21.3		
pyrene-d_{10}	GC	212.1	106	26.0		
hexachlorobenzene-$^{13}C_6$	GC	289.9	254.8	18.7		
diazinon-d_{10}	LC/GC	314	183 / 138	20.4		
4-NP-d_4	GC	224.2	111.1	21.8		

blank: detections already in the not spiked samples, thus no method LDC could be derived

7.2.4 Sample preparation

7.2.4.1 Urine

Urine samples were thawed at room temperature. Following homogenization by vortexing aliquots were taken for the different types of sample preparation.

For a direct injection the urine was centrifuged for 10 min at 4000 x g and 10% of MeOH was added. For spike experiments the urine standard mixture was spiked at different concentrations. Spikes into bidistilled water mixed with 10% of MeOH were used for the calculation of recoveries due to matrix effects.

For the QuEChERS extraction 10 mL of urine were thoroughly mixed with 10 mL of ACN. Addition of 4 g of $MgSO_4$ and 1 g of NaCl was followed by vortexing and centrifugation (10 min at 4000 x g). The supernatant was divided. 3 mL supernatant were taken and concentrated to 300 µL under a N_2 stream (called LLE fraction). 5 mL were mixed with 125 mg of PSA (primary secondary amine) and 750 mg of $MgSO_4$ for clean-up (called dSPE fraction). This fraction was again vortexed and centrifuged (10 min at 4000 x g). The supernatant (3 mL) was concentrated to 300 µL under a N_2 stream.

Spike experiments for method development were conducted by spiking the urine standard mixture at different concentrations into 10 mL of urine before addition of ACN. Matrix spikes were prepared by spiking into the final extracts before concentration under N_2. External standards (urine-standard-mixture diluted in ACN) were used for the calculation of absolute recoveries.

7.2.4.2 Blood

Blood samples were thawed at room temperature. Following homogenization by vortexing, samples were extracted using the QuEChERS method as described for the urine samples. 5 mL of blood was taken and thoroughly mixed with 5 mL of ACN. Addition of 2 g of $MgSO_4$ and 0.5 g of NaCl was followed by vortexing and centrifugation (10 min at 3913 x g). The supernatant was divided. 1.5 mL supernatant were taken for the LLE fraction and concentrated to 150 µL under a N_2 stream. 2.5 mL were mixed with 75.5 mg of PSA (primary secondary amine) and 375 mg of $MgSO_4$ for the dSPE fraction. This was again vortexed and centrifuged (10 min at 3913 x g). The supernatant (1.5 mL) was concentrated to 150 µL under a N_2 stream. For analysis with the QTrap no concentration step (neither for LLE nor dSPE) was necessary due to the high sensitivity of the instrument.

Spike experiments for method development were conducted by spiking the blood standard mixture at different concentrations into the 5 mL of blood before addition of ACN. Matrix spikes were done by spiking into the final extracts before concentration under a N_2 stream. External standards (blood standard mixture diluted in ACN) were used for the calculation of absolute recoveries.

7.3 Results and discussion of method development

7.3.1 Urine

7.3.1.1 Instrumental method

Instrumental lowest detectable concentrations (instrLDCs) and retention times (RT) can be found in Table 12. They were determined both for 100 µL injections of standard solutions in water:MeOH (90:10) and for 10 µL injections of standard solutions in acetonitrile, to compare them to the direct injections and the QuEChERS extract injections, respectively. Some compounds showed high LDCs with the instrument used, thus they were spiked at higher concentrations when doing the recovery experiments (concentrations of

each analyte in the spike standards are listed in Table 8 and Table 9). Peak shapes of all compounds were good, except for the aromatic amines, which showed two peaks. A fraction was not retained and eluted at the dead time. The second peak was taken for quantification.

Table 12: Instrumental and method lowest detectable concentrations (LDCs) and retention times (RT) of all urine target analytes.

	instr. LDC [ng/mL]		method LDC [ng/mL]			RT [min]	
	DI	Q	DI	Q LLE	Q dSPE	DI	Q
2,4-TDI	10	100	20	200	50*	19.33	18.97
CMIT	1	5	-	25	25	13.69	12.97
citronellal	20	200	-	50*	50*	23.14	22.74
4,4-thiodianiline	1	5	5	100	5	15.21	14.38
4,4-methylendi-o-toluidine	1	10	5	100	5*	11.59	10.72
solvent yellow	<1	1	1	<5	<5	23.84	23.48
o-dianisidine	<10	50	10	1000	250	8.46	7.57
mercaptobenzothiazole	25	50	100	100	-	20.89	20.60
2-(methylthio)benzothiazole	1	5	5	5	5	24.47	24.11
octhilinone	<1	1	1	<5	<5	25.20	24.86
4-hydroxybenzotriazole	1	25	5	25	25	12.54	11.60
p-toluene-sulfonamide	50	50	-	1000	- (P)	16.12	15.64
5-methylbenzotriazole	<1	1	1	<5	5	19.03	18.69
ethylosylamide	1	1	5	5	blank	20.58	20.26
2-ethoxyethyl acetate	10	50	-	250	50	15.21	14.66
HEMA	10	250	1000	1000	- (P)	3.94	2.84
TMDD	2	2	20	10	10	25.69	25.28
isophorone diamine	5	25	25	100	- (P)	2.14	2.02
1-hydroxypyrene	1	5	1	25	5	26.19	25.83
3-OH-PCB	5	5	10	100	5	26.28	25.98
4-OH-PCB	<1	<1	5	25	<5	27.34	26.99
pyrimidinol	1	5	5	25	5	12.01	10.95
diazinon	<1	1	1	<5	<5	26.06	25.68
diphenyl phosphate	1	5	blank	blank	5	23.23	22.42
PFBA	<1	5	1	5	5	18.53	17.98
MEP	5	1	blank	blank	5	19.94	19.60
MBP	<1	1	blank	blank	blank	22.89	22.51
MBzP	1	1	5	5	5	23.17	22.80
triethylcitrate	<1	1	25	25	<5	21.04	20.68
triclosan	<1	5	5	25	<5*	26.98	26.62
ethyl paraben	1	25	25	100	5*	21.38	21.08
butyl paraben	1	5	10	25	5	24.31	23.94
tetraoxadodecan	1	5	5	5	blank	14.54	13.86
cinnamate	5	10	-	25	5*	29.74	29.06
homosalate	100	2000	-	500*	500*	30.77	29.83
benzophenone-3	1	1	10	5	<5	25.78	25.43

	instr. LDC [ng/mL]		method LDC [ng/mL]			RT [min]	
4-MeUmb-gluc	1	10	100	100	- (P)	15.83	15.31
4-MeUmb-sul	1	10	5	25	25	17.86	17.47
estrone-gluc	2	20	50	200	- (P)	21.89	21.54
estrone-sul	1	5	blank	blank	blank	24.40	23.52

blank: detections already in the not spiked samples, thus no method LDC could be derived

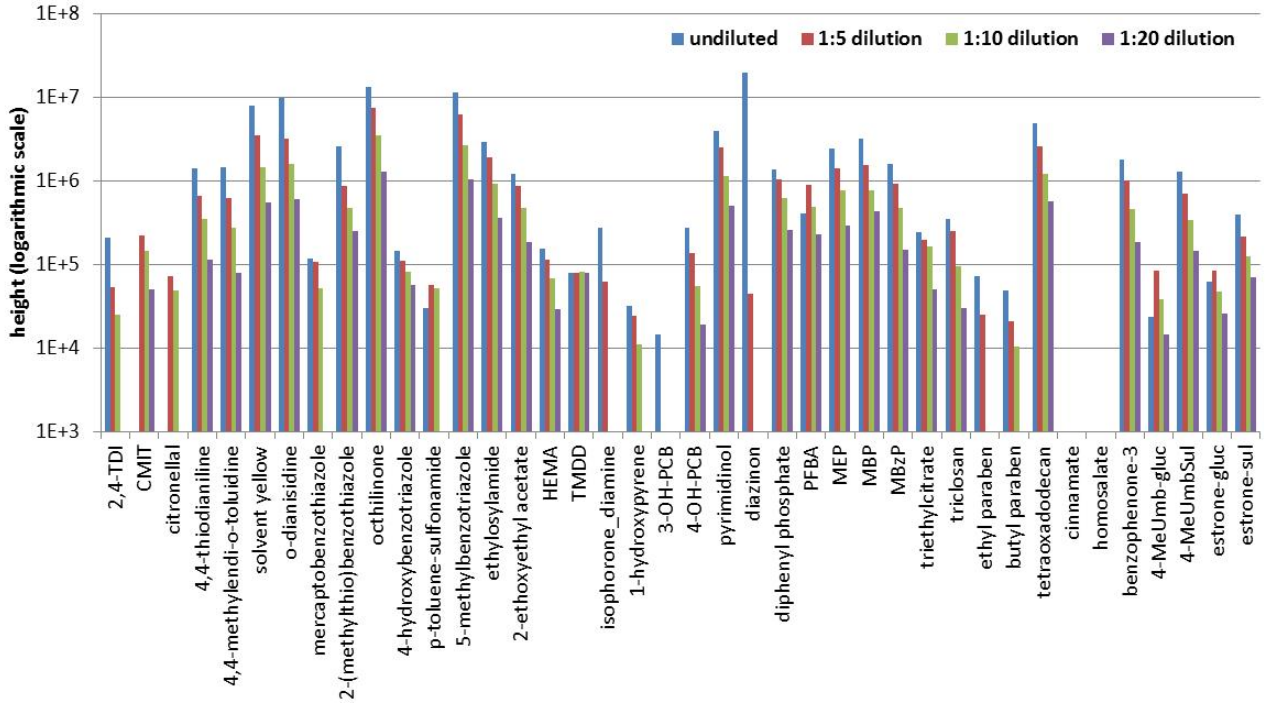
* method LDCs are lower than instrumental LDCs, probably due to matrix enhancements

7.3.1.2 Direct injection

Although urine contains a large amount of matrix constituents many direct injection methods have been described, see section 5.2. Urine is either filtered or centrifuged before injection. Tests with filtration of spiked urine showed that some target analytes were lost to some extent. Therefore all urine samples for direct injection were centrifuged at 4000 x g for 10 min.

Many publications describe a dilution of the urine with water to decrease matrix effects. Therefore tests with dilutions of spiked urine were conducted. Calculated recoveries were best for most target analytes for a 1:5 dilution with water:MeOH (90:10). This is due to a dilution not only of the analyte concentration, but also of the matrix present. However, for a non-target screening not the recoveries themselves but the peak heights and mass spectra are most decisive. Figure 6 shows the peak heights of the target analytes in undiluted urine compared to three different dilutions. Peak heights for most substances were largest for the undiluted urine injections. Only for CMIT and citronellal it seems to be necessary to dilute the urine, as they were not detected in the undiluted sample injections. Thus all further DI experiments were conducted with undiluted urine only mixed with 10% of MeOH to resemble the starting gradient of the LC method.

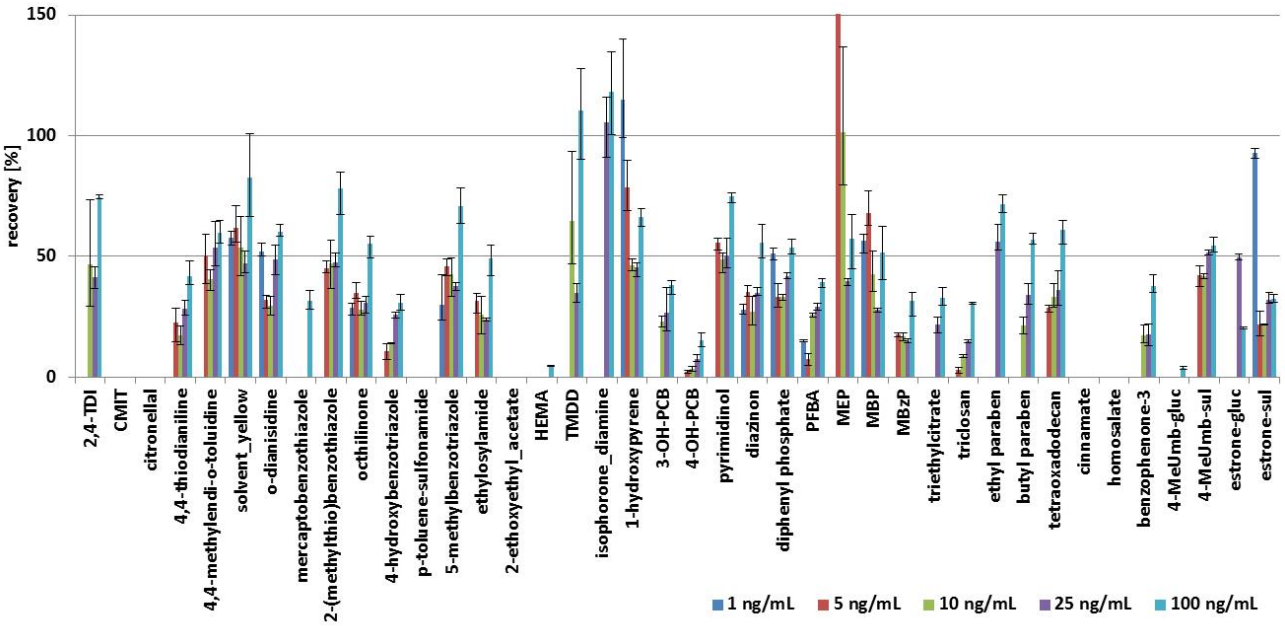
Figure 6: Peak heights of DI tests with urine at different dilutions (diluted with water:MeOH 9:1). Note the logarithmic scale.



To determine the connection between injection volumes and matrix effects, tests with different volumes (10, 40, 100 and 200 µL) and different concentrations were conducted. Stronger matrix effects were detected for larger injection volumes when injecting the same on-column concentration of 1 ng (peak heights were lower the higher the injection volume). However, when injecting different volumes of the same 5 ng/mL concentration (thus having different on-column concentrations), higher peaks were observed for larger injection volumes. As mentioned above, for a non-target screening the peak height is a determining factor, thus an injection volume of 100 µL was chosen for all further tests.

Recoveries due to matrix effects of 3 spiked concentrations compared to spiked water samples are illustrated in Figure 7. For many analytes the highest concentration of 100 ng/mL shows best and higher recoveries than the lower concentrations (it should be noted here that the mentioned concentrations arethe ones of most target analytes, however, some are higher, see Table 8 and Table 9). This could be due to stronger ion suppression for the lower concentrations. 19 analytes show recoveries of ≥ 50% for the injection of 100 ng/mL spikes. Six analytes could not be detected at all (CMIT, 2-ethoxyethyl-acetate, p-toluene-sulfonamide, citronellal, cinnamate and homosalate), HEMA and 4-MeUmb-gluc showed very low recoveries only for the 100 ng/mL level. LDCs of spiked urine samples can be found in Table 12 and are ≤ 5 ng/mL for 21 of the 40 target analytes. Diphenyl phosphate, MEP, MBP and estrone-sulfate were already present in the pooled urine and thus no method LDC could be determined. This demonstrates that with a simple DI several analytes if present at 5 ng/mL or more would be detectable in human urine.

Figure 7: DI average recoveries at 5 different concentrations in urine compared to standard injections in water, error bars represent the maximum and minimum values (n=3).



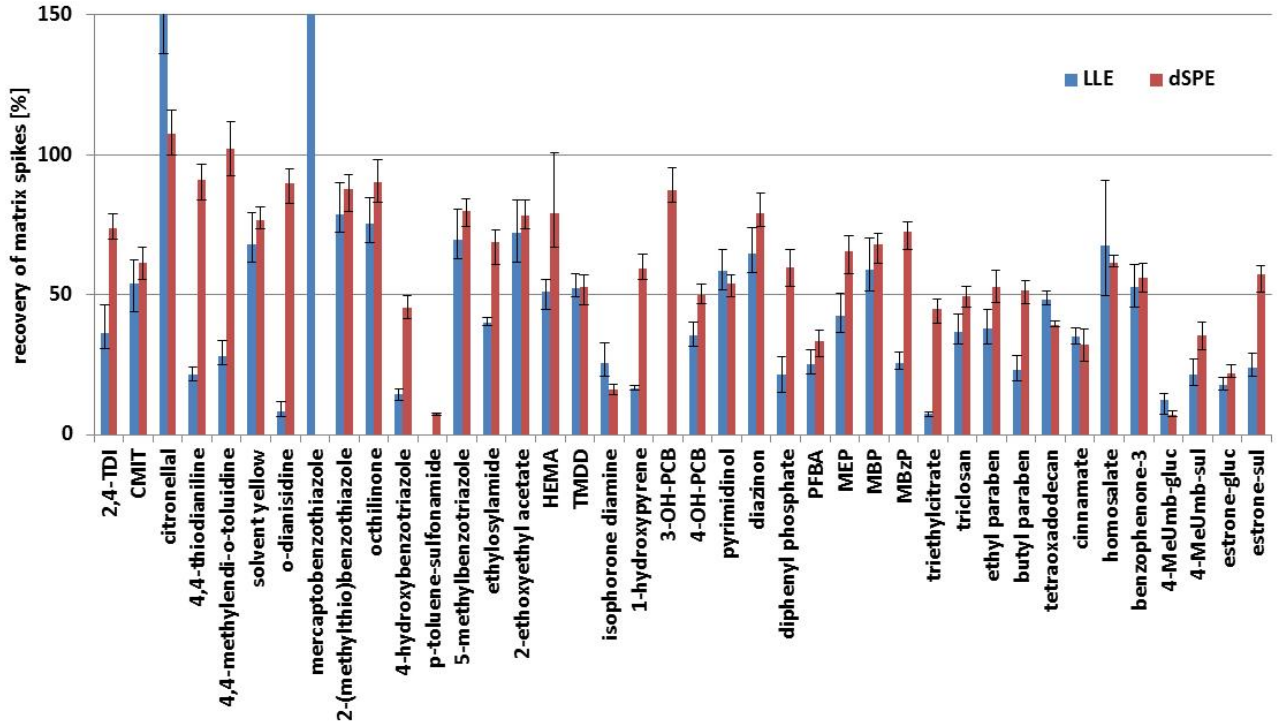
7.3.1.3 QuEChERS extraction

LDCs of spiked urine samples can be found in Table 12. Often method LDCs were lower in dSPE extracts than in LLE extracts, showing the effectiveness of the additional clean-up step. Method LDCs were ≤5 ng/mL for 24 of the 40 analytes for dSPE extracts.

Matrix spike recoveries shown in Figure 8 showed still quite some suppression for most analytes, which was more pronounced in the LLE extracts. Thus the clean-up step proved to be an advantage for all analytes except mercaptobenzothiazole, which could not be found when spiked into a dSPE extract. Also

p-toluene-sulfonamide was prone to huge matrix suppression, it could only be found with a recovery of 7.3% in a spiked dSPE matrix extract. However, 28 of all analytes showed satisfactory matrix effects (≥50% recoveries of matrix spikes) spiked into a dSPE extract.

Figure 8: Matrix spike recoveries for QuEChERS LLE and dSPE extractions, error bars represent the maximum and minimum values (n=3).



Relative recoveries, describing the sample preparation step, of LLE extracts (Figure 9) were only between 75 – 140% for all 3 spiked concentrations for the target analytes 5-methylbenzotriazole, 2-methylthiobenzothiazole and PFBA. Many other analytes could only be detected at the 25 and 100 ng/mL concentrations, 19 analytes showed recoveries between 50 -150% for the 100 ng/mL level. As the LLE extracts also showed fairly large standard deviations, this step did not result in any improvement compared to the simple DI.

Comparing the additional dSPE step (relative recoveries in Figure 10) to the LLE, the picture looked better. The only substances with no or very low recoveries were the glucuronides, HEMA, phthalates, isophorone diamine, p-toluene-sulfonamide and mercaptobenzothiazole. Relative recoveries of the lowest spiked concentration of 5 ng/mL were very high with large standard deviations for many analytes, which is due to uncertainties resulting from levels close to the LDCs. However, for most analytes the clean-up step proved to be important to determine them without any major matrix suppression.

Experiments with the addition of a buffer (addition of NaCOOH and HCOOH instead of NaCl) during the first step of ACN extraction showed lower recoveries for most of the analytes, however, for a few ones like HEMA and the phthalates recoveries were improved. However, the improved recoveries were still not very good, thus the buffer addition did not result in an overall improvement of the extraction method and was therefore not applied any during the non-target screening.

Figure 9: Relative recoveries compared to matrix spikes of the LLE fraction for 3 spiked concentrations, error bars represent the maximum and minimum values (n=3).

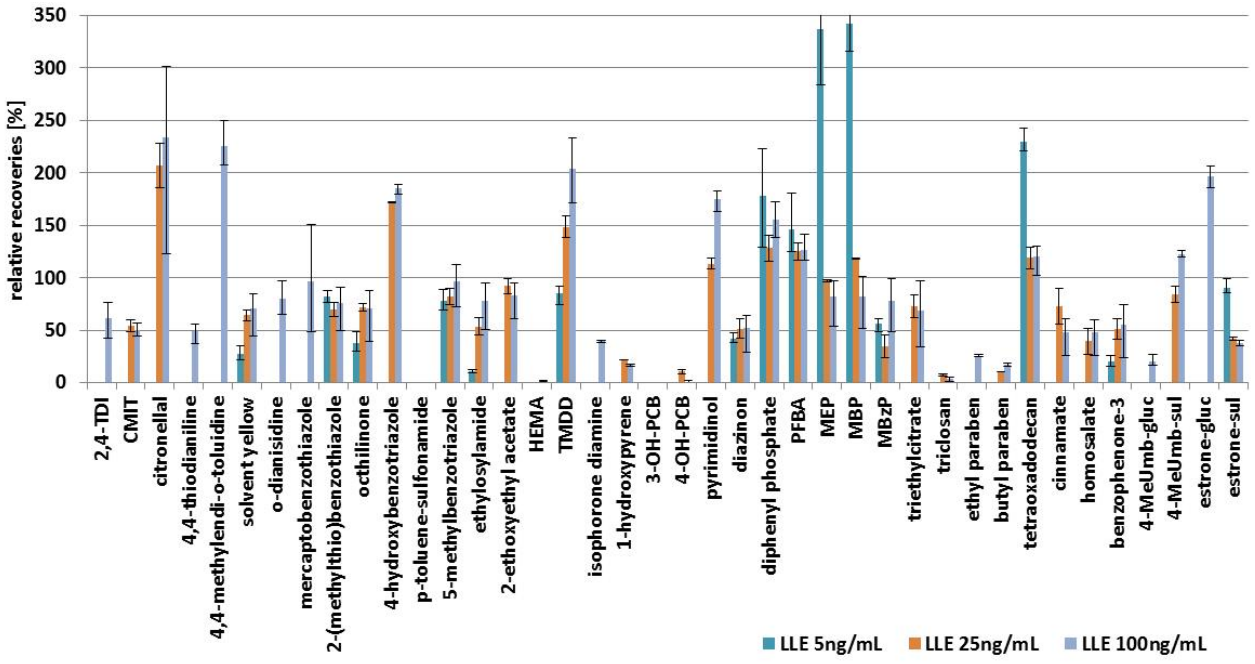
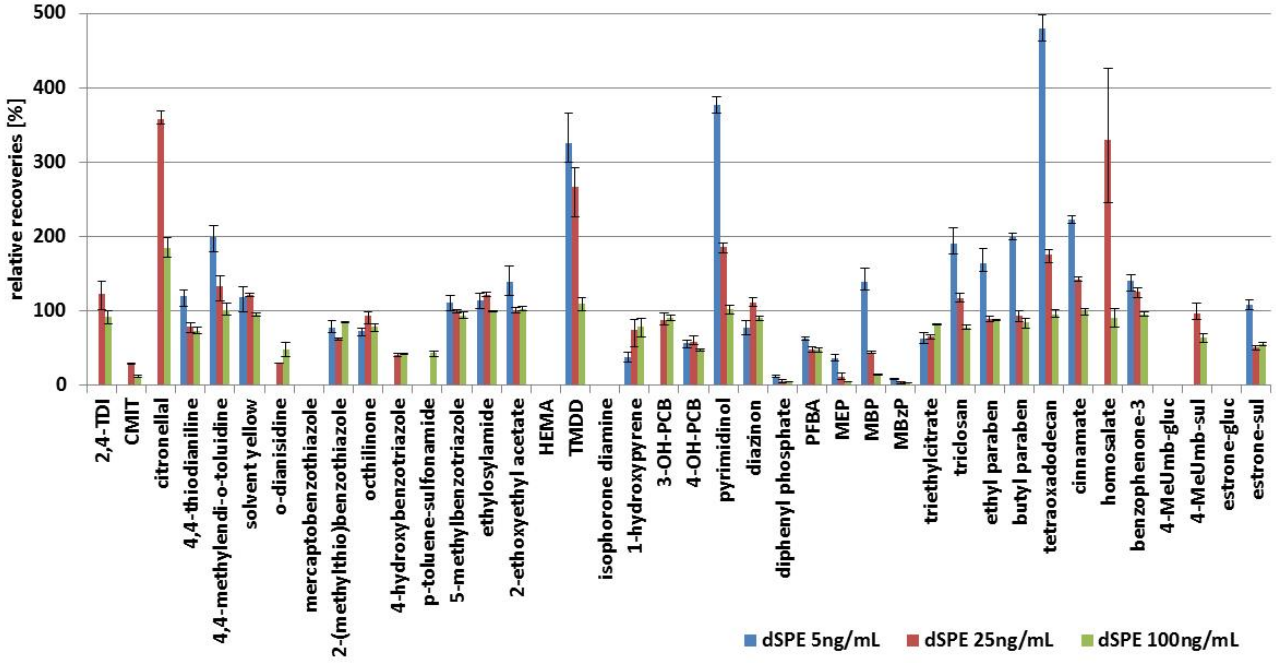


Figure 10: Relative recoveries compared to matrix spikes of the dSPE fraction for 3 spiked concentrations, error bars represent the maximum and minimum values (n=3).



7.3.1.4 Comparison of DI and QuEChERS

A summary over which target analytes could be found in which extracts (DI, LLE or dSPE) can be found in Table 13. Substances adsorbing to the dSPE material like glucuronides and phthalates were not present in the dSPE extracts, but could be detected with the DI. Considering only the DI and dSPE extracts 38 of the 40 target analytes were present with absolute recoveries between 30 - 150%. Only citronellal and p-toluene-sulfonamide could not be found. These however, could neither be found in the LLE fraction. Overall recoveries for many target analytes were in the range of 30 - 50%, which was due to matrix effects. However, further clean-up would result in losses of several targets, as could already be seen for the phthalates during the dSPE step.

A combination of DI and dSPE resulting in satisfactory recoveries of most compounds was to be applied for the non-target screening. The analysis of the LLE extract was not necessary, as it did not improve recoveries compared to the DI. Also no addition of a buffer was included in the method.

Comparing only DI and dSPE two targets could only be detected in the dSPE extracts, the UV-filters cinnamate and homosalate. Their calculated $\log K_{OW}$ seemed to be out of the application range of the DI method. Benzophenone-3 (also a UV-filter) was detectable during DI of a spiked sample, as it has a lower calculated $\log K_{OW}$ value of 3.52. Thus the $\log K_{OW}$ range for compounds detectable with a DI of urine lies between -1 and 5.2. On the other hand there were some very hydrophilic compounds (the glucuronides and HEMA), which could not be detected in the spiked dSPE extracts. However, by a combination of DI and dSPE the property ranges are very broad with calculated $\log K_{OW}$ values between -0.9 and 6.2 and Henry constants between $8*10^{-22}$ and $2.5*10^{-2}$ atm*m^3/mol. These ranges seemed to be sufficiently broad for a general non-target screening.

Table 13: Summary of recovery tests with DI and QuEChERS LLE and dSPE extracts; x: absolute recoveries 50-150%, (x): absolute recoveries 30-50%, -: absolute recoveries outside of 30-150%, nd: not detected

target analyte	DI	LLE	dSPE
2,4-TDI	x	-	x
CMIT	nd	-	-
citronellal	nd	-	-
4,4'-thiodianiline	(x)	-	x
4,4'-methylendi-o-toluidine	x	x	x
solvent yellow	x	(x)	x
o-dianisidine	x	-	(x)
mercaptobenzothiazole	(x)	-	nd
2-(methylthio)benzothiazole	x	x	x
octhilinone	(x)	x	x
4-hydroxybenzotriazole	(x)	-	-
p-toluene-sulfonamide	nd	-	-
5-methylbenzotriazole	x	x	x
ethyltosylamide	(x)	(x)	x
2-ethoxyethyl acetate	(x)	x	x
HEMA	(x)	-	nd
TMDD	(x)	x	x
isophorone diamine	x	-	nd
1-hydroxypyrene	x	-	(x)
3-OH-PCB	(x)	nd	x
4-OH-PCB	x	-	-
pyrimidinol	x	x	x
diazinon	x	(x)	x
diphenyl phosphate	x	(x)	-
PFBA	x	(x)	-
MEP	x	(x)	-
MBP	x	(x)	-
MBzP	(x)	-	-
triethylcitrate	(x)	-	(x)
triclosan	x	-	(x)
ethyl paraben	x	-	(x)
butyl paraben	x	-	(x)
tetraoxadodecan	x	x	(x)
cinnamate	nd	-	(x)
homosalate	nd	(x)	x
benzophenone-3	(x)	-	x
4-MeUmb-gluc	(x)	-	nd

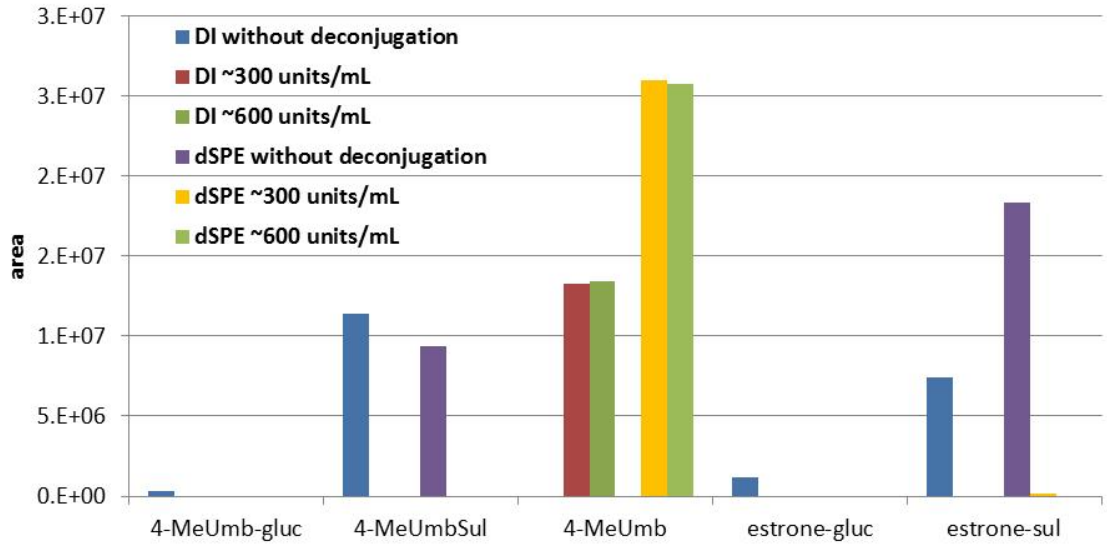
target analyte	DI	LLE	dSPE
4-MeUmb-sul	x	-	-
estrone-gluc	(x)	(x)	nd
estrone-sul	x	-	(x)

7.3.1.5 Deconjugation step

The deconjugation of glucuronides and sulfates was tested with the 4-methyl umbelliferyl and estrone metabolites. Different amounts of β-glucuronidase addition were tested, ~300 units/mL and ~600 units/mL (β-glucuronidase dissolved in 1 M ammonium acetate buffer), followed by incubation at 37°C over night (19 hours). For comparison one sample was analysed without the deconjugation step. In addition 3 blanks were analysed (without conjugation step, and with the two different β-glucuronidase amounts). During analysis also the formation of 4-methyl-umbelliferyl from the deconjugation of 4-MeUmb-gluc and –sul was monitored. Estrone formed from the other two conjugates was not detectable with the LC method used.

In all extracts incubated with β-glucuronidase, none of the four metabolites was found, except for a very small fraction of estrone-sul in the dSPE extract when using 300 units/mL β-glucuronidase, see Figure 11. The formation of 4-methyl-umbelliferyl has been detected in the incubated samples. This shows that the deconjugation step worked with both the low and high amounts of β-glucuronidase.

Figure 11: Peak areas for conjugates (4-MeUmb-glucurunide and –sulfate, estrone-glucuronide and sulfate) and the unconjugated 4-MeUmb in samples without and with deconjugation by β-glucuronidase.

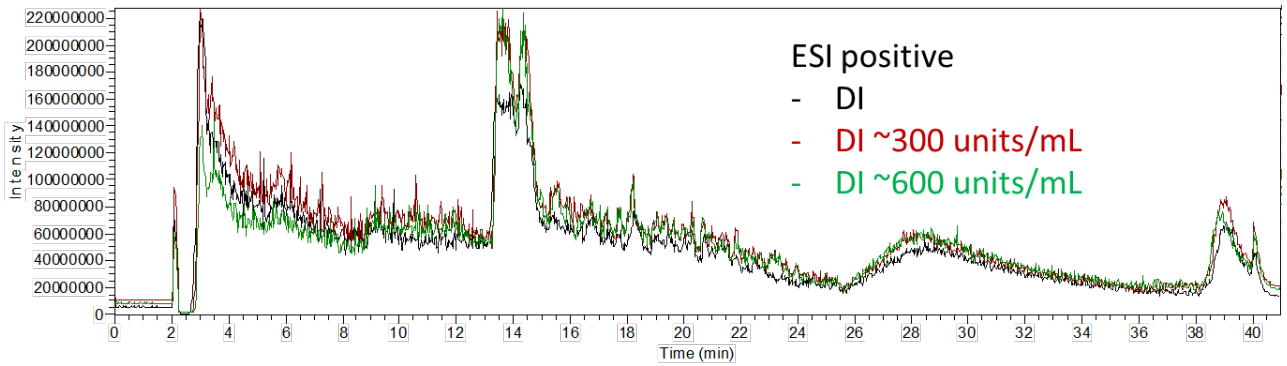


To check whether the addition of the β-glucuronidase-buffer solution led to an increase in matrix, the TICs of the samples were compared. Figure 12 and Figure A 1 in the Annex show the TICs of the DI and QuEChERS, where the runs do look very similar. However, the background of the dSPE extract was higher for the analysis of the deconjugated samples. Thus the addition of the β-glucuronidase buffer does not add anything to the background of the DI, however, it adds some matrix to the dSPE extracts.

For the non-target screening we concluded that a deconjugation step had to be included in the sample preparation. The main reason was that it is simpler to identify the original substances compared to their metabolites. Due to the good results regarding matrix, it was decided to apply the deconjugation to all

samples for the non-target screening, with some samples additionally without a deconjugation step for comparison.

Figure 12: TIC of DI LC-MS analysis in ESI positive mode, comparing the deconjugated and not-deconjugated samples. TICs for negative mode and QuEChERS dSPE extracts can be found in the Annex.



7.3.2 Blood

Initial tests with whole blood samples were conducted to test the phase separation using the QuEChERS method and applying different amounts of salts and dSPE sorbent. By using the same method as for the urine samples (described by Anastassiades 2003 [27]), clean extracts were produced. One essential addition to the method was the usage of stainless steel beads (8 mm) during the first extraction step. This was necessary to improve mixing and phase separation by destroying the blood clog formed upon ACN and salt addition. Taking higher amounts of salt resulted in very low amounts of supernatants and was thus not applicable. A test with the addition of a buffer (2 g of $MgSO_4$, 0.5 g of NaCOOH and 340 mg of HCOOH after ACN addition) and one with the addition of salts listed in the DIN EN 15662:2008 (2 g of $MgSO_4$, 0.5 g of NaCl, 0.5 g of trisodium-citrate-dihydrate and 0.25g of disodium-hydrogencitrate-sesquihydrate after the addition of ACN) resulted in coloured and turbid extracts, see Figure 13. Thus the original method was kept for tests with different blood types (whole blood, heparin blood and plasma). Absolute recoveries for the three blood types spiked with the target analytes at a concentration of 50 ng/mL can be found in Figure 14 for LC and in Figure 15 for GC substances.

Figure 13: Extraction of whole blood samples. Left side: LLE; right side: dSPE. The three tubes from left to right: with normal procedure according to [27], with DIN EN 15662:2008 procedure, with addition of buffer.

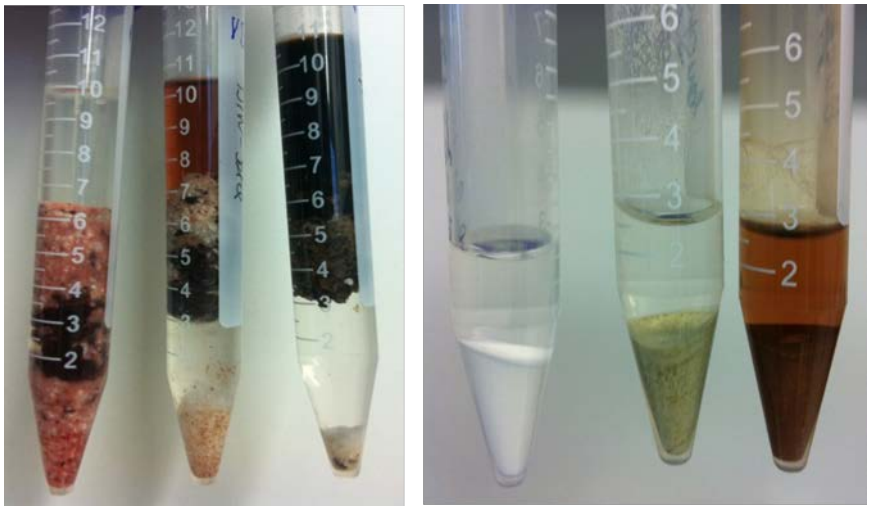


Figure 14: LC-MS analysis: absolute recoveries of target analytes spiked into different blood sample types (50 ng/mL) and extracted by LLE and dSPE: WB – whole blood, HpB – heparin blood, plasma.

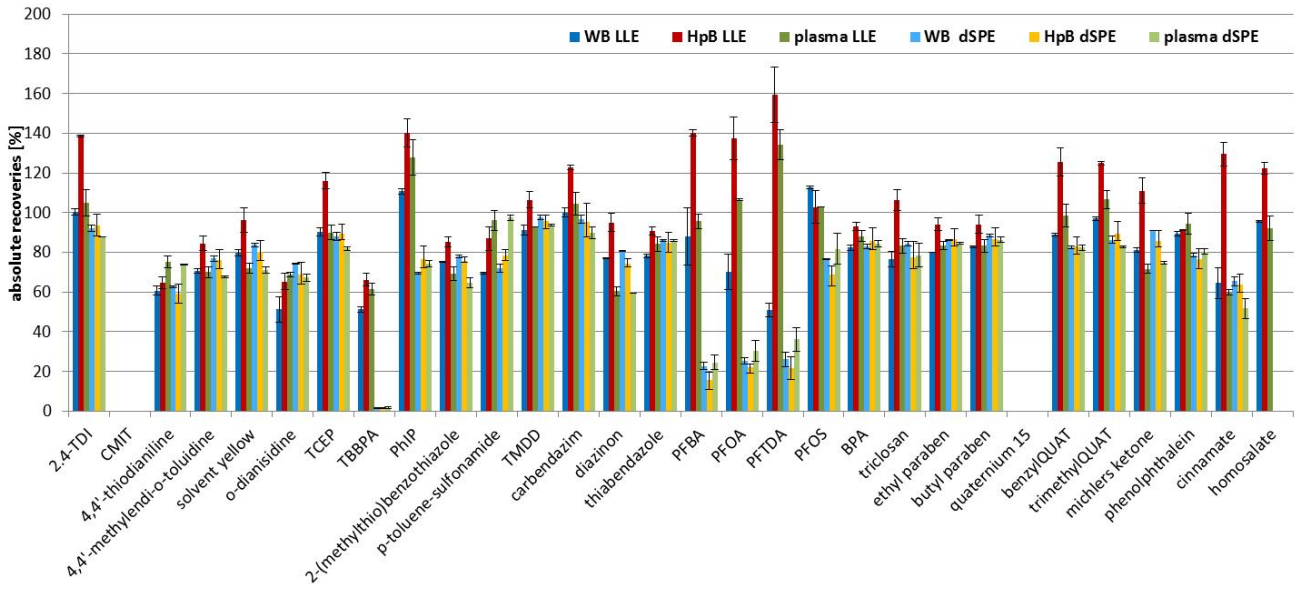
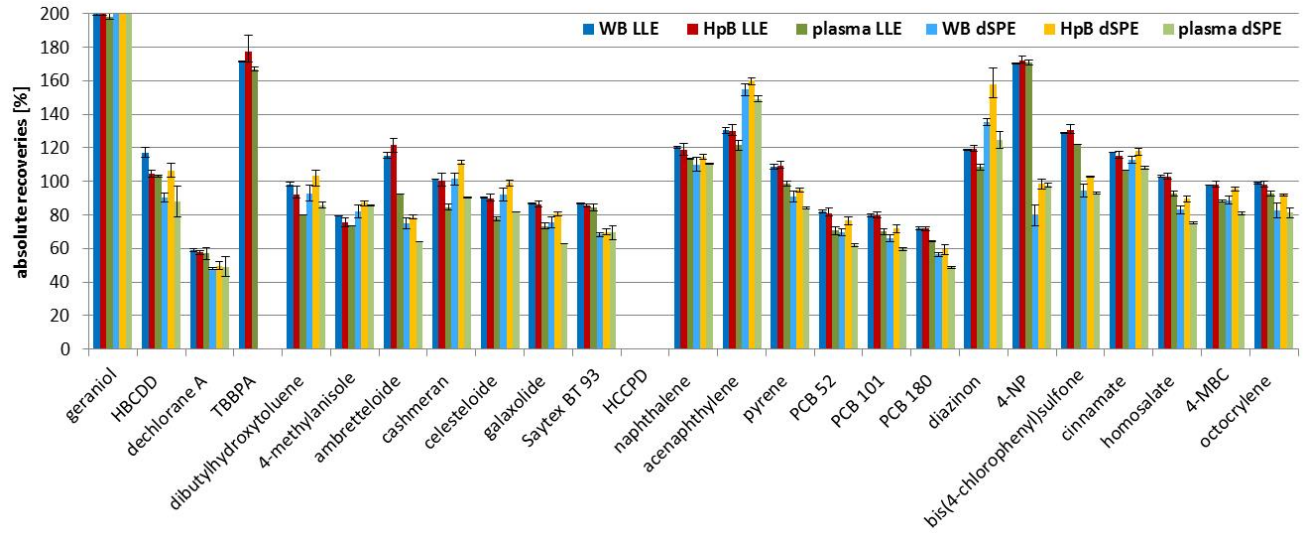


Figure 15: GC-MS analysis: absolute recoveries of target analytes spiked into different blood sample types (50 ng/mL) and extracted by LLE and dSPE: WB – whole blood, HpB – heparin blood, plasma.



Most compounds showed absolute recoveries above 70%, which was similar for all three blood samples types. CMIT, quaternium 15 and HCCPD were not detected. This was due to losses during the extraction, as matrix spikes (spike into the final extract) showed good recoveries. The absolute recoveries were similar for most compounds comparing LLE and dSPE fractions. A few compounds were lost during the dSPE clean-up, due to sorption to the PSA sorbents. These are the perfluorinated carboxylates (PFBA, PFOA and PFTDA) and TBBPA.

Comparing the LC and GC-MS analysis for the 4 analytes that were determined with both instruments, the results look a bit different. TBBPA shows matrix enhancement during GC analysis, while recoveries for LC analysis were around 60 %. Recoveries of diazinon and cinnamate were similar with both methods. Homosalate was not found during LC analysis of the dSPE extracts, while during GC analysis good

recoveries were observed. This can be explained by high matrix effects during LC analysis in the dSPE extract.

LLE relative recoveries for spiked heparin blood samples at concentrations of 2, 10 and 50 ng/mL can be found in Figure 16 and Figure 17, for LC and GC substances, respectively. At concentrations of 10 ng/mL all substances, except o-dianisidine, were detectable, for 2 ng/mL some compounds were below the method detection limit. As the recoveries for the dSPE extracts were not better and in some cases even worse than the ones for the LLE extracts, some scan measurements of the extracts were conducted, to see if there were large differences regarding background and number of peaks. The TICs of the LC-HRMS scans can be seen in Figure 18. The LLE extract for heparin blood only showed slightly larger background than the dSPE extract. Also the GC-MS TICs for heparin blood LLE and dSPE looked fairly similar. Some peaks were higher in the dSPE extract, others only appear in the LLE fraction. There is no visible change in the background signal, see Figure 19. Thus no further clean-up of the LLE extracts was done for the non-target screening of human blood samples.

Figure 16: Relative recoveries compared to a matrix spike of LC blood targets spiked into heparin blood at different concentrations – LLE fraction.

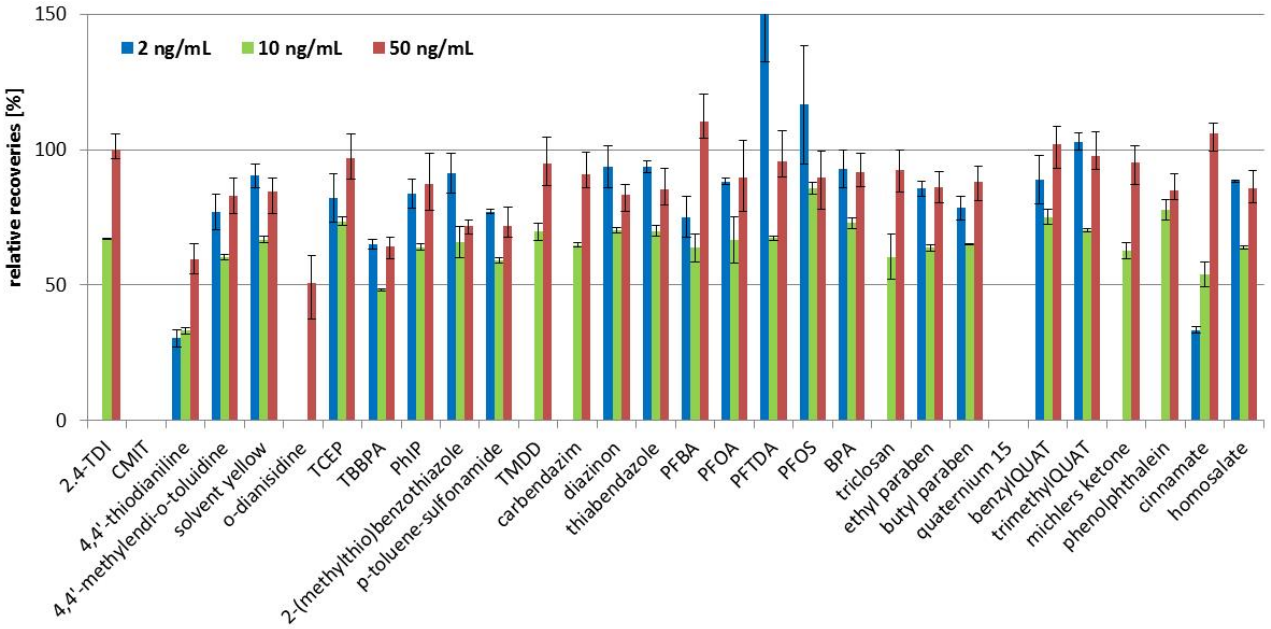


Figure 17: Relative recoveries compared to a matrix spike of GC blood targets spiked into heparin blood at different concentrations – LLE fraction.

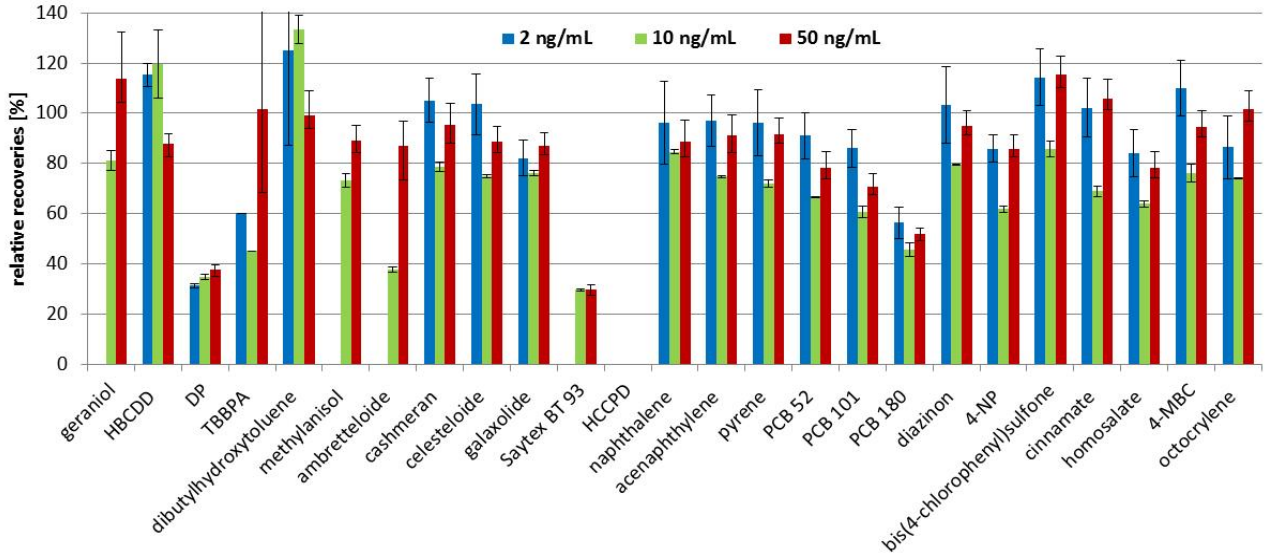


Figure 18: LC-LTQ-Orbitrap TICs of heparin blood extracted with LLE and after dSPE.

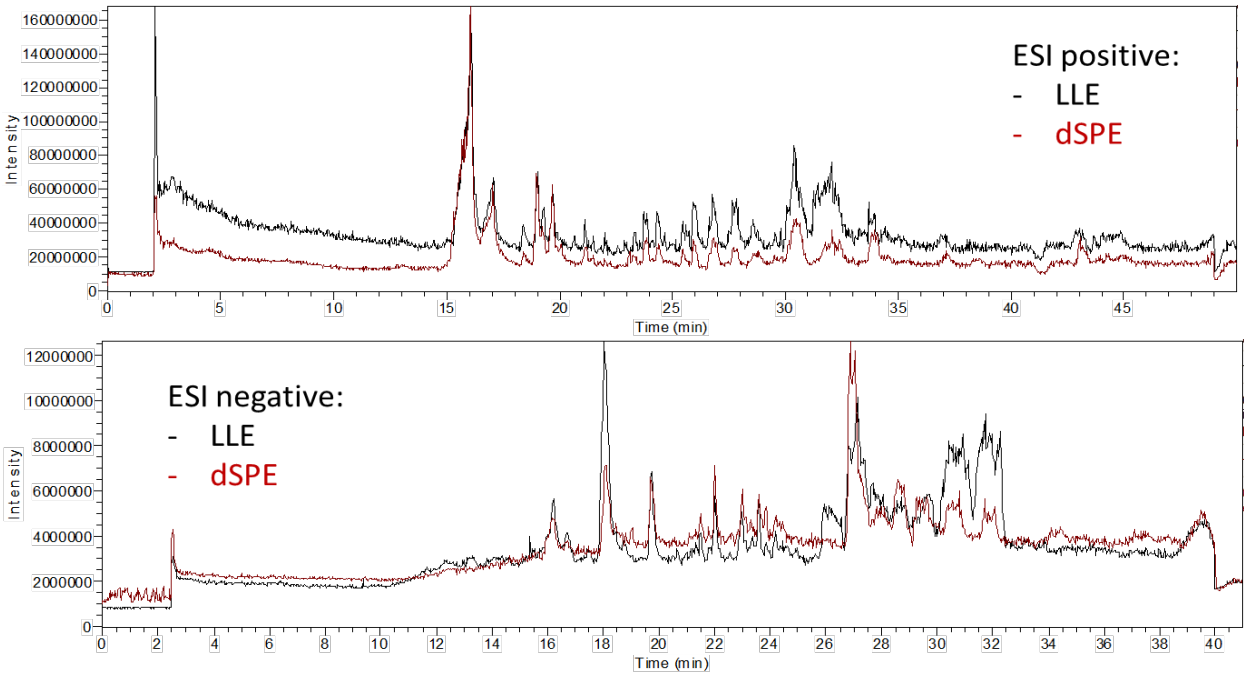
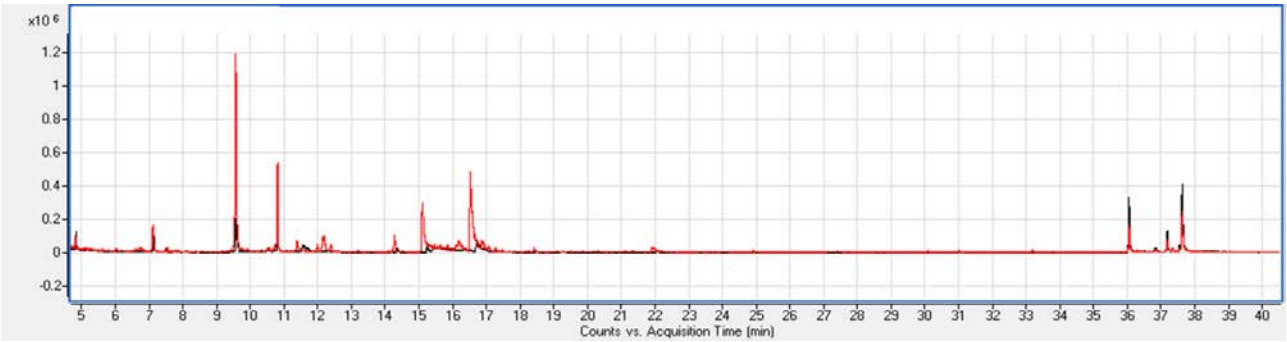


Figure 19: GC-TICs of heparin blood extracted with LLE (black line) and after dSPE (red line).

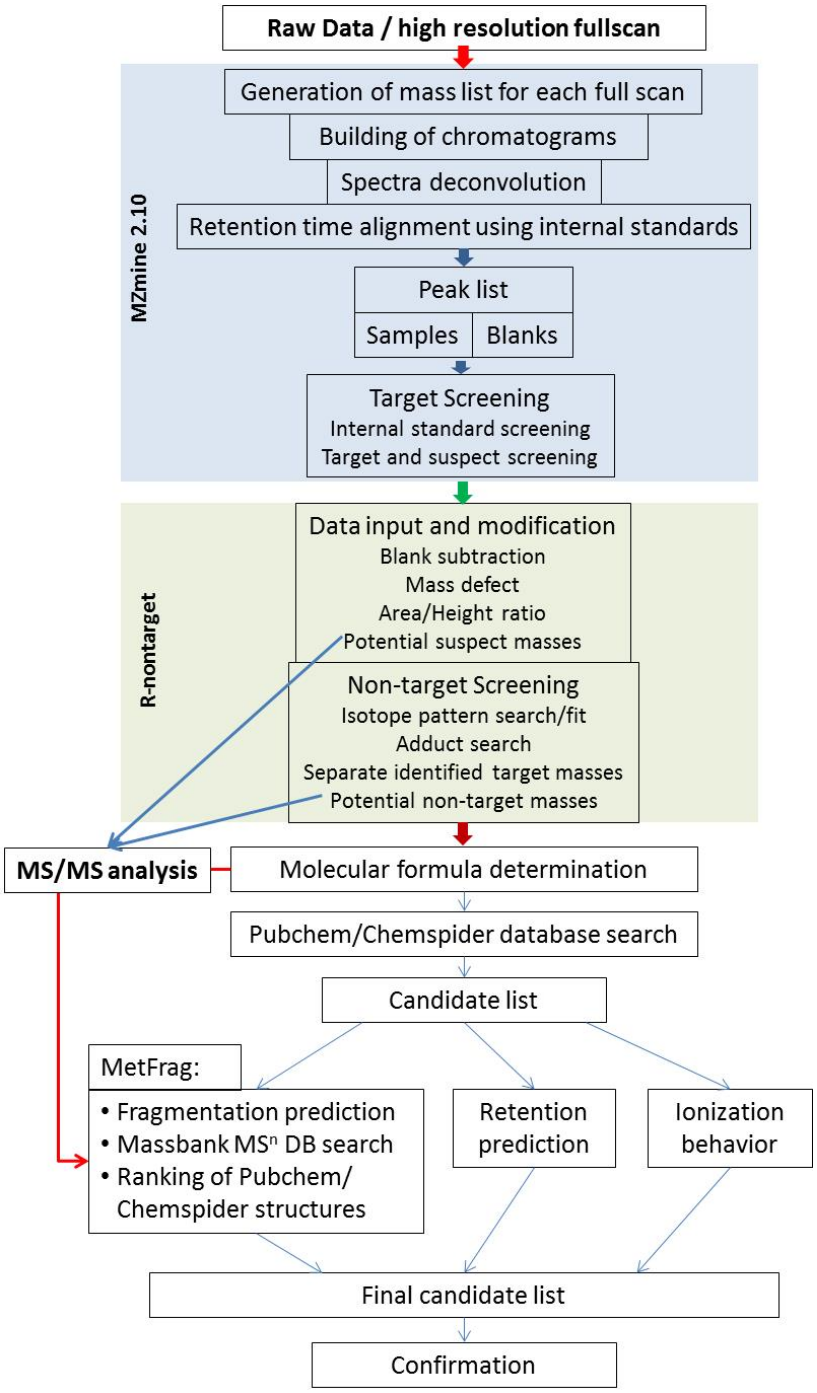


8 Data evaluation (suspect + non-target)

8.1 LC-MS

This chapter describes the software and parameters applied and to implement some quality control by reviewing the results of the target compounds analysed in the external standards and the IS spiked into all samples, all processed by the same methods.

Figure 20: Scheme of data evaluation for LC-MS analysis.



The evaluation scheme applied in this project is given in Figure 20 and was adapted from Hug et al. [80] with some changes. Exact mass chromatogram files from the full scan analysis with the LC-Orbitrap system of samples, standards and method blanks were imported into the open access program MZmine 2.10 [164] for the generation of peak lists. Mass detection with a noise cut-off of 200 was followed by a FTMS shoulder peak detection assuming a mass resolution of 100 000 (the actual resolution depends strongly on the mass). Chromatograms were build using a minimum time span of 0.1 minutes for blood samples and 0.2 minutes for urine samples (higher values tested took away too many isotopic peaks that were very narrow), a minimum height of 5000 a.u. and a mass tolerance of 0.002 m/z. Resulting peaks were smoothed with a filter width of 7 and deconvoluted using the local minimum search (chromatographic threshold 30%, search minimum in retention time range 0.2 min, minimum relative height 5%, minimum absolute height 5000, minimum ratio of peak top/edge 2 and retention duration range 0.1 to 10 minutes). Using the retention normalization the retention times of all samples were normalized against each other taking a mass tolerance of 0.001 m/z, retention time tolerance of 0.2 and a minimum standard intensity of 500,000 and 100,000 for positive and negative mode, respectively. The resulting peak lists were aligned by the join aligner, setting the mass tolerance to 0.002 m/z and the retention time tolerance to 0.5 min. The weighing did not play any role as no differences in results were observed with tests of 1:1 and 100:1. Six aligned peak lists were conducted, for urine DI, urine QuEChERS and blood sample extracts, each in negative and positive mode. The aligned peak lists contained from 11 300 up to 39 000 peaks.

In MZmine the aligned peak lists were searched for the IS list, the target list and the suspect list, each contained in a separate csv file, using the “compare against custom database” option. The results were added into the “identity” column of the aligned peak list. One problem here is that if the identity is already defined as being a target or an IS, a possible suspect with the same mass will not be listed (as the peak is already defined). Thus each time 3 aligned lists were build, each searched for IS+targets, suspects and HMDB suspects (human metabolite database suspects – extracted from the Human Metabolite Database: www.hmdb.ca/). The lists were then copied into excel files and the identity columns were combined into one, containing all information from the three lists.

The number of detected IS and target compounds in the aligned lists is given in Table 14. All IS were detected in all analysed external standards. Only 4 of the 15 IS were not detected in all analysed samples. Chlormequat-d_9 was not found in some of the urine samples due to the low retention time and possible ion suppression. For the other 3 it might have been a shift in retention time or mass, so that MZmine did not find them, or they were suppressed by matrix effects. Average recoveries with standard deviations in the different matrices are depicted in the Annex in Figure A 2. All target analytes were found in the external standards. Detections of target analytes in the samples are described in section 9.1.

Table 14: Internal standards detected after MZmine peak list alignment.

compound	ESI mode	blood RT / detected		urine: DI RT / detected		urine: QuEChERS RT / detected	
PFOS-$^{13}C_4$	-	27.5	all samples + stds	27.5-29	all samples + stds	30	all samples + stds
PFBA-$^{13}C_3$	-	16.3	all samples + stds	16-19	all samples + stds	17-19	all samples + stds
triclosan-d_3	-	26.8	all samples + stds	27.3	all samples + stds	26.9	all samples + stds
benzotriazole-d_4	+/-	15.9	all samples + stds	16.1	all samples + stds	15.9	all samples + stds
MBP-d_4	+/-	22.9	all samples + stds	23.3	all samples + stds	23	all samples + stds
chlormequat-d_9	+	2.3	all samples + stds	2.4	4 samples (1 con+ 3 decon) + stds	2.3	all samples (except 5) + stds
p-toluene-sulfonamide-d_4	+	16.1	all samples (except 3) + stds	16.3	only stds	16.1	all samples + stds
carbendazim-d_4	+	13.3	all samples + stds	13.4	all samples + stds	13.2	all samples + stds
benzophenone-3-d_5	+	25.6	all samples + stds	26.1	all samples (except 5) + stds	25.7	all samples + stds
PhIP-d_3	+	17.4	all samples + stds	17.6	all samples + stds	17.4	all samples + stds
cotinine-d_3	+	2.5	all samples + stds	3.7	all samples + stds	2.4	all samples + stds
tri-butyl-d_{27}-phosphate	+	26.5	all samples + stds	27.0	all samples + stds	26.6	all samples + stds
tonalide-d_3	+	28.6	all samples + stds	29.3	only stds	28.7	all samples (except 2) + stds
diazinon-d_{10}	+	25.9	all samples + stds	26.4	all samples + stds	26	all samples + stds
atrazine-$^{13}C_3$	+	22.7	all samples + stds	23.1	all samples + stds	22.8	all samples + stds

The aligned lists were imported into an R script designed for further processing. In a first step peaks showing a peak shape resulting from integration of background noise were filtered out if their area/height ratio was above 100. In a second step blank peaks were subtracted from the peak lists, if the intensity in a sample was lower than 2 times the blank intensity and if the area was lower than 2 times the blank area. In a final step peaks with an unreasonable mass defect for singly charged ions were deleted. The mass defect describes the difference between the monoisotopic mass of a compound and its nominal mass [165, 166].

Table 15 shows the number of peaks deleted for the different samples in each step, taken the blood analysis in ESI positive and negative mode as an example. From the R script peak lists for each sample and standard run were generated and additionally one peak list with all samples still aligned. The aligned list containing all 16 samples and two standards included 36 702 and 11 830 peaks after MZmine and 23 323 and 9 037 peaks after peak removal using the R script, for the positive and negative mode, respectively.

Table 15: Number of peaks present after MZmine and after each removal step using the R script for the analysis of a standard and a blood sample (W17) in ESI positive and negative mode.

	standard ESI pos	standard ESI neg	sample W17 ESI pos	sample W17 ESI neg
after MZmine	4813	1180	7790	3290
area/height ratio	4400	1097	7448	3237
intensity blank	3347	776	5678	2642
area blank	3347	776	5678	2642
mass defect	2919	714	5010	2557

A suspect search was conducted using the aligned peak lists. Suspects identified by MZmine were further processed if they were detected in at least 5 of the 16 samples and if they showed a reasonable peak shape.

The R package “nontarget” was used to search for isotope patterns. Here, each sample has to be processed separately; therefore this was only done for two samples, namely W17 and M14. Peaks showing Cl, Br, N and S isotopes and being present with intensities above 100,000 (for urine samples in negative mode peaks with intensities above 500,000) were further processed.

m/z values for the suspects and the peaks with Cl, Br, N and S isotopes were taken to conduct MS/MS fragmentation measurements, details see section 7.2.3.1. Samples W17 and M14 were analysed again using the LC-Orbitrap in HR-MS/MS mode. All suspects and peaks with Cl or Br isotope pattern of which HR-MS/MS spectra could be recorded were processed further. Peaks were looked up in the original chromatogram again and the relative intensities of the isotopic peaks were determined.

All suspects for which the fragmentation could not be explained by the structure were sorted out. Here MetFrag was applied for the in-silico generation of mass spectra. This software calculates mass spectra from structures and compares them to measured ones [74]. Thus the input parameters were the analysed molecular m/z value and the fragment ions detected at different collision energies. MetFrag searches for compounds with the given molecular mass in different databases. The first one is KEGG (Kyoto Encyclopedia of Genes and Genomes - http://www.genome.jp/kegg/ - which, amongst others lists small molecules with biological roles like organic acids, lipids, carbohydrates etc.), representing naturally occurring compounds. This one was used first for the in-silico fragmentation to rule out the possibility of the suspect rather being a natural product. However, some of the suspects were already included in the KEGG database. The second option was the search in PubChem, which mostly results in very large numbers of compounds fitting the molecular masses. These were then all in-silico fragmented and the results are listed as matching scores and fragment peaks identified. For the remaining suspects that seemed interesting reference standards were obtained if available. Results from the suspect search can be found in section 9.2.1.

In the non-target analysis possible molecular formula for the m/z values were determined allowing the elements C, H, O, P, N, S and Cl or Br depending on the isotope pattern in Xcalibur (analysis software from Thermo) with a 7 ppm mass tolerance. The resulting molecular formula were put into the program Seven-Golden-Rules, which tests for plausible molecular formulas according to [71]. The remaining molecular formulas were searched in the database ChemSpider and if interesting structures were found, it was further investigated, results see section 9.2.2.

8.2 GC-MS

For the non-target evaluation of GC-MS data the open access program AMDIS was used for deconvolution of mass spectra and search in the commercial NIST (version 11) and WILEY (version 9) spectral libraries. First chromatograms were deconvoluted and searched against the target and internal standard library. Settings used can be found in Table 16. All five IS were found in all standards and samples with two exceptions. $^{13}C_6$-hexachlorobenzene was not detected by AMDIS in sample W79, although it was found by manual search. The peak shape however was not good, which might be the reason why AMDIS did not deconvolute the peak. The other exception is 4-NP-d_4, which was not found in the last standard run for the same reason as for $^{13}C_6$-hexachlorobenzene. Section 9.1.2 shows the results for the detection of target analytes in the samples. The target search was followed by a search in the NIST library of the peaks not yet identified by the target search (parameters for this are also listed in Table 16). The results were checked and can be found in section 9.3.

Table 16: AMDIS analysis settings for non-target analysis of GC-MS data.

Parameter group	Parameter	Settings
Identification	Minumum match factor	80
	Multiple idendifications	On
	Show standards	Off
	Only reverse search	Off
	Type of analysis	Use RI Calibr. Data + Internal Std.
	RI window	10 + 0 x 0.01 RI
	Match factor penalties	Average
	Maximum penalty	25
Deconvolution	Component width	20
	Omit m/z	On, 0
	Adjacent peak subtraction	Two
	Resolution	High
	Sensitivity	Low
	Shape requirements	Medium
Search NIST library parameters	Hits reported per search	Min match factor: 80
	Use instrument m/z limits	On
	Build combined results	On
	Libraries	NIST and Wiley
	Select from	Only unidentified components Consider all models: On
	Number of components searched	All above threshold: 0%
	Search mode	Normal identity

9 Analysis of real samples

Human urine and blood samples for non-target screening were kindly provided by the German Environmental Specimen Bank. Urine and blood samples were collected from students in Greifswald in 2013. Eight samples from male and eight samples from female students were supplied on dry ice, urine and blood stemming from the same persons. Upon arrival samples were frozen at -80°C. For sample preparation they were thawed at room temperature, aliquots were taken and directly extracted.

For the non-target screening 5 µL of IS mixture was added for DI of 1 mL of urine and 50 µL before the ACN addition during QuEChERS extraction of 10 mL of urine. Of the 16 samples, 4 were processed without a deconjugation step (con samples) and 16 samples with a deconjugation step (decon samples). The 16 samples processed with a deconjugation step were spiked with the IS and also with 4-MeUmb-glucuronide and 4-MeUmb-sulfate. Additionally one method blank (consisting of bidistilled water) was processed without and three method blanks were processed with a deconjugation step. 5 mL of the blood samples were spiked with 50 µL of IS mixture. Additionally three method blanks (consisting of bidistilled water) were processed. The extraction procedures were described in section 7.2.4.

9.1 Target analysis

9.1.1 LC-MS

The target analytes were qualitatively detected using MZmine 2.10. Only an approximate quantification was conducted using a single point calibration in Xcalibur, results are listed in Table 17. Compounds detected in some or all urine samples were 2-ethoxyethyl acetate, HEMA, diphenyl phosphate, the phthalate metabolites, triclosan, ethyl paraben, benzophenone-3 and estrone-sulfate. All of these compounds have been detected in urine previously.

Table 17: LC-MS target analytes detected in blood and urine samples, with approximate concentrations in ng/mL; nd: not detected.

compound	detected blood	detected urine DI	detected urine QuEChERS
2-ethoxyethyl acetate	-	3 samples: M14, Md28, Md125*: 1.6 – 15.3 ng/mL	nd
HEMA	-	1 sample: M14: 9.6 ng/mL	nd
TCEP	1 sample (peak height >2x method blank peak height): M125: 0.23 ng/mL	-	-
PhIP	blank	-	-
pyrimidinol	-	blank	blank
diphenyl phosphate	-	7 samples: W17, W38, Wd17, Wd38, Wd46, Wd57, Wd 84, Wd122, Md21: 0.1-0.3 ng/mL	1 sample: Md21: 0.4 ng/mL
PFOA	all samples: 0.6 – 4.4 ng/mL	-	-
PFTDA	sample M 14: 0.8 ng/mL	-	-
PFOS	all samples: 0.9 – 7 ng/mL	-	-
MEP	-	all samples (except Md118): 2.3 - 163 ng/mL	all samples (except Wd121, Md118, Md125): 2.5 – 57 ng/mL
MBzP	-	4 samples: Wd17, Wd38, Md21, Md55: 0.5 – 9.5 ng/mL	1 sample: Wd17: 2.1 ng/mL
MBP	-	blank	blank
triclosan	nd	3 samples: W17, Wd17, Md14, Md105: 3.3 – 145 ng/mL	3 samples: W17, Wd17, Md14, Md105: 1.6 – 62 ng/mL
ethyl paraben	nd	5 samples: Wd17, Wd38, Wd121, Wd129, Md21: 11 – 310 ng/mL	samples: M21, Wd17, Wd38, Wd57, Wd121, Wd129, Md21: 6.6 – 316 ng/mL
butyl paraben	nd	2 samples: Wd17, Wd121: 4.4 – 11 ng/mL	2 samples: Wd17, Wd121: 6.3 – 13 ng/mL
tetrachlorosalicylanilide	sample M 14: 0.3 ng/mL	-	-
benzylQUAT	blank	-	-
tetraoxadodecan	-	blank	nd
benzophenone-3	-	1 sample: Md21: 272 ng/mL	2 decon samples: Wd129, Md21: 0.8 – 111 ng/mL
estrone-sul	-	2 samples: W38, M14: 0.4 – 0.7 ng/mL	2 samples: M14, M21: 1 – 2.2 ng/mL
estrone-gluc	-	1 sample: W38: 99 ng/mL	nd

* W stands for samples from women, M for samples from men. An additional d behind it means that the sample has been analysed after a deconjugation step.

Compounds detected in some or all blood samples were TCEP, PFOA, PFTDA, PFOS and tetrachlorosalicylanilide. Results from the quantitative analysis of blood samples by MRM measurements using the LC-QTrap showed detections below the quantification limit for cinnamate, ethyl paraben and MeFOSA. The perfluorinated compounds PFOS and PFOA, however, were detected in all samples and could be quantified, see Table 18. The concentrations are in general agreement with the concentrations derived by the one-point calibration using HR-MS analysis. A recent analysis of samples from the Environmental specimen bank from 2001-2010 showed concentrations of PFOS with 4 ng/mL in plasma and PFOA with concentrations of 4.8 – 6.3 ng/mL in plasma with a decreasing trend [167]. The levels found here in whole blood from 2013 are thus in agreement with the ones from earlier years.

Table 18: Concentrations of detected target analytes in human blood samples in ng/mL, analysed by the LC-QTrap MRM method. Values in brackets are below the lowest calibration level, but showed good peak shapes.

sample	PFOA	PFOS
W 17	2.8	3.4
W 38	2.0	14
W 46	2.8	3.1
W 57	3.3	4.8
W 84	(0.3)	2.6
W 121	(0.9)	2.5
W 122	1.1	2.8
W 129	1.5	4.1
M 14	0.5	1.8
M 21	(0.9)	3.0
M 28	(0.5)	2.1
M 55	1.3	4.7
M 79	1.4	3.6
M 105	1.1	3.2
M 118	1.3	6.9
M 125	(0.8)	5.7

9.1.2 GC-MS

Qualitative results from the GC-MS measurements detected only two compounds in the blood samples, namely dibutylhydroxytoluene and naphthalene. Dibutylhydroxytoluene was detected in 13 of 16 samples. This compound is widely used as antioxidant in food, plastics and cosmetics and has been monitored in occupational monitoring [104]. It has been detected in human breast adipose tissue during a non-target screening by Hernandez et al. [29]. Its approximate concentration calculated by one-point-calibration ranges from 6.9 to 15 ng/mL in the 13 samples. It is discussed further in section 9.3. Naphthalene was detected in 3 samples (W17, M14, M125) with low abundances, calculated concentrations were about 2-3 ng/mL. It has also been detected as the most abundant PAH in blood samples by Pleil et al. 2010 [145].

9.2 Non-target analysis – LC-MS

9.2.1 Suspects

The numbers of suspects identified by exact mass search in MZmine and present in 5 or more samples are listed in Table 19. From these suspects those with a recorded MS/MS fragment spectrum and additionally interesting ones like the perfluorinated carboxylates were picked out and processed further. Table 20 lists the tentatively identified suspects. Those that might have environmental and health relevance will be discussed more detailed in the following chapters, while the others are mostly endogenous compounds or stem from food consumption.

Table 19: Number of suspects detected by MZmine, with recorded MS/MS spectra, and tentatively identified ones.

	Number detected	recorded MSMS	tentatively identified
Urine ESIpos	112	36	6
Urine ESIneg	64	44	11
Blood ESIpos	20	13	6
Blood ESIneg	43	29	11

Table 20: Suspect chemicals tentatively identified in urine and blood samples.

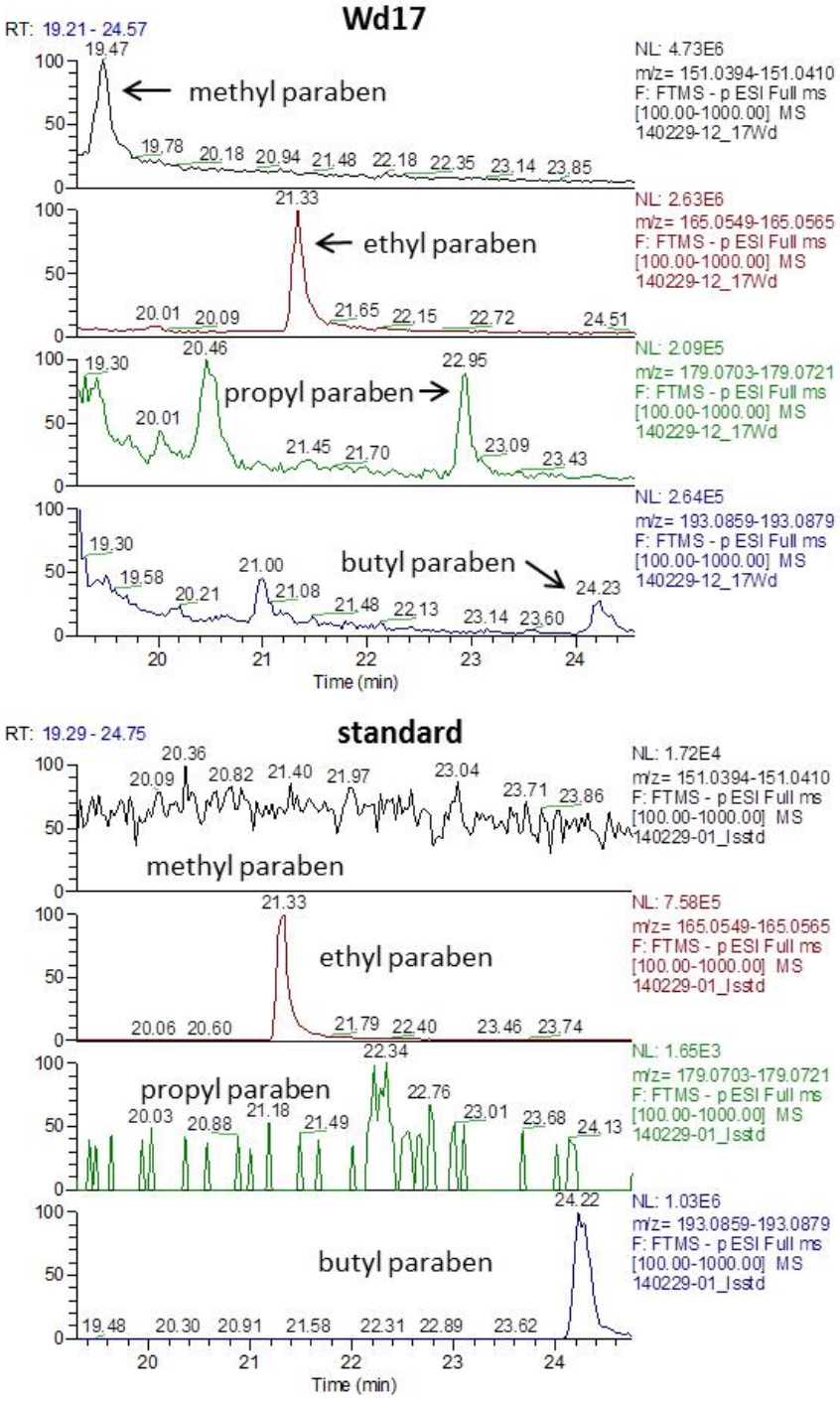
mode	suspect	m/z	comment
Urine			
-	methyl paraben	151.0401	see discussion in section 9.2.1.1
-	propyl paraben	179.0712	see discussion in section 9.2.1.1
-	enterodiol	301.1446	MetFrag: explains 9 fragment ions, thus tentatively identified
-	phenylglyoxylic acid	149.0246	MetFrag: ranked first (KEGG 3 hits, PC 167 hits), explaining 2 main fragment ions, thus tentatively identified
-	dihydroxybenzophenone	213.0556	see discussion in section 9.2.1.2
-	trihydroxybenzophenone	229.0503	see discussion in section 9.2.1.2
-	tetrahydroxybenzophenone	245.0452	see discussion in section 9.2.1.2
-	3,4-dihydroxy-chlorobenzene	142.9906	see discussion in section 0
+/-	hippuric acid	180.0651 / 178.0511	MetFrag: pos: 4 hits in KEGG, ranked first, explains 3 main fragment ions; neg: 4 hits in KEGG, ranked first, explains all 4 fragment ions, thus tentatively identified
+/-	daidzein	255.0649 / 253.0508	MetFrag: pos: the first 5 ranks are dihydroxyflavones (one of them being daidzein), explaining 5/6 fragment ions (most of the major ones); neg: 7 explained fragment ions
+/-	enterolactone	299.1275 / 297.1133	MetFrag: pos: first hit in KEGG with 6 main fragment ions explained, listed in HMDB and detected in urine; neg: explains 12 fragment ions
+	triethyl phosphate	183.0779	see discussion in section 9.2.1.4
+	p-aminophenol	110.0596	see discussion in section 9.2.1.5
+	cotinine	177.1019	see discussion in section 9.2.1.6
Blood			
-	(benzophenone-4)	307.0273	see discussion in section 9.2.1.2
-	salicylic acid	137.0245	RT: 19.9
-	hippuric acid	178.0507	RT: 13.9
-	estrone 3-sulfate	349.1105	MetFrag: 2 hits in KEGG, estrone-sul explains the fragment ion 269.1545 with 0.7 ppm, the other does not explain it; RT: 22.5 (in urine DI/Q: 24.6)
-	estrone 3-glucuronide	445.1891	RT: 20.6 (in urine DI/Q: 22)
-	perfluorohexanesulfonic acid	398.9355	see discussion in section 9.2.1.7
-	perfluoroheptanesulfonic acid	448.9321	see discussion in section 9.2.1.7
-	perfluorononanoic acid	462.9623	see discussion in section 9.2.1.7
-	plus other PFCAs: C10-C13		see discussion in section 9.2.1.7
+	triethyl phosphate	183.0781	see discussion in section 9.2.1.4
+	di-cresyl phosphate	278.0699	see discussion in section 9.2.1.4
+	diethylhexyl phosphate	323.2347	see discussion in section 9.2.1.4
+	2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-b]pyridine	241.1072	MetFrag: both the 4-OH-PhIP and 2-Hydroxyamino-PhIP explain the fragment ion 209.0923

mode	suspect	m/z	comment
+	N,N'-diacetylbenzidine	269.1288	MetFrag: 3 hits in KEGG, all explain the 3 main fragments (the other compounds were N-butyl-beta-carboline-3-carboxylate and lysergic acid)
+	oleic acid	283.2635	MetFrag: 5 hits in KEGG, oleic acid explains 3 main fragment ions

9.2.1.1 Parabens

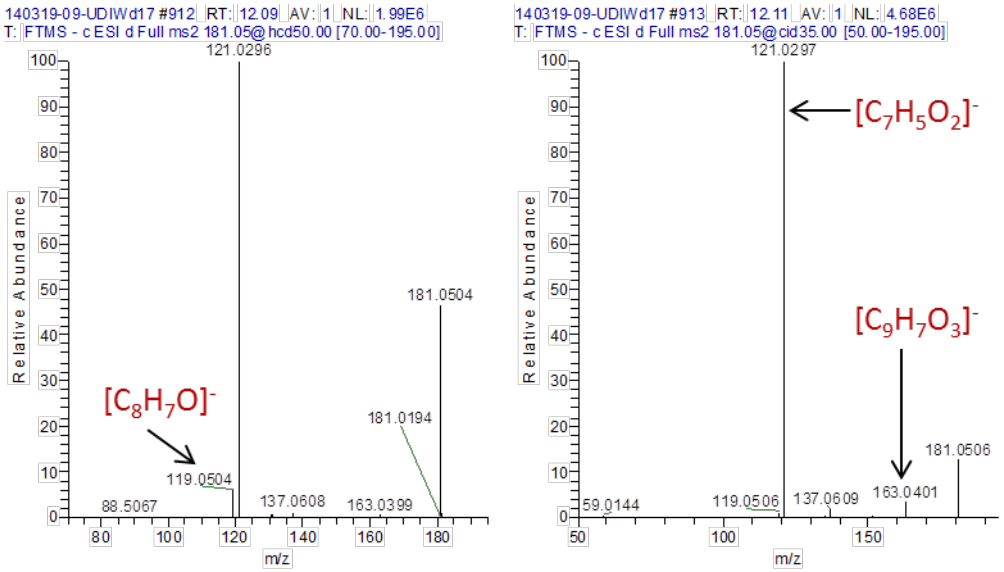
Both ethyl and butyl paraben were detected and quantified in urine samples during the target analysis. Additionally by the suspect screening methyl and propyl paraben were likely detected in urine samples. Their RTs fit very well to the ones of ethyl and butyl paraben, see Figure 21. There were, however, also many other peaks present with the same masses in the chromatogram. Thus it would be hard to determine parabens without knowing the exact RTs during a suspect screening of urine. For propyl paraben also an MS/MS spectrum could be recorded. Applying the in-silico fragmentation with MetFrag isopropyl paraben is ranked first of 3 compounds listed in KEGG, explaining 4 of the 5 fragment ions (the other two compounds explain 3 and 2 fragment ions). By using PubChem as database, iso- and n-propyl paraben are on rank 5 and 6 (of 1655 compounds). Methyl paraben was detected in 10 samples, while propyl paraben was detected in 12 samples. For a quantification and final identification reference standards would be necessary. However, their peak heights compared to ethyl and butyl paraben suggest similar concentrations around a few ng/mL. Parabens are widely used as preservatives in cosmetics, pharmaceuticals and food and concentrations in 2500 samples from US citizens showed concentrations in the low ng/mL range [130].

Figure 21: Extracted chromatograms of the four detected parabens (methyl, ethyl, propyl and butyl paraben) in the sample W17 (deconjugated extract) and a standard, showing matching RTs.



Another suspect detected in urine samples by MZmine was ethyl-protocatechuate, a metabolite of ethyl paraben. The m/z value of 181.0505 was found in all urine extracts analysed in ESI negative mode. Due to the detection of ethyl paraben the presence of its metabolite might be reasonable. However, MZmine also defined the peak as being the metabolite homovanillic acid listed in the Human Metabolite Database. This metabolite stems from human dopamine and is released via the urine.

Figure 22: HR-MS/MS spectra of 181.05, left: HCD 50, right: CID 35 with tentatively identified fragment ions for homovanillic acid.



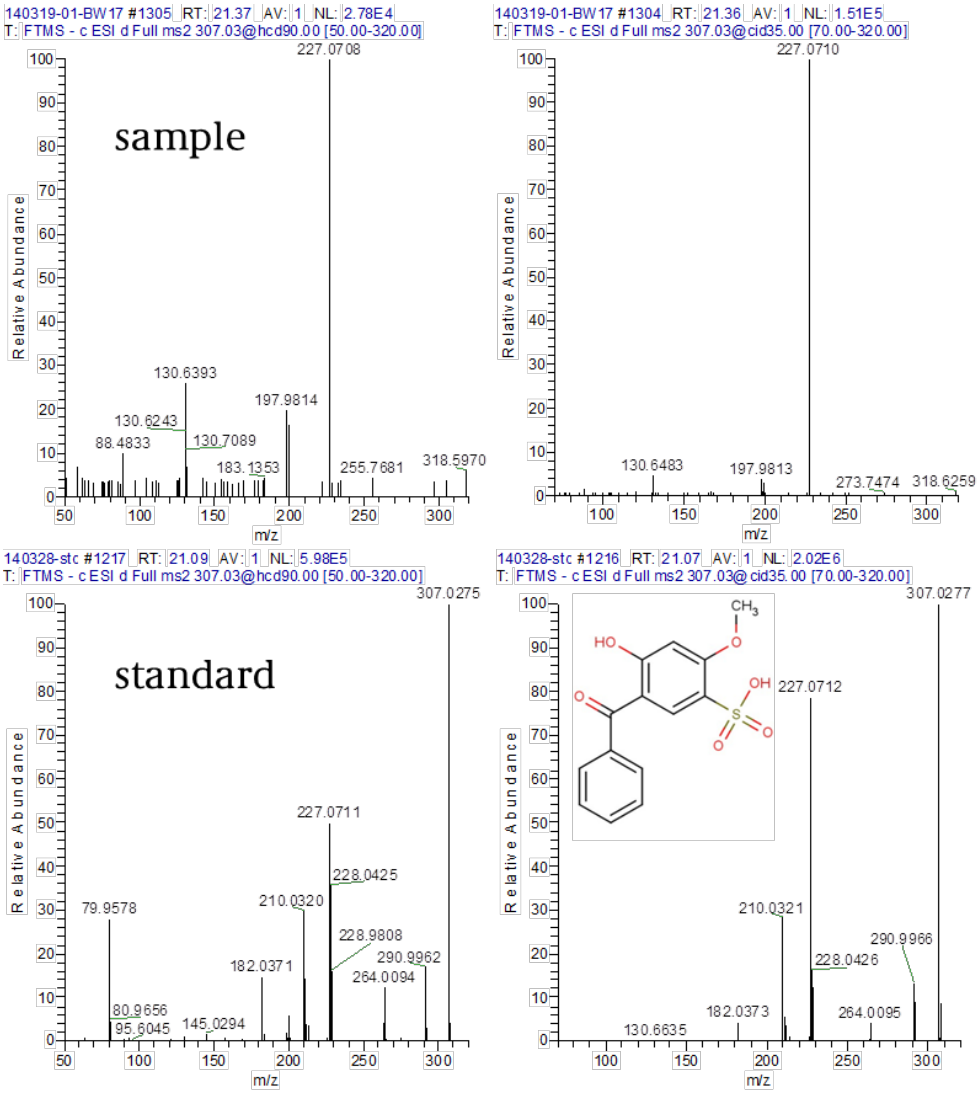
Using MetFrag the fragmentation of these two compounds was compared by taking the recorded HR-MS/MS spectra of this compound (see Figure 22). For the parent mass 182.0574 and PubChem as database reference 867 hits resulted. MetFrag was set to [M-H] mode and negative charge, applying a m/z variation of 0.005 absolute and 10 ppm. For the merged spectrum homovanillic acid had a score of 0.971 and 4 explained fragments, while ethyl protocatechuate had a score of 0.784 with 2 explained fragments. Looking at the MetFrag results the peak with m/z value of 181.0505 is more likely to be homovanillic acid than ethyl protocatechuate.

This example shows the importance to consider naturally occurring substances and their metabolites during non-target screening of biological samples. One solution might be to take the HMDB as a reference and to include the in-silico fragmentation of compounds listed in the KEGG database. In the above example 8 compounds with the m/z 181.0505 are listed in the KEGG database, and homovanillic acid is one of them. This at least gives a hint about the presence of endogenous substances with matching exact masses.

9.2.1.2 UV-filters

Benzophenone-4 was identified as a suspect by MZmine analysed in ESI negative mode. It was detected in 5 blood samples, W17, W46, M14, M105 and M118. MetFrag counts 598 hits for the parent mass using PubChem as reference database. Benzophenone-4 had a score of 0.863 with 1 explained fragment (227.0710) for the CID 35 spectrum of the sample. There were, however, 130 additional compounds explaining this one fragment ion resulting from a loss of SO_3. As benzophenone-4 was available as a reference standard, it was analysed in data-dependent HR-MS/MS mode together with one sample to check the RT and fragmentation pattern. The RT was 21.57 and 21.66 min in the reference standard and the sample, respectively. The intensity of the m/z value 307.0273 was 100-fold lower in the sample compared to the standard (5 µg/mL). A HR-MS/MS spectra containing several fragment ions was observed for the reference standard, while in the sample the only fragment ion was 227.0710, see Figure 23. As neither of the other fragment ions nor the molecular ion observed in the standard was visible in the sample, we cannot identify the peak as being benzophenone-4.

Figure 23: HR-MS/MS spectra of m/z 307.03 in the blood sample W17 (first row) and a reference standard containing benzophenone-4 (second row). Left: HCD 90, right: CID 35.



Due to the detection of benzophenone-3 in urine during the target analysis, we also checked the urine samples again for any UV-filters and their metabolites. Some of them were detected as suspects in less than 5 samples, thus they did not appear in the original search when setting the limit to detections in at least 5 samples. However, after checking the raw data again, di-, tri- and tetrahydroxybenzophenone could all be tentatively identified in the sample Md21. The RTs for the three compounds fitted very well with each other, tetrahydroxybenzophenone eluting first with 19.6 and 21 minutes (probably 2 isomers), trihydroxybenzophenone with a RT of 21.66 min and dihydroxybenzophenone with 23.6 min. Tetrahydroxybenzophenone was detected in several samples, where the first peak was found in 7 samples and the second peak only in 3 samples. Dihydroxybenzophenone was detected both in the conjugated and the deconjugated analysis of sample M21, however, the intensity in the still conjugated extract was about 50 fold lower than the one in the deconjugated sample. Thus it seemed that the hydroxybenzophenones are most probably present as conjugated forms in the samples. Therefore the sulfate and glucuronide metabolites of the di-, tri- and tetrahydroxybenzophenones were calculated and searched for in the raw data of the unconjugated extracts. This way, both the sulfate and the glucuronide metabolite of dihydroxybenzophenone were detected in the sample M21 when analysed by DI (no detection during QuEChERS extraction, as expected from method development results), with RTs of 23.23 and 21.15 min,

respectively, see Figure A 3 in the Annex. The RT difference of approximately 2 min between the different conjugates fits with the conjugates of 4-methylumbelliferyl and estrone as analysed during method development. Thus the conjugated forms are tentatively identified in sample M21.

In summary it seems that the benzophenone UV-filters might be relevant for human biomonitoring studies. Some of the here detected UV-filters and others have been analysed in human samples before [168, 169]. This large compound group, though, seems to be of interest for further studies.

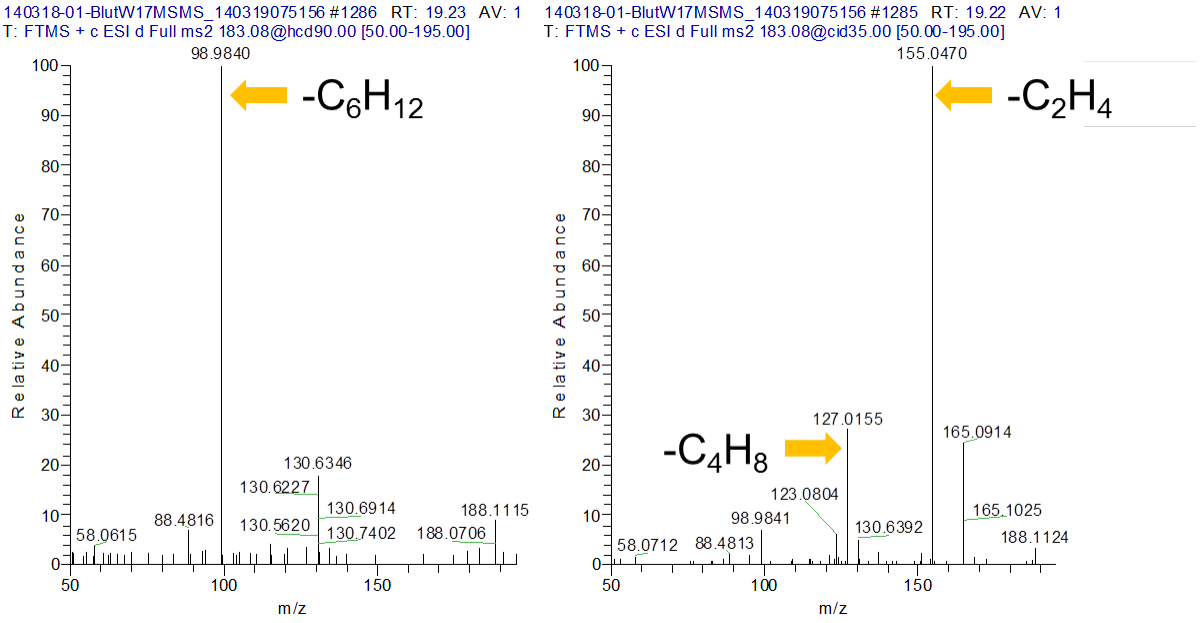
9.2.1.3 Dihydroxy-chlorobenzene

3,4-dihydroxy-chlorobenzene was identified by MZmine in 12 deconjugated samples analysed by QuEChERS (RT 18.9 min), while it was only detected in 2 deconjugated samples analysed by DI (RT 19.2 min). The chlorine pattern was visible in all samples, it was however, not detected by R „nontarget“, probably due to the low peak height in Wd17 with 12 000 (it was not detected in Md14). This shows that it is necessary to deconvolute also peaks with low intensity during MZmine peak detection, as otherwise the isotopes are being lost for further identification. For the final identification, a reference standard would be necessary.

9.2.1.4 Organophosphate flame retardants

One by MZmine in ESI positive mode identified suspect was triethylphosphate, a flame retardant and plasticizer. By taking a closer look at the fragmentation pattern, see Figure 24, one can easily assign the three most abundant ions found: $C_4H_{12}O_4P$ (155.0473: M-C_2H_6), $C_2H_8O_4P$ (127.0160: M-C_4H_8) and H_4O_4P (98.9847: M-C_6H_{12}). This fragmentation is similar to the one observed for the target analyte TCEP (tris(2-chloroethyl)phosphate), which also shows the fragments M-C_2ClH_3 and M-C_4ClH_5 and could be identified during the target analysis. However, for a final identification a reference standard would be necessary. Looking at the raw data again, triethylphosphate is also present in the method blank samples. Thus this compound could only be detected in significantly higher amounts than in the method blanks (at least four times higher peak heights than the highest method blank peak) in 6 blood samples and 3 urine samples.

Figure 24: HR-MS/MS spectra of 183.08 in the blood sample W17, tentatively identified as triethylphosphate, left: HCD 90, right: CID 35.



Another suspect identified by MZmine is diethylhexylphosphate detected in all blood samples, but not in any method blanks. The recorded MS/MS spectra did not contain any fragment ions, probably due to too low concentrations. However, the ^{13}C isotope peak was present at about 14% of the molecular ion and the RT at 27.5 min is much later than the one of triethylphosphate with 19.5 min due to its longer carbon chains.

Due to the detection of three phosphate flame retardants during target and suspect search we looked for further compounds only present in less than 5 samples. Tributylphosphate was detected in all samples, but was also present in all method blanks. Only two samples showed 2 times higher peak heights than the highest peak in the method blanks. Also TCPP (or tris(2-chloro-1-methylethyl)phosphate) was present in all method blanks. Thus during the analysis of phosphate flame retardants care has to be taken regarding blank concentrations. At least for TCPP and tributylphosphate the blank contamination seemed to stem mostly from the LC system and the solvents used, as already high peaks were detected in simple solvent injections.

One additional in blood tentatively identified compound is dicresylphosphate, where no detections were present in the method blanks. A peak at 19.5 minutes was detected in two samples (W121 and M28), while another peak at 20.6 min was detected in M79. These different RTs could be due to the different isomers of this compound (di-m-, di-o- and di-p-cresylphosphate). The ^{13}C isomer was detected with about 13% and the mass deviation was -1.25 ppm. No MS/MS spectrum could be obtained due to low peak heights of 23 000 to 84 000.

In summary many different organophosphate flame retardants seem to be present in human blood samples, which might be reasonable due to their intense use after phasing out the brominated flame retardants. So far, mostly the diester metabolites have been analysed in human urine samples [170-172], to the best of our knowledge no flame retardants have been analysed so far in human blood samples.

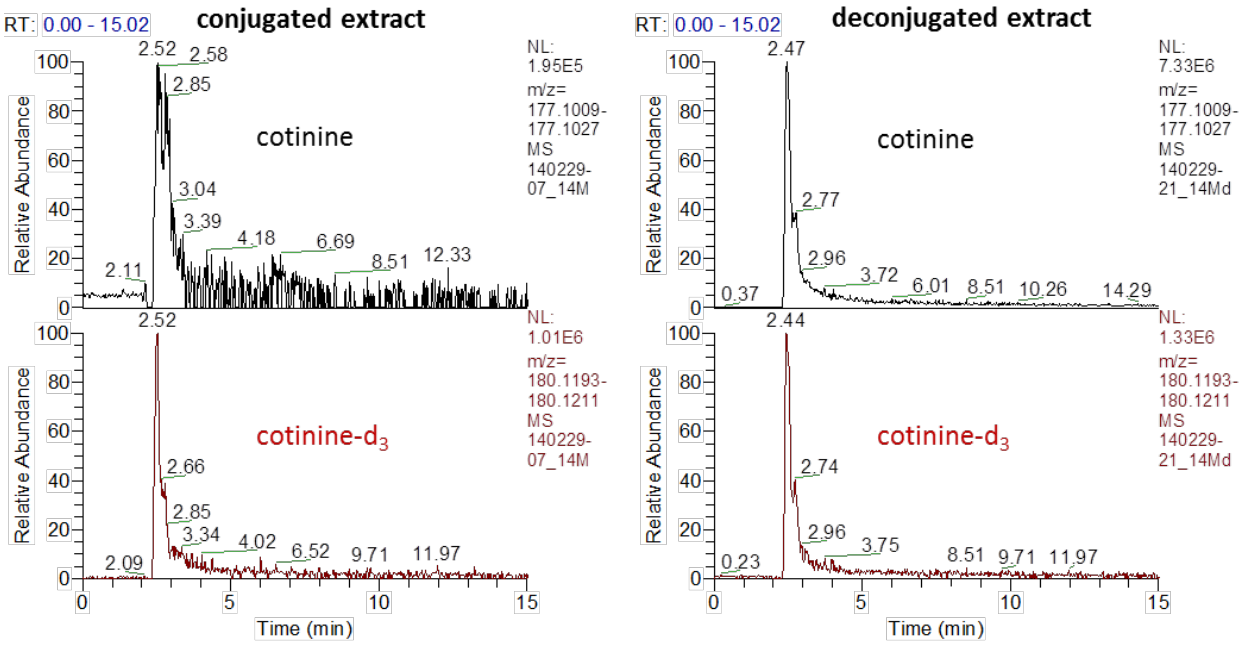
9.2.1.5 Aminophenol

Aminophenol was identified by MZmine in all urine samples and the peaks were less intense in the still conjugated samples than in the deconjugated ones. When looking at the MS/MS fragments and using MetFrag for in-silico fragmentation, 4 hits appear when using KEGG as the database. Of these 4 three are the different isomers, p-, m- and o-aminophenol, which all explain 5 of the 8 detected fragments, while the other compound only explains 2 fragments. Due to the wide usage of aminophenols as hair dye, in the production of dyes for textiles and constituents in pharmaceuticals and due to them being metabolites of anilin, anisidin, nitrobenzol and degradation products of paracetamol it seems likely for them to be present in human urine samples. However, for the final identification, reference standards are necessary.

9.2.1.6 Cotinine

Cotinine was detected in 9 urine samples analysed by the QuEChERS method. It showed the same RT as cotinine-d_3 and showed higher peaks in the deconjugated extracts compared to the still conjugated extracts, Figure 25. The highest values were found in samples M14, 118 and 125, which were the smokers. Peak heights in non-smokers were between a factor 2 and 20 lower than in the samples from smokers.

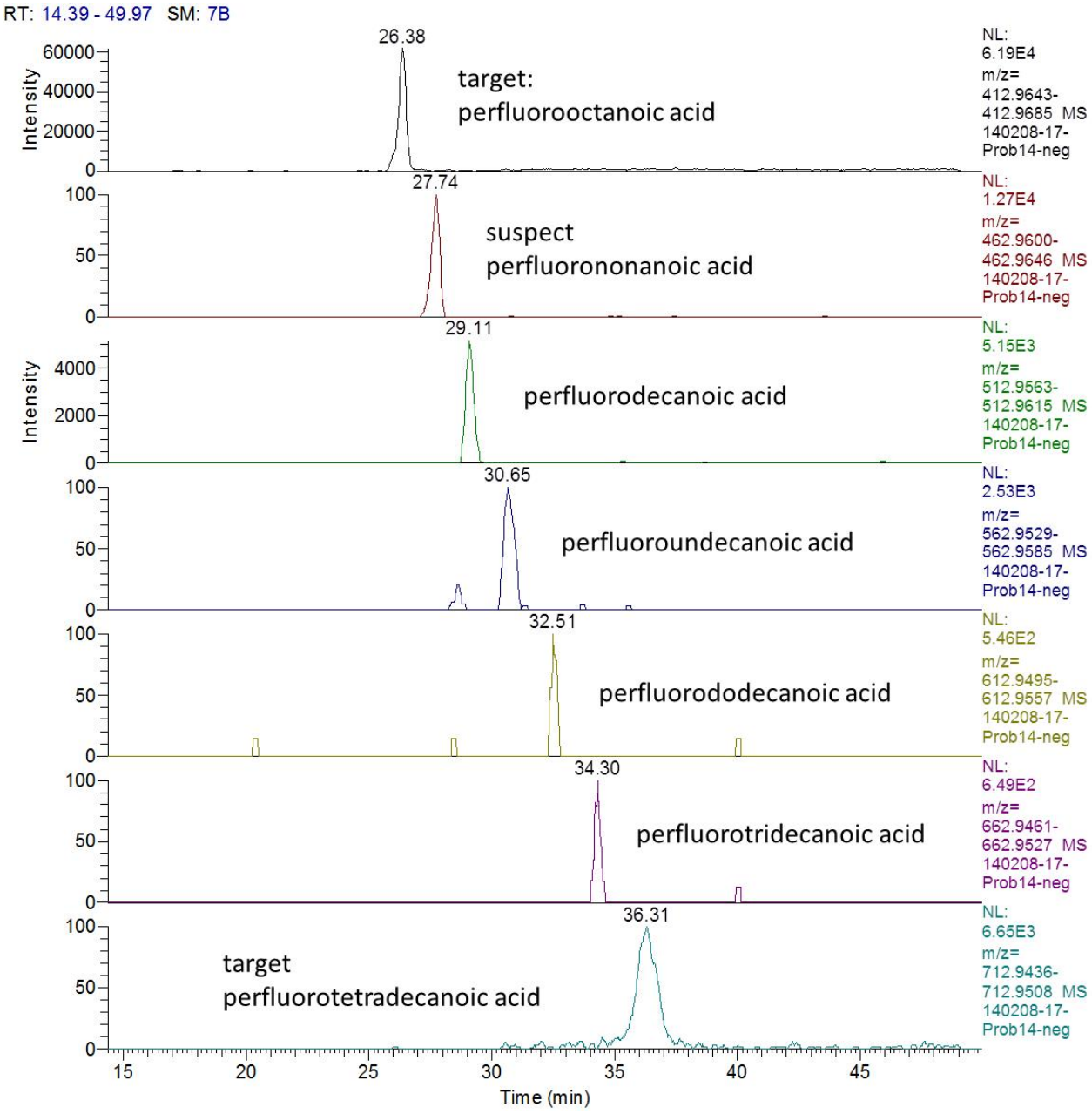
Figure 25: Chromatograms of cotinine and cotinine-d_3 in a urine sample of a smoker, conjugated extract (left) and deconjugated extract (right), showing the same RT of the compound and the IS and lower peak height in the conjugated sample extract.



9.2.1.7 PFASs

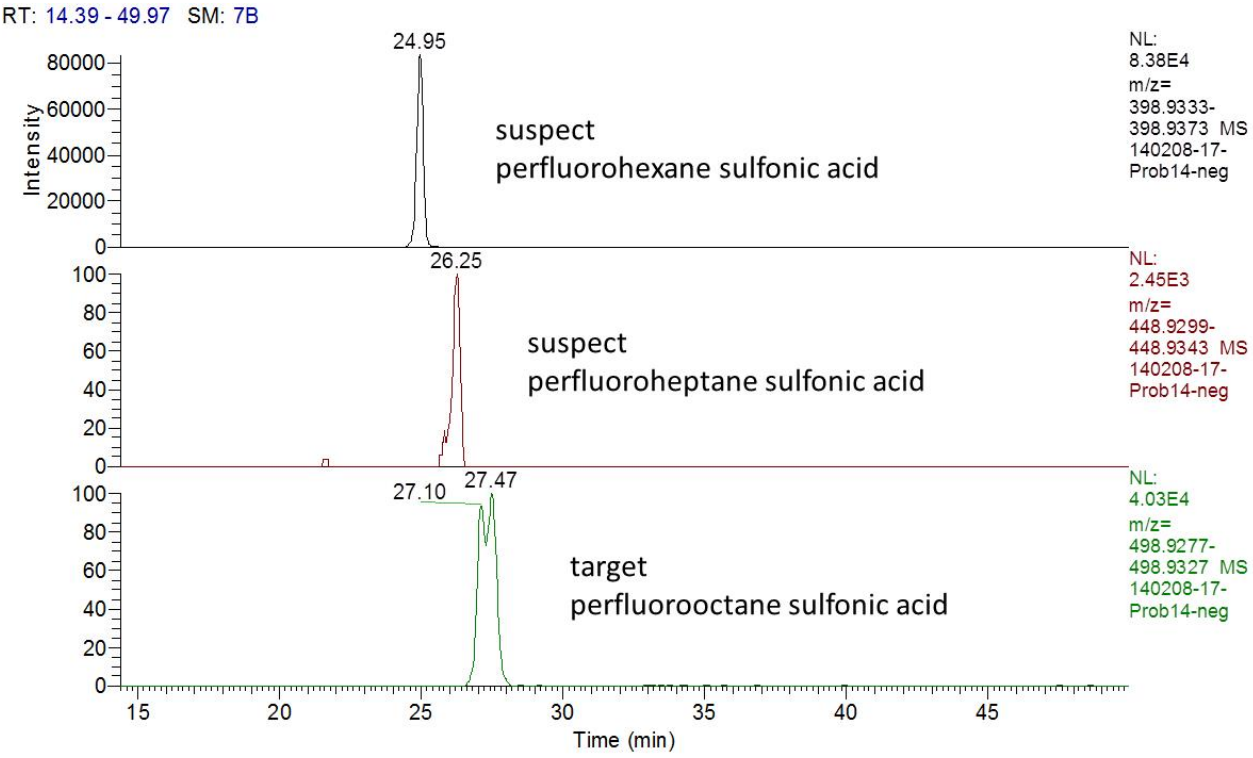
Perfluorononanoic acid was identified in blood samples as a suspect by MZmine in ESI negative mode, however, due to relatively low abundance in the samples, no MS/MS spectra could be recorded with the method used. As PFNA and other homologues have already been detected in human blood samples (e.g. [173]) and the RT fits well into the homologues row of other perfluorinated alkyl acids, it was next to the other homologues tentatively identified. PFOA and PFTDA were as targets also analysed in the external standards and their RT is the same as in the samples. Thus in sample M14 perfluorocarboxylic acids with chain length of C8 to C14 could be tentatively identified, see Figure 26.

Figure 26: Extracted ion chromatograms of tentatively identified perfluorinated carboxylic acids in sample 14, showing matching RTs of the homologues row of C8 to C14 carbon chain lengths.



The same can be applied to the perfluorinated sulfonic acids, where perfluorohexane sulfonic acid and perfluoroheptane sulfonic acid have been identified by MZmine as suspects. Looking at the target perfluorooctane sulfonic acid (see Figure 27), one can conclude the same as for the perfluorocarboxylic acids.

Figure 27: Extracted ion chromatograms of tentatively identified perfluorinated sulfonic acids in sample M14 showing matching RTs of the homologues row of C6 to C8 carbon chain lengths.



9.2.2 Non-targets

Numbers of non-target peaks in the two with R nontarget processed samples W17 and M14 showing a Cl or Br isotope pattern and intensities >100,000 are listed in Table 21. For these ions molecular formulas were generated in Xcalibur and between 2 and more than 400 formula were obtained using a 10 ppm mass tolerance. By application of the Seven-Golden-Rules software these were reduced to mostly 1 or 2 plausible ones. The resulting formulas were searched for in ChemSpider. Lists of 1 up to several hundred structures resulted. Many structures could be sorted out due to their non-ionisability in the respective ion mode. Additionally, most of the structures had less than 5 references, thus it was not possible to retrieve any further information about their production and usage. One explanation for this is that the detected peaks are metabolites that have not been described or are simply not present in the database ChemSpider. Thus only two compounds could be identified further, as described below. To be able to identify more of the detected peaks, it would be necessary to go back to the suspect list and calculate metabolites of the compounds containing Cl or Br. The resulting masses could then be compared to the m/z values detected. This, however, was beyond the scope of this project.

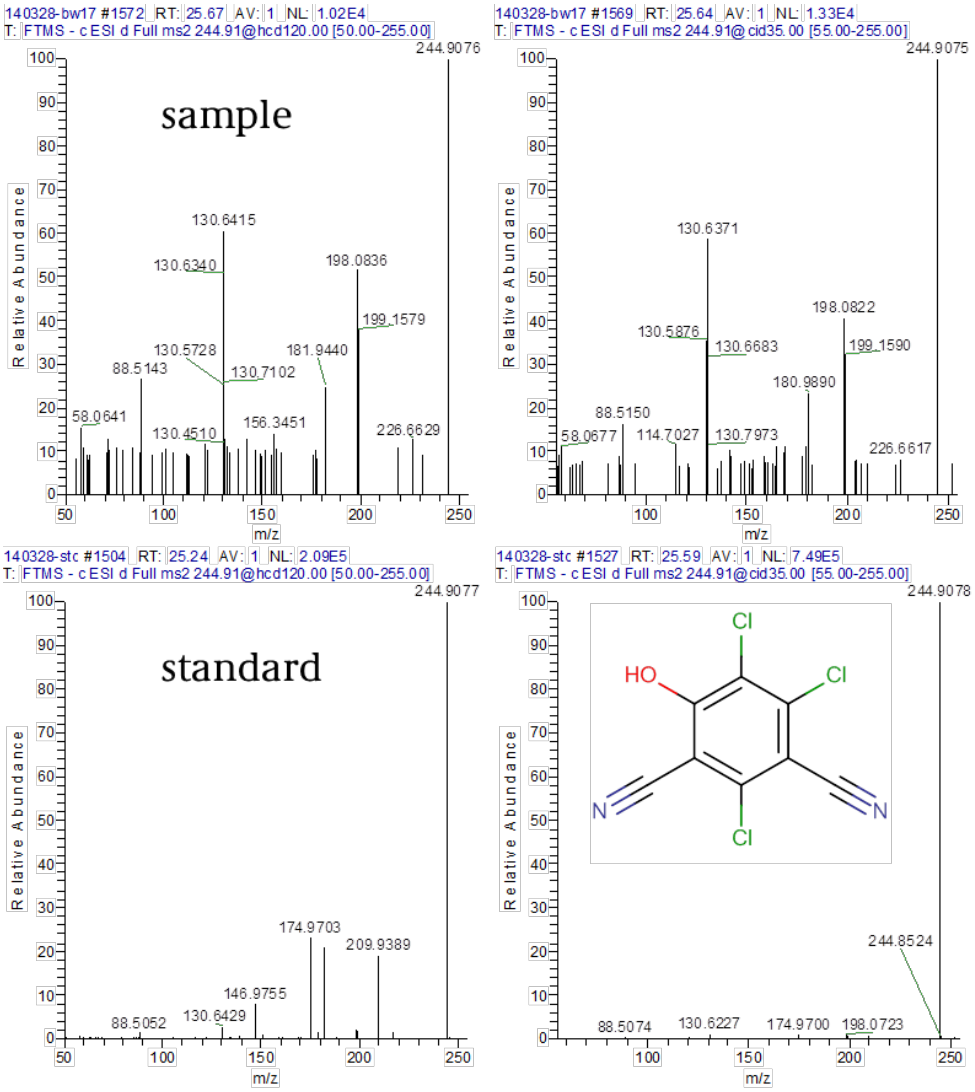
Table 21: Numbers of detected peaks in the samples W17 and M14 with a Cl or Br pattern.

	Urine		Blood
	DI	QuEChERS	
ESIpos	11	6	12
ESIneg	32	20	24

One search in ChemSpider resulting in a plausible hit was the chlorothalonil metabolite 4-hydroxy-chlorothalonil with 18 references. A reference standard of this compound was available and

thus the RTs, isotope pattern and MS/MS spectra were compared. The RTs were similar with 25.7 and 25.9 min in a solvent standard and in the blood extract, respectively. The RT shift of 0.2 minutes is due to a broad peak and a different maximum. The isotope pattern in the standard and the blood extract were identical, showing the distinctive isotope pattern of 3 Cl ions. The HR-MS/MS spectra in the sample did not show extensive fragmentation of the molecular ion, except with the highest collision energy HCD 120, see Figure 28. However, in the sample the MS/MS spectra contained only background noise, due to the lower abundance of the molecular ion. Due to the matching RT and isotope pattern, we consider this non-target as identified. The peak was observed in all blood samples and a rough quantification using a one-point-calibration resulted in amounts of about 12 and 17 ng/mL in M14 and W17, respectively.

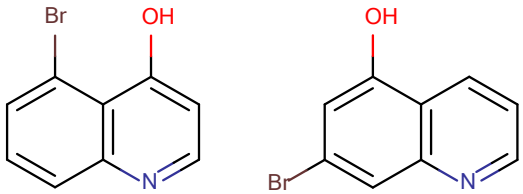
Figure 28: **HR-MS/MS spectra of the blood sample W17 (first row) and a reference standard containing 4-hydroxy-chlorothalonil (second row). Left side: HCD 120, right side: CID 35.**



One peak was detected in the urine sample M14 in negative mode with a m/z of 221.9557 and a Br isotope pattern. By taking the exact mass and the isotope pattern the only possible molecular formula could be identified as C_9H_6ONBr (mass deviation of -1.35 ppm). By visual inspection a peak with the same RT and Br isotope pattern was found in positive mode with a m/z of 223.9703. Thus the compound was ionisable both in positive and negative mode. When searching in ChemSpider for the molecular formula 123

substances were listed. This list could be reduced to 34 substances by deleting all compounds that were most probably not ionisable in both positive and negative mode (for example by sorting out all compounds where the O was present in the ring system, as an OH group is necessary for the compound to be ionized in negative mode). From these 34 substances all except 3 were bromo-quinolinoles, see Figure 29. The other three were listed with only 1, 4 or 8 references compared to up to 109 references for the bromo-quinolinoles. Possible precursors to the bromo-quinolinoles, bromo-quinolines are used as intermediates in the production of pharmaceutical compounds [174], while derivatives of bromo-quinolinoles are also applied as dyes for jeans and other cotton textiles [175]. Thus it seems reasonable that these compounds occur in human samples. However, a final identification by a reference standard is necessary to confirm the finding. By checking the other samples the same peak was found in all urine and also all blood samples, but it was not present in any of the blank samples.

Figure 29: Possible structures of the non-target peak detected with Br isotope pattern.

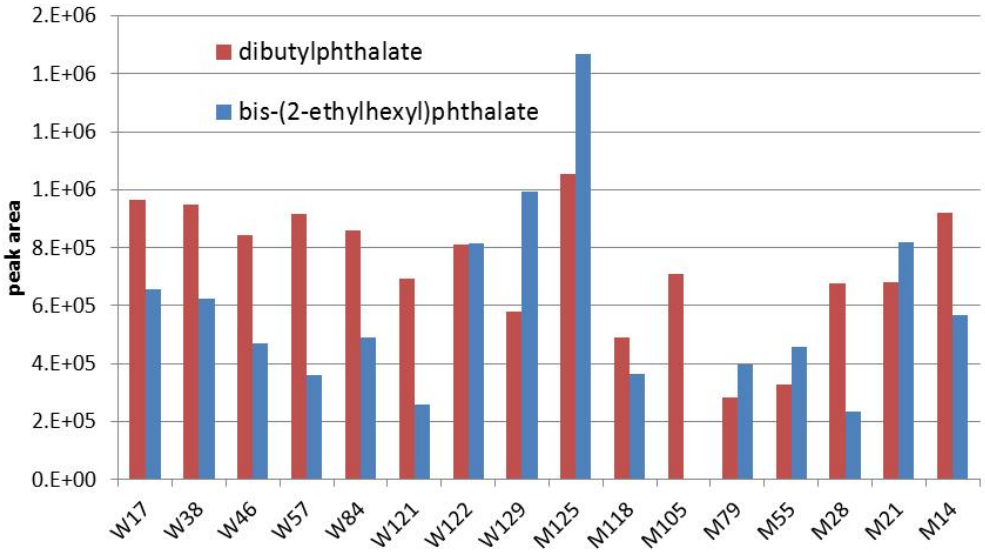


9.3 Non-target analysis – GC-MS

Several non-target compounds could be tentatively identified using the program AMDIS. Seven of the detected compounds showed very good matches with their recorded mass spectra in NIST and their occurrence in human blood samples was reasonable due to their usages.

The two phthalates dibutylphthalate and di(2-ethylhexyl)phthalate were detected in all samples, see Figure 30. In most studies phthalates metabolites are analysed in human urine and not in blood samples. Their metabolites are also routinely analysed in the samples from the environmental specimen bank and metabolites of both of these compounds are found in all samples; detailed concentrations can be searched for at the homepage of the German Environmental Specimen Bank: www.umweltprobenbank.de. During the target analysis of the urine samples MBP - the metabolite of dibutylphthalate - could not be analysed due to blank detections.

Figure 30: Peak areas of the two detected phthalates in the 16 blood samples.



Another group of interesting unknowns detected by AMDIS were di-tert-butylbenzene, di-tert-butylphenol and 7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione. These compounds are similar to dibutylhydroxytoluene, which has been detected in some of the blood samples during target analysis. Table 22 shows the structures of these 4 compounds, together with RT and Kovats RI, while Figure 31 shows the peak areas in the different samples, integrated by AMDIS. These, however, are not indicative of the concentrations, as the ionization efficiency can be different for the different compounds. All four substances have been detected as leaching from plastic tubes used for drinking water supply [176], thus it seems reasonable to detect them in human samples. One of the three tentatively identified compounds could be finally identified using a reference standard of 2,4-di-tert-butylphenol. Using a 2-point-calibration, an approximate concentration in W46 and M14 could be calculated as being 180 and 160 ng/mL, respectively.

Table 22: Di-tert-butylbenzene compounds detected in blood samples by GC-MS analysis.

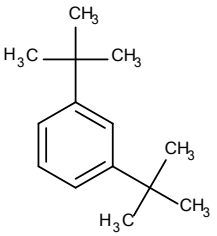
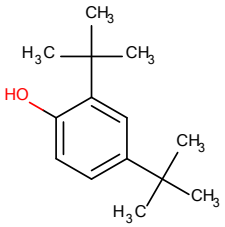
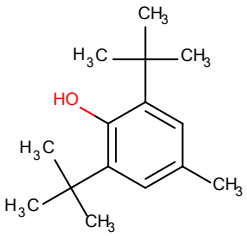
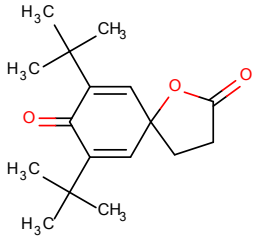
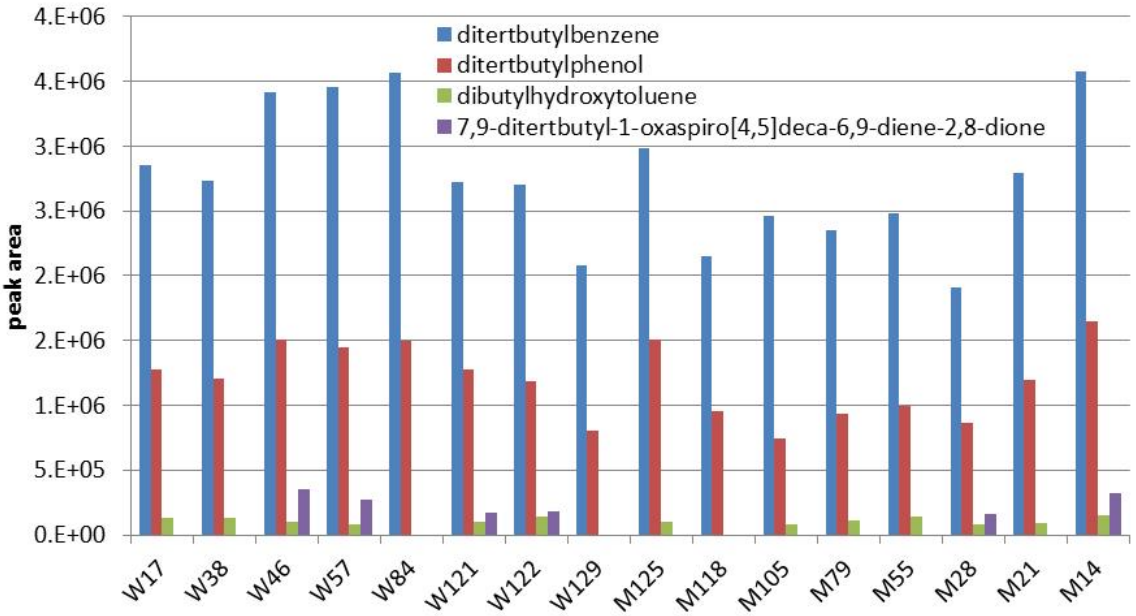
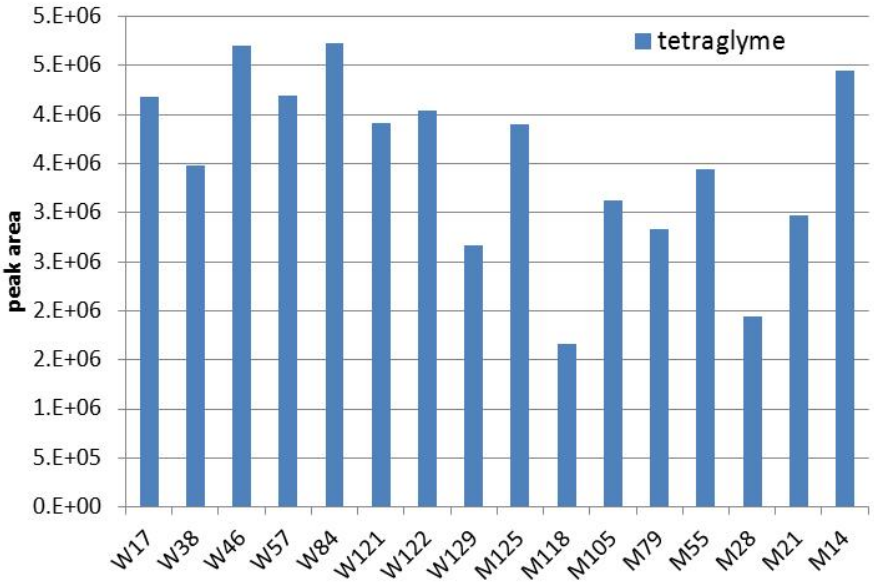
	di-tert-butylbenzene	di-tert-butylphenol	dibutylhydroxytoluene (BHT)	7,9-di-tertbutyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione
Structure				
CAS	1014-60-4	96-76-4	128-37-0	82304-66-3
RT (min)	19.4	30.3	30.8	44.8
Kovats RI analysed / NIST	1246 / 1245	1515 / 1539	1500 / 1504	1898 / 1929

Figure 31: Peak areas of the four different tentatively identified di-tert-butylbenzenes.



Furthermore, tetraglyme was detected in all samples, see Figure 32. The substance triglyme or triethylenglycol dimethylether (tetraoxadodecan) (CAS: 112-49-2) has only been analysed for in the urine samples during LC-MS analysis, where it could not be detected due to blank detections. A final identification of tetraglyme was possible by the repeated analysis of W46 and M14 and a reference standard of tetraglyme. The approximate concentration calculated by one point calibration in both samples was 300 ng/mL.

Figure 32: Peak areas of tetraglyme tentatively identified by GC-MS in all blood samples.



One more interesting tentatively identified substance is niacidamine (CAS: 98-92-0) or Vitamin B3 (listed in the HMDB ID: 01406), which is present in some food items, but also widely used in cosmetics [177]. The

peak shape of this compound was not very good, thus AMDIS was not able to detect it in all samples, although a manual check revealed the presence in all blood samples.

Additionally other substances resulting from food consumption have been tentatively identified, namely benzaldehyde (HMDB ID: 06115, listed as additive in cosmetics and also food, used as denaturant, flavoring agent, and as fragrance; expected in blood, but so far not quanitfied), hydroxy-benzaldehyde (HMDB ID: 11718, found in the benzoate degradation via hydroxylation, bisphenol A degradation, toluene and xylene degradation, and biosynthesis of phenylpropanoids pathways), caffeine, theobromine (cocoa consumption) and methyl salicylate (HMDB ID: 34172, present in beverages and used as artificial flavouring agent). As these compounds are not relevant in the context of environmental pollutants we will not go into their detection in detail. However, the detections show that the developed method is able to detect a wide range of compounds. On the other hand we are not able to distinguish between endogenous and exogenous compounds, making the data processing and identification process tedious.

10 Summary and outlook

Within this project we developed and evaluated a non-target screening approach for human samples. The sample preparation method QuEChERS was for the first time tested for the extraction of a wide range of environmental pollutants and metabolites from human urine and blood. This extraction method together with a direct injection of urine was able to detect most of the tested target analytes. Especially for the extraction of blood samples the recoveries were very good. Also the non-target measurement and data processing was able to detect all target analytes if present above certain concentrations (typically in the low ng/mL range). Some of the target analytes could also be detected in the human urine and blood samples from the German Environmental Specimen Bank. Both in blood and urine several suspect chemicals were tentatively identified by taking MS/MS spectra into account and in a few cases even a final identification using reference standards was possible. Among the (tentatively) identified compounds were four parabens, the UV-filter benzophenone-3 and three benzophenone metabolites, five organophosphate flame retardants, ten perfluoroalkyl acids, two phthalates and some phthalate metabolites, four antioxidants used in plastic materials similar to dibutylhydroxytoluene (BHT), and tetraglyme. It was further shown that using the isotopic information one non-target peak could be tentatively identified as 4-hydroxy-chlorothalonil and afterwards be verified using a reference standard. Another peak showing a Br-pattern could be tentatively identified as being a bromo-quinolinole isomer.

The data evaluation process for the non-targets however, is very laborious. Therefore the preparation of suspect lists can be suggested as an important part of a non-target or suspect screening. Here it is an advantage to collect additional information about the suspects like reports of previous detections, production and application data besides the chemical information about structure and mass. After a positive detection due to peak findings for the exact mass this additional information can be used to plausibilize the presence of the suspect. Using measured and in-silico generated MS/MS spectra (MetFrag) the identified suspects can further be compared to other chemicals listed in databases.

With a further development of software, the data processing workflow will become more efficient. The suspect and non-target compounds found are only a small portion of the peaks detected in the human blood and urine samples. During this project it was only possible to look for suspects present in 5 or more samples, however, there are many more peaks present in less than 5 samples. Thus with additional time and work there is a potential to detect more contaminants. This also implies for a non-target screening used in a broader application during human biomonitoring studies, that it is important to analyse individual rather than pooled samples.

An aspect that has to be taken into account during non-target screening of biological samples is the occurrence of natural substances and endogenous metabolites. To the best of our knowledge there is no way to generally distinguish between endogenic and exogenic substances. We tried to take this aspect into account by including a search for endogenous substances listed in the Human Metabolome Database, containing data about small molecules detected in the human body.

To decrease the amount of data and peaks that need to be identified, there might be interesting follow-up studies. One could be the examination of time trends using samples from the German Environmental Specimen Bank stemming from several years. By identifying peaks showing a significant time trend (increasing or decreasing), especially compounds with increasing levels becoming interesting for inclusion into human biomonitoring studies could be identified. Another way to reduce the amount of peaks would be the comparison of different cohorts, for example with and without a certain disease or certain exposure groups. The latter could include occupational exposure, leading to the identification of peaks that could subsequently be searched for in the general population. By statistical methods one could

determine peaks which occur predominantly in one of the cohorts and subsequently identify solely these compounds. This would help to handle the large amount of peaks present in the samples.

A third way to reduce the number of relevant peaks is to determine compounds being of toxicological relevance. Here an effect directed analysis (EDA) [178] of human blood and urine samples could be developed. A fractionation with a subsequent toxicological test like for example on endocrine disruption could be applied, where the fractions showing effects are filtered out. In these fractions a non-target analysis could be used to identify the compounds responsible for the detected effects. This way the several hundred to thousand peaks found in a sample can be reduced to the relevant ones regarding effects on human health. One study on polar bear blood already showed the applicability of EDA to blood [179], which suggests also an application to human blood.

11 References

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12 Annex

Table A 1: Urine target analytes and their calculated properties.

Compound name	logD[1]	pK_a[1]	pK_b[1]	$logK_{OW}$[2]	BCF[2]	$logK_{OA}$[2]	Henry's law constant[2]
4-methyl-m-phenylene diisocyanate (2,4-TDI)	2.31			3.74	136	7.08	$1.1E^{-05}$
5-chloro-2-methyl-4-isothiazolin-3-one (CMIT)	1.35			-0.34	3.16	5.50	$3.6E^{-08}$
citronellal	2.71	16.1		3.53	156	5.39	$6.8E^{-04}$
geraniol	2.50	16.3		3.47	90.5	6.80	$1.2E^{-05}$
resorcinol	1.37	9.26		1.03	3.16	9.19	$8.1E^{-11}$
2,4-diaminoanisole	0.15			-0.31	3.16	8.48	$4E^{-11}$
4,4'-thiodianiline	2.50			2.46	12.8	12.0	$3.9E^{-12}$
4,4'-methylendi-o-toluidine	3.43			3.28	67.4	11.8	$7.3E^{-11}$
4-aminoazobenzene (solvent yellow)	3.55			3.19	10.0	10.1	$5.2E^{-09}$
o-dianisidine	1.65			2.08	7.27	12.9	$1.8E^{-13}$
mercaptobenzothiazole	2.88	10.9		1.83	7.48	5.07	$1.1E^{-05}$
2-(methylthio)benzothiazole	3.43		1.13	3.22	55.6	9.50	$1.1E^{-08}$
8-octyl-4-isothiazolin-3-one (octhilinone)	3.33			2.61	19.2	8.52	$2.1E^{-08}$
4-hydroxybenzotriazole	0.82	7.70		0.69	3.16	9.89	$1.5E^{-11}$
p-toluene-sulfonamide	1.09	10.5		0.92	3.16	5.54	$4.7E^{-07}$
5-methylbenzotriazole	1.76	8.87		1.71	6.28	6.89	$1.6E^{-07}$
ethyltosylamide	1.67	10.4		1.87	8.01	6.13	$1.4E^{-06}$
dibutylhydroxytoluene	4.03	10.5		4.08	229	8.26	$1.6E^{-06}$
2-ethoxyethyl acetate	0.23			0.59	3.16	4.47	$3.2E^{-06}$
hydroxyethyl mercapturic acid (HEMA)	-4.53	3.82		-0.36	3.16	15.6	$2.5E^{-18}$
2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD)	3.17	13.2		3.61	112	8.61	$2.4E^{-07}$
3-aminomethyl-3,5,5-trimethylcyclohexyl-amine (isophorone diamine)	-4.16		10.5	1.90	8.96	8.65	$4.4E^{-09}$
1-hydroxypyrene	3.98	9.50		4.45	403	11.9	$8.6E^{-10}$
1-naphthol	2.66	9.60		2.69	35.3	8.46	$6.0E^{-08}$
3-OH-4,4'-dichloro-biphenyl (3-OH-PCB)	4.38	7.78		4.57	478	10.6	$2.4E^{-08}$
4-OH-3,4',5-trichloro-biphenyl (4-OH-PCB)	4.14	6.42		5.21	1273	11.4	$1.8E^{-08}$
2-isopropyl-6-methyl-pyrimidin-4-ol (IMPY)	0.56			1.20	2.90	8.25	$2.2E^{-09}$
diazinon	4.19			3.86	152	9.15	$1.1E^{-07}$
dimethylthiophosphate (DMTP)	-1.75			1.11	2.50	5.94	$3.6E^{-07}$
diphenyl phosphate	0.68			2.88	5.50	11.2	$1.1E^{-10}$
perfluorobutanoic acid (PFBA)	-1.22	1.07		2.14	3.16	4.45	$1.2E^{-04}$
bisphenol A	4.04	9.78		3.64	72.0	12.7	$9.2E^{-12}$
monoethyl-phthalate (MEP)	-1.47	3.08		1.86	3.16	9.28	$9.3E^{-10}$
mono-iso-butyl-phthalate (MBP)	-0.59	3.08		2.77	3.16	9.95	$1.6E^{-09}$
monobenzyl-phthalate (MBzP)	-0.11	3.08		3.07	3.16	11.7	$5.6E^{-11}$
triethylcitrate	-5.40	3.66		1.09	2.03	15.8	$4.6E^{-17}$
triclosan	4.80	7.68		4.66	642	11.5	$5.0E^{-09}$
ethyl paraben	2.00	8.50		2.49	19.8	9.18	$4.8E^{-09}$
butyl paraben	2.96	8.50		3.47	105	10.0	$8.5E^{-09}$
triethylenglycol dimethylether (tetraoxadodecan)	-0.02			-0.76	3.16	6.12	$3.2E^{-09}$
2-ethylhexyl-p-methoxycinnamate (cinnamate)	5.38	8.07		5.80	3128	9.94	$1.5E^{-08}$

Compound name	logD[1]	pK_a[1]	pK_b[1]	$logK_{OW}$[2]	BCF[2]	$logK_{OA}$[2]	Henry's law constant[2]
salicylic acid 3,3,5-trimethcyclohexyl ester (homosalate)	4.99			6.16	5403	9.26	$1.8E^{-06}$
benzophenone-3	3.54	9.72		3.52	38.2	10.0	$1.9E^{-05}$
4-methylumbelliferyl glucuronide (4-MeUmb-gluc)	-3.65	2.97		-0.267	0.50	18.7	$2.5E^{-21}$
4-methylumbelliferyl sulfate (4-MeUmb-sul)	-1.07	-2.32		-0.903	0.50	8.70	$6.1E^{-12}$
estrone 3-(β-D-glucuronide) (estrone-gluc)	-1.06	3.30		1.58	3.16	21.0	$8.5E^{-22}$
estrone 3-sulfate (estrone-sul)	1.46	-1.75		0.95	3.16	11.03	$2.0E^{-12}$
creatinine	-3.26	5.58		-1.77	3.16	8.24	$2.4E^{-12}$

[1] calculated with JChem: logD (partition coefficient between octanol and water at the pysiological pH of 7.4), pK_a and pK_b (acidic and basic dissociation constants); [2] calculated with EPIWIN: $logK_{OW}$ (partition coefficient between octanol and water), BCF (bioconcentration factor), $logK_{OA}$ (partition coefficient between octanol and air)

Table A 2: Blood target analytes and their calculated properties.

Compound name	logD[1]	pK_a[1]	pK_b[1]	$logK_{OW}$[2]	BCF[2]	$logK_{OA}$[2]	Henry's law constant[2]
4-methyl-m-phenylene diisocyanate (2,4-TDI)	2.31			3.74	136	7.08	$1.1E^{-05}$
5-chloro-2-methyl-4-isothiazolin-3-one (CMIT)	1.35			-0.34	3.16	5.50	$3.6E^{-08}$
citronellal	2.71	16.1		3.53	156	5.39	$6.8E^{-04}$
geraniol	2.50	16.3		3.47	90.5	6.80	$1.2E^{-05}$
resorcinol	1.37	9.26		1.03	3.16	9.19	$8.1E^{-11}$
2,4-diaminoanisol	0.15		5.71	-0.31	3.16	8.48	$4E^{-11}$
4,4'-thiodianiline	2.50		4.24	2.46	12.8	12.0	$3.9E^{-12}$
4,4'-methylendi-o-toluidine	3.43		4.68	3.28	67.4	11.8	$7.3E^{-11}$
4-aminoazobenzene (solvent yellow)	3.55		3.06	3.19	10.0	10.1	$5.2E^{-09}$
o-dianisidine	1.65		4.55	2.08	7.27	12.9	$1.8E^{-13}$
2,2',4,4'-tetrabromodiphenyl ether (BDE 47)	6.55			6.77	14000	10.7	$3E^{-06}$
2,2',4,4',5-pentabromodiphenyl ether (BDE 99)	7.32			7.66	15000	11.2	$1.2E^{-06}$
2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether (BDE 209)	11.2			12.11	41.7	18.4	$1.2E^{-08}$
hexabromocyclodo-decane (HBCDD)	7.21			7.86	5019	12.0	$1.7E^{-06}$
syn and anti dechlorane plus (DP)	9.07			11.3	108	14.8	$7.4E^{-06}$
tris(2-chloroethyl)phosphate (TCEP)	2.11			1.63	0.62	5.31	$3.3E^{-06}$
tetrabromobisphenol A (TBBPA)	5.87	6.57		7.20	10000	18.2	$2.3E^{-13}$
decabromodiphenyl ethane (DBDPE)	12.2			13.64	7.43	19.2	$6.4E^{-08}$
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)	2.09		5.43	2.16	13.8	13.2	$2.8E^{-13}$
mercaptobenzothiazole	2.88	10.9		1.83	7.48	5.07	$1.2E^{-05}$
2-(methylthio)benzothiazole	3.43		1.13	3.22	55.6	9.50	$1.1E^{-08}$
p-toluene-sulfonamide	1.09	10.5		0.92	3.16	5.54	$4.7E^{-07}$
dibutylhydroxytoluene	4.03	10.4		4.08	229	8.26	$1.6E^{-06}$
2,4,7,9-tetramethyl-5-decyne-4,7-diol (TMDD)	3.17	13.2		3.61	112	8.61	$2.4E^{-07}$
4-methylanisole	2.33			2.62	26.4	4.50	$4.7E^{-03}$
ambrettolide	5.12			5.37	1628	6.33	$2.7E^{-03}$
cashmeran	3.57			4.49	426.4	6.73	$1.4E^{-04}$
celestolide	4.67	16.2		5.93	983.7	8.82	$3.2E^{-05}$
galaxolide	4.72			6.26	3629	8.17	$1.3E^{-04}$
1,2-bis(2-ethylhexyl) 3,4,5,6-tetrabromobenzene-1,2-dicarboxylate (pyronil 45)	11.1			11.95	12.6	16.9	$3E^{-07}$
N,N-ethylene-bis(tetrabromophthalimide) (saytex BT 93)	7.81			9.80	567	28.6	$3.6E^{-21}$
hexachlorocyclopenta-1,3-diene (HCCPD)	3.57			4.63	983	5.00	$2.7E^{-02}$
naphthalene	2.96			3.17	69.9	5.05	$4.4E^{-04}$
acenaphthylene	3.33			3.94	184.8	6.27	$1.1E^{-04}$
pyrene	4.28			4.93	770.6	8.19	$1.2E^{-05}$
2,2',5,5'-tetrachlorobiphenyl (PCB 52)	6.04			6.34	18700	8.18	$2.0E^{-04}$
2,2',4,5,5'-pentachlorobiphenyl (PCB 101)	6.64			6.98	54900	9.23	$9.0E^{-05}$
2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB 180)	7.85			8.27	12300	11.7	$1.0E^{-05}$
carbendazim	1.80	9.70	4.28	1.55	4.68	10.6	$2.1E^{-11}$
diazinon	4.19		4.19	3.86	152	9.15	$1.1E^{-07}$
thiabendazole	2.33	10.3	4.08	2.00	19.8	11.5	$2.1E^{-11}$

Compound name	logD[1]	pK_a[1]	pK_b[1]	$logK_{OW}$[2]	BCF[2]	$logK_{OA}$[2]	Henry's law constant[2]
perfluorobutanoic acid (PFBA)	-1.22	1.07		2.14	3.16	4.45	$1.2E^{-04}$
perfluorooctanoic acid (PFOA)	5.11			4.81	3.16	4.24	$9.1E^{-02}$
perfluorotetradecanoic acid (PFTDA)	5.79			8.83	10.0	3.94	$1.9E^{+03}$
perfluorooctane sulfonic acid (PFOS)	5.43			4.49	3.16	4.84	$1.1E^{-02}$
2-N-methylperfluoro-octanesulfonamide (MeFOSA)	4.14	3.47		6.27	6345	4.05	4.04
perfluorooctyl phosphonic acid (C8-PFPA)	2.33	0.46		4.40	371	7.76	$1.1E^{-05}$
8:2 fluorotelomer alcohol (8:2 FTOH)	5.61	15.8		5.75	2232	3.35	4.14
10:2 fluorotelomer alcohol (10:2 FTOH)	7.01	15.8		7.08	12100	3.41	114
4-nonylphenol (4-NP)	5.74	10.3		5.99	123.9	8.62	$3.4E^{-05}$
bisphenol A	4.04	9.78		3.64	72.0	12.7	$9.2E^{-12}$
bis(4-chlorophenyl) sulfone	4.14			3.90	173	9.15	$1.4E^{-07}$
triclosan	4.80	7.68		4.66	642	11.5	$5E^{-09}$
ethyl paraben	2.00	8.50		2.49	19.8	9.18	$4.8E^{-09}$
butyl paraben	2.96	8.50		3.47	105	10.0	$8.5E^{-09}$
3,5-dichloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide (tetrachlorosalicylanilide)	3.81	5.95		5.87	3492	14.6	$4.8E^{-11}$
quaternium 15	-2.72		3.70	-5.92	3.16	6.04	$1.8E^{-08}$
benzyldimethyldodecylammonium chloride (benzylQUAT)	2.63	18.1		4.93	70.8	15.6	$4.8E^{-13}$
trimethyloctyl-ammonium bromide (trimethylQUAT)	-0.87			1.26	3.16	11.3	$1.9E^{-12}$
michlers ketone	3.65		3.73	3.50	43.2	11.6	$4.9E^{-10}$
phenolphthalein	4.35	9.16		3.06	18.1	15.8	$9E^{-16}$
2-ethylhexyl-p-methoxycinnamate (cinnamate)	5.38			5.80	3128	9.94	$1.8E^{-06}$
salicylic acid 3,3,5-trimethcyclohexyl ester (homosalate)	4.99	9.72		6.16	5403	9.26	$1.9E^{-05}$
4-methyl-benzylidene camphor (4-MBC)	5.12			5.92	3744	9.97	$2.2E^{-06}$
octocrylene (OC)	6.78			6.88	16100	13.8	$3.0E^{-09}$

[1] calculated with JChem: logD (partition coefficient between octanol and water at the pysiological pH of 7.4), pK_a and pK_b (acidic and basic dissociation constants); [2] calculated with EPIWIN: $logK_{OW}$ (partition coefficient between octanol and water), BCF (bioconcentration factor), $logK_{OA}$ (partition coefficient between octanol and air)

Figure A 1: TICs of DI and QdSPE extracts, comparing the deconjugated and not-deconjugated samples.

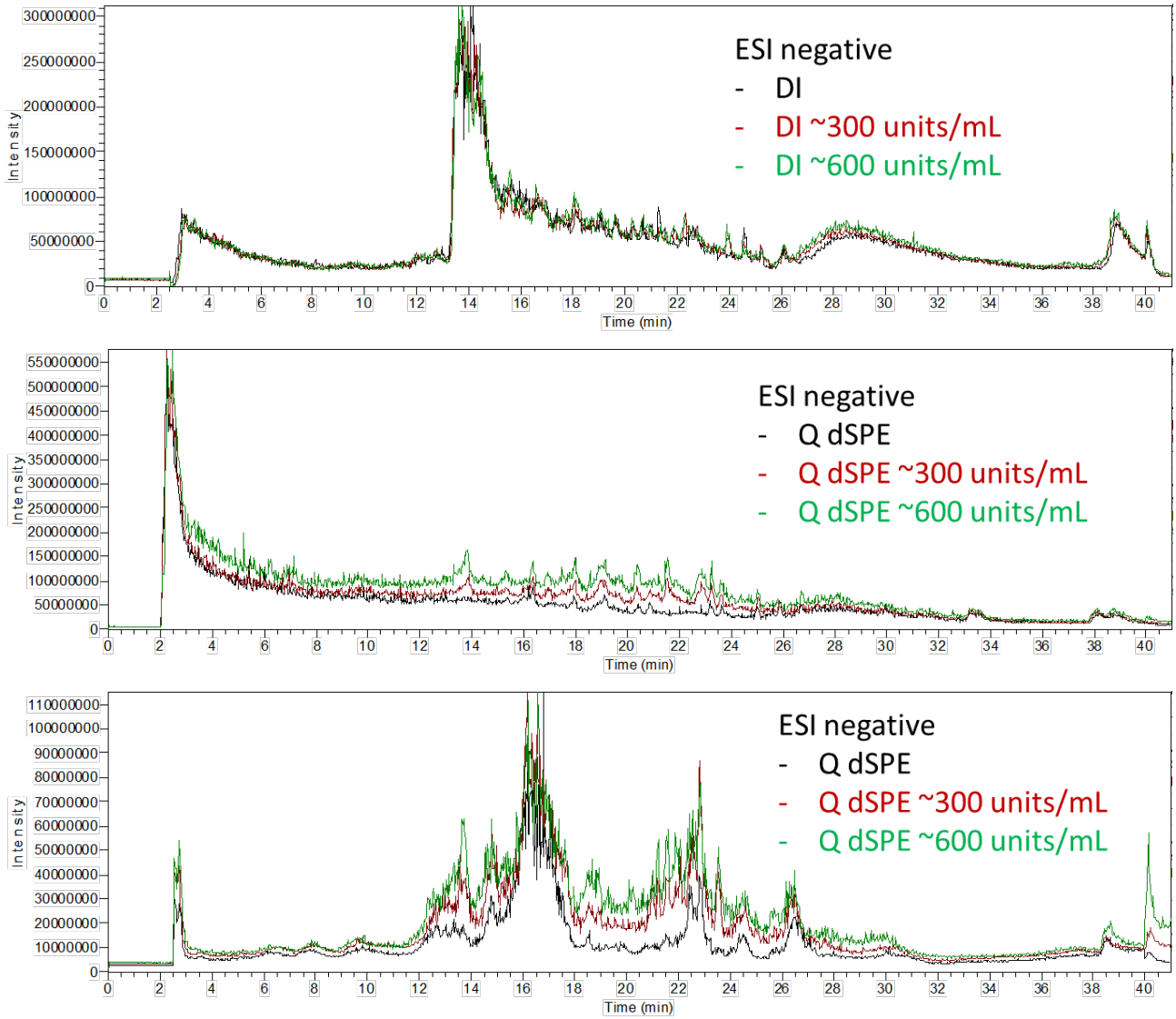

Table A 3: MRM transitions and instrument parameters for blood target analytes analysed on the LC-QTrap system.

Compound name	modus	Q1 (Da) > Q3 (Da)	DP (volts)	CE (volts)	CXP (volts)	instr LDC [ng/mL extract]
2,4-TDI	pos	175.0 > 147.0	161	17	16	< 1
		175.0 > 77.0	161	37	10	
		175.0 > 131.9	161	21	14	
CMIT	pos	149.9 > 86.9	81	51	10	5
		149.9 > 134.9	81	31	14	
		149.9 > 114.9	81	27	12	
(citronellal)	pos	155.0 > 113.8	1	11	18	
		155.0 > 72.8	1	25	20	
		155.0 > 109.0	1	15	12	
(resorcinol)	pos	111.0 > 55.0	1	21	14	
		111.0 > 68.8	1	13	18	
		111.0 > 92.9	1	21	28	
4,4'-thiodianiline	pos	217.0 > 123.9	1	29	16	< 1
		217.0 > 200.0	1	25	22	
		217.0 > 79.9	1	57	18	
4,4'-methylendi-o-toluidine	pos	227.1 > 120.0	126	33	12	< 1
		227.1 > 77.0	126	73	10	
		227.1 > 178.0	126	35	10	
solvent yellow	pos	198.1 > 77.0	101	25	12	< 1
		198.1 > 50.9	101	67	12	
		198.1 > 104.9	101	19	12	
o-dianisidine	pos	245.0 > 230.0	156	25	12	< 1
		245.0 > 187.0	156	43	10	
		245.0 > 213.0	156	25	10	
TCEP	pos	284.8 > 222.9	91	17	26	< 1
		284.8 > 98.9	91	29	16	
		284.8 > 160.9	91	21	18	
TBBPA	neg	540.6 > 417.6	-125	-54	-19	10
		540.6 > 445.7	-125	-46	-19	
		540.6 > 443.7	-125	-46	-21	
PhIP	pos	224.8 > 115.0	161	65	22	< 1
		224.8 > 113.9	161	63	22	
		224.8 > 112.9	161	87	24	
(mercaptobenzothiazol)	neg	165.8 > 133.8	-85	-28	-11	
		165.8 > 57.9	-85	-54	-7	
		165.8 > 101.9	-85	-28	-11	
2-(methylthio)benzothiazole	pos	181.9 > 166.9	91	31	20	< 1
		181.9 > 122.9	91	45	14	
		181.9 > 108.9	91	47	12	
p-toluene-sulfonamide	neg	169.8 > 106.0	-75	-24	-5	< 10

Compound name	modus	Q1 (Da) > Q3 (Da)	DP (volts)	CE (volts)	CXP (volts)	instr LDC [ng/mL extract]
		169.8 > 78.9	-75	-34	-11	
		169.8 > 79.9	-75	-28	-9	
TMDD	pos	224.9 > 98.9	146	25	16	1
		224.9 > 160.9	146	15	8	
		224.9 > 162.9	146	15	16	
carbendazim	pos	192.0 > 159.9	61	27	8	< 1
		192.0 > 131.9	61	41	14	
		192.0 > 105.0	61	49	12	
diazinon	pos	305.0 > 169.0	136	29	8	< 1
		305.0 > 153.0	136	29	16	
		305.0 > 96.9	136	45	12	
thiabendazole	neg	199.8 > 172.8	-85	-30	-21	1
		199.8 > 140.9	-85	-48	-9	
		199.8 > 171.8	-85	-46	-17	
PFBA	neg	212.8 > 168.9	-10	-12	-11	5
		212.8 > 168.3	-10	-16	-55	
PFOA	neg	412.9 > 368.8	-10	-14	-47	1
		412.9 > 168.8	-10	-24	-19	
		412.9 > 218.8	-10	-22	-27	
PFTDA	neg	712.8 > 668.8	-35	-20	-29	< 10
		712.8 > 218.9	-35	-34	-13	
		712.8 > 268.8	-35	-32	-15	
PFOS	neg	498.6 > 79.7	-90	-130	-9	1
		498.6 > 229.8	-90	-50	-13	
		498.6 > 98.7	-90	-130	-11	
MeFOSA	neg	511.9 > 218.8	-200	-38	-21	1
		511.9 > 169.0	-200	-38	-21	
		511.9 > 268.8	-200	-38	-21	
(PFOPA)	neg	498.8 > 78.8	-200	-38	-21	1
		498.8 > 62.9	-200	-38	-21	
BPA	neg	227.0 > 212.0	-90	-24	-7	< 10
		227.0 > 132.9	-90	-32	-15	
		227.0 > 211.0	-90	-38	-15	
triclosan	neg	286.9 > 35.0	-60	-8	-18	10
		288.8 > 141.7	-60	-44	-17	
ethyl paraben	neg	164.9 > 91.9	-70	-28	-11	1
		164.9 > 136.9	-70	-20	-7	
		164.9 > 92.8	-70	-26	-9	
butyl paraben	neg	193.0 > 91.8	-80	-32	-11	< 1
		193.0 > 135.8	-80	-22	-15	
		193.0 > 136.9	-80	-22	-13	
(tetrachlorosalicylanilide)	pos	348.9 > 272.8	76	21	14	

Compound name	modus	Q1 (Da) > Q3 (Da)	DP (volts)	CE (volts)	CXP (volts)	instr LDC [ng/mL extract]
		348.9 > 139.0	76	29	6	
quaternium 15	pos	215.0 > 172.0	26	17	20	< 10
		215.0 > 101.9	26	29	12	
		215.0 > 42.0	26	53	10	
benzylQUAT	pos	303.9 > 90.9	96	53	10	< 1
		303.9 > 212.2	96	29	10	
		303.9 > 65.0	96	97	8	
trimethylQUAT	pos	171.9 > 60.0	81	27	16	< 1
		171.9 > 43.0	81	41	12	
		171.9 > 41.0	81	51	10	
michlers ketone	pos	269.0 > 148.0	151	29	14	< 1
		269.0 > 76.9	151	75	14	
		269.0 > 120.0	151	41	12	
phenolphthalein	pos	318.9 > 225.0	-75	-18	-11	< 1
		318.9 > 114.9	-75	-24	-11	
		318.9 > 141.0	-75	-32	-17	
cinnamate	pos	291.0 > 161.0	101	25	20	2
		291.0 > 179.0	101	11	10	
		291.0 > 133.0	101	43	16	
homosalate	pos	263.0 > 230.9	26	19	12	< 10
		263.0 > 74.9	26	43	10	
		263.0 > 155.0	26	31	18	
Internal Standards						
BPA-d16	neg	242.2 > 224.2	-200	-38	-21	
PFOS-13C4	neg	502.9 > 79.7	-200	-38	-21	
		502.9 > 98.7	-200	-38	-21	
PFBA-13C3	neg	215.8 > 171.9	-200	-38	-21	
chlormequat-d9	pos	131.1 > 68.1	130	44	10	
		131.1 > 95.1	130	44	10	
p-toluene-sulfonamide-d4	neg	174.1 > 110.0	-200	-38	-21	
		174.1 > 79.9	-200	-38	-21	
		174.1 > 78.9	-200	-38	-21	
carbendazim-d4	pos	196.0 > 163.9	130	44	10	
		196.0 > 135.9	130	44	10	
DEET-d3	pos	195.2 > 93.0	130	44	10	
PhIP-d3	pos	227.8 > 115.0	130	44	10	
		227.8 > 113.9	130	44	10	
		227.8 > 112.9	130	44	10	
diazinon-d10	pos	315.0 > 163.0	130	44	10	
		315.0 > 96.9	130	44	10	
		315.0 > 169.0	130	44	10	

Figure A 2: Recoveries of internal standards in blood and urine samples (error bars represent the standard deviation).

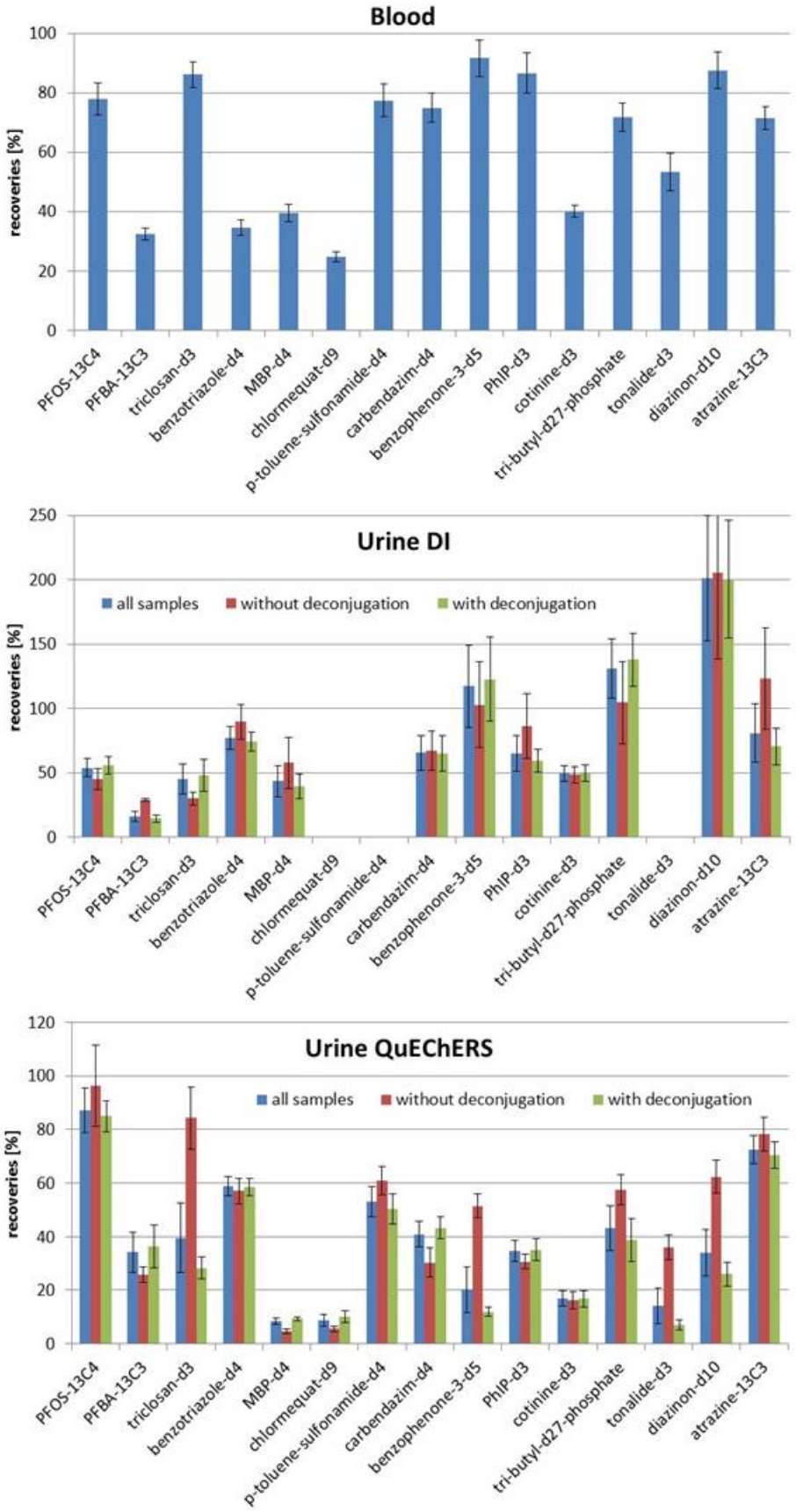

Figure A 3: Extracted ion chromatograms of the calculated m/z values for dihydroxy-benzophenone-sulfate and glucuronide analysed by DI in the still conjugated extract of sample 21.