DETERMINATION OF POLYBROMINATED DIPHENYL ETHERS AND STUDY OF CHROMIUM BEHAVIOR IN HUMAN BLOOD

Matic Bergant

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Prof. Dr. Janez Ščančar, Jožef Stefan Institute, Ljubljana, Slovenia

Evaluation Board:
Prof. Dr. Maja Ponikvar-Svet, Chair, Jožef Stefan Institute and IPS, Ljubljana, Slovenia
Prof. Dr. Jernej Iskra, Member, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia
Prof. Dr. Aleš Podgornik, Member, Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL

Matic Bergant

DETERMINATION OF POLYBROMINATED DIPHENYL ETHERS AND STUDY OF CHROMIUM BEHAVIOR IN HUMAN BLOOD

Doctoral Dissertation

DOLOČITEV POLIBROMIRANIH DIFENIL ETROV IN ŠTUDIJA OBNAŠANJA KROMA V ČLOVEŠKI KRVI

Doktorska disertacija

Supervisor: Prof. Dr. Janez Ščančar

Ljubljana, Slovenia, September 2020

Acknowledgments

First, I would like to thank my supervisor Prof. Dr. Janez Ščančar for his guidance and support during my studies. Together with Prof. Dr. Radmila Milačič they gave me the opportunity to work in their lab. Without their encouragement and help I would not be able to finish this thesis.
I am also grateful for the financial support of the Ministry of Higher Education, Science and Technology of the Republic of Slovenia (Programme group P1-0143). This work was also supported by the European Union funded project European Human Biomonitoring Initiative – HBM4EU, Grant Agreement 733032 and the MASSTWIN project which received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement no. 692241.
I want to thank the members of the evaluation board: Prof. Dr. Maja Ponikvar-Svet, Prof. Dr. Jernej Iskra and Prof. Dr. Aleš Podgornik for reviewing the manuscript and evaluating the work of my thesis.
I want to thank Assist. Prof. Dr. Tea Zuliani, Dr. Kelly Peters, Dr. Janja Vidmar, Dr. Petra Novak and Dr. Ana Drinčić for their help and guidance when I started working in the lab.
I want to thank Katarina and Stefan for their help in these last three years. Together with Marija, they were a great company to work with.
I also want to thank dr. Jožko Cesar, who first gave me the opportunity and freedom to work in the lab on my own.
Lastly, I want to thank my family and friends for their help and support, especially during difficult times.

Abstract

Human biomonitoring is a scientific technique that allows us to assess whether and to what extent particular environmental substances have entered our bodies and how our exposure to them is changing over time. Polybrominated diphenyl ethers (PBDEs) and Cr(VI) were both identified as priority substances in the European human biomonitoring initiative (HBM4EU) because of their well-known toxicity and presence in the environment. In order to reliably determine these substances in different environmental and biological samples, simple, sensitive and robust analytical methods must be developed. Such methods provide important data to our knowledge on the behavior of potentially toxic substances in the environment and in the human body.

Polybrominated diphenyl ethers (PBDEs) are flame retardants that are added to a wide range of consumer products. Due to their extensive use in the past, their presence has been documented in multiple environmental compartments and living organisms, including humans. Most analytical procedures for the determination of PBDEs in human serum require large amounts of sample and tedious clean-up steps that consume large amounts of organic solvents. Therefore, a new simple, reliable, and sensitive method was developed for the determination of six PBDE congeners (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, BDE 154) in human serum by gas chromatography–inductively coupled plasma mass spectrometry (GC-ICP-MS). The PBDEs were extracted from 1 mL of serum by 30 min of mechanical shaking with 1 mL of formic acid. Subsequently, 2 mL of iso-octane was added and 30 min of mechanical shaking applied. For clean-up of the extract, Florisil column was applied. The analytical method was validated by analysis of human serum standard reference materials SRM 1957 (Non-Fortified Human Serum) and SRM 1958 (Fortified Human Serum). The results showed that the method is accurate, repeatable and reproducible. The limits of detection (LODs) for the PBDEs analyzed were between 0.0016 and 0.0039 ng mL^{-1} wet weight (ww). The feasibility of the method was tested by analyzing human serum samples. The determined concentrations were similar to those reported for certain other European countries.

Although the transport and interactions of Cr species with blood constituents have attracted the interest of researchers, there are only a few studies available on Cr speciation in the human serum. Therefore, in order to investigate the kinetics of interactions of Cr(VI) and Cr(III) with serum constituents, and to perform Cr speciation at physiological concentration levels, a novel analytical method based on monolithic convective interaction media (CIM) chromatography coupled to UV and inductively coupled plasma mass spectrometry (ICP-MS) detectors was developed. Cr(VI) was separated from Cr-transferrin (Cr-Tf) and Cr-albumin (Cr-HSA) on a CIM diethylamino (DEAE) column using linear gradient elution from 100% buffer A (50 mM Tris-HCl + 10 mM $NaHCO_3$, pH 7.4) to 60% buffer B (buffer A + 2 M NH_4Cl) in 10 min at a flow rate of 1 mL min^{-1}. Good column recovery of separated Cr species (close to 100%) and satisfactory method repeatability (RSDs below 8%) was obtained. Kinetics of interaction of Cr(VI) and Cr(III) with serum constituents was followed after the human serum was doubly spiked with enriched ^{50}Cr(VI) and ^{53}Cr(III) solutions, and speciation analysis applied from 5 min up to 48 h after spiking.

The results showed that in the serum, ^{53}Cr(III) rapidly interacted with Tf, while ^{50}Cr(VI) reduction was slow. 48 h after spiking, more than 90% of added ^{53}Cr(III) was bound to Tf and the remaining 10% associated with HSA. About 20% of spiked ^{50}Cr(VI) was still present in the serum, while the resulting ^{50}Cr(III) was bound predominantly to Tf. High sensitivity of the developed speciation procedure enabled the detection of the Cr-Tf complex as the main Cr species in the human serum at physiological concentration levels.

Povzetek

Humani biomonitoring je znanstvena metoda, ki nam omogoča, da ocenimo, ali so določene spojine iz okolja vstopile v naše telo, v kakšnem obsegu in kako se naša izpostavljenost tem spojinam spreminja skozi čas. Polibromirane difenil etre in Cr(VI) spojine smo v Evropski skupnosti zaradi njihove strupenosti ter pogoste pojavnosti v okolju uvrstili na prioritetni seznam Evropske iniciative za humani biomonitoring (HBM4EU). Za zanesljivo določitev omenjenih spojin v bioloških vzorcih in vzorcih iz okolja potrebujemo robustne analizne metode, ki morajo biti dovolj preproste in občutljive. Novo razvite metode pomembno prispevajo k novemu znanju o obnašanju preučevanih spojin v okolju in človeškem organizmu.

Polibromirani difenil etri so zaviralci gorenja, ki so dodani velikemu številu izdelkov široke potrošnje. Zaradi njihove obsežne uporabe v preteklosti so pogosto prisotni v okolju in živih organizmih, vključno s človekom. Večina analiznih postopkov za določitev polibromiranih difenil etrov v krvnem serumu zahteva velike količine vzorca. Del postopkov so zahtevne metode čiščenja ekstrakta, kjer se porabi velike količine organskih topil. V okviru doktorske naloge smo razvili zanesljivo, preprosto in občutljivo metodo za določitev šestih analogov PBDE v človeškem serumu z uporabo plinske kromatografije in masne spektrometrije z induktivno sklopljeno plazmo. Za ekstrakcijo PBDE iz 1 mL krvnega seruma smo uporabili 1 mL mravljinčne kisline in 30-minutno mehansko stresanje vzorca. Nato smo vzorcu dodali 2 ml izooktana ter ga mehansko stresali še dodatnih 30 minut. Ekstrakt smo očistili z uporabo kolon, polnjenih s Florisilom. Točnost analiznega postopka smo preverili z analizo standardnega referenčnega materiala SRM 1957 (ne-obogateni humani serum) in SRM 1958 (obogateni humani serum). Rezultati so pokazali, da je analizen postopek točen, ponovljiv in obnovljiv. Meje detekcije analiziranih spojin so bile med 0.0016 in 0.0039 ng mL^{-1}. Metodo smo uporabili za analizo realnih vzorcev seruma. Izmerjene koncentracije PBDE so bile v enakem velikostnem razredu kot v nekaterih drugih evropskih državah.

V preteklosti so raziskovalci že preučevali transport in interakcije kromovih zvrsti v človeški krvi. Malo pa je študij, ki so se ukvarjale s kromovo speciacijo v človeškem serumu. Da bi preučili kinetiko interakcij Cr(VI) in Cr(III) spojin v človeškem serumu in določili koncentracije posameznih kromovih zvrsti na fizioloških nivojih, smo razvili novo analizno metodo, ki temelji na uporabi monolitske kromatografije, sklopljene z UV in ICP-MS detektorjema. Cr(VI), Cr-transferin (Cr-Tf) in Cr-albumin (Cr-HSA) smo ločili na CIM dietilamino (DEAE) koloni z uporabo linearnega gradientnega spiranja kolone od 100 % pufra A (50 mM Tris-HCl + 10 mM $NaHCO_3$, pH 7.4) do 60 % pufra B (Pufer A + 2 M NH_4Cl) v 10 minutah pri pretoku 1 mL min^{-1}. Kromove zvrsti so se na koloni ločile z izkoristkom, ki je bil blizu 100 %, ponovljivost metode pa je bila sprejemljiva z RSD pod 8 %. Kinetiko interakcij Cr(VI) in Cr(III) v serumu smo spremljali v časovnem intervalu od 5 minut do 48 ur po dodatku obogatenih izotopskih sledilcev ^{50}Cr(VI) in ^{53}Cr(III). Rezultati so pokazali, da se ^{53}Cr(III) hitro veže na Tf, medtem ko je redukcija ^{50}Cr(VI) počasna. Po 48 urah je bilo več kot 90 % dodanega ^{53}Cr(III) vezanega na Tf, preostalih 10 % pa na albumin. 20 % dodanega ^{50}Cr(VI) je bilo še vedno prisotnega v serumu, preostali

reducirani ^{50}Cr(III) pa je bil v večini vezan na Tf. Visoka občutljivost razvite analizne metode je omogočila detekcijo kompleksa Cr-Tf na fizioloških koncentracijskih nivojih.

Contents

List of Figures **xvii**

List of Tables **xix**

Abbreviations **xxi**

1 Introduction **1**
1.1 Human Biomonitoring ... 1
1.2 PBDEs ... 2
1.2.1 Production, use and regulations of PBDEs ... 3
1.2.2 Toxicology ... 3
1.2.3 Occurrence of PBDEs in the environment ... 4
1.2.3.1 Exposure of humans to PBDEs ... 6
1.2.3.1.1 Body burden ... 6
1.2.3.1.2 Temporal body burden trends ... 7
1.2.4 Analytical methods for determination of PBDEs ... 7
1.2.4.1 Extraction methods ... 7
1.2.4.2 Clean-up methods ... 8
1.2.4.3 Separation methods ... 8
1.2.4.4 Detection methods ... 9
1.3 Chromium ... 10
1.3.1 Chromium in the environment ... 11
1.3.2 Toxicity ... 11
1.3.2.1 Molecular mechanisms of toxicity of Cr(VI) ... 12
1.3.3 Legislation and regulation ... 12
1.3.4 Chromium in the human body ... 12
1.3.4.1 Redox chemistry ... 12
1.3.4.2 Essentiality ... 13
1.3.4.3 Absorption ... 13
1.3.4.4 Blood ... 13
1.3.4.5 Uptake into tissues ... 14
1.3.4.5.1 Cr(VI) ... 14
1.3.4.5.2 Cr(III) ... 14
1.3.5 Analytical methods for chromium speciation in blood ... 15
1.4 ICP-MS ... 15
1.4.1 Signal acquisition ... 15
1.4.2 Hyphenated systems ... 16

2 Aims and Hypothesis **19**

3 Materials and Methods **21**
3.1 PBDEs ... 21

3.1.1 Materials 21
3.1.2 Instrumentation 21
3.1.3 Cleaning procedure 22
3.1.4 Methods 22
3.1.4.1 Sample preparation 22
3.1.4.2 Total lipid content 22
3.1.4.3 Optimization experiments 22
3.1.4.4 Optimized analytical procedure 22
3.1.4.5 GC-ICP-MS parameters for separation and determination of PBDEs 23
3.2 Chromium 23
3.2.1 Materials 23
3.2.2 Instrumentation 24
3.2.3 Methods 25
3.2.3.1 Sample preparation 25
3.2.3.1.1 Buffer solutions 25
3.2.3.1.2 Serum samples and standard serum proteins 25
3.2.3.1.3 Preparation of ^{50}Cr(VI) and ^{53}Cr(III) isotopic spike solutions 26
3.2.3.2 Optimized analytical procedure for the determination of total Cr concentration in serum 26
3.2.3.3 Optimized analytical procedure for speciation of Cr in serum 26
3.2.3.4 Quantification of separated Cr species by the post-column ID-ICP-MS 26

4 Results and Discussion 29
4.1 PBDEs 29
4.1.1 Optimization of Extraction Procedure 29
4.1.1.1 Influence of different extraction agents on extraction efficiency 29
4.1.1.2 Selection of extracting agent volume 31
4.1.1.3 Influence of different modes of extraction on extraction efficiency 32
4.1.1.3.1 Microwave-assisted extraction 32
4.1.1.3.2 Mechanical shaking and ultrasound-assisted extraction 33
4.1.2 Optimization of analytical procedure for determination of PBDEs in non-spiked human serum 35
4.1.3 Performance of analytical procedure 35
4.1.3.1 Limits of detection and quantification, linearity, repeatability and reproducibility of measurements 35
4.1.3.2 Accuracy check 36
4.1.4 Analysis of PBDEs in human serum 38
4.2 Chromium 38
4.2.1 Method development and optimization 38
4.2.2 Kinetics of interactions of Cr(VI) and Cr(III) with serum constituents 40
4.2.3 Analytical figures of merit 42
4.2.3.1 Column recovery 42
4.2.3.2 Repeatability of measurement 42
4.2.3.3 Accuracy check for the determination of total Cr concentration in serum 42

4.2.3.4 Accuracy check for Cr speciation analysis in serum 43
4.2.3.5 Linearity of measurement 43
4.2.3.6 Limits of detection 44
4.2.4 Speciation of Cr in human serum at physiological concentration levels .. 44

5 Conclusions **47**

References **53**

Bibliography **63**

Biography **65**

List of Figures

Figure 1: Most prevalent PBDE congeners in the environment. 2
Figure 2: Transport and fate of PBDEs in the environment. 5
Figure 3: Schematic representation of ICP-MS system.....16
Figure 4: Influence of different extracting agents on signal intensities of PBDEs in spiked human serum in the first phase of extraction 30
Figure 5: Influence of different extracting agents on signal intensities of PBDEs in spiked human serum in the second phase of extraction 31
Figure 6: Influence of the amount of formic acid on signal intensities of PBDEs in spiked human serum 32
Figure 7: Influence of microwave-assisted extraction on signal intensities of PBDEs in spiked human serum 33
Figure 8: Influence of mechanical shaking and ultrasound-assisted extraction on signal intensities of PBDEs in spiked human serum 34
Figure 9: Chromatograms of (A) the mixture of six PBDE congeners and internal standard BDE 77 in iso-octane and (B) Non-Fortified Human Serum SRM 1957 spiked with internal standard BDE 77. 37
Figure 10: Typical chromatograms of separation of (A) Cr(VI) solution, (B) 5-times diluted mixture of serum proteins, (C) 5-times diluted Cr-Tf solution and (D) Cr-HSA solution... 39
Figure 11: Separation of Cr species of 5-times diluted human serum doubly spiked with 25 ng mL^{-1} ^{50}Cr(VI) and 20 ng mL^{-1} ^{53}Cr(III) 40
Figure 12: Kinetics of interaction of (A) ^{53}Cr(III) with serum proteins, and (B) reduction of ^{50}Cr(VI) and interaction of formed ^{50}Cr(III) with serum proteins.....41
Figure 13: Speciated isotope dilution of Cr in 5-times diluted spiked human serum 43
Figure 14: Overlays of chromatograms of (A) blank and 5-times diluted serum sample No. 3, (B) 5-times diluted serum sample No. 3 and sample No. 3 spiked with 1 ng mL^{-1} Cr(III), and (C) 5-times diluted mixture of serum proteins 45

List of Tables

Table 1: Total concentrations of PBDEs in seafood sampled in different countries. 6
Table 2: Most commonly used detection methods for the determination of PBDEs. 10
Table 3: GC and ICP-MS operation parameters. 23
Table 4: ICP-MS operating parameters for Cr speciation and determination of total Cr content 25
Table 5: LODs, LOQs and correlation coefficients (R^2) for the determination of six PBDEs in SRM 1958 . 36
Table 6: Extraction recoveries for PBDEs from SRM 1957 and SRM 1958. 36
Table 7: Concentrations of PBDEs in human serum samples (collected in 2017) 38
Table 8: Repeatability of measurement of Cr species 42
Table 9: Cr concentrations in human serum samples determined by ICP-MS and contents of Cr species associated with serum proteins determined by CIM DEAE-ICP-MS. 45
Table 10: Repeatability of measurement tested for SRM 1958.....49
Table 11: Reproducibility of measurement tested for SRM 1958.....50
Table 12: Total lipid content (TLS) in samples analyzed 50
Table 13: Median values of selected PBDE congeners in the human serum found reported for some European countries.....51

Abbreviations

APCI	...	Atmospheric pressure chemical ionization
BDE	...	Brominated diphenyl ether
CIM DEAE	...	Convective interaction media diethylamino
DFG	...	German research foundation
ECD	...	Electron capture detector
ECNI	...	Electron capture negative ionization
EI	...	Electron impact
FPLC	...	Fast protein liquid chromatography
GC	...	Gas chromatography
GPC	...	Gel permeation chromatography
HBM4EU	...	European Human biomonitoring initiative
HDPE	...	High-density polyethylene
HECM	...	High energy collision mode
HPLC	...	High performance liquid chromatography
HR	...	High resolution
HSA	...	Human serum albumin
ICP-MS	...	Inductively coupled plasma mass spectrometry
ID	...	Isotope dilution
LMM	...	Low molar mass
lw	...	Lipid weight
MAE	...	Microwave-assisted extraction
MES	...	Mechanical shaking
MS	...	Mass spectrometry
MS-MS	...	Tandem mass spectrometry
NIST	...	National institute of standards and technology
OEL	...	Occupational exposure limit
PBDE	...	Polybrominated diphenyl ethers
PCB	...	Polychlorinated biphenyl
PCDD	...	Polychlorinated dibenzo-p-dioxins
PCDF	...	Polychlorinated dibenzofurans
PLE	...	Pressurized liquid extraction
POP	...	Persistent organic pollutant
PTV	...	Programmable temperature vaporizing
RBC	...	Red blood cell
REACH	...	Registration, Evaluation, Authorization and Restriction of Chemicals
SFE	...	Super critical fluid extraction
SPE	...	Solid phase extraction
SRM	...	Standard reference material
Tf	...	Transferrin
TMAH	...	Tetramethylammonium hydroxide

UAE	...	Ultrasound-assisted extraction
UV-Vis	...	Ultra violet-visible
ww	...	Wet weight

Chapter 1

Introduction

1.1 Human Biomonitoring

Every day, humans are exposed to numerous environmental pollutants and other man-made chemicals present in the environment, food, water, cosmetics and other consumer products. Exposure can occur through different routes, such as inhalation, ingestion and dermal contact. The total body burden of a specific chemical depends on its concentration in the environment, its physical and chemical properties, timing of exposure and also on its pharmacokinetic properties such as uptake, metabolism and excretion rates. Because many of these chemicals can produce adverse effects on human health, we need to monitor their concentrations in the environment and also in humans [1].

Human biomonitoring is defined as "the method for assessing human exposure to chemicals or their effects by measuring these chemicals, their metabolites or reaction products in human specimens". Biomonitoring is performed in different bodily fluids, such as blood, urine, saliva, breast milk, sweat and also feces, hair, teeth, and nails. Biomonitoring data provide information on the total body burden or biological effect arising from all routes of exposure and interindividual differences in exposure levels, metabolism and excretion rates. This data is crucial for assessing the impact of chemicals on human health, especially for persistent organic pollutants (POPs), lead and cadmium which are stored in the body for longer periods. For chemicals that are excreted more rapidly, cross-sectional biomonitoring data can tell us only about recent exposure, while information about long-term exposure requires repetitive sampling. Biomonitoring can be used for acquiring spatial and time trends and identify lifestyle contributing factors and population groups that are at risk. It can also be employed in epidemiological studies together with health data to show an association between levels of pollutants in the body and their (adverse) health effects. Biomonitoring can thus be a valuable tool in policy decision-making [1].

The European human biomonitoring initiative (HBM4EU) has been launched in 2017 with the aim to address knowledge gaps and promote innovative approaches in human biomonitoring in Europe. In the first year of the project, nine prioritized substance groups were identified, among them polybrominated diphenyl ethers (PBDEs) that belong to a wider group of flame retardants, and also Cr(VI). Biomonitoring for PBDEs is well-established. Analytical methods are known, and analytical standards and reference materials are widely available. Data from a large number of studies in different European countries in a range of human matrices is available. Human serum and maternal milk are the two main matrices for analysis, as they are easily obtained and act as good proxies for assessing total PBDE body burden. On the other hand, biomarkers for Cr(VI) exposure are not well-established. After occupational exposure via inhalation, distribution of Cr(VI)

throughout the body allows biological monitoring of total Cr in urine, whole blood, plasma and red blood cells. Urinary, plasma and whole blood Cr levels are indicative of total Cr exposure while Cr levels inside the erythrocytes are indicative of Cr(VI) exposure. In any case, all these biomarkers of exposure are not sufficiently validated and advancements in this field are needed. Nevertheless, DFG (German Research Foundation) proposed two regulatory methods for biomonitoring of Cr in occupational setting. The first is the measurement of total Cr in urine, and the second is the measurement of total Cr in plasma, whole blood and erythrocytes [2], [3].

1.2 PBDEs

PBDEs are brominated aromatic compounds consisting of two phenyl rings linked by an ether bond. They are a group of persistent organic pollutants that were added to plastics, paints, varnishes and textile materials to reduce their inherent flammability. Since PBDEs are not covalently bound to the matrix of plastics, they are susceptible to leaching out of consumer products during their production, transport and life time. Therefore, the presence of PBDEs has been confirmed worldwide in all environmental compartments, including water, air, soil, sediments, animals and humans. In theory, there are 209 possible compounds, referred to as PBDE congeners. They differ in the number and position of bromine atoms attached to the ether skeleton. Congeners with equal numbers of bromine atoms are known as homologs. Chemical structures of the most prevalent congeners in the environment are shown in Figure 1.

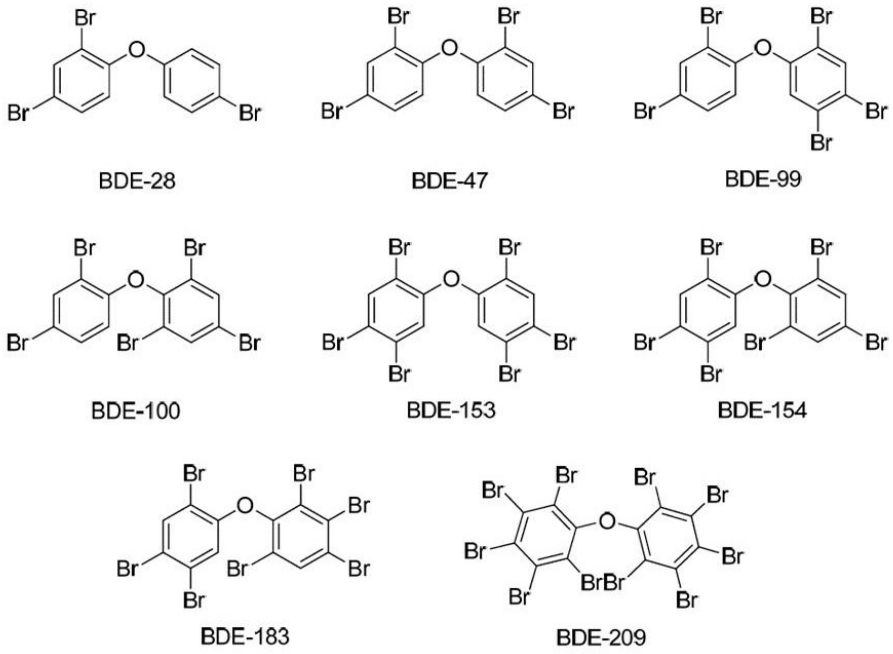


Figure 1: Most prevalent PBDE congeners in the environment.

PBDEs are semi-volatile compounds. Their volatility is decreasing with increasing molecular weight of PBDEs. They show a high resistance to chemical, physical and biological degradation. Nevertheless, higher brominated PBDEs are known to degrade to lower brominated PBDEs through debromination, especially in living organisms. PBDEs are non-ionizable compounds. Because of their low water solubility, they tend to bind to

organic fraction of particulate matter, soils and sediments and are capable of long-range transport, bioaccumulation and biomagnification [4].

1.2.1 Production, use and regulations of PBDEs

PBDEs have been produced and sold in three commercial mixtures: Penta, Octa and Deca-BDE, which are named after the dominating homologue group in the mixture. The total historical production of PBDEs has been estimated in 1.3– 1.5 million tons between 1970 and 2005. Penta-BDE mixture was used mainly in furniture (60 %) and in transport (36 %). Around 95 % of Octa-BDE supplied in the EU was used in the manufacture of acrylonitrile–butadiene–styrene resins. The main use of these resins was predominantly in housings of electrical and electronic equipment, particularly for cathode ray tube housings, and office equipment. Deca-BDE was employed as a back coating on products such as nylon, polypropylene, acrylics and polyester cotton.

In 1999, Norén and Meironyté first reported that the concentrations of PBDEs in the breast milk of Swedish women were increasing exponentially since the 1970s. Following this and other findings, the European Union and 10 US states introduced a ban on the use and production of Penta and Octa BDE commercial mixtures in 2004. In 2008, European Court of Justice also issued a ban on Deca BDE mixture for the use in electrical and electronic applications, while it is still allowed to use DecaBDE in plastics, according to the EU directive. In 2013, large-scale producers have ceased the manufacturing of all commercial mixtures. Despite regulations put in place, PBDEs will continue to enter the environment from existing products during their life-time. Combined with the fact that PBDEs are highly persistent pollutants, they will be present in the environment and living organisms for many years to come [4], [5].

1.2.2 Toxicology

Commercial PBDE mixtures showed low acute toxicity in rats. In contrast, sub-chronic and chronic toxic effects are well-documented. Numerous studies have demonstrated that PBDEs have the potential to adversely affect endocrine functions and the central nervous and reproductive systems. Animal studies showed that exposure to PBDEs during gestation or after birth can cause developmental and reproductive effects, including impaired spermatogenesis and changes in female reproductive organs and perturbation of thyroid hormone regulation. Most human epidemiological studies on thyroid homeostasis suggest an association between the exposure to PBDEs and altered thyroid hormone regulation. While exact mechanism is unknown, several studies have shown that PBDEs are potent competitors for thyroid hormone serum transporters. In addition, increased expression and activity of thyroid hormone clearance systems may also be involved. An association between neuropsychological functioning and exposure to PBDEs has also been described by epidemiologists. Motor, cognitive and behavioral performance, as well as mental and physical development were decreased in children that were exposed to higher levels of PBDEs. Cryptorchidism in male infants, low birth weights and abnormal levels of reproductive hormones were also associated with higher levels of PBDEs in human serum or breast milk. All this data raises concerns about infants' exposure to PBDEs which begins in utero and can increase during the first years of life from ingestion of breast milk and house dust [6].

1.2.3 Occurrence of PBDEs in the environment

PBDEs are contaminants that are ubiquitous in the environment. They are used primarily as additives and therefore migrate readily from products within which they are incorporated. These chemicals can be released continuously from products such as polyurethane, computers, television, and furnishings into indoor air and dust. At the end of their life time cycle, these products are then disposed in landfills, where temperatures can sometimes reach as high as 80–90°C, due to the heat released during aerobic degradation. Consequently, PBDEs are emitted to air via volatilization. Emissions of PBDEs are also plausible as a result of combustion of waste, either accidentally (e.g. landfill fires) or intentionally via incineration of municipal solid waste. At temperatures between 250 and 500°C, PBDEs are not completely destroyed and are able to transform to polybrominated dibenzo-p-dioxins and dibenzofurans. Recycling of products containing PBDEs can also be problematic. Particularly, uncontrolled electronic waste recycling in developing countries has been the ongoing primary source of PBDEs in the world [7]–[9]. Additional significant source of PBDEs may be municipal sewage. In municipal wastewater treatment plants, up to 90 % of PBDEs from wastewater end up in sewage sludge. This is then disposed to landfills or applied to land for fertilization and regeneration of soil. As we can see, sources of PBDEs are related to human activities and it was found that concentrations of PBDEs in air, water and soil are elevated near urban areas and are decreasing moving outward from the dense population areas. In non-urban locations, long-range environmental movements of PBDEs via air and water become more significant. PBDEs are partitioning between the gaseous and the particulate phase in the atmosphere and this is a key factor affecting their mobility and atmospheric fate. PBDEs have a tendency to bind to finer particles, which enables them to be transported over longer distances in the environment since fine aerosols experience less wet and dry deposition. Living organisms can also contribute to a long-range transport with their migration from contaminated sites where they acquire high levels of PBDEs to previously non-contaminated sites. This is known as “bio transport”. Because of long-range transport potential of PBDEs, they have been found in arctic environment and bioaccumulated in arctic organisms. Figure 2 depicts how PBDEs are transported in the environment. Major processes in fresh water ecosystems involve dry/wet deposition on fresh waters, air-water exchange, water sediment exchange and sediment-biota or water-biota exchange [10], [11].

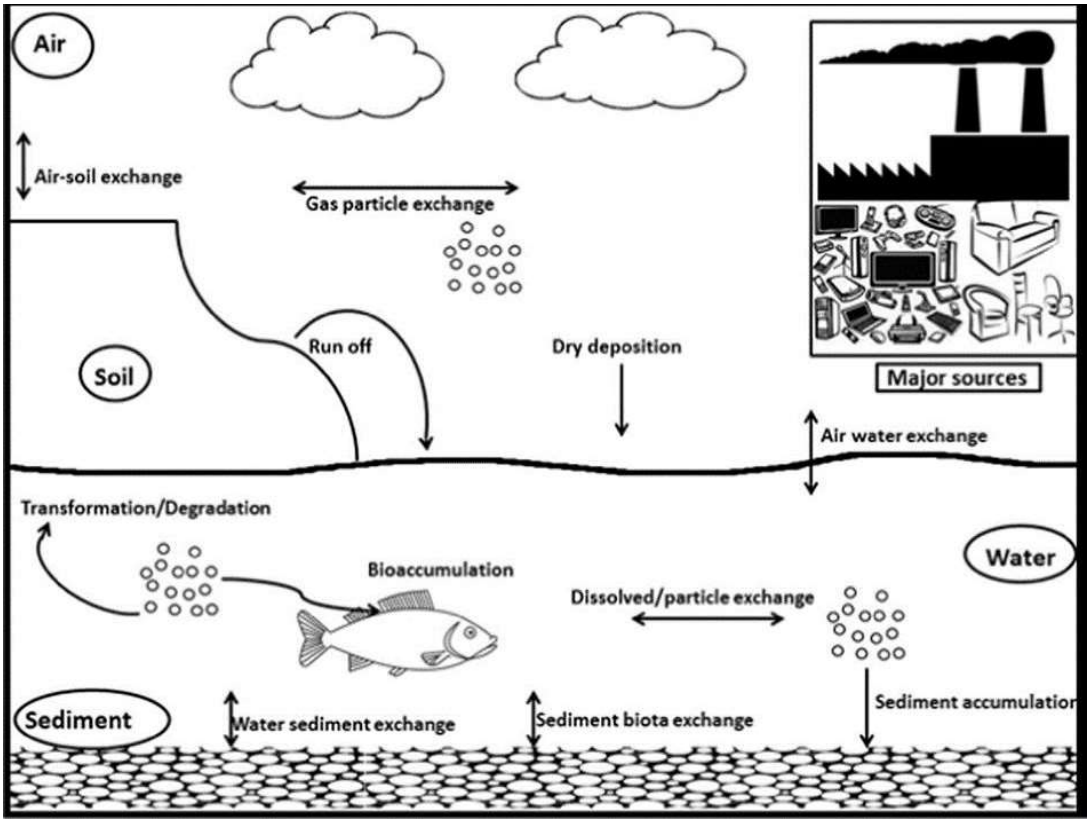


Figure 2: Transport and fate of PBDEs in the environment. Adapted from [11].

1.2.3.1 Exposure of humans to PBDEs

Humans are exposed to PBDEs mainly through the ingestion of food and dust and inhalation of air. Inhalation exposure occurs after PBDEs are released from household products into the air. The levels are dependent on the amount of PBDEs emitted into the air, room size, ventilation, exposure time, and inhalation rate. Electrical appliances, especially computers and televisions, are the major PBDE sources in indoor environments [12]. Ingestion of house dust can also be an important route of exposure, especially for young children because of their tendency to play on the floors and frequent hand-to-mouth contact. Ingestion of dust can lead to substantially higher exposures than average for a child with a high dust intake rate living in a home in which PBDE concentrations are elevated [13]. It has been widely accepted that indoor air and dust concentrations are higher in North America compared to Europe. This is due to higher flammability standards in North America, which led to higher amounts of PBDEs being applied to consumer products. Therefore, inhalation of contaminated air and ingestion of house dust is a major pathway of human exposure to PBDEs in North America. In Europe, ingestion of food is the predominant pathway [14], [15]. Among food items, seafood and fish in particular are considered to be the main source of exposure to PBDEs. Studies have demonstrated that the main factor for PBDE accumulation in fish is their lipid content. Other factors are trophic position, age of the fish, season and tissues chosen for analysis. Because of these factors, it is difficult to make comparisons between species in the same or different regions. Besides, most studies contain only data for the sum of PBDEs and not concentrations for specific congeners, which further hinders the ability to compare values. Nevertheless, to give an estimation of PBDEs in seafood, total concentrations of PBDEs are shown in Table 1. Other food products, such as butter, fats and oils, can contribute significantly to the total body burden of PBDEs, especially in people that consume very little seafood [16].

Table 1: Total concentrations of PBDEs in seafood sampled in different countries. Values are given in pg/g ww [16].

Matrix	Country	Σ PBDE (pg/g ww)
Clam	Japan	52.4
Shrimps	Belgium	51
Mussels	Canada	260
Cod	Belgium	40
Tuna	Japan	20.8
Herring	Denmark	2390
Salmon	USA	710
Salmon	Belgium	1570
Salmon	Japan	703
Mackerel	Japan	1443

.

1.2.3.1.1 Body burden

Numerous studies have been done worldwide in order to evaluate the human body burden of PBDEs. Sjodin et al. [17] conducted a large study in the United States which showed that geometric mean concentrations of BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, and BDE 154 in human serum were 1.2, 20.5, 5.0, 3.9, 5.7, and 2.3 ng/g lw, respectively. Levels of exposure in the United States are known to be approximately ten times higher

than those in Europe and China. Children under age 4 have higher concentrations of PBDEs compared to older children and adults due to the ingestion of mother's milk and higher exposure to house dust. The most commonly found congener in Europe and abroad was BDE 47, accounting for about 50% of total concentrations of PBDEs in the serum. It was followed by BDE 99 and 153, explaining about 10-20%. In Europe, total concentrations of PBDEs in human milk and serum are below 10 ng/g lw. In the United States, however, total concentrations in milk are near 100 ng/g lw, whereas in the serum, PBDEs are in a range of 30-100 ng/g lw. BDE 47 was the predominant congener in human milk, followed by either BDE 99 or BDE 153. Levels of PBDEs in adipose tissue are similar to levels in serum and milk. Concentrations in USA are higher compared to other countries. Also, BDE 47 predominates [4], [18]–[21].

1.2.3.1.2 Temporal body burden trends

In 1999, Swedish researchers published a study that showed an exponential increase in levels of PBDEs in human milk from 1972 to 1997. Similar findings were presented in a smaller Faroese study where a 4-fold increase of total concentrations of PBDEs was measured from 1987 to 1999 [22], [23]. Darnerud et al. [24] reported decreasing levels of BDE 47, 99, 100 in Swedish mothers in pooled serum from 1996 to 2010. Similarly, Lignel et al. [25] showed that most BDE congeners are decreasing in human milk from 2000 and forwards. It seems that the legislation put in place in 2004 rapidly resulted in reduced human exposure to PBDEs. Zota et al. [26] reported concentrations of PBDEs in two similar cohorts of pregnant women in California from 2009 and 2011. In two years, total concentrations of PBDEs dropped by 65%, from 90 to 55 ng/g lw. In contrast, the total concentrations of PCBs did not decline significantly in the same time. Because of structural similarity of PCBs to PBDEs, the authors suggested that levels of PBDEs may eventually plateau and persist for decades.

1.2.4 Analytical methods for determination of PBDEs

Given the fact that PBDEs are present in all environmental compartments and living organisms at trace level concentrations together with other persistent organic pollutants, the analytical methods for their determination must be highly sensitive and selective. They consist of sample collection, sample preparation (extraction, clean-up, pre-concentration) and instrumental analysis consisting of separation and detection by different hyphenated techniques.

1.2.4.1 Extraction methods

Extraction is a crucial step in the analysis of PBDEs as complex matrices such as sediments, biota, house dust, food and human tissues require careful optimization of multiple extraction parameters in order to achieve high extraction efficiency. The following parameters should be studied thoroughly [27]:

- Type of extracting agent: properties of the extracting agent determine its ability to release PBDEs from the sample matrix. Its role is to degrade the sample matrix in order to free PBDEs from the matrix into the organic solvent. Strong acids and bases are commonly used for this purpose.
- Type of organic solvent: properties of the solvent (density and polarity) determine its ability to penetrate the sample matrix. Its main role is to solubilize PBDEs and minimize the amount of coextracted interfering components. Toluene,

dichloromethane, hexane, iso-octane, acetone and their combinations are most commonly used for extraction of PBDEs.

- Time of extraction: extraction efficiency increases over time and reaches a plateau at a certain time point. Excluding Soxhlet extraction, most techniques can achieve efficient extraction in less than one hour.
- Temperature of extraction: extraction efficiency usually increases with increasing temperatures due to reduction of solvent viscosity that allows better permeability of solvent into the sample matrix. But too high temperature can cause degradation of higher brominated congeners.
- Mode of extraction: There are several techniques for the extraction of PBDEs that have been described in the literature. Soxhlet and multi-stage liquid-liquid extraction are slowly being replaced in recent years with more advanced techniques such as pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), solid phase extraction (SPE) and supercritical fluid extraction (SFE). For serum specifically, ultrasound-assisted extraction combined with solid phase extraction is used almost exclusively [28]–[32]. A few authors also report the employment of liquid–liquid extraction [33], [34].

1.2.4.2 Clean-up methods

Before the instrumental analysis, cleaning of PBDE extracts is often necessary in order to remove coextracted compounds such as PCBs, humic acids and lipids. For biota samples lipid removal is of crucial importance, otherwise column performance can be compromised over time. This can be achieved with destructive or non-destructive methods. Sulphuric acid or silica gel impregnated with potassium hydroxide are often used for destruction of lipids. This can also cause degradation of PBDEs so careful optimization of this approach is required. Lipids can also be removed by reducing their solubility in organic solvent by cooling the extracts in dry ice/acetone and later applying sulphuric acid. For non-destructive lipid removal, gel permeation chromatography (GPC) using a combination of silica gel and Florisil or polystyrene-divinyl benzene column is applied. Also, solid phase extraction (SPE) using Florisil or Alumina was reported to obtain good results. SPE is also used for selective removal of compounds such as PCBs and PCDD/Fs, which can produce interferences in detection of PBDEs. In this case, silica gel was found to be the best option, while Florisil and Alumina were not appropriate. Multi-layer silica columns (e.g. silica gel-acidic silica gel-silica gel-KOH-silica-silica gel) were also successfully employed for this purpose [35].

1.2.4.3 Separation methods

For separation of PBDEs, gas chromatography is used almost exclusively due to its higher inherent separation efficiency. It also provides better sensitivity compared to liquid chromatography, since PBDEs are poorly ionized using Atmospheric-pressure chemical ionization (APCI). Gas chromatography is usually performed using 15-30 meters long capillary columns with non-polar stationary phases. For injection of samples into the GC column, three injection systems are applied: splitless/pulsed splitless, on-column and programmed temperature vaporizing injector (PTV). When using splitless injection, injection port temperature is very important since higher PBDE congeners are known to degrade at high temperatures. For PBDEs, the temperature is usually between 250 and 300 °C. Therefore, long residence time in the liner should be avoided. The small volume (1-3µl) of sample that can be injected in splitless mode is considered to be the main limitation of this technique. This can be improved with pulsed splitless injection which allows higher volumes to be injected (up to 5 µl). Alternative injection technique that

allows injection of up to 125 µl of sample is PTV. It is most commonly used for human serum samples where low limits of detection are required. PTV injection is made in three steps: injection, solvent venting and splitless transfer of analytes. In the first two steps, the split exit is open to allow solvent removal with the temperature of inlet held around 50 °C. After this, the split vent is closed, the inlet temperature increased and the analytes are transferred to the column. PTV is especially suitable for PBDEs because of their low volatilities at low temperatures. The third injection technique used in PBDE analysis is the on-column injection. It is mainly used for analyzing higher PBDE congeners, especially BDE 209, which is most likely to degrade in a hot inlet. On-column injection requires clean extracts, since dirty samples will quickly deteriorate the column performance [27], [36].

1.2.4.4 Detection methods

Because of relatively low concentrations of PBDEs in environmental and biological samples, mass spectrometry is the main technique used for their detection. Electron capture negative ionization mass spectrometry (ECNI-MS) is most widely applied since ions formed there are bromine isotopes m/z 79 and 81. This provides very good selectivity, similarly to ICP-MS where the same ions are formed in the plasma. However, ICP-MS is rarely used for the detection of PBDEs. Electron impact mass spectrometry (EI-MS) has also been used often. It is considered useful due to relatively easy and low-cost maintenance of the instrumentation. It should be noted that using this technique additional sample preparation step is needed in order to remove PCBs that would otherwise interfere with the detection of PBDEs. Due to additional quadrupole mass filtering, electron impact tandem mass spectrometry (EI-MS-MS) does not suffer from any interfering compounds. It is also widely available and easy to operate. Although electron impact high resolution mass spectrometry (EI-HRMS) provides the best sensitivity and selectivity, it is not commonly used because of highly trained personnel required to operate it. Besides mass spectrometry, electron capture detector (ECD) is the only alternative for the detection of PBDEs. Despite low sensitivity, its low cost makes ECD a good solution for samples where higher concentrations of PBDEs are present. This technique is also prone to suffer interferences from other Cl and Br compounds. In Table 2, the most commonly used techniques for the detection of PBDEs are presented [27].

Table 2: Most commonly used detection methods for the determination of PBDEs.

Detection method	Disadvantages	Selectivity	Sensitivity	Cost
ECD	Cannot be applied for samples with low concentrations of PBDEs, interferences with Cl and Br compounds	Low	Medium	Low
EI-MS	Requires prior removal of interfering PCBs	Medium	Very good	Medium
ECNI-MS	Possible interferences from Br compounds	Very good	Very good	Medium
ICP-MS	Possible interferences from Br compounds	Very good	Very good	High
EI-MS-MS	/	Excellent	Very good	High
EI-HRMS	Highly trained personnel required	Excellent	Excellent	High

1.3 Chromium

Chromium is a first-row transition element and one of the most abundant metals on Earth. It is found in rocks, soils, volcanic dust and gases. Its electronic configuration allows it to exist in oxidation states from II- to VI+. Cr(0), Cr(III) and Cr(VI) are the most stable ones and therefore the most abundant. Cr(0) is present in various alloys, while Cr(III) and Cr(VI) can be found in the environment, biological fluids and various chemicals.

Chromium is produced from chromite ore. Primary products include chromium alloys with iron (thermal reduction) and sodium chromate (thermal oxidation). Secondary products include stainless steel and other Cr alloys, leather tanning solutions, medical devices, nutritional supplements, pigments, wood treatment and electroplating solutions.

The main non-occupational sources of exposure of chromium for the general population are traces in air, water and food. Consumer products, such as stainless steel and other Cr-coated surfaces, medical prostheses, tanned leather and Cr(III) nutritional supplements, also represent a part in the non-occupational exposure. Occupational sources of exposure are stainless steel welding, chrome plating, production of Cr(VI) pigments, corrosion inhibitors and wood treatment solutions [37], [38]. In the absence of known exposure, typical values reported for median total serum Cr concentration range from 0.2 to 0.5 ng mL^{-1} [39]–[41].

1.3.1 Chromium in the environment

Chromite (Cr_2O_3·FeO) is the main source of Cr(III) in soil and in ground waters. Water-soluble Cr(III) complexes, formed by interactions of chromite with ligands such as humic acids, are absorbed by plants and enter the food chain [37], [38], [42]. Cr(III) is sparingly soluble and relatively immobile. It can be bound with calcium and magnesium carbonates, iron and manganese oxides and hydroxides, sulphides, silicate minerals and organic molecules. Cr(VI) compounds with alkali and alkaline earth metals are highly soluble and mobile, but they are rarely found in the environment. Nevertheless, in soils, sediments and waters, Cr(III) can be oxidized by MnO_2 to Cr(VI). Oxidation can also occur with dissolved oxygen, but only at very high pH [43]. Another major source of Cr(III) and Cr(VI) is fly ash, which is produced during the burning of fossil fuels [44]. A lot of chromium is released into the air, water and terrestrial environments due to anthropogenic activities, especially by metallurgical, chemical and refractory brick industries. This represents an important environmental concern and a source of exposure for humans. Air concentrations of Cr in urban European areas were found to range from 4 to 70 ng m^{-3} and in industrial zones from 5 to 200 ng m^{-3}. One third of these emissions are thought to be Cr(VI). Even more concerning, indoor air concentrations of Cr due to smoking inside can reach up to 1000 ng m^{-3}. Sources of Cr in surface waters are surface runoff, deposition from air, and release of municipal and industrial wastewaters. Total chromium concentrations are generally few tens of ng mL^{-1} or lower in aquatic media. In rainwater, they usually range from 0.2 to 1 ng ml^{-1} and in groundwater and drinking water they span from < 1 ng mL^{-1} up to a few ng mL^{-1}. Because water treatment facilities use strong oxidants to potabilise water, drinking water can easily contain Cr(VI). Chromium levels in soils can vary up to three orders of magnitude, depending on the composition of the parent rock and local anthropogenic sources. Estimated total Cr concentrations in European agricultural soils were found to be quite high. From this study, 2.7% of soils were above 100 mg kg^{-1} and 1.1% of soils above 300 mg kg^{-1} [2].

1.3.2 Toxicity

Cr(VI) is established group 1 human carcinogen as it has been associated with an increased risk of lung and bronchial cancer in exposed industrial workers [45]. This is due to less efficient detoxification of Cr(VI) vapors and dust in lungs compared to the gastrointestinal tract [46]. Also, sialoglycoproteins and mucins that are present in saliva, nasal mucus, bronchial and lung fluids favor the formation of Cr(V) species which are relatively stable and highly genotoxic [47]. Manufacturing and use of Cr(VI) pigments, chrome electroplating and stainless steel welding represent the highest risk activities for exposed workers. Data on toxicity for other cancers and non-industrial exposure is inconclusive. Regarding different chromate salts, moderately water-soluble chromates (Ca, Sr, Zn) are more carcinogenic compared to highly soluble (Na, K, NH4) or insoluble (Pb, Ba) salts [45]. There has been a lot of controversy regarding the carcinogenicity of traces of Cr(VI) in ground water which are a result of environmental Cr(III) oxidation by MnO_2 and inappropriate Cr(VI) waste disposal. Recent data indicates that it does pose a significant health risk. The content of Cr(VI) in water can increase even more with chlorination or ozonation of water due to residual Cr(III) oxidation and removal of organic matter which would otherwise act as a reducing agent [48], [49]. Cr(III) and Cr(0) are considered non-toxic to humans based on all evidence, although some data from animal and cell studies raised the question about the potential danger of Cr(III) supplements due to the potential oxidation of Cr(III) in the body under chronic oxidative stress [50]–[52]. The use of Cr-Co alloys in hip prostheses has been shown to increase chromium levels in blood, which could

potentially lead to a higher risk of cancer and other diseases [53]. Allergies caused by Cr(0), Cr(III) and Cr(VI) remain a concern in chromium industries and general population [54]. Cr(VI) is also toxic to animals, plants and microorganisms, while Cr(III) is considered safe [55].

1.3.2.1 Molecular mechanisms of toxicity of Cr(VI)

Cr(VI) enters the cell via the sulphate/phosphate anion channel [56]. Once inside the cell, it is first confined to small areas in the cytoplasm and strongly co-localized with S, Cl, K and Ca. After some time, it spreads throughout the cell, and also reaches the nucleus [57]. This process is facilitated by binding of Cr(VI) to the positively charged residues of nuclear proteins, e.g. histones [58]. Reduction of Cr(VI) near the nucleus results in the formation of Cr(III) adducts with DNA. This can lead to abnormalities in gene transcription [59]. The similarity of CrO_4^{2-} to HPO_4^{2-} also causes binding of chromate to phosphate-metabolizing enzymes which leads to alterations in cell signaling. Other important cellular targets of Cr(VI) and Cr(V) include RNA, regulatory proteins and zinc finger proteins [60]–[62]. This can generate errors in gene regulation, DNA repair and cell signaling which can cause malignant transformation of the cell [59].

1.3.3 Legislation and regulation

To date, there is no EU regulation in regard to maximum levels of Cr in food items. Council Directive 98/83/EC and the Commission Directive 2003/40/EC set a maximum value of 50 ng mL^{-1} of Cr in drinking water and also in natural mineral waters. There is no specific limit for Cr(VI) however. EU proposed an OEL (Occupational exposure limit) for Cr(VI) at 25 µg $m3^{-1}$ in air. In addition, Cr(VI) is included in the revised Annex XIV to the EU REACH Regulation. Therefore, the use of chemicals containing Cr(VI) requires authorization. Since May 2015, a limit of 3 ppm of Cr(VI) has been set for leather products. Cr(VI) is also prohibited in cosmetics by the new EU Cosmetics Directive 76/768/EEC due to its allergenic potential. Finally, migration limits from toys for Cr(VI) have been set by the Toy Safety Directive 2009/48/EC.

Biological monitoring guidance levels for occupational exposure have been set on different national levels, but not by the EU. In Spain, 10 ng mL^{-1} of Cr in urine is the limit, measured during the shift and 25 ng mL^{-1} at the end of the workweek. In the UK, the limit of 10 µmol of Cr mol^{-1} creatinine in urine has been established at the end of the shift. DFG set the range of total Cr in urine from 10 to 40 ng mL^{-1}. For erythrocyte fraction, the range is from 9 to 35 ng mL^{-1} [2].

1.3.4 Chromium in the human body

1.3.4.1 Redox chemistry

Cr is most likely to be present in biological compartments (pH 7, aqueous solution) as Cr(III) or Cr(VI) [37], [38]. In normal circumstances, intra- and extra-cellular reduction of Cr(VI) to Cr(III) predominates, but oxidation of Cr(III) complexes to Cr(VI) can also occur under chronic oxidative stress [63], [64]. This is usually due to poorly controlled diabetes or chronic inflammation. In these redox reactions, reactive and relatively stable intermediates Cr(IV), Cr(V) and Cr(VI) are formed which are all bound to biological ligands. The most important ones are thiolato-Cr(VI) complexes, Cr(V) complexes with carbohydrates and glycoproteins, peroxide-Cr(V) complexes during Cr(VI) reduction with ascorbate in aerated aqueous solution and Cr(III) complexes with proteins, small ligands and DNA [47], [65]–[69]. Such complexes are known to damage DNA. In addition, reactive

oxygen species formed in these processes also contribute to cellular oxidative stress [62], [70]. Most common reductants in these redox reactions are glutathione, ascorbate, tocopherols and enzyme cofactors [56], [64–66]. Most common oxidants are hydrogen peroxide, peroxides of amino acids, proteins and lipids, hypochlorite and oxidized forms of metalloenzymes [74]–[76]. Dynamic redox equilibrium exists between Cr(III) and Cr(IV), Cr(V) and Cr(VI) which is dependent on the balance between the above-mentioned reductants and oxidants.

Cr(VI) species are stable at pH above 7, in the absence of strong reductants and chelators for Cr(III) [77], [78]. Cr(V) and Cr(IV) are more stable in a slightly acidic environment (pH 4-6) [63]. The majority of Cr(III) complexes are stable across the whole biological pH range [74].

1.3.4.2 Essentiality

Although there are a lot of claims about Cr(III) essentiality in the nutritional literature, considering all current evidence, Cr(III) is probably not an essential element [52], [79], [80]. With the advancement of analytical techniques, it became clear that chromium levels in the human body and in typical food sources are much lower than previously thought [81], [82]. Therefore, the effects of Cr(III) on the glucose metabolism in past studies were pharmacological, not nutritional [52]. Achieving chromium deficiency in experimental animals has been extremely difficult and no genetic Cr deficiency disease has been identified [52], [81]. There is also no conclusive evidence for Cr homeostasis and no functional Cr-containing molecule has been found unambiguously [52], [80], [81].

1.3.4.3 Absorption

Given the fact that the essentiality of chromium remains questionable, current recommended intake values for Cr are based on measurements of Cr content in typical food items [83]. Cr in food is ingested mainly as a complex with oxalic, citric acid or amino acids [55]. The extent of absorption of Cr in the gastrointestinal tract remains controversial. Some studies report low values of absorption (around 1%) for picolinato, nicolinato, propionato and amino acid complexes [84]. In contrast, there are also reports of higher absorption values (around 20%) for some amino acid complexes [85].

Experiments with monolayers of human intestinal cells suggested that Cr(III) crosses the intestinal barrier via passive diffusion [86]. On the other hand, Cr(VI) is capable of active transport using sulphate/phosphate ion channels. Although Cr(VI) is reduced in the gastrointestinal tract to a significant extent because of the acidic environment and food, part of it can still survive this reductive environment and pass into the blood as Cr(VI). Therefore, Cr(VI) in drinking water can represent a significant part of total chromium intake in humans [49]. No active regulatory mechanism (homeostasis) has been proven for the absorption of Cr from the gastrointestinal tract [87].

1.3.4.4 Blood

Cr(VI) in blood comes from four main sources: inhalation of Cr(VI) (e.g. welding, smoking), corrosion of Cr-based medical implants [88], regular ingestion of Cr(VI)-contaminated water [49] and oxidation of Cr(III) complexes (e.g. Cr supplements for weight loss or anti-diabetics) [74], [75], [78]. The erythrocyte fraction (RBC) is responsible for most of the reduction of Cr(VI) in blood due to the fast transport of Cr(VI) through RBC anion channels and much higher glutathione content of RBC compared to plasma [89]. The resultant Cr(III) is bound to the main RBC protein, hemoglobin [90], [91]. Because of kinetic inertness of Cr(III)-hemoglobin complex and inability of Cr(III) compounds to cross

from plasma to the RBC in a significant extent, the content of Cr in RBC can be used as a biomarker of a recent Cr(VI) exposure [92]. For example, distribution of chromium between plasma and RBC can tell us if the chromium from medical implants is leaching as Cr(III) or Cr(VI) [92]. Recent human studies involving implant-wearing patients suggested that chromium is released as Cr(III) [93]. On the other hand, older animal studies showed that chromium can be released as Cr(VI) under specific conditions (e.g. inflammation, chronic oxidative stress) [76].

Oral or intravenous administration of Cr(III) compounds to animals resulted in binding of Cr to plasma proteins, mainly transferrin, and little uptake by RBC [94]–[96]. The role of other compounds in Cr transport in blood, like albumin and LMM binders (small peptides, citrate, phosphate), still has to be elucidated [97].

1.3.4.5 Uptake into tissues

1.3.4.5.1 Cr(VI)

Cr(VI) compounds have a great ability of entering mammalian cells. This process is one of the most efficient of all metal complexes [63]. Soluble CrO_4^{2-} enters the cell mainly through Na/SO_4 co- transporter [56]. On the other hand, poorly soluble chromates can adsorb onto the surface of the cell and slowly dissolve and eventually cross the lipid bilayer [98]. Also, cells can transfer such chromates inside the cell via endocytosis [99]. After Cr(VI) enters the cell, it is reduced to Cr(III) mainly by glutathione and ascorbate, but also by proteins such as nitric oxide synthase, cytochrome b and metallothioneins [56], [64–66]. Once reduced, the majority of Cr(III) is bound to carboxylate, amine and imine residues of proteins [100]. These products are very similar across different human and animal cells originating from different tissues [50], [100]. They are also kinetically inert, therefore their efflux from the cell is very slow [59].

1.3.4.5.2 Cr(III)

Cr(III) enters the cell bound to transferrin via Tf receptor on the cell surface. After binding to the receptor, the complex enters the cell via endocytosis [97]. The mechanism of Cr release from the endosomes and its distribution in the cell afterwards remains unknown. The extent of uptake is similar to Fe(III), which shows that Cr(III) binding to Tf is selective [97], [101], [102]. It is expected that Cr(III) shares cellular metabolic pathways with other trivalent metals with no known biological function, e.g. Al(III) and Ga(III) [97], [103]. It is currently not known whether anionic Cr(III) complexes can enter the cell via anion transporter proteins, similar to Al(III). There are reports of high cellular uptake of some Cr complexes with small peptides [104], [105].

The study of cellular metabolism of Cr(III) nutritional supplements revealed that all Cr complexes underwent extensive ligand exchange prior to entering the cells. Therefore, none of the complexes remained intact. Further analysis showed that products in the cells and in the cell media mostly consisted of Cr(III)-amino acid and Cr(III)-protein complexes. These complexes were different from those resulting from Cr(VI) uptake and its subsequent reduction [50].

Excretion of chromium is led by binding of Cr(III) inside the cell to chromodulin. Chromodulin is an oligopeptide composed of glutamate, aspartate, cysteine and glycine. It is capable of binding 4 equivalents of chromium ions. Once the complex is formed, it is excreted from the cell into the bloodstream and then into the urine as this species [106].

1.3.5 Analytical methods for chromium speciation in blood

Hopkins et al. [94] first reported that 99% of chromium in blood was associated with non-cellular components after $CrCl_3$ was given by stomach tube to rats. After performing starch gel electrophoresis, 90% of chromium in serum was associated with the beta globulin fractions and 80 % precipitated with transferrin. Next, using size exclusion chromatography, Sayato et al. [95] confirmed that chromium is bound mainly with transferrin in human serum. In addition, they also found that a small proportion of chromium in serum is associated with low molar mass (LMM) species. Vincent et al. [106] hypothesized that this species could be chromodulin. Bourget et al. [107] studied the distribution of Cr(III) complexes by combining cation and anion-exchange fast protein liquid chromatography (FPLC) in ^{51}Cr-labelled plasma. The results revealed that Cr(III) binds preferentially to transferrin (Tf) and to a significantly lesser extent to human serum album (HSA). After 1-6 hours, ^{51}Cr shifted from albumin to an unidentified low molar mass complex, but this was not characterized further.

1.4 ICP-MS

ICP-MS (inductively coupled plasma mass spectrometry) is a highly sensitive elemental detection technique. As the name suggests, the instrument is a combination of inductively coupled plasma and mass spectrometer. It is able to detect over 90% of elements in the periodic table at concentrations below 0.01 µg L^{-1}. It possesses a wide dynamic range and can be easily hyphenated online with gas chromatography, liquid chromatography and laser ablation systems. It can provide information on the isotopic composition and is therefore suitable for isotope dilution quantification. It is capable of simultaneous multielement and rapid quantitative analysis. These properties make ICP-MS a preferred instrumental technique for the use in metallomics, especially in on-line hyphenation with different separation techniques. ICP-MS also has some minor downsides. It is subject to spectral interferences, although most of them can be removed during or prior to sample analysis. It is a destructive analytical technique and requires a relatively large amount of sample, especially for measuring total concentrations. Finally, it is a rather expensive instrument compared to other elemental detection systems [108].

1.4.1 Signal acquisition

Liquid sample is introduced into the spray chamber by a peristaltic pump where it is turned into aerosol via a pneumatic nebulizer. Inside the spray chamber, bigger droplets are directed to waste and smaller droplets are introduced into the torch where the droplets are dried, vaporized, atomized and ionized by argon plasma. High-temperature plasma (cca. 10,000 K) is formed when argon gas inside the torch interacts with the magnetic field created by radiofrequency time-varying electric current passing through the copper coil which surrounds the torch. The torch made of three concentric quartz tubes is used to generate positively charged ions. Argon gas for the generation of plasma is delivered through the middle tube, the inner tube is for delivering sample aerosol and the outer tube is for cooling (also argon). After the sample is ionized inside the plasma, representative beam of ions is extracted into the low-pressure interface region (1 mbar) of the ICP-MS instrument through the sampler and skimmer cones. Next, the positive ions are separated from photons and neutral particles by electrostatic ion lenses and accelerated into collision reaction cell. There, removal of polyatomic interferences is done using helium gas and kinetic energy discrimination. After that, ions are filtered through the mass analyzer. There

are three main types of mass analyzers: quadrupole, sector field and time-of-flight. The most common is quadrupole, which is made out of four cylindrical rods arranged in a square. By altering the current, only ions with a specific m/z ratio are allowed to be transmitted, the rest are lost from the ion beam. Finally, ions reach the detector consisting of a series of dynodes with increasing potential. On every dynode, the signal is multiplied which enables detection of measurable signal from a single positive ion hitting the detector. The detector possesses high sensitivity, wide dynamic range and low background noise [108].

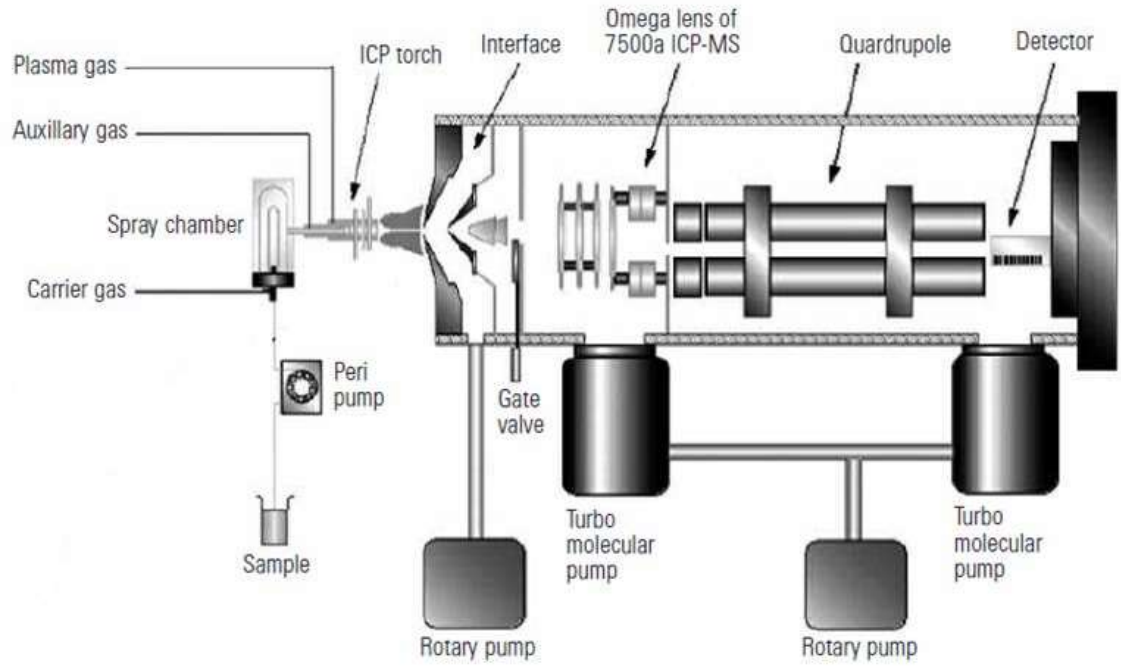


Figure 3: Schematic representation of ICP-MS system.

1.4.2 Hyphenated systems

ICP-MS by itself does not give information on the chemical or structural form of the analytes present in the sample. Therefore, hyphenated techniques are an excellent way to overcome this shortcoming. Hyphenation is achieved through coupling of ICP-MS with different separation techniques, normally chromatography. Most common ones are gas chromatography, liquid chromatography, ion chromatography and also capillary electrophoresis. In this way, elements or compounds in the sample are separated before elemental analysis. All hyphenated systems must meet certain conditions. First, the connecting interface must physically transfer the sample from the chromatographic system to the ICP-MS in a form that the plasma can tolerate without losing sample integrity. The temporal resolution of the sample analytes must not be changed significantly. Second, the chromatograph and ICP-MS have to communicate to permit synchronous separation and detection. Third, ICP-MS must be capable of acquiring transient signal at sufficient sampling frequency and over sufficient dynamic range to adequately measure all the species in all concentration ranges. Finally, tuning of ICP-MS should be done under conditions similar to those encountered during the chromatographic run.

For liquid samples, which contain non-volatile compounds or ions, liquid or ion chromatography is employed. The connection between the chromatographic column and

ICP-MS is straightforward. It is basically just an inert tube with the smallest possible internal volume compatible with the column flow combined with standard low dead volume HPLC fittings.

For gaseous samples, gas chromatography is applied. In this case, the transfer line between the outlet of the GC column and the tip of the ICP injector at the base of the plasma must be inert, short as possible and uniformly heated to adequate temperature to avoid sample condensation. This is accomplished by using two independent heated zones and extensive thermal insulation. Because typical GC column flows are too low to obtain annular plasma, 1 L min^{-1} of make-up gas is added inside the GC oven at the end of the GC column. This also helps to maintain uniformly high temperature inside the transfer line and sweeps the sample rapidly and inertly into the plasma. The addition of oxygen to the nebulizer gas flow is also recommended to avoid deposition of carbon on the sampler cone originating from the sample solvent [109].

Chapter 1

Aims and Hypothesis

Human biomonitoring is a scientific technique that allows us to assess whether and to what extent particular environmental substances have entered our bodies and how exposure to them is changing over time. It can provide valuable information on environmental exposures and identify potential health risks. Polybrominated diphenyl ethers (PBDEs) and Cr(VI) were both identified as priority substances in the European human biomonitoring initiative (HBM4EU) because of their well-known toxicity and presence in the environment. In order to reliably determine these substances in different environmental and biological samples, robust analytical methods that are simple and sensitive must be developed. Such methods contribute important data to our knowledge on the behavior of potentially toxic substances in the environment and in the human body.

Most analytical procedures for the determination of PBDEs in human serum require large amounts of sample and tedious clean-up steps that consume large amounts of organic solvents. Therefore, aims of the work were:

- To develop a simple, reliable, and sensitive analytical method for the determination of the six PBDE congeners (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, and BDE 154) by GC-ICP-MS in small amounts of human serum.
- To improve the extraction efficiency of PBDEs into iso-octane by testing different extracting agents.
- To lower the amount of serum used in the analytical procedure to facilitate large biomonitoring studies.
- To lower the amount of organic solvents used for cleaning the extract in order to simplify the procedure and to minimize the impact on the environment.

Although the transport and interactions of Cr species with blood constituents have attracted the interest of researchers, there are only a few studies available on Cr speciation in the human serum. The results revealed that Cr(III) binds preferentially to transferrin (Tf) and to a significantly lesser extent to human serum albumin (HSA) and low molar mass (LMM) species. The lack of literature data on speciation of Cr in human serum at physiological concentration levels is related mainly to very low total Cr concentrations. Typical values reported for median total serum Cr concentration range from 0.2 to 0.5 ng mL^{-1}. Therefore, aims of the work were:

- To develop a reliable analytical method for the separation of Cr species in the human serum by anion-exchange monolithic chromatography combined with ICP-MS.
- To investigate the kinetics of interaction of Cr(VI) and Cr(III) with human serum by the use of ^{50}Cr(VI) and ^{53}Cr(III) isotopic tracers.

- To test different buffers with adequate chemical purity to achieve low background levels of Cr and consequently perform speciation of Cr in the human serum at physiological concentration levels.

Chapter 2

Materials and Methods

3.1 PBDEs

3.1.1 Materials

All the reagents used were of analytical reagent grade. Ultrapure water (Milli-Q, 18.2 MΩ cm) obtained from a Direct-Q 5 Ultrapure water system (Millipore Watertown, MA, USA) was used.

Individual standards of seven BDE congeners (28, 47, 77, 99, 100, 153 and 154) at a concentration of 50 µg mL^{-1} were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Standard stock solutions (5 µg mL^{-1}) of the PBDEs were prepared in iso-octane and stored in the dark at 4 °C. The working standard solutions were prepared daily in acetone. The standard reference materials SRM 1957 (freeze-dried Non-Fortified Human Serum) and SRM 1958 (freeze-dried Fortified Human Serum) were purchased from the National Institute of Standards & Technology (NIST) (Gaithersburg, MD, USA). Formic acid, tetramethylammonium hydroxide (TMAH), hydrochloric acid (HCl), acetone, citric acid monohydrate, 25% potassium hydroxide (KOH) and tris(hydroxymethyl)aminomethane (Tris) were obtained from Merck (Darmstadt, Germany).

Hexane, methanol (MeOH), 2-propanol and iso-octane were purchased from J. T. Baker (Deventer, Holland). Tris-citrate buffer (pH 6.0) was prepared daily from a 0.2 mol L^{-1} solution of Tris with appropriate addition of citric acid. The absorbent columns (500 mg, 3 mL) for cleaning the extracts were Strata FL-PR Florisil (170 μ m, 80 A) purchased from Phenomenex, Inc. (Torrance, CA, USA).

3.1.2 Instrumentation

The analysis of the PBDEs was carried out on an Agilent 6890 GC Agilent Technologies (Santa Clara, CA, USA) equipped with an Agilent 6890 Series Autosampler Injector. The GC was coupled to an Agilent 7700x ICP-MS via a heated transfer line and fitted with a 15 m × 0.25 mm DB-5MS capillary column (film thickness 0.25 μ m) coated with 5% phenylmethylpolysiloxane (Agilent J&W Scientific, Palo Alto, CA, USA). Hyphenated instrumental set-up was controlled by Agilent MassHunter software.

The mechanical shaking of the samples was performed on a Vibromix 40 orbital shaker (elliptical table shaker) Tehtnica (Železniki, Slovenia), ultrasound-assisted extraction on a 550D VWR International ultrasonic bath (West Chester, PA, USA), and microwave-assisted extraction on a CEM MARS 6 microwave accelerated reaction system CEM

(Matthews, NC, USA). The sample extracts were centrifuged on Hettich Universal 320 Centrifuge Hettich GmbH & Co., KG (Tuttlingen, Germany).

3.1.3 Cleaning procedure

To prevent contamination, all the glassware was rinsed three times with tap water, soaked in 10% nitric acid for 48 h, rinsed three times with tap water and three times with MilliQ water and heated at 400 °C for 4 h. Prior to use, all the glassware was rinsed with hexane and acetone and dried at room temperature.

3.1.4 Methods

3.1.4.1 Sample preparation

Venous blood (venous puncture) from a transplanted renal patient was taken during clinical examination after informed consent was obtained, and collected into a Pyrex glass bottle without additives. After the collection, 10 mL aliquots of blood were transferred into glass centrifuge tubes and centrifuged for 10 min at 855 g. The serum aliquots were collected and stored at -20 °C. Prior to analysis, serum was thawed and equilibrated to room temperature.

3.1.4.2 Total lipid content

The total lipid content (TLS) was obtained on the basis of enzymatic assays by summing the individual lipid species measured: total cholesterol (TC) and triglycerides (TG), using the formula proposed by Bernet et al. [110].
(1)TLS = (2.27 x TC) + TG + 62.3 mg dL^{-1}

3.1.4.3 Optimization experiments

To study the influence of the different extraction agents on the extraction efficiency, to select the extracting agent volume and to verify the influence of the different modes of extraction on extraction efficiency, human serum was spiked with the six PBDE congeners (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153 and BDE 154) and BDE 77 as an internal standard, at a concentration of 27 ng g^{-1} each. The spiking procedure comprised the addition of standards (in acetone) to the serum and leaving the sample 15 min for the PBDEs' equilibration. If not stated otherwise, the same amount of PBDEs was applied in the spiking experiments.

3.1.4.4 Optimized analytical procedure

1 mL of human serum was transferred into a 50 mL glass reactor to which the internal standard BDE 77 (0.50 ng mL^{-1}) and 1 mL of formic acid were added. The vessels were closed and subjected to 30 min of mechanical shaking (20 °C, 300 rpm). Next, 2 mL of iso-octane was added and the samples mechanically shaken for additional 30 min (20 °C, 300 rpm). The organic phase containing the dispersed emulsion was collected into a 10 mL glass vial with a Pasteur pipette. For separating the emulsion and organic phase, 0.5 mL of 25% potassium hydroxide (KOH) in MeOH was added and the samples centrifuged for 5 min at 5000 g. The collected organic phase was passed through a Florisil column (preconditioned with 1 mL of iso-octane) for removing of the co-extracted lipids [111]. The column was washed with 2 mL of iso-octane and the entire organic phase concentrated under nitrogen stream to approximately 200 µL. Then, the samples were transferred to 2 mL amber vials containing glass inserts using Pasteur pipette. The samples were further

evaporated under a nitrogen stream to incipient dryness and reconstituted with 10 µL of iso-octane. 2 µL of iso-octane was injected onto the column and analysis performed by GC-ICP-MS. The concentrations of the PBDEs were calculated using the standard addition calibration method. To prepare the calibration curve, 1 mL of human serum was spiked with six PBDEs standards (in acetone) between 0.05 and 5 ng mL^{-1} for each BDE, whereas the concentration of BDE 77 (internal standard) remained constant (0.50 ng mL^{-1}). The samples were left for 15 min for PBDEs' equilibration. If not stated otherwise, all the analyses were conducted in triplicate.

3.1.4.5 GC-ICP-MS parameters for separation and determination of PBDEs

For separating PBDEs, the following GC temperature program was used: the temperature was raised from 120 °C to 300 °C at a heating rate of 30 °C min^{-1} and held there for 5 min. The inlet temperature and transfer line were held at 280 °C. Helium was used as the carrier gas and set at a flow rate of 1.5 mL min^{-1}. The injection mode was splitless and the injection volume 2 µL. The operating parameters of the GC-ICP-MS are presented in Table 3. Their optimization is described in the previous work of our research group [112].

Table 3: GC and ICP-MS operation parameters.

ICP-MS	Unit
RF power	1300 W
Sample depth	7.0 mm
Carrier gas (Ar)	1.50 L min^{-1}
Optional gas (20 % $^{v}/_{v}$ O_2 in Ar)	10 %
Integration time per isotope	0.1 s
Isotopes measured	^{79}Br, ^{81}Br
Tune gas	100 ppm Xe in Ar
Total acquisition time	666 s
GC	Unit
Injection volume	2 µL
Mode	Splitless
Carrier gas (He)	1.50 mL min^{-1}
Inlet temperature	280 °C
Transfer line temperature	280 °C

3.2 Chromium

3.2.1 Materials

Ultrapure water (Milli-Q, 18.2 MΩ cm) obtained from a Direct-Q 5 Ultrapure water system (Millipore Watertown, MA, USA) was used.

Tris(hydroxymethyl)aminomethane (Tris) ultrapure from Goldbio (St. Louis, Missouri, USA) and (4-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Sigma Aldrich (St. Louis, Missouri, USA) buffers were used. Hydrochloric acid (HCl) s.p., sodium hydrogen carbonate ($NaHCO_3$) p.a., ammonium chloride (NH_4Cl) s.p., 25% ammonia solution (NH_3) p.a. sodium chloride (NaCl) s.p. and sodium hydroxide (NaOH) p.a. were obtained from Merck (Darmstadt, Germany).

Human serum apo-transferrin (Tf, 77 000 Da), albumin (HSA, 66 000 Da) and immunoglobulins (IgG, 150 000 Da) were purchased from Sigma-Aldrich (Steinheim, Germany).

Merck stock Cr solution (1000 ± 2 mg L^{-1} in 5 % HNO_3) was used for the preparation of calibration standard solutions for the determination of the total Cr concentrations in serum samples. To control the stability of the ICP-MS, Ge and Rh (1000 ± 2 mg L^{-1} in 5 % nitric acid), both purchased from Merck, were aspirated along with the sample into the plasma by a peristaltic pump via a T-piece.

A natural abundance Cr(VI) solution (K_2CrO_4 in water) containing 1000 ± 2 mg L^{-1} Cr and a natural abundance Cr(III) nitrate nonahydrate ($Cr(NO_3)_3 \cdot 9H_2O$) were purchased from Sigma- Aldrich.

The enriched ^{50}Cr and ^{53}Cr isotopes in the form of enriched oxides ($^{50}Cr_2O_3$ and $^{53}Cr_2O_3$) were obtained from Oak Ridge National Laboratory (Oak Ridge, TN, USA) and were used for the preparation of ^{50}Cr(VI) and ^{53}Cr(III) isotopic spike solutions. The declared composition of enriched ^{50}Cr isotope was 96.82 ± 0.05% for isotope 50, 2.95 ± 0.02 % for isotope 52, 0.18 ± 0.01 % for isotope 53 and 0.05 ± 0.01% for isotope 54. The declared composition of enriched ^{53}Cr isotope was 0.03 ± 0.005 % for isotope 50, 2.65 ± 0.02 % for isotope 52, 97.20 ± 0.02% for isotope 53 and 0.12 ± 0.005% for isotope 54.

The accuracy of the determination of the total Cr content in serum was checked by the analysis of standard reference material Seronorm Trace Elements Serum L-2 from Sero (Billingstad, Norway).

3.2.2 Instrumentation

Chromatographic separations were performed using an Agilent (Tokyo, Japan) series 1200 quaternary HPLC pump equipped with Agilent 1260 Bio-inert Manual Injector fitted with a 0.5 mL or 1 mL high-density polyethylene (HDPE) injection loop. A UV-Vis detector (Agilent 1200 series diode array and multiple-wavelength detector, DAD/MWD) was used on-line with HPLC for absorption measurements at 278 nm. Weak anion-exchange convective interaction media diethylamino (CIM DEAE) short monolithic column disks (12.0 mm i.d, length 3.0 mm, bed volume 0.34 mL, seated into a nonporous self-sealing fitting ring, matrix support made of highly porous polyglycidyl methacrylate-co-ethylene dimethacrylate, pH stability 3–9) from Bia Separations (Ajdovščina, Slovenia) were used for the separation of Cr species. The outlet of the UV-Vis detector was coupled on-line with an Agilent 7700x ICP-MS instrument. ICP-MS was used also for the determination of the total Cr content in serum samples. Data was processed with Agilent MassHunter software. The ICP-MS operating parameters were optimized for plasma robustness and for introducing minimum amounts of salts used in the separation procedure (Table 4).

To eliminate polyatomic interferences of chlorine and carbon at m/z 52 and 53, the High Energy Collision Mode (HECM) was applied, using helium as a collision gas [113].

A Mettler Toledo MS104 (Zürich, Switzerland) analytical balance was used for the weighing.

A WTW (Weilheim, Germany) 330 pH meter was employed to determine the pH.

Table 4: ICP-MS operating parameters for Cr speciation and analysis of total Cr content.

Parameter	Cr speciation analysis	Analysis of total Cr content
Sample introduction		
Nebulizer	Miramist	Miramist
Spray chamber	Scott	Scott
Skimmer and sampler	Ni	Ni
Plasma conditions		
Forward power	1550 W	1550 W
Plasma gas flow (Ar)	15.0 L min^{-1}	15.0 L min^{-1}
Carrier gas flow (Ar)	0.40 L min^{-1}	1.00 L min^{-1}
Dilution gas flow (Ar)	0.80 L min^{-1}	/
Make up gas flow (Ar)	/	0.15 L min^{-1}
Total carrier gas flow	1.20 L min^{-1}	1.15 L min^{-1}
He gas flow	10 mL min^{-1}	10 mL min^{-1}
Oct bias	-100 V	-100 V
Cell entrance	-129 V	-100 V
Cell exit	-150 V	-150 V
Deflect	-74 V	-75 V
Plate bias	-150 V	-150 V
Sample uptake rate	1.0 mL min^{-1}	0.3 mL min^{-1}
Data acquisition parameters		
m/z of isotopes monitored	^{50}Cr, ^{52}Cr,^{53}Cr	^{52}Cr
m/z of internal standards*	^{72}Ge, ^{103}Rh	^{72}Ge, ^{103}Rh
Total acquisition time	600 or 1200 s	/

*In speciation analysis, internal standards were not used when the ID-ICP-MS procedure was applied

3.2.3 Methods

3.2.3.1 Sample preparation

3.2.3.1.1 Buffer solutions

The following buffer solutions were used in the chromatographic procedure: Buffer A was composed of 50 mM Tris-HCl + 10 mM $NaHCO_3$ (pH 7.4). Buffer B was composed of buffer A + 2 M NH_4Cl (pH 7.4). Buffer C was composed of 0.2 M Tris-HCl (pH 7.4). When HEPES was used instead of Tris, pH was adjusted to pH 7.4 with 25% NH_3 solution.

3.2.3.1.2 Serum samples and standard serum proteins

Venous blood from 5 individual transplanted renal patients was taken during clinical examination by venous puncture after informed consent was obtained (this blood is normally discarded). The sample was centrifuged for 10 min at 855 g. Serum aliquots were

transferred into 2 mL polyethylene tubes with a polyethylene pipette and stored in a freezer at -20 °C.

Standard serum proteins (25 g L^{-1} of HSA, 5 g L^{-1} of IgG and 2.5 g L^{-1} of Tf) used for the optimization of the analytical procedure were dissolved in buffer A.

Before the speciation analysis, serum samples and standard serum proteins were diluted 5-times with buffer A.

3.2.3.1.3 Preparation of ^{50}Cr(VI) and ^{53}Cr(III) isotopic spike solutions

^{50}Cr(VI) and ^{53}Cr(III) isotopic spike solutions were prepared from ^{50}Cr and ^{53}Cr enriched oxides according to the procedure previously developed in our laboratory [114]. The declared composition of the enriched ^{50}Cr and ^{53}Cr isotopes is provided in 3.2.1.

3.2.3.2 Optimized analytical procedure for the determination of total Cr concentration in serum

Before the analysis, serum samples were diluted 5 times with water and Cr concentration determined by ICP-MS using the standard addition calibration method.

3.2.3.3 Optimized analytical procedure for speciation of Cr in serum

0.5 mL of 5-times diluted spiked serum proteins or spiked serum sample (20–25 ng mL^{-1} of Cr(VI) or Cr(III), or mixture of both) was injected onto the column. 5-times dilution of serum prior to speciation analysis is necessary due to high serum density. To separate Cr species, linear gradient elution from 100% buffer A to 60% buffer B was applied for 10 min at a flow rate of 1 mL min^{-1}. The eluate from the CIM DEAE column was passed through a UV detector (set at 278 nm) for protein monitoring and was introduced to ICP-MS. Quantification of the separated Cr species was based on the peak area using the post-column ID-ICP-MS or standard addition calibration method. After the separation step, the column was regenerated by rinsing with 100% buffer C for 3 min at a flow rate 6 mL min^{-1}, followed by elution with buffer B for 3 min at a flow rate 6 mL min^{-1}. After that, the column was equilibrated with buffer A at a flow rate of 6 mL min^{-1} for 5 min. Finally, the column was rinsed for 0.5 min with buffer A at a flow rate of 1 mL min^{-1}. The eluate from the regeneration and equilibration steps was directed to waste through a software-controlled six-port valve. After approximately 30 injections of serum samples, rigorous cleaning of the CIM DEAE column was performed at a flow rate of 5 mL min^{-1} with 20 mL of 1 M NaOH, followed by rinsing with 20 mL of water, 20 mL of buffer C, 20 mL of 2 M NaCl, 20 mL of buffer C and finally with 20 mL of buffer A. After cleaning, the column was ready for further use.

To achieve adequate sensitivity for performing speciation analysis of Cr at physiological concentration levels, 1 mL of 5-times diluted sample was injected onto the column. The same analytical procedure as described above was carried out, with the exception that for separation of Cr species, linear gradient elution from 100% buffer A to 60% buffer B was applied for 20 min. The longer time was necessary for obtaining selective separation of serum proteins when applying a higher sample load onto the column. Due to a higher sample load, more frequent rigorous column cleaning was also required.

3.2.3.4 Quantification of separated Cr species by the post-column ID-ICP-MS

For quantification of separated Cr species by the post-column ID-ICP-MS, isotopically enriched ^{50}Cr(VI) (5.59 ng mL^{-1}) was added continuously by a peristaltic pump via a T-piece after the separation of Cr species. To calculate the content of the eluted Cr species,

the mass flow of Cr was plotted versus time throughout the chromatographic run. Calculations were performed using equations derived for species-unspecific post-column ID-ICP-MS analysis [115].

If not stated otherwise, all the experiments were performed in duplicate.

Chapter 3

Results and Discussion

4.1 PBDEs

4.1.1 Optimization of Extraction Procedure

The human serum consists of water, proteins, lipids, electrolytes, hormones, nutrients and waste products [116]. To release hydrophobic PBDEs from such a complex matrix, efficient extraction and clean-up procedures must be developed prior to instrumental analysis by GC–ICP-MS. Hence, the effect of different extracting agents and modes of extraction on the selected PBDEs' extraction efficiencies was systematically studied and the co-extracted lipids from the organic phase removed by the clean-up procedure (using Florisil) that was developed previously by our group [111].

Due to the potential adverse effects of PBDEs on humans, particularly neonates and children, there is increasing demand for sensitive analytical methods that require low amounts of serum. Most of the published methods that have achieved satisfactory detection limits and accuracy require between 2 and 5 mL of serum [28]–[30], [117]. An exception is the method developed by Lin et al. who used 0.5 mL of serum [31]. However, it should be noted that this method was suitable for the determination of levels of PBDEs in North America, which are approximately 10 times higher on average than those observed in the European countries. ICP-MS, which was applied in the present study, is a highly sensitive and selective detector. Therefore, the possibility of achieving satisfactory limits of detection (LODs) for the selected PBDE congeners by using only 1 mL of serum for the analysis was investigated. This volume is also low enough to obtain a minimal amount of blood samples from the general population.

4.1.1.1 Influence of different extraction agents on extraction efficiency

First, the effect of four different extracting agents on the extraction efficiency, selected on the basis of the cited literature, was examined. For the extraction, 1 mL of formic acid, 1 mL mixture of formic acid in 2-propanol (4:1, v/v), 2.5 mL of 0.1 M HCl in MeOH or 5 mL of 25% TMAH were added to 1 mL of spiked human serum as described in Section 3.1.4.3. Subsequently, a specified extracting agent was added and the samples mechanically shaken for 2 h (first phase of extraction). Then, 15 mL of Tris-citrate buffer (pH 6) and 2 mL of iso-octane were added and the samples mechanically shaken for another 2 h (second phase of extraction). The dispersed emulsion formed in the organic phase was separated from iso-octane by adding 25 % KOH in MeOH and passed through a Florisil column (preconditioned with 1 mL of iso-octane) for removing the co-extracted lipids, as described in Section 3.1.4.4. The clear organic phase was collected and 2 µL of it injected onto GC–ICP-MS for analysis. In the first extraction step, the PBDEs were quantitatively extracted

from the serum by partial degradation of the sample matrix, whereas in the second extraction phase, the PBDEs released from the matrix into the aqueous phase were extracted into the organic phase (iso-octane). The instrumental signal intensities were compared for assessing the extraction efficiency of a specified extraction agent. The results are shown in Figure 4, which shows that formic acid and formic acid in 2-propanol (4:1, v/v) extract heavier PBDE congeners more efficiently than the other extracting agents. There was no significant difference in the extraction efficiency between formic acid and formic acid in 2-propanol (4:1, v/v). Considering practicality, formic acid was chosen as the extracting agent in the first phase of extraction.

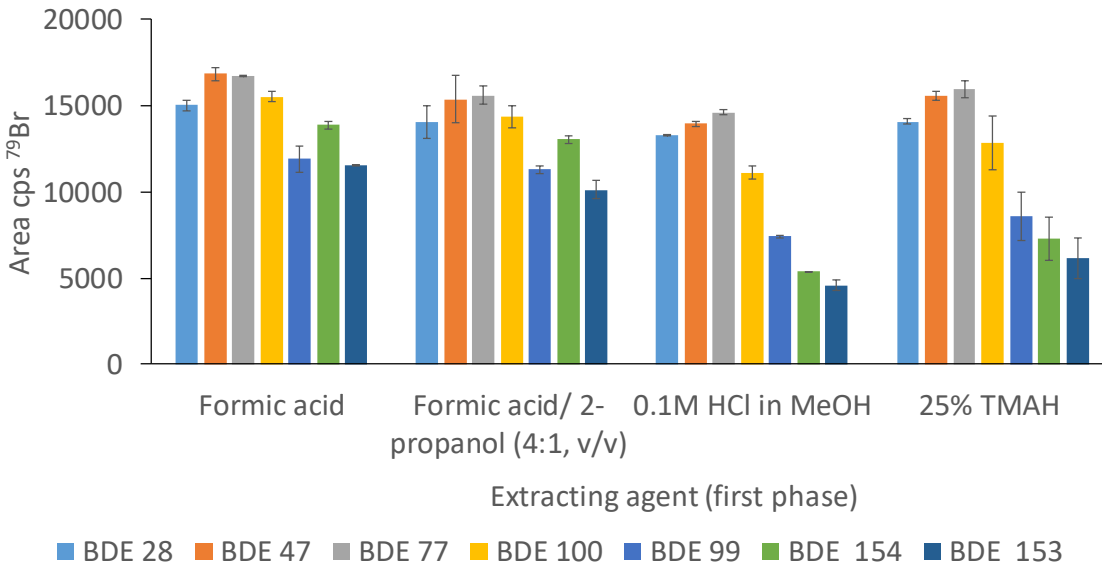


Figure 4: Influence of different extracting agents on signal intensities of PBDEs in spiked human serum (27 ng mL^{-1} each). In the first phase of extraction, samples were mechanically shaken for 2 h with different extracting agents. In the second phase, Tris-citrate buffer and iso-octane were added and samples mechanically shaken for another 2 h. Results represent the average ± standard deviation of three replicates.

After selecting the extracting agent for the first phase of extraction, the impact of Tris-citrate buffer (pH 6), which was observed to facilitate the extraction of PBDEs from complex sample matrices, such as fish and sewage sludge, was studied [111], [118]. To find out if Tris-citrate buffer also facilitates the extraction of PBDEs from the serum, 15 mL of this buffer and 2 mL of iso-octane were added to serum samples after the first phase of extraction. For comparison, 15 mL of MilliQ water (to keep the same volume as in the case of Tris-citrate buffer) and 2 mL of iso-octane, or 2 mL of iso-octane solely, were added to the serum samples, which were mechanically shaken for additional 2 h. The organic phase was collected and cleaned up before the instrumental analysis. The resulting GC–ICP-MS signal intensities are presented in Figure 5. It is evident that the addition of Tris-citrate buffer exerts no significant beneficial effect on the extraction efficiency. Hence, 2 mL of iso-octane without co-extracting agent was added in the second phase of the extraction procedure.

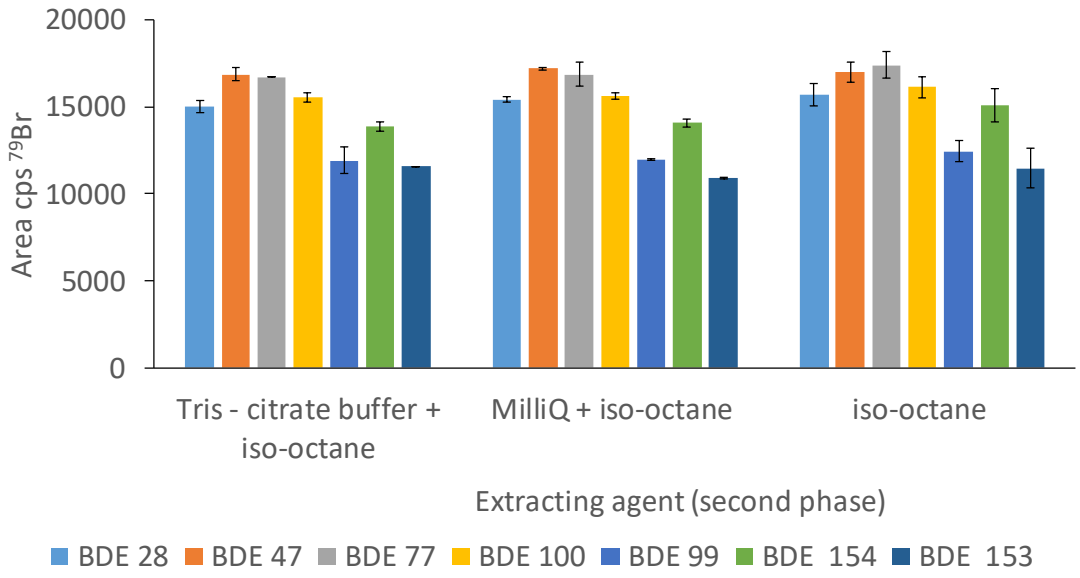


Figure 5: Influence of different extracting agents on signal intensities of PBDEs in spiked human serum (27 ng mL^{-1} each). In the first phase of extraction, samples were mechanically shaken for 2 h with formic acid. In the second phase, Tris-citrate buffer and iso-octane, MilliQ water and iso-octane or iso-octane were added and samples mechanically shaken for another 2 h. Results represent the average ± standard deviation of three replicates.

4.1.1.2 Selection of extracting agent volume

To select the optimal volume of extracting agent 0.5, 1 or 3 mL of formic acid were added to 1 mL of spiked serum. The samples were mechanically shaken for 2 h and for another 2 h after adding 2 mL of iso-octane. Before the analysis, the organic phase was collected and cleaned up. The results are presented in Figure 6. The addition of 0.5 mL of formic acid resulted in the formation of a thick slurry (due to the small volume of formic acid). Consequently, it was not possible to collect the organic phase (data are not shown). The data from Figure 6 exhibit no significant difference in signal intensities between using 1 and 3 mL of formic acid for extracting PBDEs from serum. Therefore, 1 mL of formic acid was used in subsequent experiments.

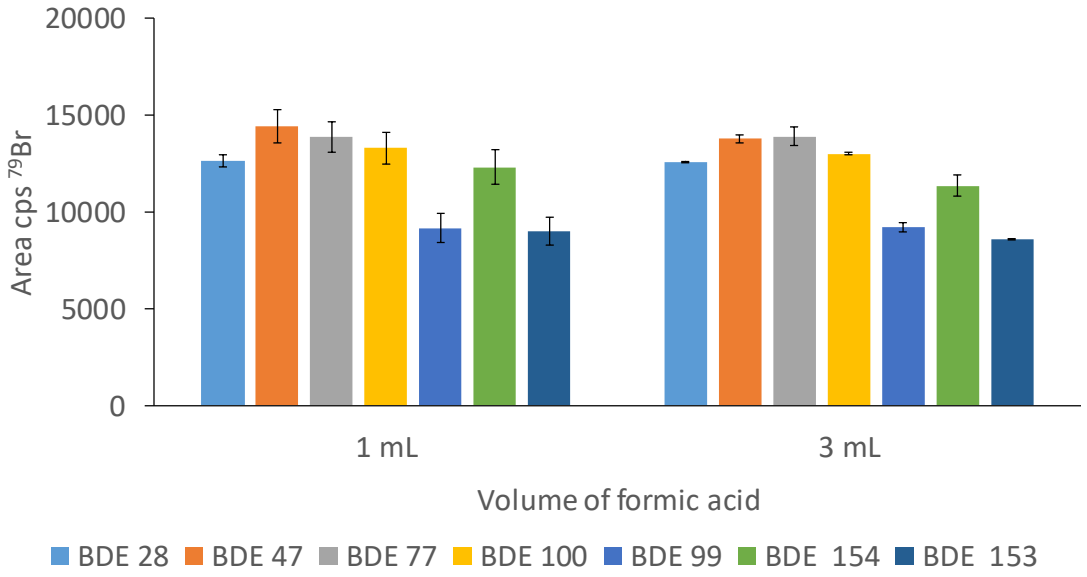


Figure 6: Influence of the amount of formic acid on signal intensities of PBDEs in spiked human serum (27 ng mL^{-1} each). In the first phase of extraction, samples were mechanically shaken for 2 h with different volumes of formic acid. In the second phase, iso-octane was added and samples mechanically shaken for another 2 h. Results represent the average ± standard deviation of three replicates.

4.1.1.3 Influence of different modes of extraction on extraction efficiency

To select the optimal mode of extraction, microwave-assisted extraction (90 °C, 1200 W) mechanical shaking (20 °C, 300 rpm) and ultrasound-assisted extraction (20 °C, 240 W) were applied over different time intervals and their efficiencies compared.

4.1.1.3.1 Microwave-assisted extraction

To optimize microwave-assisted extraction, 1 mL of formic acid was added to 1 mL of spiked human serum in Teflon vessels. The vessels were closed and the PBDEs extracted in accordance with the extraction method wherein the temperature was gradually increased over 2 min to 90 °C (1200 W) and held there for 3, 5, or 10 min. Subsequently, 2 mL of iso-octane was added, and the extraction was continued at 90 °C (ramp to 90 °C in 2 min) for 10 min. The organic phase was collected and cleaned up before the instrumental analysis (Figure 7A). As can be seen, there is no significant difference in signal intensities when the temperature was maintained at 90 °C for 3 or 5 min, whereas when maintained for 10 min, the signal intensities started to decrease. Hence, 3 min of the extraction in the first phase was selected. In the second phase, 2 mL of iso-octane was added and the samples subjected to 1, 5, or 10 min of extraction at 90 °C. The organic phase was collected and cleaned up prior to instrumental analysis. These results are shown in Figure 7B. No significant differences in signal intensities were observed.

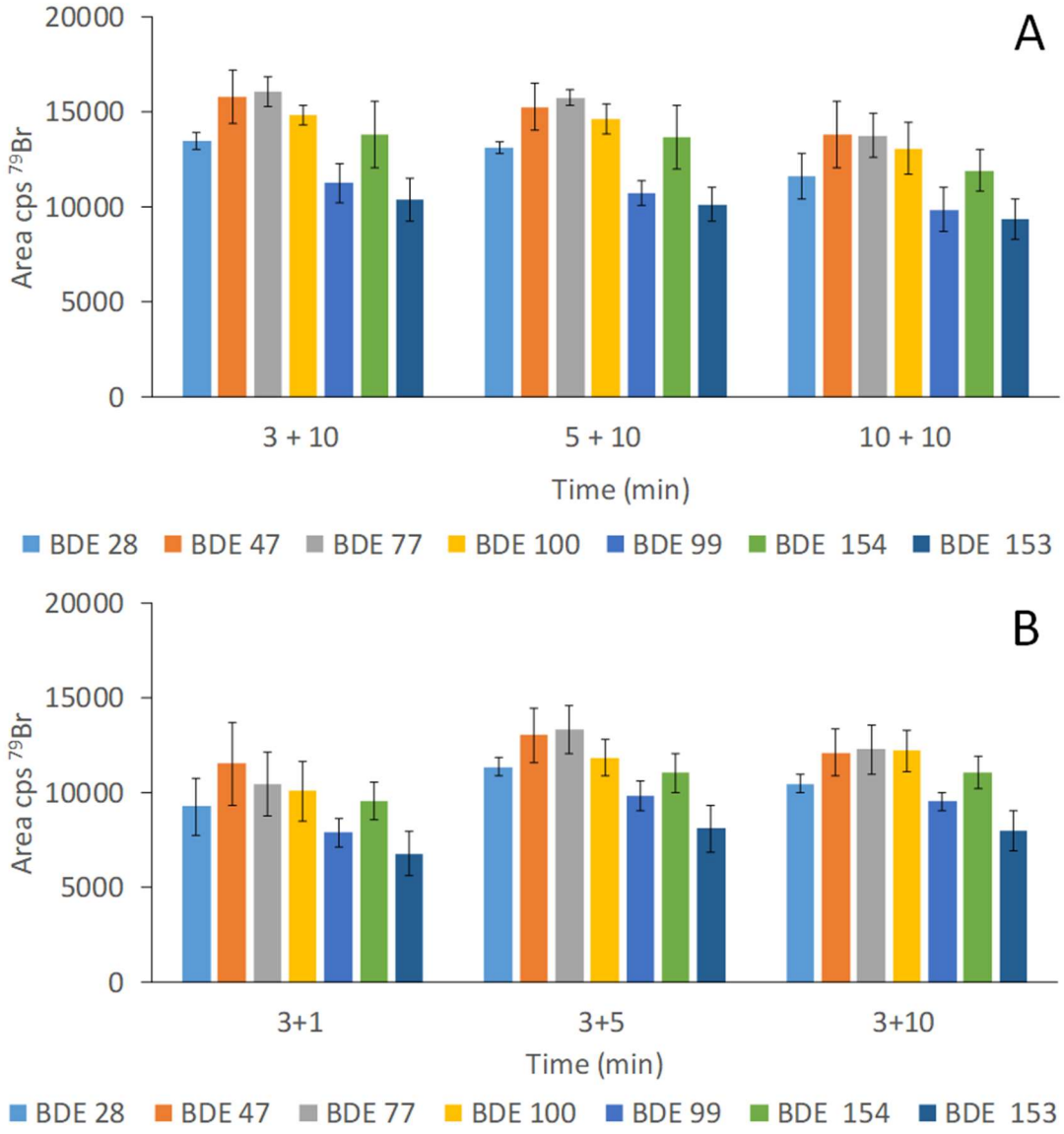


Figure 7: Influence of microwave-assisted extraction on signal intensities of PBDEs in spiked human serum (27 ng mL^{-1} each).

(A) In the first phase, samples were subjected to different time intervals of extraction with formic acid at 90 °C. In the second phase, iso-octane was added and the samples subjected to extraction for 10 min at 90 °C.

(B) In the first phase, samples were subjected to 3 min extraction with formic acid at 90 °C. In the second phase, iso-octane was added and the samples subjected to different time intervals of extraction at 90 °C.

Results represent the average ± standard deviation of three replicates.

4.1.1.3.2 Mechanical shaking and ultrasound-assisted extraction

To optimize mechanical shaking and ultrasound-assisted extraction, 1 mL of formic acid was added to 1 mL of spiked human serum. In the first phase, the influence of different time intervals on signal intensities was tested (30, 60 or 120 min for mechanical shaking,

or 15, 30, or 60 min for ultrasound-assisted extraction). In the second phase, iso-octane was added and the samples were subjected to 2 h of mechanical shaking. The organic phase was collected and cleaned up prior to instrumental analysis. These results are presented in Figure 8A. As can be seen, there are no significant differences in signal intensities between different times and modes of extractions applied. Due to the marginally better repeatability of measurements, 30 min of mechanical shaking in the first phase of extraction was selected. To optimize the second phase of extraction, 2 mL of iso-octane was added and the samples subjected to 30, 60, or 120 min of mechanical shaking or 5, 15, or 30 min of ultrasound-assisted extraction. The organic phase was collected and cleaned up prior to instrumental analysis (Figure 8B). It is evident that ultrasound-assisted extraction is inefficient for the extraction of PBDEs into iso-octane. The efficiency of mechanical shaking is significantly higher. No significant differences were observed between the different times of extraction, therefore, 30 min of mechanical shaking was selected as optimal.

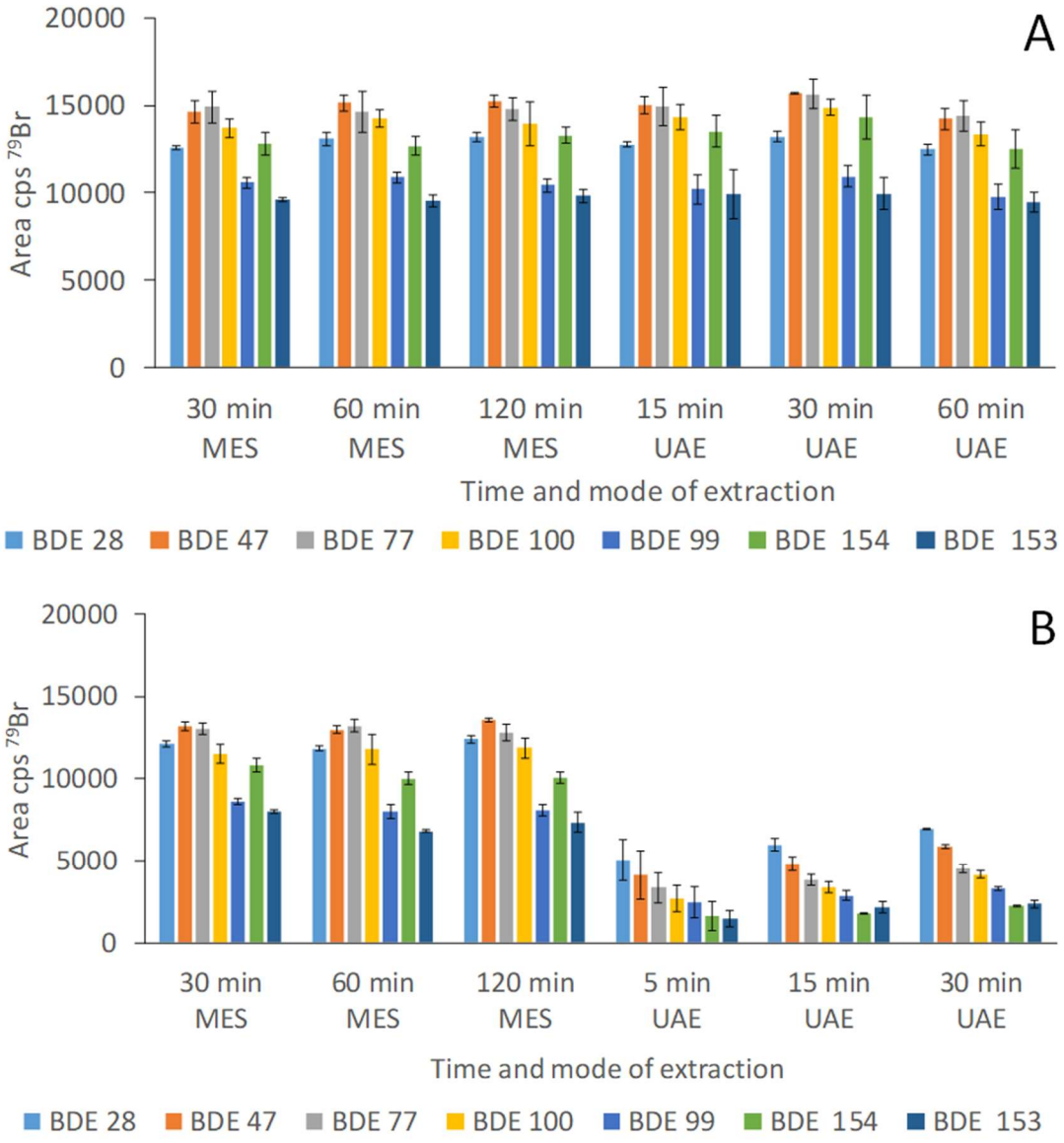


Figure 8: Influence of mechanical shaking and ultrasound-assisted extraction on signal intensities of PBDEs in spiked human serum (27 ng mL^{-1} each).

(A) In the first phase, samples were subjected to different time intervals of mechanical shaking (MES) and ultrasound-assisted extraction (UAE) with formic acid at 20 °C. In the second phase, iso-octane was added and the samples mechanically shaken for 2 hours.

(B) In the first phase, samples were subjected to 30 min of mechanical shaking with formic acid at 20 °C. In the second phase, iso-octane was added and the samples subjected to different time intervals of mechanical shaking and ultrasound-assisted extraction.

Results represent the average ± standard deviation of three replicates.

The data of the above experiments indicate that among the three modes of extraction tested, microwave-assisted extraction and mechanical shaking yielded similar results, while ultrasound-assisted extraction was determined to be inefficient (second phase of extraction). Due to the easier sample manipulation and better repeatability and reproducibility of measurements, mechanical shaking (30 min in the first phase and 30 min in the second phase of extraction) was concluded to be superior over microwave-assisted extraction.

4.1.2 Optimization of analytical procedure for determination of PBDEs in non-spiked human serum

After the extraction procedure was optimized, the possibilities of achieving the highest possible sensitivity for the determination of PBDEs in non-spiked human serum were examined. For this purpose, the optimal extraction procedure was applied (mechanical shaking, 30 min in the first phase and 30 min in the second phase of extraction), the organic phase (iso-octane) was cleaned up and concentrated approximately 200 times by evaporation under nitrogen stream to incipient dryness and reconstituted with 10 µL of iso-octane. 2 µL of iso-octane was injected onto the column and analysis performed by GC-ICP-MS. The concentrations of PBDEs were calculated using the standard addition calibration method. The optimal analytical procedure is described in Section 3.1.4.4.

4.1.3 Performance of analytical procedure

The performance of the optimized analytical procedure (Section 3.1.4.4.) was evaluated by determining the LODs and the limits of quantification (LOQs), linearity, repeatability and reproducibility of measurements, and by an accuracy check.

4.1.3.1 Limits of detection and quantification, linearity, repeatability and reproducibility of measurements

The LODs and LOQs were calculated on the basis of 3 times (3s) and 10 times (10s) the standard deviation, respectively, of the signal intensities of the blank sample, measured in six replicates. To evaluate the linearity of the calibration curves, SRM 1958 (Fortified Human Serum) was spiked with six PBDEs between 0.050 and 5 ng mL^{-1} for each BDE. The results are presented in Table 5. Based on the analysis of 1 mL of the serum sample, the LODs for the six PBDE congeners were between 0.0016 and 0.0039 ng mL^{-1}, whereas the LOQs ranged between 0.0053 and 0.0129 ng mL^{-1}. These LODs are similar or better than those reported by other authors [28]–[30], [117]. The correlation coefficients of the calibration curves (R^2) were between 0.9996 and 1.

Table 5: LODs, LOQs and correlation coefficients (R^2) for the determination of six PBDEs in SRM 1958 (Fortified Human Serum) by GC-ICP-MS using the standard addition calibration method.

BDE congener	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	R^2 (SRM 1958)
BDE 28	0.0018	0.0061	0.9996
BDE 47	0.0016	0.0053	0.9997
BDE 99	0.0026	0.0086	0.9999
BDE 100	0.0023	0.0075	0.9999
BDE 153	0.0039	0.0129	1
BDE 154	0.0032	0.0105	0.9998

The repeatability of the measurements was evaluated by performing 7 independent analyses of SRM 1958 on the same day. The reproducibility was examined by analyzing 7 independent samples over a period of 7 days. The results are presented in Table 10 and Table 11 (Appendix). The repeatability (RSD between 1.4 and 5.9%) and reproducibility (RSD between 1.7 and 6.1%) of the method were better than those reported by other authors (RSDs of approximately 10% or higher) [28]–[31].

4.1.3.2 Accuracy check

The accuracy of the method was verified by analyzing the PBDEs in Non-Fortified Human Serum SRM 1957 and Fortified Human Serum SRM 1958. The recoveries calculated as the ratio between the determined and certified PBDE concentrations are presented in Table 6. It is evident that the determined concentrations were in good agreement with the certified ones. The recoveries were between 96 and 101% for SRM 1957 and from 89% to 114% for SRM 1958. These recoveries are comparable or better than those reported in the cited literature [28]–[34].

Table 6: Extraction recoveries for PBDEs from SRM 1957 (Non-Fortified Human Serum) and SRM 1958 (Fortified Human Serum) determined by GC-ICP-MS using the standard addition calibration method. Results represent the mean value of three replicate determinations of a selected BDE with the standard deviation of the measurements.

Sample	BDE congener	Determined (ng g^{-1})	Certified (ng g^{-1})	Recovery (%)
SRM 1957	BDE 28	0.0192±0.0007	0.0200±0.0024[a]	96±4
	BDE 47	0.257±0.005	0.268±0.014	96±2
	BDE 99	0.0730±0.0050	0.0760±0.0038	96±7
	BDE 100	0.0496±0.0011	0.0497±0.0027	100±2
	BDE 153	0.0616±0.0029	0.0610±0.0032	101±5
	BDE 154	<LOQ	0.0070±0.0010 [a]	/
SRM 1958	BDE 28	0.416±0.004	0.462±0.019	90±1
	BDE 47	0.582±0.033	0.651±0.029	89±5
	BDE 99	0.440±0.007	0.492±0.015	89±1
	BDE 100	0.433±0.013	0.475±0.027	91±3
	BDE 153	0.486±0.015	0.455±0.054	107±3
	BDE 154	0.501±0.020	0.441±0.039	114±5

[a] Reference value

Typical chromatograms of the mixture of six PBDE congeners and internal standard BDE 77 in iso-octane (6.0 ng mL^{-1} each) and Non-Fortified Human Serum SRM 1957 spiked with internal standard BDE 77 (0.20 ng mL^{-1}) are shown in Figure 9. Before the GC–ICP-MS analysis of SRM 1957, the organic phase was concentrated approximately 200 times.

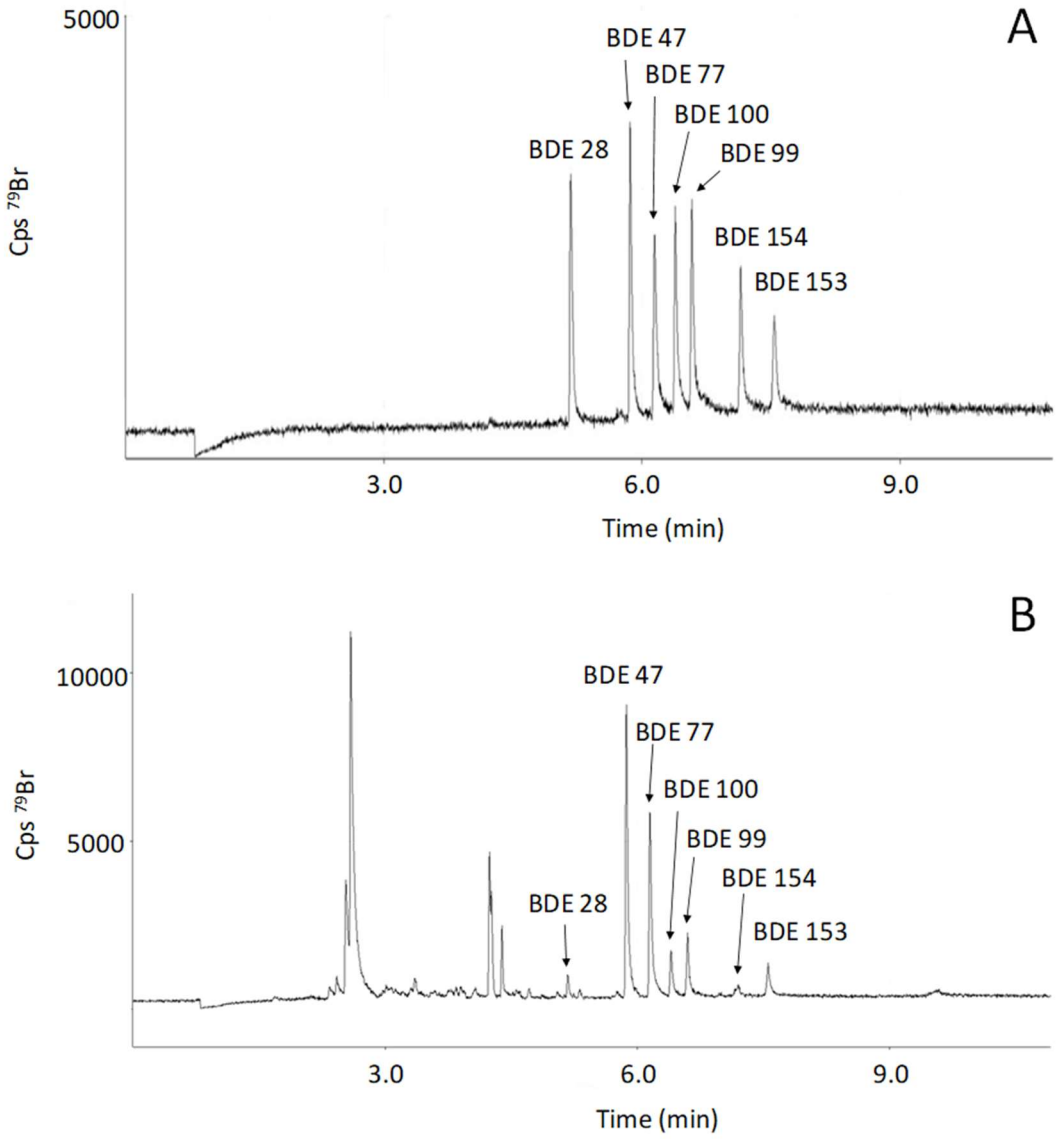


Figure 9: Chromatograms of (A) the mixture of six PBDE congeners and internal standard BDE 77 in iso-octane (6.0 ng mL^{-1} each) and (B) Non-Fortified Human Serum SRM 1957 spiked with internal standard BDE 77 (0.20 ng mL^{-1}). Before GC-ICP-MS analysis of SRM 1957, organic phase was concentrated for about 200 times. Chromatograms were recorded at m/z 79.

It can be seen that the retention times obtained for the standard solution of PBDEs and internal standard solution BDE 77 match those of SRM 1957. It is further evident that the peaks are well-resolved, thus confirming the selectivity of the developed analytical procedure.

4.1.4 Analysis of PBDEs in human serum

To demonstrate the applicability of the developed analytical method, six human serum samples collected in 2017 were analyzed. The results calculated on lipid weight basis are presented in Table 7, whereas the data on the total lipid content of the individual serum samples are provided in Table 12 (Appendix).

Table 7: Concentrations of PBDEs in human serum samples (collected in 2017) determined by GC-ICP-MS. Concentrations are given in ng of PBDEs per g of lipid weight.

Sample number	BDE 28	BDE 47	BDE 99	BDE 100	BDE 153	BDE 154
1	<LOD	1.12	0.76	0.78	1.38	<LOD
2	<LOD	1.53	<LOD	0.94	<LOD	<LOD
3	0.74	1.89	1.64	1.48	2.61	1.46
4	<LOD	0.84	0.80	1.00	1.19	<LOD
5	<LOD	1.50	0.91	0.83	<LOD	<LOD
6	<LOD	0.66	<LOD	<LOD	<LOD	<LOD

As can be seen from Table 7, the concentrations of PBDEs were from below LOD to 2.61 ng g^{-1} lw, which is in the same concentration range as values reported for other European countries [24], [119]–[123] (Table 13, Appendix). With regard to specific PBDE congeners, BDE 47 was most common as it was detected in all the samples. BDE 99 and BDE 100 were also largely frequent. The highest determined concentration was for BDE 153 (2.61 ng g^{-1} lw). These results are similar to those reported for other European countries, where BDE 47, BDE 99, BDE 100 and BDE 153 were found to be present in the highest concentrations (Table 13, Appendix).

4.2 Chromium

4.2.1 Method development and optimization

In order to investigate the kinetics of interaction of Cr(VI) and Cr(III) with serum constituents and to perform speciation of Cr in human serum at physiological concentration levels, it was necessary to develop a selective speciation procedure, which enables separation of Cr(VI) and serum proteins in a single chromatographic run. To reduce reagent blanks of eluents used in the chromatographic separation, chemicals of analytical reagent grade, suprapur or ultrapure quality, obtained from different producers, were tested. Finally, those with the highest purity, with regard to Cr content, were selected (see reagents listed in 3.2.1). First, strong anion-exchange FPLC MonoQ particle-packed column was examined for separation of Cr(VI) from serum proteins, applying the procedure for the determination of Cr(VI) [113]. Despite the fact that separation was possible, the main drawback of the FPLC procedure was that when 0.5 mL of 5-times diluted serum was loaded onto the column, rigorous column cleaning, as reported by Novotnik et al. [113], was necessary after two chromatographic runs, which made the analysis difficult.

Therefore, the applicability of the monolithic chromatography, which allows separation of biomolecules and proteins, was investigated [124]. Monolithic chromatographic stationary phases are made of a single piece of a rigid porous material. Convective mass transport enables fast and efficient chromatographic separations at low backpressure. The important advantage is also that the porosity of monolithic stationary phases enables the

application of higher sample loads onto the column and easier cleaning of the column supports than is possible using particle-packed columns. Based on our experiences on the separation of metals bound to human serum proteins [125], a potential of monolithic chromatography was investigated for the separation of Cr(VI) from Cr complexes with serum proteins. To increase the loading capacity, and consequently to lower the limit of detection (LOD) of the separated Cr species, 4 weak anion-exchange CIM DEAE disks were assembled into a single housing, forming a CIM DEAE (1.36 mL) monolithic column. The ionic strength of the eluent (buffer B) was optimized so that quantitative elution of Cr species from the column was obtained. Under the optimized analytical procedure (3.2.3.3), Cr(III) species associated with serum proteins were separated from Cr(VI). Cr(VI) was eluted from 7.2 to 9.4 min (Figure 10A), while immunoglobulins G (IgG), Tf and HSA were eluted from 1.1 to 2.6 min, 2.8 to 4.2 min and 4.2 to 7.0 min, respectively (Figure 10B).

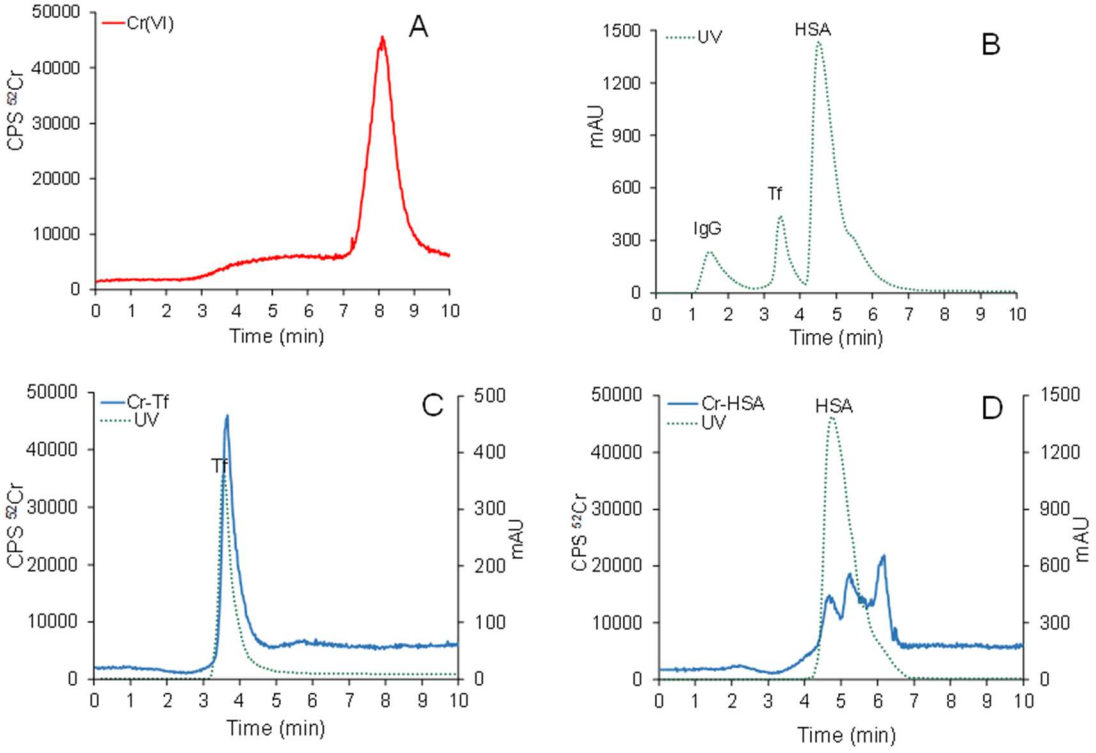


Figure 10: Typical chromatograms of separation of (A) Cr(VI) solution (5 ng mL^{-1}), (B) 5-times diluted mixture of serum proteins (25 g L^{-1} HSA, 5 g L^{-1} IgG, 2.5 g L^{-1} Tf), (C) 5-times diluted Cr-Tf solution (25 ng mL^{-1} Cr(III) + 2.5 g L^{-1} Tf, 4 h incubation) and (D) Cr-HSA solution (25 ng mL^{-1} Cr(III) + 25 g L^{-1} HSA, 4 h incubation) on the CIM DEAE monolithic column followed by ICP-MS (m/z 52) and UV (278 nm) detection. Solutions in Tris-HCl + $NaHCO_3$ buffer, pH 7.4.

As evident from Figure 10A and Figure 10B, Cr(VI) was selectively separated from serum proteins. To demonstrate the ability of the developed CIM DEAE procedure to separate Cr-Tf from Cr-HSA complex, Cr(III)-nitrate (25 ng mL^{-1} Cr) was added to the solution of standard serum Tf or standard serum HSA (dissolved in buffer A). Samples were incubated at 37 °C for 4 hours and, before speciation analysis, diluted 5-times with buffer A. The results presented in Figure 10C show that Cr peak monitored by ICP-MS detection is overlapped with the UV profile of Tf, which confirmed the formation of Cr-Tf complex.

Interaction of Cr(III) with HSA resulted in 3 unresolved Cr peaks that coincide with the UV elution profile of HSA (Figure 10D). Data from Figure 10A, Figure 10C and Figure 10D further demonstrate that Cr-Tf is separated from Cr-HSA, the latter being also well-separated from Cr(VI). This enables the use of the developed speciation procedure in the investigations of the kinetics of interactions of Cr(VI) and Cr(III) with serum constituents. The experiments performed with HEPES (pH 7.4), instead of Tris buffer (pH 7.4), showed the same elution profiles of Cr, which confirmed that Tris does not influence the Cr speciation. Therefore, in all experiments of the present investigation, Tris buffer, which is longer stable in solution than HEPES, was used.

4.2.2 Kinetics of interactions of Cr(VI) and Cr(III) with serum constituents

To investigate the kinetics of interactions of Cr(VI) and Cr(III) with serum constituents, human serum sample was doubly spiked with 25 ng mL^{-1} of enriched ^{50}Cr(VI) and 20 ng mL^{-1} of enriched ^{53}Cr(III). The sample was incubated at 37 °C and aliquots were taken for the speciation analysis after 5 min, 30 min, 1 h, 2 h, 4 h, 24 h and 48 h. A representative chromatogram of separation of Cr species in human serum doubly spiked with ^{50}Cr(VI) and ^{53}Cr(III) after incubation for 4 h is presented in Figure 11.

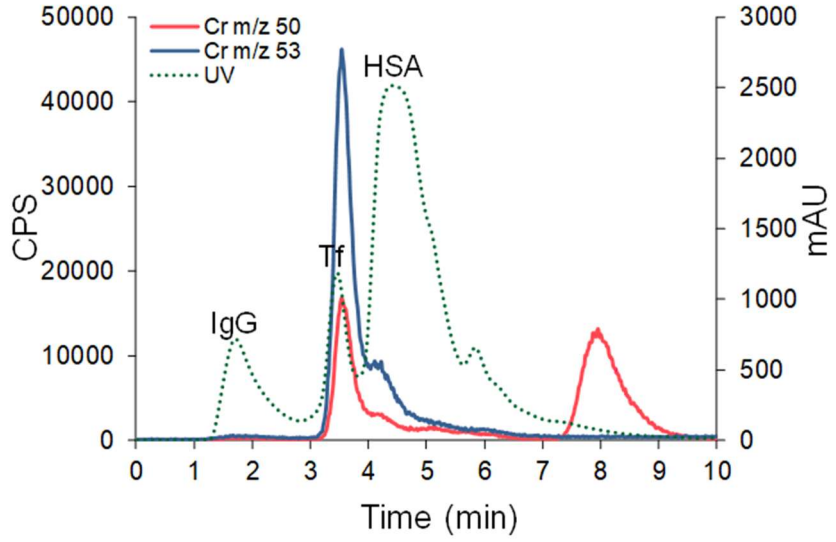


Figure 11: Separation of Cr species of 5-times diluted human serum doubly spiked with 25 ng mL^{-1} ^{50}Cr(VI) and 20 ng mL^{-1} ^{53}Cr(III) after incubation for 4 h on CIM DEAE monolithic column followed by ICP-MS (m/z 50 and 53) and UV (278 nm) detection.

By comparing Cr elution profiles of the spiked human serum with the protein elution patterns, it is evident that the added ^{53}Cr(III) is bound preferentially to Tf, while its interaction with HSA is weak. This can also be seen from the elution profile of ^{50}Cr, showing that the added ^{50}Cr(VI) is partially reduced, and the resultant ^{50}Cr(III) predominantly binds to Tf, and in a very small proportion to HSA. Kinetics of interaction over 48 hours are presented in Figure 12.

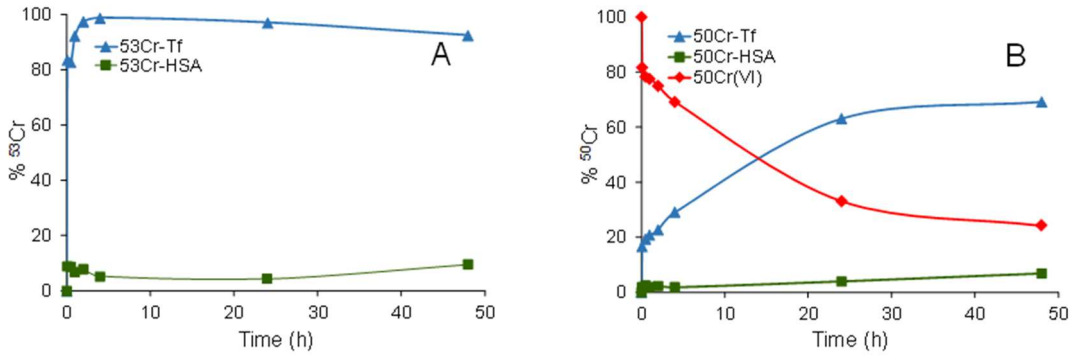


Figure 12: Kinetics of interaction of (A) ^{53}Cr(III) with serum proteins, and (B) reduction of ^{50}Cr(VI) and interaction of formed ^{50}Cr(III) with serum proteins. Human serum was doubly spiked with 25 ng mL^{-1} ^{50}Cr(VI) and 20 ng mL^{-1} ^{53}Cr(III), and speciation analysis of 5-times diluted serum performed at selected time intervals by CIM DEAE monolithic chromatography with ICP-MS (m/z 50 and 53) and UV (278 nm) detection.

Data from Figure 12A show that the added ^{53}Cr(III) interacted rapidly with serum Tf. In 5 min, 80% of ^{53}Cr(III) was bound to Tf, approximately 10% reacted with HSA, while about 10% remained unbound. After 4 h, ^{53}Cr(III) was almost exclusively present in the form of ^{53}Cr-Tf complex. With time elapsed, a small amount of ^{53}Cr(III) was transferred from Tf to HSA. After 48 h, about 90% of ^{53}Cr(III) in human serum existed in the form of ^{53}Cr-Tf and the remaining part as ^{53}Cr-HSA complex. Similarly to Fe(III), Tf is the main Cr(III) binding protein in the human serum. Since Tf is only 30% occupied with Fe(III), unoccupied sites are available for other metals. Fe(III) binds 10-times more strongly to Tf than to HSA, and the same is expected also for Cr(III) [126]. Due to the very high stability constant of Cr-Tf complex (log K = 16.4) [36], HSA does not compete with Tf for binding Cr(III) in serum. Present investigation confirmed this hypothesis and is in accordance with the study on the distribution of ^{51}Cr(III) radioactive tracer in the blood of peritoneal dialysis patients, which showed that Cr(III) binds preferentially to Tf and in a small portion to HSA [107]. The main result, i.e. rapid and overwhelmingly large binding to Tf, was the same as found in the group of Vincent who did the experiments with rats treated with a ^{51}Cr(III) radioactive tracer [106], [127], [128]. However, in rats treated orally with ^{51}Cr(III) [128] and also in rats treated intravenously with ^{51}Cr(III) containing Tf, a small amount of ^{51}Cr(III) was bound to LMM ligands [106], [127], [128]. Since the interaction of Cr(III) with HSA is weak, it is possible that with time elapsed, Cr(III) is released from HSA and complexed by LMM ligands present in the serum. To confirm this hypothesis, additional research is needed. From data presented in Figure 12B, it can be seen that the Cr(VI) reduction is slow. After 48 h, about 80% of the added ^{50}Cr(VI) was reduced. Cr(VI) in the serum is mainly reduced by the ascorbic acid and reducing sugars [129], which have a much lower reducing capacity than glutathione in RBC, where Cr(VI) is rapidly reduced [89]. Data from Figure 12B further indicate that the reduced ^{50}Cr(VI) was complexed mainly with Tf and to a very small extent to HSA. When Cr(VI) was measured 14 days after spiking, its signal was still gradually decreasing, due to on-going reduction. 90% of added ^{50}Cr(VI) was reduced by serum constituents, but due to serum instability (denaturation of serum proteins), it was not possible to perform speciation analysis to reveal the interaction of the resultant ^{50}Cr(III) with serum proteins.

The developed speciation procedure in combination with ^{53}Cr(III) and ^{50}Cr(VI) isotopic tracers provided quantitative data on the kinetics of reduction of Cr(VI) and interaction

of Cr(III) with serum proteins, which supported previously presumed mechanisms of interactions and behavior of Cr species in the human serum [107], [126].

4.2.3 Analytical figures of merit

4.2.3.1 Column recovery

Column recovery was verified by the speciation analysis of 5-times diluted human serum doubly spiked with ^{50}Cr(VI) (25 ng mL^{-1} Cr) and ^{53}Cr(III) (20 ng mL^{-1} Cr) after incubation at 37 °C for 48 h. For this purpose, fractions under the chromatographic peaks containing Cr were collected and Cr concentrations determined by ICP-MS. Column recoveries calculated as the ratio between the Cr concentration determined in the fractions eluted and the Cr concentration injected onto the column were 98% and 109% for ^{50}Cr and ^{53}Cr, respectively. These data confirmed that the developed CIM DEAE procedure enables quantitative elution of the separated Cr species from the column.

4.2.3.2 Repeatability of measurement

Repeatability of measurement of Cr species was tested by performing six consecutive speciation analyses of 5-times diluted human serum doubly spiked with ^{50}Cr(VI) (25 ng mL^{-1} Cr) and ^{53}Cr(III) (20 ng mL^{-1} Cr) after incubation at 37 °C for 48 h. Separated Cr species were monitored by ICP-MS at m/z 50 and 53. Good repeatability of measurement was obtained for the separated Cr species. The relative standard deviations (RSDs) were found to be ±1.2% for ^{50}Cr(VI), ±2.9% for ^{50}Cr-Tf, ±6.8% for ^{50}Cr-HSA, ±2.8% for ^{53}Cr-Tf and ±8.0% for ^{53}Cr-HSA (
Table 8).

Table 8: Repeatability of measurement of Cr species tested for six consecutive speciation analyses of 5-times diluted human serum doubly spiked with 25 ng mL^{-1} ^{50}Cr(VI) and 20 ng mL^{-1} ^{53}Cr(III) after incubation for 48 h. Separation of Cr species was performed on CIM DEAE monolithic column followed by ICP-MS (m/z 50 and 53) detection.

Parameter	^{50}Cr(VI)	^{50}Cr-Tf	^{50}Cr-HSA	^{53}Cr-Tf	^{53}Cr-HSA
Peak area (CPS)	345362	1473770	66957	1575600	79400
	349123	1449610	58120	1569900	68400
	343230	1447760	62930	1558450	65900
	346232	1384610	59000	1484780	71780
	353364	1481510	61600	1586210	79800
	352599	1510640	55230	1617000	77245
RSD (%)	1.2	2.9	6.8	2.8	8.0

4.2.3.3 Accuracy check for the determination of total Cr concentration in serum

The accuracy of the determination of total Cr concentration in human serum was checked by the analysis of the certified reference material Trace Elements in Serum L-2. Good agreement between the determined Cr concentration (5.96 ± 0.26 ng mL^{-1}) and the certified

value (5.81 ± 0.30 ng mL^{-1}) was obtained, confirming the accurate determination of total Cr concentration in serum by ICP-MS.

4.2.3.4 Accuracy check for Cr speciation analysis in serum

Since there are no certified reference materials available, the accuracy of the determination of Cr species in serum was checked by the speciated isotope dilution procedure and by the spike recovery test. For this purpose, serum sample containing 0.25 ng mL^{-1} of Cr was spiked with 1 ng mL^{-1} of Cr(III), so that the total Cr concentration was 1.25 ng mL^{-1}. To quantify separated Cr species by the post-column ID-ICP-MS, isotopes at m/z 50 and 52 were monitored. The mass flow of ^{52}Cr was plotted versus time throughout the chromatographic run (Figure 13).

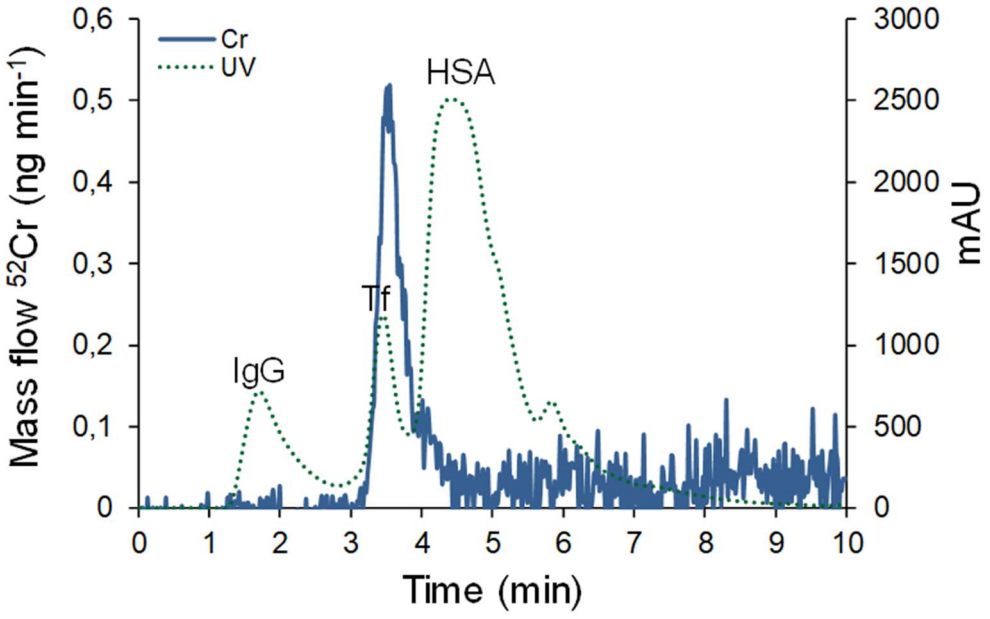


Figure 13: Speciated isotope dilution of Cr in 5-times diluted spiked human serum (spike 1 ng mL^{-1} Cr(III)) on the CIM DEAE monolithic column followed by ICP-MS and UV (278 nm) detection. Cr mass flow is based on measurements of isotope ratios m/z 50 and 52.

The determined Cr concentration by ID-ICP-MS eluted under the peak of Cr-Tf was 1.22 ng mL^{-1}, while the one based on the standard addition calibration method was 1.18 ng mL^{-1}. Since results obtained by the two different calibration approaches agreed well, standard addition calibration method was applied for quantification of Cr species in human serum at physiological concentration levels.

4.2.3.5 Linearity of measurement

The linearity of measurement for total Cr ranged from 0.33 to 1000 ng mL^{-1}, with a coefficient of determination (R^2) better than 0.998.

The linearity of measurement for separated Cr species was confirmed over the concentration range from 0.7 to 100 ng mL^{-1}, with a coefficient of determination (R^2) better than 0.997.

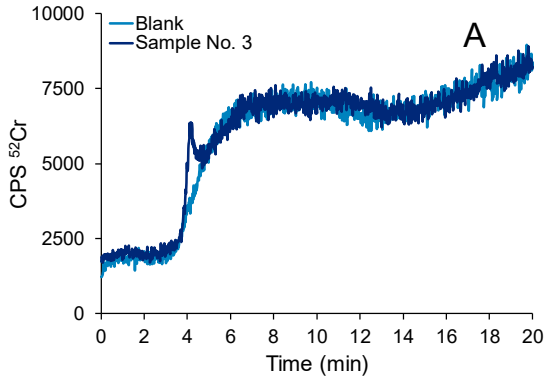
4.2.3.6 Limits of detection

The LOD and LOQ for the determination of total Cr concentration in serum was calculated as the concentration that provided a signal equal to 3s or 10s of the blank sample, respectively. To calculate the LOD and LOQ, 8 blank samples (MilliQ water) were analyzed by ICP-MS. The LOD for 5-times diluted serum sample was 0.10 ng mL^{-1} Cr, while LOQ was 0.33 ng mL^{-1} Cr.

The LODs and LOQs for the determination of Cr(VI), Cr-Tf and Cr-HSA were calculated as the concentrations that provided a signal (peak area) equal to 3s or 10s of the blank sample in the chromatogram, respectively. To calculate the LODs and LOQs, 8 blank samples (buffer A) were injected, and the speciation procedure applied. When 1 mL of 5-times diluted serum sample was analyzed, the LODs for the determination of Cr(VI), Cr-Tf and Cr-HSA were 0.15 ng mL^{-1} Cr, 0.13 ng mL^{-1} Cr and 0.20 ng mL^{-1} Cr, respectively, while LOQs were 0.50 ng mL^{-1} Cr, 0.43 ng mL^{-1} Cr and 0.67 ng mL^{-1} Cr, respectively.

4.2.4 Speciation of Cr in human serum at physiological concentration levels

Speciation of Cr in human serum is difficult due to very low Cr concentrations. To make speciation analysis at physiological concentration levels possible, the extraneous contamination should be reduced to a minimum. This can be achieved by the use of reagents of the highest purity regarding Cr content (see reagents listed in 2.1.), efficient cleaning of the CIM DEAE column (see cleaning procedure described in 2.4.2.) and replacement of stainless steel parts of the HPLC with bio-inert and HDPE materials. To enhance the sensitivity of Cr speciation in the human serum, 1 mL of 5-times diluted serum was injected onto the column. For obtaining selective separation of proteins, the time of chromatographic separation was prolonged to 20 min (see 2.4.2.). Representative chromatograms for overlays of blank, 5-times diluted human serum, 5-times diluted spiked human serum monitored by ICP-MS, and 5-times diluted mixture of serum proteins followed by UV detection are presented in Figure 14.



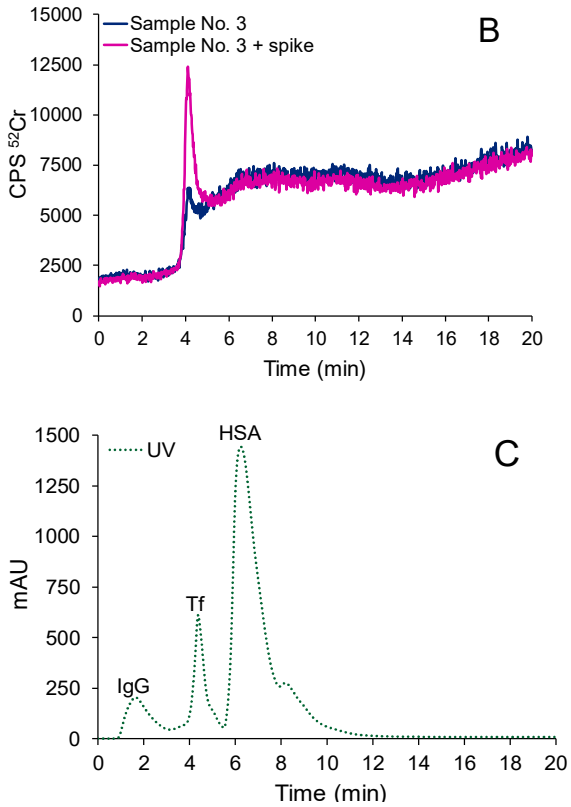


Figure 14: Overlays of chromatograms of (A) blank and 5-times diluted serum sample No. 3, (B) 5-times diluted serum sample No. 3 and sample No. 3 spiked with 1 ng mL^{-1} Cr(III), and (C) 5-times diluted mixture of serum proteins (25 g L^{-1} HSA, 5 g L^{-1} IgG, 2.5 g L^{-1} Tf) on the CIM DEAE monolithic column followed by ICP-MS (m/z 52) and UV (278 nm) detection.

As can be seen, Cr from reagent blank contributes to the background in ICP-MS detection at m/z 52 (this is not the case when Cr is monitored at naturally low abundant isotopes at m/z 50 and 53, Figure 11). Despite this, detection of Cr species was possible at physiological concentration levels (Figure 14A and Figure 14B). Based on the elution time of separated proteins (Figure 14C) and separated Cr species, it is evident that Cr present in the human serum of non-exposed individuals is bound mainly to Tf. Speciation analysis data for 5 serum samples along with total Cr concentration are provided in Table 9.

Table 9: Cr concentrations in human serum samples determined by ICP-MS and contents of Cr species associated with serum proteins determined by CIM DEAE-ICP-MS.

Sample No.	Total Cr (ng mL^{-1})	Cr-Tf (ng mL^{-1})	Cr-HSA (ng mL^{-1})
1	0.46 ± 0.07	0.39 ± 0.15	<0.20
2	0.23 ± 0.09	0.19 ± 0.09	<0.20
3	0.20 ± 0.08	0.20 ± 0.09	<0.20
4	0.19 ± 0.08	0.24 ± 0.09	<0.20
5	0.40 ± 0.06	0.50 ± 0.15	<0.20

As can be seen, total Cr concentration ranges from 0.19 to 0.46 ng mL^{-1}, which is in accordance with the reported data for Cr concentrations in the serum of non-exposed population [39]–[41], [130] . Only two out of five results for total Cr concentrations were above LOQ, while three were between LOD and LOQ, which increased the measurement uncertainty. Speciation data indicate that Cr in all the analyzed samples was bound mainly to Tf. Cr-HSA was not detected since its concentrations were below LOD. Although Cr-Tf concentrations of real human serum samples analyzed ranged between LOD and LOQ, such low Cr-Tf concentrations have never been measured at any degree of reliability. High capacity and robustness of the CIM DEAE column coupled to UV and ICP-MS enabled the detection of Cr species in the human serum at physiological concentration levels. To the best of our knowledge, this is the first report on the detection of Cr species in the human serum at physiological concentration levels.

Chapter 4

Conclusions

In the presented doctoral dissertation, two new analytical methods were developed. The first one is for the determination of PBDEs in the human serum. The second one is for studying the kinetics of interaction of Cr(VI) and Cr(III) with human serum constituents and detection of chromium species at physiological concentration levels.

A new simple, reliable and sensitive method for the determination of the six PBDE congeners (BDE 28, BDE 47, BDE 99, BDE 100, BDE 153, and BDE 154) by GC-ICP-MS in 1 mL of human serum was developed. For that purpose, the effect of different extracting agents (formic acid, formic acid in 2-propanol (4:1, v/v), 0.1 M HCl in MeOH, and 25% TMAH) and subsequent addition of iso-octane on the extraction efficiency, when applying different modes of extraction (mechanical shaking, microwave- and ultrasound-assisted extraction), was tested. Formic acid was found to be the best for extracting PBDEs from the human serum into iso-octane. Among the three modes of extraction tested, microwave-assisted extraction and mechanical shaking yielded similar results, while ultrasound-assisted extraction was inefficient (second phase of extraction). Due to the easier sample manipulation and better repeatability and reproducibility of measurements, mechanical shaking (30 min in the first phase and 30 min in the second phase of extraction) was chosen as the best. The co-extracted lipids were removed by a rapid and low solvent consuming clean-up step previously developed in our group applying the Florisil adsorbent. This clean-up step is much more simple than the available cleaning procedures commonly used in GC–MS techniques, which are laborious and require large amounts of organic solvents. Validation of the analytical method was done by the analysis of SRM 1957 (Non-Fortified Human Serum) and SRM 1958 (Fortified Human Serum), which showed good accuracy, repeatability and reproducibility of the developed procedure. The method is sensitive, with LODs comparable to or better than those reported by other authors. The feasibility of the method was tested by analyzing human serum samples. The concentrations of PBDEs in these samples were similar to those determined in other European countries. Due to its simplicity, sensitivity, reliability, and requirement of a low amount of sample for the analysis, the method can be used in future biomonitoring studies.

A novel analytical method based on monolithic convective interaction media (CIM) chromatography coupled to UV and inductively coupled plasma mass spectrometry (ICP-MS) detectors delivered first quantitative data on the kinetics of reduction of Cr(VI) and interaction of Cr(III) with human serum proteins. The results revealed that the added ^{53}Cr(III) rapidly interacted with serum Tf. After 5 min, about 80%, and after 1 h, more than 90% of added ^{53}Cr(III) was complexed with Tf, while the remaining portion interacted with HSA. Since the interaction with HSA is weak, it is possible that with time elapsed, Cr(III) is released from HSA and is complexed by LMM ligands present in the serum. However, to confirm this hypothesis, additional research is needed. Data from the present

study further demonstrated that Cr(VI) reduction in the serum is slow. 48 h after spiking, about 20% of the added ^{50}Cr(VI) remained in the serum, while resulting ^{50}Cr(III) was bound predominantly to Tf. The present investigation significantly contributes to the knowledge on the behavior of Cr in the human serum and confirms the reported mechanisms of interactions of Cr(VI) and Cr(III) species in this complex biological compartment. The developed analytical procedure, which importantly contributes also to the field of metallomics, enables the detection of Cr species in the human serum at physiological concentration levels. It was confirmed that Cr in the serum of unexposed individuals is bound mainly to Tf. The main challenge for the future is to achieve quantitative speciation of Cr in the human serum at physiological concentration levels. For this purpose, reagent blanks, which contribute to the baseline in the chromatogram, should be reduced to absolute minimum. This can only be achieved by developing better cleaning procedures that will be able to completely remove traces of Cr from reagents. Nevertheless, such complex investigation, delivering quantitative data on the kinetics of interactions of Cr species in the human serum using enriched Cr isotopic tracers, and detection of Cr species in the serum at physiological concentration levels, has not been reported yet.

Appendix A

■

Table 10: Repeatability of measurement tested for SRM 1958 (Fortified Human Serum) by GC-ICP-MS using the standard addition calibration method.

Sample	BDE congener	Concentration (ng g^{-1})							RSD (%)
		1.	2.	3.	4.	5.	6.	7.	
SRM 1958	BDE 28	0.417	0.419	0.411	0.420	0.447	0.411	0.403	3.4
	BDE 47	0.608	0.592	0.545	0.615	0.611	0.572	0.582	4.3
	BDE 99	0.443	0.445	0.432	0.443	0.445	0.449	0.434	1.4
	BDE 100	0.418	0.441	0.440	0.427	0.453	0.431	0.431	2.6
	BDE 153	0.471	0.488	0.500	0.561	0.506	0.516	0.535	5.9
	BDE 154	0.489	0.490	0.523	0.527	0.507	0.513	0.517	3.0

Table 11: Reproducibility of measurement tested for SRM 1958 (Fortified Human Serum) by GC-ICP-MS using the standard addition calibration method.

Sample	BDE congener	Concentration (ng g^{-1})							RSD (%)
		1.	2.	3.	4.	5.	6.	7.	
SRM 1958	BDE 28	0.417	0.419	0.411	0.420	0.385	0.422	0.388	3.8
	BDE 47	0.608	0.592	0.545	0.615	0.567	0.621	0.560	5.1
	BDE 99	0.443	0.445	0.432	0.443	0.426	0.428	0.440	1.7
	BDE 100	0.418	0.441	0.440	0.427	0.440	0.435	0.437	2.0
	BDE 153	0.471	0.488	0.500	0.561	0.498	0.500	0.487	5.7
	BDE 154	0.489	0.490	0.523	0.527	0.494	0.500	0.436	6.1

Table 12: Total lipid content (TLS) in samples analyzed was obtained on the basis of the enzymatic assays by summing the individual lipid species measured: total cholesterol (TC) and triglycerides (TG), using a formula proposed by Bernet et al. [110]. TLS = (2.27 * TC) + TG + 62.3 mg dL^{-1}.

Sample	Total lipids (mg dL^{-1})
1	643
2	502
3	555
4	511
5	661
6	573

Table 13: Median values of selected PBDE congeners in the human serum found reported for some European countries in the last 15 years. Concentrations are given on the lipid weight basis (lw).

Location	Sampling year	BDE 28 (ng g^{-1})	BDE 47 (ng g^{-1})	BDE 99 (ng g^{-1})	BDE 100 (ng g^{-1})	BDE 153 (ng g^{-1})	BDE 154 (ng g^{-1})	Reference
France	2004-06	0.12	2.83	1.94	0.37	0.72	0.07	[119]
Greece	2007	0.01	0.16	0.09	0.11	0.51	0.02	[120]
Denmark	2007	0.03	0.38	0.11	0.10	1.13	0.02	[121]
Sweden	2010	0.06	0.36	0.05	0.18	0.72	0.12	[24]
Norway	2012	0.33	0.49	0.13	0.14	0.82	0.07	[122]
Germany	2013	0.02	0.38	0.16	0.13	0.97	0.02	[123]
Slovenia	2017	0.17	1.31	0.78	0.88	0.79	0.30	This study

References

[1] "Human biomonitoring: facts and figures. Copenhagen: WHO Regional Office for Europe, 2015." .

[2] "HBM4EU Prioritiy Substance Group: Cadmium (Cd) and Heaxavalent Chromium (Cr(VI)). Scoping document."

[3] "HBM4EU Prioritiy Substance Group: Flame retardants. Scoping document."

[4] "US EPA (2010). An exposure assessment of polybrominated diphenyl ethers. EPA/600/R-08/086F. Washington DC: Office of Research and Development National Center for Environmental Assessment."

[5] "EFSA (2011). Scientific opinion on polybrominated diphenyl ethers (PBDEs) in food. EFSA Journal 9(5), 2156-2427."

[6] V. Linares, M. Bellés, and J. L. Domingo, "Human exposure to PBDE and critical evaluation of health hazards," *Arch. Toxicol.*, vol. 89, no. 3, pp. 335–356, 2015.

[7] W. A. Stubbings and S. Harrad, "Extent and mechanisms of brominated flame retardant emissions from waste soft furnishings and fabrics: A critical review," *Environ. Int.*, vol. 71, pp. 164–175, 2014.

[8] S. Rupp and J. W. Metzger, "Brominated-chlorinated diphenyl ethers formed by thermolysis of polybrominated diphenyl ethers at low temperatures," *Chemosphere*, vol. 60, no. 11, pp. 1644–1651, 2005.

[9] C. Agrell, A. F. H. Ter Schure, J. Sveder, A. Bokenstrand, P. Larsson, and B. N. Zegers, "Polybrominated diphenyl ethers (PBDES) at a solid waste incineration plant I: Atmospheric concentrations," *Atmos. Environ.*, vol. 38, no. 30, pp. 5139–5148, 2004.

[10] C. A. de Wit, "An overview of brominated flame retardants in the environment," *Chemosphere*, vol. 46, no. 5, pp. 583–624, Feb. 2002.

[11] M. Iqbal *et al.*, "Legacy and emerging flame retardants (FRs) in the freshwater ecosystem: A review," *Environ. Res.*, vol. 152, no. July 2016, pp. 26–42, 2017.

[12] K. Ni *et al.*, "A review of human exposure to polybrominated diphenyl ethers (PBDEs) in China," *Int. J. Hyg. Environ. Health*, vol. 216, no. 6, pp. 607–623, Nov. 2013.

[13] D. Fischer, K. Hooper, M. Athanasiadou, I. Athanassiadis, and Å. Bergman, "Children Show Highest Levels of Polybrominated Diphenyl Ethers in a California Family of Four: A Case Study," *Environ. Health Perspect.*, vol. 114, no. 10, pp. 1581–1584, Oct. 2006.

[14] S. Harrad, S. Hazrati, and C. Ibarra, "Concentrations of Polychlorinated Biphenyls in Indoor Air and Polybrominated Diphenyl Ethers in Indoor Air and Dust in

Birmingham, United Kingdom: Implications for Human Exposure," *Environ. Sci. Technol.*, vol. 40, no. 15, pp. 4633–4638, Aug. 2006.

[15] M. Lorber, "Exposure of Americans to polybrominated diphenyl ethers," *J. Expo. Sci. Environ. Epidemiol.*, vol. 18, no. 1, pp. 2–19, 2008.

[16] M. Frederiksen, K. Vorkamp, M. Thomsen, and L. E. Knudsen, "Human internal and external exposure to PBDEs – A review of levels and sources," *Int. J. Hyg. Environ. Health*, vol. 212, no. 2, pp. 109–134, Mar. 2009.

[17] A. Sjödin, D. G. Patterson, and Å. Bergman, "A review on human exposure to brominated flame retardants—particularly polybrominated diphenyl ethers," *Environ. Int.*, vol. 29, no. 6, pp. 829–839, Sep. 2003.

[18] A. Kotz, R. Malisch, K. Kypke, and M. Oehme, "PBDE, PBDD/F and mixed chlorinated-brominated PXDD/F in pooled human milk samples from different countries," *Organohalogen Compd*, vol. 67, pp. 1624–1627, 2005.

[19] K. Jakobsson, M. Athanasiadou, A. Christiansson, A. Ioannis, A. Bergman, and L. Hagmar, "Polybrominated diphenyl ethers (PBDEs) in serum from Swedish men 1988-2002. A longitudinal study," no. Report, pp. 2–10, 2005.

[20] C. Naert, M. Piette, N. Bruneel, and C. Van Peteghem, "Occurrence of polychlorinated biphenyls and polybrominated diphenyl ethers in belgian human adipose tissue samples," *Arch. Environ. Contam. Toxicol.*, vol. 50, no. 2, pp. 290–296, 2006.

[21] B. Johnson-Restrepo, K. Kannan, D. P. Rapaport, and B. D. Rodan, "Polybrominated diphenyl ethers and polychlorinated biphenyls in human adipose tissue from New York.," *Environ. Sci. Technol.*, vol. 39, no. 14, pp. 5177–5182, Jul. 2005.

[22] D. Meironyté, K. Norén, and A. Bergman, "Analysis of polybrominated diphenyl ethers in Swedish human milk. A time-related trend study, 1972-1997.," *J. Toxicol. Environ. Health. A*, vol. 58, no. 6, pp. 329–341, Nov. 1999.

[23] B. Fängström, A. Strid, P. Grandjean, P. Weihe, and A. Bergman, "A retrospective study of PBDEs and PCBs in human milk from the Faroe Islands.," *Environ. Health*, vol. 4, p. 12, Jul. 2005.

[24] P. O. Darnerud *et al.*, "Time trends of polybrominated diphenylether (PBDE) congeners in serum of Swedish mothers and comparisons to breast milk data," *Environ. Res.*, vol. 138, pp. 352–360, Apr. 2015.

[25] S. Lignell, M. Aune, P. O. Darnerud, S. Cnattingius, and A. Glynn, "Persistent organochlorine and organobromine compounds in mother's milk from Sweden 1996-2006: Compound-specific temporal trends," *Environ. Res.*, vol. 109, no. 6, pp. 760–767, 2009.

[26] A. R. Zota *et al.*, "Erratum: A temporal comparison of PBDEs, OH-PBDEs, PCBs, and OH-PCBs in the serum of second trimester pregnant women recruited from San Francisco General Hospital, California (Environmental Science and Technology (2013) 47:20 (11776-11784) DOI: 10.1021/es," *Environ. Sci. Technol.*, vol. 48, no. 4, pp. 2512–2513, 2014.

[27] S. Król, B. Zabiegała, and J. Namieśnik, "PBDEs in environmental samples: Sampling and analysis," *Talanta*, vol. 93, pp. 1–17, 2012.

[28] A. Covaci and S. Voorspoels, "Optimization of the determination of polybrominated diphenyl ethers in human serum using solid-phase extraction and gas chromatography-electron capture negative ionization mass spectrometry," *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 827, no. 2, pp. 216–223, 2005.

[29] C. Thomsen, V. H. Liane, and G. Becher, "Automated solid-phase extraction for the determination of polybrominated diphenyl ethers and polychlorinated biphenyls in serum-application on archived Norwegian samples from 1977 to 2003," *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 846, no. 1–2, pp. 252–263, 2007.

[30] E. Cequier, R. M. Marc??, G. Becher, and C. Thomsen, "Determination of emerging halogenated flame retardants and polybrominated diphenyl ethers in serum by gas chromatography mass spectrometry," *J. Chromatogr. A*, vol. 1310, pp. 126–132, 2013.

[31] Y. P. Lin, I. N. Pessah, and B. Puschner, "Simultaneous determination of polybrominated diphenyl ethers and polychlorinated biphenyls by gas chromatography-tandem mass spectrometry in human serum and plasma," *Talanta*, vol. 113, pp. 41–48, 2013.

[32] C. M. Butt, M. L. Miranda, and H. M. Stapleton, "Development of an analytical method to quantify PBDEs, OH-BDEs, HBCDs, 2,4,6-TBP, EH-TBB, and BEH-TEBP in human serum," *Anal. Bioanal. Chem.*, vol. 408, no. 10, pp. 2449–2459, 2016.

[33] D. Lu, D. Wang, H. S. S. Ip, F. Barley, R. Ramage, and J. She, "Measurements of polybrominated diphenyl ethers and polychlorinated biphenyls in a single drop of blood," *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 891–892, pp. 36–43, 2012.

[34] D. Lu *et al.*, "Multi-analyte method development for analysis of brominated flame retardants (BFRs) and PBDE metabolites in human serum," *Anal Bioanal Chem*, 2017.

[35] A. Covaci, A. Dirtu, S. Voorspoels, L. Roosens, and P. Lepom, "Sample Preparation and Chromatographic Methods Applied to Congener-Specific Analysis of Polybrominated Diphenyl Ethers," vol. 16, 2010, pp. 55–94.

[36] P. Berton, N. B. Lana, J. M. Ríos, J. F. García-Reyes, and J. C. Altamirano, "State of the art of environmentally friendly sample preparation approaches for determination of PBDEs and metabolites in environmental and biological samples: A critical review," *Anal. Chim. Acta*, vol. 905, pp. 24–41, 2016.

[37] "J.O. Nriagu and E. Nieboer, ed., Chromium in the Natural and Human Environments. New York: John Wiley & Sons, 1988."

[38] "J. Guertin, J.A. Jacobs and C.P. Avakian, ed., Chromium (VI) Handbook. Boca Raton, FL: CRC Press, 2005."

[39] B. R. Levine *et al.*, "Ten-Year Outcome of Serum Metal Ion Levels After Primary Total Hip Arthroplasty," *J. Bone Jt. Surg.*, vol. 95, no. 6, pp. 512–518, 2013.

[40] T. Zima *et al.*, "Chromium levels in patients with internal diseases.," *Biochem. Mol. Biol. Int.*, vol. 46, no. 2, pp. 365–374, Oct. 1998.

[41] E. C. Alexopoulos, X. Cominos, I. P. Trougakos, M. Lourda, E. S. Gonos, and V.

Makropoulos, “Biological monitoring of hexavalent chromium and serum levels of the senescence biomarker apolipoprotein J/Clusterin in welders,” *Bioinorg. Chem. Appl.*, vol. 2008, no. V, 2008.

[42] J. G. Farmer, R. P. Thomas, M. C. Graham, J. S. Geelhoed, D. G. Lumsdon, and E. Paterson, “Chromium speciation and fractionation in ground and surface waters in the vicinity of chromite ore processing residue disposal sites,” *J. Environ. Monit.*, vol. 4, no. 2, pp. 235–243, 2002.

[43] J. Ščančar and R. Milačič, “A critical overview of Cr speciation analysis based on high performance liquid chromatography and spectrometric techniques,” *J. Anal. At. Spectrom.*, vol. 29, no. 3, pp. 427–443, 2014.

[44] P. Shah, V. Strezov, and P. F. Nelson, “Speciation of chromium in Australian coals and combustion products,” *Fuel*, vol. 102, pp. 1–8, 2012.

[45] “International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol.100. A review of Human Carcinogens. Part C: Arsenic, Metals, Fibres, and Dusts. Lyon, France: IARC, 2012.”

[46] S. De Flora, A. Camoirano, M. Bagnasco, C. Bennicelli, G. E. Corbett, and B. D. Kerger, “Estimates of the chromium(VI) reducing capacity in human body compartments as a mechanism for attenuating its potential toxicity and carcinogenicity,” *Carcinogenesis*, vol. 18, no. 3, pp. 531–537, 1997.

[47] R. Codd, J. A. Irwin, and P. A. Lay, “Sialoglycoprotein and carbohydrate complexes in chromium toxicity,” *Curr. Opin. Chem. Biol.*, vol. 7, no. 2, pp. 213–219, 2003.

[48] R. M. Sedman, J. Beaumont, T. A. McDonald, S. Reynolds, G. Krowech, and R. Howd, “Review of the evidence regarding the carcinogenicity of hexavalent chromium in drinking water,” *J. Environ. Sci. Heal. - Part C Environ. Carcinog. Ecotoxicol. Rev.*, vol. 24, no. 1, pp. 155–182, 2006.

[49] A. Zhitkovich, “Chromium in drinking water: Sources, metabolism, and cancer risks,” *Chem. Res. Toxicol.*, vol. 24, no. 10, pp. 1617–1629, 2011.

[50] A. Nguyen, I. Mulyani, A. Levina, and P. A. Lay, “Reactivity of chromium(III) nutritional supplements in biological media: An X-ray absorption spectroscopic study,” *Inorg. Chem.*, vol. 47, no. 10, pp. 4299–4309, 2008.

[51] “D.M. Stearns, ‘Evaluation of Chromium (III) Genotoxicity with Cell Culture and In Vitro Assays’, in The Nutritional BioChemistry of Chromium(III), J.B. Vincent, Ed. Amsterdam: Elsevier, 2007, pp. 209-224.”

[52] “J. B. Vincent, The bioinorganic chemistry of chromium. Chichester, UK: John Wiley & Sons, 2013.”

[53] D. Cohen, “How safe are metal-on-metal hip implants?,” *BMJ*, vol. 344, no. 7846, pp. 1–7, 2012.

[54] J. P. Thyssen and T. Menné, “Metal allergys-A review on exposures, penetration, genetics, prevalence, and clinical implications,” *Chem. Res. Toxicol.*, vol. 23, no. 2, pp. 309–318, 2010.

[55] P. Taylor, D. E. Kimbrough, Y. Cohen, A. M. Winer, L. Creelman, and D. E. Kimbrough, “Critical Reviews in Environmental Science and Technology A Critical Assessment of Chromium in the Environment A Critical Assessment of Chromium

in the Environment," *Crit. Rev. Environ. Sci. Technol.*, vol. 29, no. 1, pp. 1–46, 1999.

[56] B. J. Collins *et al.*, "Exposure to hexavalent chromium resulted in significantly higher tissue chromium burden compared with trivalent chromium following similar oral doses to male F344/N rats and female B6C3F1 mice," *Toxicol. Sci.*, vol. 118, no. 2, pp. 368–379, 2010.

[57] H. H. Harris *et al.*, "Time-dependent uptake, distribution and biotransformation of chromium(VI) in individual and bulk human lung cells: Application of synchrotron radiation techniques," *J. Biol. Inorg. Chem.*, vol. 10, no. 2, pp. 105–118, 2005.

[58] A. Levina, H. H. Harris, and P. A. Lay, "Binding of chromium(VI) to histones: Implications for chromium(VI)-induced genotoxicity," *J. Biol. Inorg. Chem.*, vol. 11, no. 2, pp. 225–234, 2006.

[59] "P.A. Lay and A. Levina, 'Metal Carcinogens', in Comprehensive Inorganic Chemistry II, J. Reedijk and K. Poeppelmeier, Ed. Oxford: Elsevier, 2013, pp. 835-856."

[60] R. Krupa, M. Stañczak, and Z. Walter, "Chromium incorporated in RNA and DNA," *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.*, vol. 57, no. 9–10, pp. 951–953, 2002.

[61] A. Levina, A. M. Bailey, G. Champion, and P. A. Lay, "Reactions of Chromium (VI / V / IV) with Bis (O-ethyl- L -cysteinato-N , S) zinc (II): A Model for the Action of Carcinogenic Chromium on Zinc-Finger Proteins 1," *Interactions*, vol. 3, no. Ii, pp. 6208–6216, 2006.

[62] A. Hartwig, "Metal interaction with redox regulation: An integrating concept in metal carcinogenesis?," *Free Radic. Biol. Med.*, vol. 55, pp. 63–72, 2013.

[63] A. Levina, R. Codd, C. T. Dillon, and P. A. Lay, *Chromium in biology: Toxicology and nutritional aspects*, vol. 51. 2003.

[64] "A. Levina, I. Mulyani and P.A. Lay, 'Redox Chemistry and Biological Activities of Chromium (III) Complexes', in The nutritional Biochemistry of Chromium(III), J.B. Vincent, Ed. Amsterdam: Elsevier, 2007, pp. 225-256."

[65] A. Levina and P. A. Lay, "Solution Structures of Chromium(VI) Complexes with Glutathione and Model Thiols," *Inorg. Chem.*, vol. 43, no. 1, pp. 324–335, 2004.

[66] L. Zhang and P. A. Lay, "EPR spectroscopic studies of the reactions of Cr(VI) with L-ascorbic acid, L-dehydroascorbic acid, and 5,6-O-isopropylidene-L-ascorbic acid in water. Implications for chromium(VI) genotoxicity," *J. Am. Chem. Soc.*, vol. 118, no. 50, pp. 12624–12637, 1996.

[67] P. H. Connett and K. E. Wetterhahn, "In Vitro Reaction of the Carcinogen Chromate with Cellular Thiols and Carboxylic Acids," *J. Am. Chem. Soc.*, vol. 107, no. 14, pp. 4282–4288, 1985.

[68] L. F. Sala, J. C. González, S. I. García, M. I. Frascaroli, and S. Van Doorslaer, *Detection and structural characterization of Oxo-Chromium(V)-sugar complexes by electron paramagnetic resonance*, vol. 66, no. 1. 2011.

[69] A. Zhitkovich, "Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI)," *Chem. Res. Toxicol.*, vol. 18, no. 1, pp. 3–11, 2005.

[70] P. A. Lay and A. Levina, "Activation of molecular oxygen during the reactions of

chromium(VI/V/IV) with biological reductants: Implications for chromium-induced genotoxicities," *J. Am. Chem. Soc.*, vol. 120, no. 27, pp. 6704–6714, 1998.

[71] R. Porter, M. Jáchymová, P. Martásek, B. Kalyanaraman, and J. Vásquez-Vivar, "Reductive activation of Cr(Vi) by nitric oxide synthase," *Chem. Res. Toxicol.*, vol. 18, no. 5, pp. 834–843, 2005.

[72] G. R. Borthiry, W. E. Antholine, J. M. Myers, and C. R. Myres, "Addition of DNA to CrVI and cytochrome b5 containing proteoliposomes leads to generation of DNA strand breaks and CrIIcomplexes," *Chem. Biodivers.*, vol. 5, no. 8, pp. 1545–1557, 2008.

[73] D. Krepkiy, W. E. Antholine, and D. H. Petering, "Properties of the reaction of chromate with metallothionein," *Chem. Res. Toxicol.*, vol. 16, no. 6, pp. 750–756, 2003.

[74] A. Levina and P. A. Lay, "Chemical properties and toxicity of chromium(III) nutritional supplements," *Chem. Res. Toxicol.*, vol. 21, no. 3, pp. 563–571, 2008.

[75] A. Levina and P. A. Lay, "Metal-based anti-diabetic drugs: Advances and challenges," *Dalt. Trans.*, vol. 40, no. 44, pp. 11675–11686, 2011.

[76] C. C. Winterbourn, M. B. Hampton, J. H. Livesey, and A. J. Kettle, "Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: Implications for microbial killing," *J. Biol. Chem.*, vol. 281, no. 52, pp. 39860–39869, 2006.

[77] F. Y. Salem, T. F. Parkerton, R. V. Lewis, J. H. Huang, and K. L. Dickson, "Kinetics of chromium transformations in the environment," *Sci. Total Environ.*, vol. 86, no. 1–2, pp. 25–41, 1989.

[78] I. Mulyani, A. Levina, and P. A. Lay, "Biomimetic oxidation of chromium(III): Does the antidiabetic activity of chromium(III) involve carcinogenic chromium(VI)?," *Angew. Chemie - Int. Ed.*, vol. 43, no. 34, pp. 4504–4507, 2004.

[79] J. B. Vincent, "Chromium: Celebrating 50 years as an essential element?," *Dalt. Trans.*, vol. 39, no. 16, pp. 3787–3794, 2010.

[80] "P.A. Lay and A. Levina, 'Chromium: Biological Relevance', in Encyclopedia of Inorganic and Bioinorganic Chemistry, R.A. Scott, Ed. Chichester, UK: John Wiley & Sons, 2012."

[81] W. Mertz, "Chromium Research from a Distance: From 1959 to 1980," *J. Am. Coll. Nutr.*, vol. 17, no. 6, pp. 544–547, 1998.

[82] C. Veillon and K. Y. Patterson, "Analytical issues in nutritional chromium research," *J. Trace Elem. Exp. Med.*, vol. 12, no. 2, pp. 99–109, 1999.

[83] "B.J. Stoecker. 'Basis for Dietary Recommendations for Chromium', in The Nutritional Biochemistry of Chromium(III), J.B. Vincent, Ed. Amsterdam : Elsevier, 2007, pp. 43-55."

[84] N. Laschinsky, K. Kottwitz, B. Freund, B. Dresow, R. Fischer, and P. Nielsen, "Bioavailability of chromium(III)-supplements in rats and humans," *BioMetals*, vol. 25, no. 5, pp. 1051–1060, 2012.

[85] R. A. Anderson, M. M. Polansky, and N. A. Bryden, "Stability and absorption of chromium and absorption of chromium histidinate complexes by humans," *Biol. Trace Elem. Res.*, vol. 101, no. 3, pp. 211–218, 2004.

[86] L. Y. Zha, Z. R. Xu, M. Q. Wang, and L. Y. Gu, "Chromium nanoparticle exhibits higher absorption efficiency than chromium picolinate and chromium chloride in Caco-2 cell monolayers," *J. Anim. Physiol. Anim. Nutr. (Berl).*, vol. 92, no. 2, pp. 131–140, 2008.

[87] R. A. Anderson and M. M. Polansky, "Dietary and metabolite effects on trivalent chromium retention and distribution in rats," *Biol. Trace Elem. Res.*, vol. 50, no. 2, pp. 97–108, 1995.

[88] K. Merritt and S. A. Brown, "Release of hexavalent chromium from corrosion of stainless steel and cobalt—chromium alloys," *J. Biomed. Mater. Res.*, vol. 29, no. 5, pp. 627–633, 1995.

[89] G. E. Corbett *et al.*, "In vitro reduction kinetics of hexavalent chromium in human blood," *Environ. Res.*, vol. 78, no. 1, pp. 7–11, 1998.

[90] J. Aaseth, J. Alexander, and T. Norseth, "Uptake of 51Cr-Chromate by Human Erythrocytes – A Role of Glutathione," *Acta Pharmacol. Toxicol. (Copenh).*, vol. 50, no. 4, pp. 310–315, 1982.

[91] H. Sakurai, K. Takechi, H. Tsuboi, and H. Yasui, "ESR characterization and metallokinetic analysis of Cr(V) in the blood of rats given carcinogen chromate(VI) compounds," *J. Inorg. Biochem.*, vol. 76, no. 1, pp. 71–80, 1999.

[92] B. D. Kerger, B. L. Finley, G. E. Corbett, D. G. Dodge, and D. J. Paustenbach, "Ingestion of chromium(vi) in drinking water by human volunteers: Absorption, distribution, and excretion of single and repeated doses," *J. Toxicol. Environ. Health*, vol. 50, no. 1, pp. 67–95, 1997.

[93] A. W. Newton, L. Ranganath, C. Armstrong, V. Peter, and N. B. Roberts, "Differential distribution of cobalt, chromium, and nickel between whole blood, plasma and urine in patients after metal-on-metal (MoM) hip arthroplasty," *J. Orthop. Res.*, vol. 30, no. 10, pp. 1640–1646, 2012.

[94] L. L. Hopkins and K. Schwarz, "Chromium (III) binding to serum proteins, specifically siderophilin," *BBA - Gen. Subj.*, vol. 90, no. 3, pp. 484–491, 1964.

[95] Y. Sayato, K. Nakamuro, S. Matsui, and M. Ando, "Metabolic fate of chromium compounds. I. Comparative behavior of chromium in rat administered with Na251CrO4 and 51CrCl3.," *J. Pharmacobiodyn.*, vol. 3, no. 1, pp. 17–23, Jan. 1980.

[96] Y. Sun, J. Ramirez, S. A. Woski, and J. B. Vincent, "The binding of trivalent chromium to low-molecular-weight chromium- binding substance (LMWCr) and the transfer of chromium from transferrin and chromium picolinate to LMWCr," *J. Biol. Inorg. Chem.*, vol. 5, no. 1, pp. 129–136, 2000.

[97] J. B. Vincent and S. Love, "The binding and transport of alternative metals by transferrin," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1820, no. 3, pp. 362–378, 2012.

[98] H. Xie, A. L. Holmes, S. S. Wise, S. Huang, C. Peng, and J. P. Wise, "Neoplastic transformation of human bronchial cells by lead chromate particles," *Am. J. Respir. Cell Mol. Biol.*, vol. 37, no. 5, pp. 544–552, 2007.

[99] H. Xie, A. L. Holmes, S. S. Wise, N. Gordon, and J. P. Wise, "Lead chromate-induced chromosome damage requires extracellular dissolution to liberate chromium ions but does not require particle internalization or intracellular dissolution," *Chem.*

Res. Toxicol., vol. 17, no. 10, pp. 1362–1367, 2004.

[100] A. Levina, H. H. Harris, and P. A. Lay, "X-ray absorption and EPR spectroscopic studies of the biotransformations of chromium(VI) in mammalian cells. Is chromodulin an artifact of isolation methods?," *J. Am. Chem. Soc.*, vol. 129, no. 5, pp. 1065–1075, 2007.

[101] C. D. Quarles, R. K. Marcus, and J. L. Brumaghim, "Competitive binding of Fe3+, Cr3+, and Ni 2+ to transferrin," *J. Biol. Inorg. Chem.*, vol. 16, no. 6, pp. 913–921, 2011.

[102] P. Aisen, R. Aasa, and A. G. Redfield, "The chromium, manganese, and cobalt complexes of transferrin.," *J. Biol. Chem.*, vol. 244, no. 17, pp. 4628–4633, Sep. 1969.

[103] J. M. El Hage Chahine, M. Hémadi, and N. T. Ha-Duong, "Uptake and release of metal ions by transferrin and interaction with receptor 1," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1820, no. 3, pp. 334–347, 2012.

[104] N. R. Rhodes, P. A. LeBlanc, J. F. Rasco, and J. B. Vincent, "Monocarboxylate transporters are not responsible for Cr3+ transport from endosomes," *Biol. Trace Elem. Res.*, vol. 148, no. 3, pp. 409–414, 2012.

[105] "H.A. Headlam, 'The role of Cr(III) and Cr(V) peptide and amino acid complexes in Cr-induced carcinogenesis', Ph.D. dissertation, The University of Sydney , Sydney, Australia, 1998."

[106] B. J. Clodfelder and J. B. Vincent, "The time-dependent transport of chromium in adult rats from the bloodstream to the urine," *J. Biol. Inorg. Chem.*, vol. 10, no. 4, pp. 383–393, 2005.

[107] F. Borguet, R. Cornelis, J. Delanghe, M. C. Lambert, and N. Lameire, "Study of the chromium binding in plasma of patients on continuous ambulatory peritoneal dialysis," *Clin. Chim. Acta*, vol. 238, no. 1, pp. 71–84, 1995.

[108] "Nelms, S. (2005). Inductively Coupled Plasma Mass Spectrometry Handbook. Oxford, England: Blackwell."

[109] "ICP-MS. A Primer. Agilent Technologies, 2005."

[110] J. T. Bernert, W. E. Turner, D. G. Patterson, and L. L. Needham, "Calculation of serum 'total lipid' concentrations for the adjustment of persistent organohalogen toxicant measurements in human samples," *Chemosphere*, vol. 68, no. 5, pp. 824–831, 2007.

[111] P. Novak, T. Zuliani, R. Milačič, and J. Ščančar, "Development of an analytical method for the determination of polybrominated diphenyl ethers in mussels and fish by gas chromatography—Inductively coupled plasma mass spectrometry," *J. Chromatogr. A*, vol. 1524, pp. 179–187, 2017.

[112] P. Novak, T. Zuliani, R. Milačič, and J. Ščančar, "Development of an analytical procedure for the determination of polybrominated diphenyl ethers in environmental water samples by GC-ICP-MS," *Anal. Chim. Acta*, vol. 827, no. May, pp. 64–73, 2014.

[113] B. Novotnik, T. Zuliani, A. Martinčič, J. Ščančar, and R. Milačič, "Effective reduction of polyatomic interferences produced by high chloride and carbon concentrations in determination of Cr(vi) by FPLC-ICP-MS," *J. Anal. At.*

Spectrom., vol. 27, no. 3, pp. 488–495, 2012.

[114] B. Novotnik, T. Zuliani, A. Martinčič, J. Ščančar, and R. Milačič, "Preparation of Cr(VI) and Cr(III) isotopic spike solutions from 50Cr and 53Cr enriched oxides without the use of oxidizing and/or reducing agents," *Talanta*, vol. 99, pp. 83–90, 2012.

[115] P. Rodríguez-González, J. M. Marchante-Gayón, J. I. García Alonso, and A. Sanz-Medel, "Isotope dilution analysis for elemental speciation: A tutorial review," *Spectrochim. Acta - Part B At. Spectrosc.*, vol. 60, no. 2, pp. 151–207, 2005.

[116] J. R. Marrack and H. Hoch, "Serum proteins: A review," *J. Clin. Pathol.*, vol. 2, pp. 161–192, 1949.

[117] A. Sjödin, R. S. Jones, C. R. Lapeza, J.-F. Focant, E. E. McGahee, and D. G. Patterson, "Semiautomated High-Throughput Extraction and Cleanup Method for the Measurement of Polybrominated Diphenyl Ethers, Polybrominated Biphenyls, and Polychlorinated Biphenyls in Human Serum," *Anal. Chem.*, vol. 76, no. 7, pp. 1921–1927, Apr. 2004.

[118] P. Novak, T. Zuliani, R. Milačič, and J. Ščančar, "Development of an analytical method for the determination of polybrominated diphenyl ethers in sewage sludge by the use of gas chromatography coupled to inductively coupled plasma mass spectrometry," *Anal. Chim. Acta*, vol. 915, pp. 27–35, Apr. 2016.

[119] J.-P. Antignac *et al.*, "Exposure assessment of French women and their newborn to brominated flame retardants: Determination of tri- to deca-polybromodiphenylethers (PBDE) in maternal adipose tissue, serum, breast milk and cord serum," *Environ. Pollut.*, vol. 157, no. 1, pp. 164–173, Jan. 2009.

[120] O. I. Kalantzi, T. Geens, A. Covaci, and P. A. Siskos, "Distribution of polybrominated diphenyl ethers (PBDEs) and other persistent organic pollutants in human serum from Greece," *Environ. Int.*, vol. 37, no. 2, pp. 349–353, Feb. 2011.

[121] M. Frederiksen *et al.*, "Polybrominated diphenyl ethers in paired samples of maternal and umbilical cord blood plasma and associations with house dust in a Danish cohort," *Int. J. Hyg. Environ. Health*, vol. 213, no. 4, pp. 233–242, Jul. 2010.

[122] E. Cequier, R. M. Marcé, G. Becher, and C. Thomsen, "Comparing human exposure to emerging and legacy flame retardants from the indoor environment and diet with concentrations measured in serum," *Environ. Int.*, vol. 74, pp. 54–59, Jan. 2015.

[123] H. Fromme *et al.*, "PCBs, PCDD/Fs, and PBDEs in blood samples of a rural population in South Germany," *Int. J. Hyg. Environ. Health*, vol. 218, no. 1, pp. 41–46, Jan. 2015.

[124] R. Milačič, T. Zuliani, J. Vidmar, and J. Ščančar, "Monolithic chromatography in speciation analysis of metal-containing biomolecules: A review," *J. Anal. At. Spectrom.*, vol. 31, no. 9, pp. 1766–1779, 2016.

[125] A. Martincic, R. Milacic, M. Cemazar, G. Sersa, and J. Scancar, "The use of CIM-DEAE monolithic chromatography coupled to ICP-MS to study the distribution of cisplatin in human serum," *Anal. Methods*, vol. 4, no. 3, pp. 780–790, 2012.

[126] "R.B. Martin, 'Are there proteins containing chromium?', in Handbook of Metalloproteins, I. Bertini, A.Sigel, H. Sigel, Eds. Bocca Raton, FL: CCR Taylor&Francis group, 2001, pp. 181-192."

[127] B. J. Clodfelder, J. Emamaullee, D. D. D. Hepburn, N. E. Chakov, H. S. Nettles, and J. B. Vincent, "The trail of chromium(III) in vivo from the blood to the urine: The roles of transferrin and chromodulin," *J. Biol. Inorg. Chem.*, vol. 6, no. 5–6, pp. 608–617, 2001.

[128] B. J. Clodfelder, R. G. Upchurch, and J. B. Vincent, "A comparison of the insulin-sensitive transport of chromium in healthy and model diabetic rats," *J. Inorg. Biochem.*, vol. 98, no. 3, pp. 522–533, 2004.

[129] M. Capellmann and H. M. Bolt, "Chromium (VI) reducing capacity of ascorbic acid and of human plasma in vitro.," *Arch. Toxicol.*, vol. 66, no. 1, pp. 45–50, 1992.

[130] J. Penny and S. Overgaard, "Serum chromium levels sampled with steel needle versus plastic IV cannula. Does method matter?," *J. Biomed. Mater. Res. - Part B Appl. Biomater.*, vol. 92, no. 1, pp. 1–4, 2010.

Bibliography

Publications Related to the Thesis

Journal Articles

M. Bergant, R. Milačič, and J. Ščančar, "Determination of polybrominated diphenyl ethers in human serum by gas chromatography - inductively coupled plasma mass spectrometry.," J. Chromatogr. A, vol. 1572, pp. 112–118, 2018.

M. Bergant, J. Ščančar, and R. Milačič, "Kinetics of interaction of Cr(VI) and Cr(III) with serum constituents and detection of Cr species in human serum at physiological concentration levels," Talanta, vol. 218, pp. 121199, 2020.

Biography

Matic Bergant obtained his master's degree in pharmacy at Faculty of Pharmacy, University of Ljubljana in 2016. He is currently enrolled in Ecotechnologies doctoral study programme at Jožef Stefan International Postgraduate School. He works as a fellow in the group for trace element speciation in the Department of Environmental Sciences, Jožef Stefan institute. His research interests center around the development of analytical methods for trace element speciation in human serum.