May Britt Rian

Determination of Occurrence of 17 Phthalate Metabolites in Harbour Porpoises from the Norwegian Coast

Master's thesis in Natural Science with Teacher Education
Supervisor: Alexandros Asimakopoulos
June 2019

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I would like to dedicate my thanks to following people, who have made this work possible. First, a special thanks to my supervisor, Alexandros Asimakopoulos. I am grateful for the opportunity to work with this interesting and important study. This work had not been possible without you, your guidance and your enthusiasm. Thank you for guiding me through the steps of the laboratory work, teaching me how to handle and discuss the data – and for answering my thousand questions, often more than once. Thank you for being so positive, encouraging me and calming me down. I always walk out of your office super motivated!

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Abstract

Phthalates have received considerable attention due to their high potential of environmental pollution and potential adverse health effects in living organisms. Occurrence of phthalates metabolites are well established in human populations, but is less studied in in marine mammals. Harbour porpoises (*Phocoena phocoena*) bycaught along the Norwegian coast were examined for 17 phthalate metabolites. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to separate and identify the metabolites, after extensive sample preparation involving ultrasonic extraction, solid phase extraction and enzymatic deconjugation. Highest concentrations of the analytes were observed in liver, and consequently, liver was chosen as the sample matrix for determining occurrence of phthalate metabolites in 100 harbour porpoises. Phthalates metabolites were detected in all individuals of the population. A total of 14 out of 17 phthalate metabolites were detected in livers of harbour porpoises. Highest detection rates were found for monoethyl phthalate (mEP; 100 %), followed by monoisobutyl phthalate (mIBP; 99 %), mono-n-butyl phthalate (mBP; 97 %) and monomethyl phthalate (mMP; 69 %). Mono(2-ethyl-1-hexyl) phthalate (mEHP) was found in all samples in relatively high concentrations, but a high background was observed in control samples, indicating contamination. Highest mean concentrations were observed for mono-n-nonyl phthalate (mNP; 48.1 ng/g ww), mBP (37.2 ng/g ww) and mIBP (41.1 ng/g ww). To the best of my knowledge, this is the first study to report occurrence of phthalates metabolites in liver samples of marine mammals.

Sammendrag

Ftalater har mottatt betydelig oppmerksomhet grunnet høyt potensial for miljøforurensning og skadelige helseeffekter hos levende organismer. Forekomst av ftalatmetabolitter er godt etablert hos mennesker, men er i liten grad studert hos marine pattedyr. Niser (*Phocoena phocoena*) utsatt for bifangst i norsk fiskeindustri har blitt undersøkt for 17 ftalatmetabolitter. Væskekromatografi med tandem massespektrometri (LC-MS/MS) ble brukt for separasjon og identifikasjon av metabolittene, etter omfattende prøveforberedelser inkludert ultrasonisk ekstraksjon, solid fase ekstraksjon og enzymatisk dekonjugering. Lever ble valgt ut som prøvematriks fordi høyeste konsentrasjoner av de fleste metabolittene ble målt her. Leverprøver ble dermed brukt for å bestemme forekomst av ftalatmetabolitter i 100 niser. Ftalatmetabolitter ble observert i alle individer av studert populasjon. Totalt 14 av 17 metabolitter ble målt over deteksjonsgrensen i lever hos niser. Høyeste deteksjonsrate ble funnet for monoetylftalat (mEP; 100 %), etterfulgt av monoisobutylftalat (mIBP; 99 %), mono-n-butylftalat (mBP; 97 %) og monometylftalat (mMP; 69 %). Metabolitten mono(2-etyl-1-heksyl)ftalat (mEHP) ble funnet i alle prøver i relativt høye konsentrasjoner, men høyt bakgrunnsnivå i kontrollprøver indikerte kontaminering. Høyeste gjennomsnittlige konsentrasjoner ble målt for mono-n-nonylftalat (mNP; 48,1 ng/g ww), mBP (37,2 ng/g ww) og mIBP (41,1 ng/g ww). Så vidt jeg vet, er dette første studie som rapporterer forekomst av ftalatmetabolitter i leverprøver av marine pattedyr.

Table of contents

List of figures

List of tables

Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
BDL	Below detection limit
dc	Direct-current
DNA	Deoxyribonucleic acid
DR	Detection rate
ECHA	European Chemicals Agency
ESI	Electrospray ionisation
GC	Gas chromatography
HMW	High molecular weight
IR	Ion ratio
IS	Internal standard
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LMW	Low molecular weight
LOD	Limit of detection
LOQ	Limit of quantification
ME	Matrix effects
MPE	Monoalkyl phthalate esters
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass-to-charge
PCA	Principal component analysis
PM	Phthalate metabolite
PP	Poly propylene
PVC	Poly vinyl chloride
R^2	Coefficient of determination
rf	Radio-frequency
RT	Retention time
RR	Relative response

RRT	Relative retention time
RSD	Relative standard deviation
SLE	Solid liquid extraction
SPE	Solid phase extraction
SPIN	Substances in preparations in Nordic countries
STD	Standard deviation
SVHC	Substances of very high concern
TA	Trace analytes
UPLC	Ultra-performance liquid chromatography

Abbreviations of target analytes and internal standards

PA	Phthalic acid
mMP	Monomethyl phthalate
mEP	Monoethyl phthalate
mBP	Mono-n-butyl phthalate
mIBP	Monoisobutyl phthalate
mPeP	Mono-n-pentyl phthalate
mIPeP	Monoisopentyl phthalate
mHxP	Mono-n-hexyl phthalate
mCHP	Monocyclohexyl phthalate
mBzP	Monobenzyl phthalate
mHpP	Mono-n-heptyl phthalate
mOP	Mono-n-octyl phthalate
mEHP	Mono(2-ethyl-1-hexyl) phthalate
mEOHP	Mono(2-ethyl-5-oxohexyl) phthalate
mEHHP	Mono(2-ethyl-5-hydroxyhexyl) phthalate
mDP	Mono-n-decyl phthalate
mNP	Mono-n-nonyl phthalate
mEP-d_4	Monoethyl phthalate-3,4,5,6-d_4
mBP-d_4	Mono-n-butyl phthalate-3,4,5,6-d_4
mNP-d_4	Mono-n-nonyl-phthalate-3,4,5,6-d_4

Chapter 1

Introduction

Anthropogenic activity has led to extensive release of multiple organic pollutants into the environment. Studies investigating environmental concentrations of these pollutants are necessary for assessing the environmental risk. Phthalates are synthetic organic chemicals of high concern due to their widespread applications and use (AMAP, 2017; Rocha et al., 2017). They are used in polyvinyl chloride (PVC) plastics, building materials, personal care products and so on (Emmanouil et al., 2017). They are not chemically bound to the matrix thus they have a high potential for environmental pollution. Phthalate exposure of living organisms are associated with several negative health effects, including effects like endocrine disruption and oxidative stress (Asimakopoulos et al., 2016; Hart et al., 2018). It is therefore important to obtain information of phthalates occurrence in the biota. Quantification of phthalates have been problematic due to high contamination risk since the compounds are abundant in the environment and in the laboratory (Emmanouil et al., 2017). In biological samples, metabolites of the phthalates have been described as suitable biomarkers for assessing phthalate exposure. Occurrence of phthalates metabolites has been well established in human populations (Frederiksen et al., 2013; Asimakopoulos et al., 2016; Rocha et al., 2017), but has been less studied in marine mammals. Harbour porpoises (*Phocoena phocoena*) inhabit coastal waters and are therefore susceptible to chemical pollution from urban areas (Bjørge & Tolley, 2018). The focus in this work was to determine occurrence of phthalates metabolites in tissue of harbour porpoises from the Norwegian coast.

The main aims of the work were 1) to examine what matrix tissue is most suitable for analysis of phthalate metabolites in harbour porpoises by liquid chromatography tandem mass spectrometry (LC-MS/MS); and 2) to establish occurrence and concentrations of phthalate metabolites in target tissue(s) of harbour porpoises (N=100), which were collected along the coast of Norway.

Chapter 2

Theoretical background

2.1 Phthalates

Recently, phthalates have received increasingly public and scientific interest due to their wide array of occurrence, suspicion of exposure risk for humans and the biota and observed adverse effects on animals (Casarett, Klaassen & Doull, 2013; AMAP, 2017). Phthalates are synthetic organic chemicals used in a wide array of applications in high amounts (AMAP, 2017; Rocha et al., 2017). In this thesis the name phthalates refers to *o*-phthalates, the most biologically active phthalates, which are diesters of 1,2-benzenedicarboxylic acid, also called phthalic acid (PA) (National Research Council (NRC), 2008; Emmanouil et al., 2017). Chemical structure consists of one benzene ring linked to two ester functional groups at position 1 and 2 on the ring (Katsikantami et al., 2016). Relevant phthalates and their functional groups are presented in table 2.1. The ester side-chains can vary in length and structure, and the two chains can be identical, such as in di-n-butyl phthalate, or different, as in butyl benzyl phthalate (NRC, 2008). General structure and examples of phthalate esters are shown in figure 2.1 and figure 2.2.

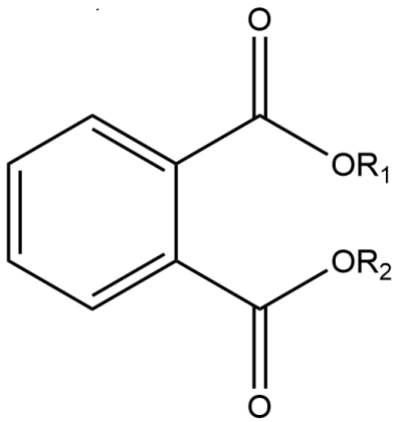

Figure 2.1: General structure of phthalates.

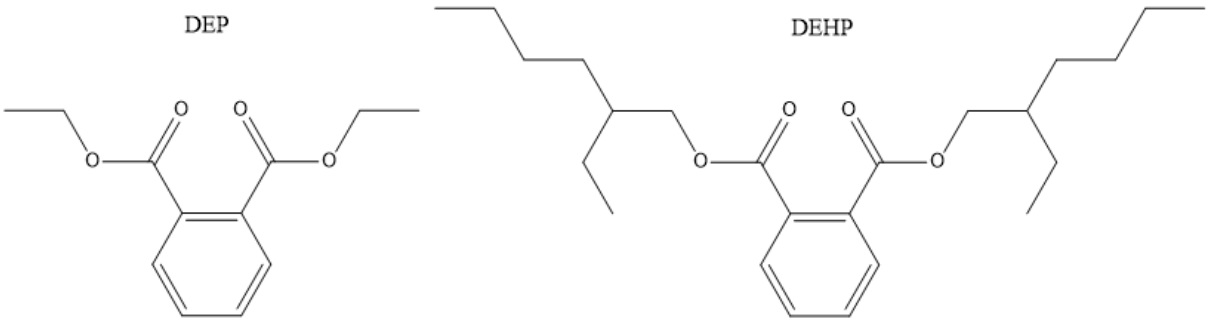

Figure 2.2: Examples of phthalates: diethyl phthalate (DEP) and di(2-ethyl-1-hexyl) phthalate (DEHP).

Table 2.1: Phthalates, abbreviations (Abb.), examples of metabolic products of the phthalate (Met.) and R groups.

Phthalate	**Abb.**	**Met.**	**R group**	
Dimethyl phthalate	DMP	mMP	R_1, R_2	$-CH_3$
Diethyl phthalate	DEP	mEP	R_1, R_2	$-CH_2CH_3$
Di-n-butyl phthalate	DBP	mBP	R_1, R_2	$-CH_2(CH_2)_2CH_3$
Diisobutyl phthalate	DIBP	mIBP	R_1, R_2	$-CH_2CH(CH_3)_2$
Di-n-pentyl phthalate	DnPeP	mPeP	R_1, R_2	$-CH_2(CH_2)_3CH_3$
Di-iso-pentyl phthalate	DIPeP	mIPeP	R_1, R_2	$-CH_2CH_2CH(CH_3)_2$
Di-n-hexyl phthalate	DHxP	mHxP	R_1, R_2	$- CH2(CH_2)_4CH_3$
Dicyclohexyl phthalate	DCHP	mCHP	R_1, R_2	$-C_6H_{11}$
Benzyl butyl phthalate	BBP	mBzP	R_1	$-CH_2C_6H_5$
		mBP	R_2	$-CH_2(CH_2)_2CH_3$
Di-n-octyl phthalate	DnOP	mOP	R_1, R_2	$-CH_2(CH_2)_6CH_3$
Di-n-nonyl phthalate	DnNP	mNP	R_1, R_2	$-CH_2(CH_2)_7CH_3$
Di-n-decyl phthalate	DnDP	mDP	R_1, R_2	$-CH_2(CH_2)_8CH_3$
Di(2-ethyl-1-hexyl)	DEHP	mEHP	R_1, R_2	$-CH_2CH(CH_2CH_3)(CH_2(CH_2)_2CH_3)$
phthalate		mEOHP		
		mEHHP		

Phthalates are categorised into two groups according to the length of their ester side-chains, named low molecular weight (LMW) and high molecular weight (HMW) phthalates (Emmanouil et al., 2017). LMW phthalates can be defined as phthalates with ester side-chain lengths of one to four carbons, including dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP) and diisobutyl phthalate (DIBP). HMW phthalates can be defined as phthalates with ester side-chain lengths of five or more carbon, including di-(2-ethyl-1-

hexyl) phthalate (DEHP), diisononyl phthalate (DINP) and di-*n*-octyl phthalate (DnOP) (NRC, 2008; Rocha et al., 2017). Generally, HMW phthalates are used as plasticizers in polyvinyl chloride (PVC) plastics, and LMW phthalates are applied in different personal care products (AMAP, 2017). In plastics, they have the function to increase the flexibility and other desired properties of the product (Hart et al., 2018). Out of the total global plasticizer production, phthalates stand for 70 % of the global production (Emmanouil et al., 2017). As an additive in personal care products, they often function as an emulsifier and increase solubility of the components in the product (Hart et al., 2018). In addition, phthalates are used in other application areas, as in pharmaceuticals, paint, glue, cleaning and building materials and so on (NRC, 2008; Emmanouil et al, 2017).

Phthalates are produced by a reaction between phthalic anhydride and alcohols as shown in figure 2.3 (Katsikantami et al., 2016). Type of alcohols determines the obtained ester groups in the phthalate and are responsible for the different properties among the different phthalates.

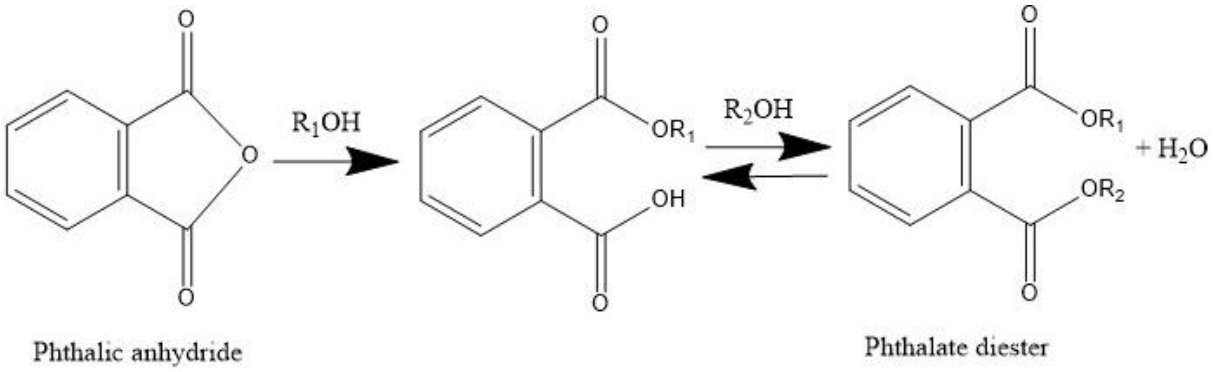

Figure 2.3: Reaction between phthalic anhydride and alcohols in phthalate production (Reproduced from Katsikantami et al., 2016).

2.1.1 Use and production of phthalates in Norway and Europe

In 2011, the global annual production of phthalates was estimated to 5000 million tonnes (Lowell Center for Sustainable Production, 2011). Data obtained from the European Chemicals Agency (ECHA) shows that phthalates are manufactured and imported in high amounts in Europe, even though many of the chemicals are categorised as substances of very high concern (SVHC) and are strictly regulated (table 2.2; ECHA, 2019).

Table 2.2: Reported amounts of phthalates manufactured and/or imported in the European Economic Area, in tonnes/year, hazard classification and regulations of the substance (ECHA, 2019).

	Tonnes/year	**Hazard classification and regulations**	
DMP	1 000 – 10 000	No hazards notified	No regulations
DEP	1 000 – 10 000	No hazards notified	No regulations
DBP	1 000 – 10 000	Categorised as SVHC	Requires authorization; restricted for some uses
DIBP	1 - 10	Categorised as SVHC	Requires authorization
DnPeP	No data	Categorised as SVHC	Requires authorization
DIPeP	1 - 10	Categorised as SVHC	Requires authorization
DHxP	No data	Categorised as SVHC	Requires authorization
DCHP	1 000 – 10 000	Categorised as SVHC	Requires authorization
BBP	1 - 10	Categorised as SVHC	Requires authorization
DnOP	No data	Hazards classified	Restricted for some uses
DnNP	No data	No hazards notified	No regulations
DINP	100 000 – 1 000 000	No hazards notified	Restricted for some uses
DnDP	No data	No hazards notified	No regulations
DEHP	10 000 – 100 000	Categorised as SVCH	Requires authorization; restricted for some uses

SVHC: substances of very high concern.

The use in Norway is predominantly from plastic products imported from other countries. The Norwegian Environment Agency (2018) points out that not much information about use and release of phthalates in Norway is available. Total use of some phthalates in Norwegian products from 2000 to 2017 is showed in figure 2.4. Importantly, the figure is based on numbers from the Substances of Preparations in Nordic Countries (SPIN) database (total use of substances in products in Nordic Countries based on Product Registries), which is only listing ingredients of chemical preparations, not in finished consumer applications which is the predominant use of phthalates (AMAP, 2017). The data in figure 2.4 will therefore only indicate relative use among the different types of phthalates. A change in phthalates use since 2000 is evident in the usage data from Norway (SPIN, n.d.). Exemplified with the decreases of DEHP in the early 2000s and an increase of DINP, especially with the high amounts used in 2016. At the same time, the Norwegian Environment Agency (2019) reports that even though the use of DEHP in Norwegian products have been reduced with 50 % since 1995, the phthalate is still used in high quantities due to an increased use of imported products and the amount released into the environment is difficult to quantify. This might also be the case for other phthalates.

The reason for the shifts of phthalates in use the last decades are the inclusion of phthalates of concern in authority's priority lists (Norwegian environment agency, 2019). ECHA have included some phthalates, including DEHP, BBP, DBP and DIBP, in the candidate list of substances of very high concern (SVHC) due to their reproductive toxicity and endocrine disrupting properties (ECHA, 2016). These are therefore strictly regulated and proposed to be restricted. Companies are obligated to follow the regulations, and a reduction of these phthalates are therefore seen.

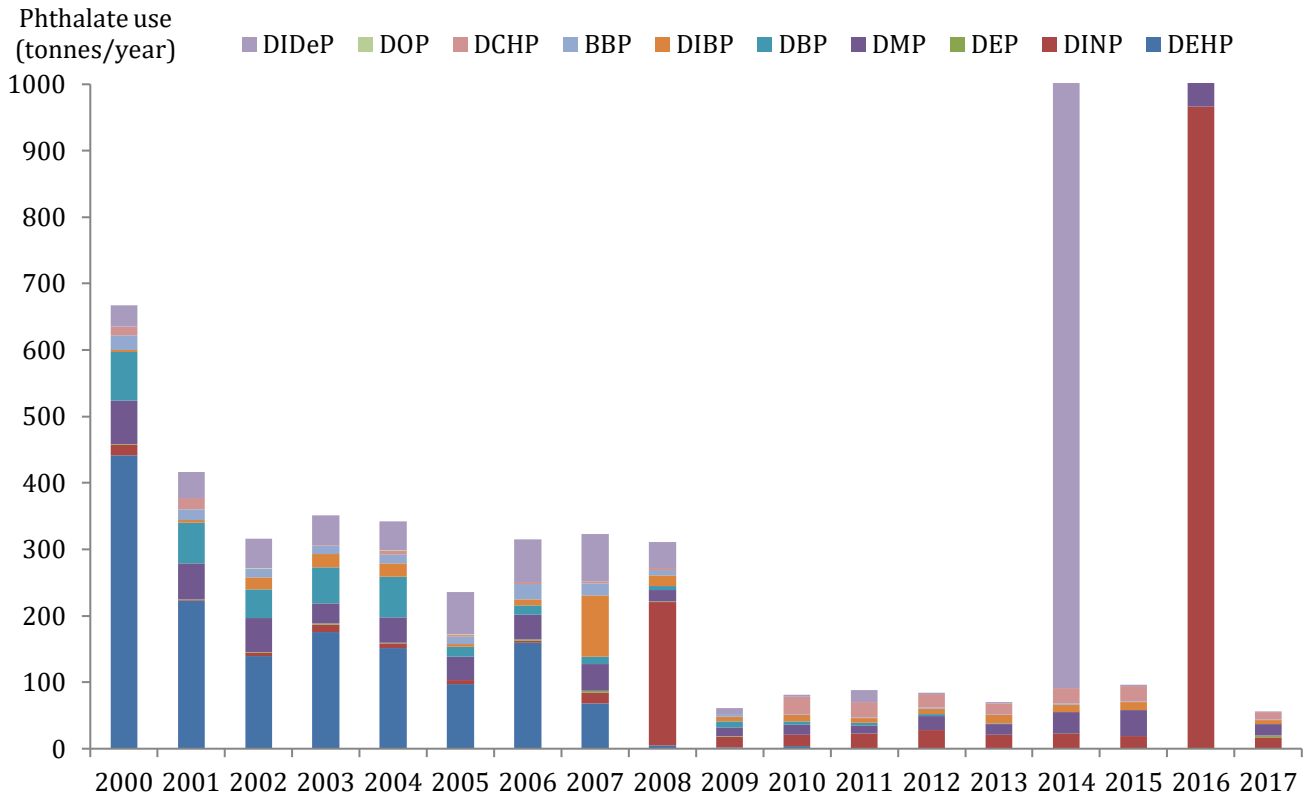

Figure 2.4: Total use of some phthalates in Norway from 2000 to 2017 in tonnes per year. The data is obtained from the SPIN database (n.d.).

2.1.2 Physical-chemical properties

Phthalates physical-chemical properties vary with the length and structural differences of the ester side-chain, including the partition coefficient, vapour pressure and solubility (AMAP, 2017; NRC, 2008). They are considered hydrophobic, where generally more branched and

isomeric phthalates are more hydrophobic (Frederiksen, Skakkebaek & Andersson, 2007). They comprise a huge group of compounds and therefore also exhibit different behaviours. Longer chained phthalates have a greater evaporative potential than low chained, in addition to a higher potential to sorption to suspended particles in waters (Cousins, Mackay & Parkerton, 2013). Due to varying properties, phthalates have varied lifetime in the environment. Degradation and biotranformation of phthalates can occur through different pathways, with monoalkyl phthalate esters (MPEs) as the primary product (AMAP, 2017). Transformation processes include photodegradation in the atmosphere, biotransformation by aquatic and terrestrial biota and anaerobic degradation in sediments and soil (Hu et al., 2016; AMAP, 2017).

2.1.3 Release into the marine environment

Increased anthropogenic activity and the associated industrialization is accompanied with release of pollutions into marine environment. During the last decade, organic pollutants have been observed in marine organisms (Weijs et al., 2010; Fourgous et al., 2016). The marine environment is exposed to phthalate contamination, as reported by the Arctic Monitoring and Assessment Programme (AMAP, 2017). Various phthalates have been detected in Arctic marine waters, sediments and biota. Phthalates can be released into the environment from manufacturing, use of household products and other applications, and disposal (AMAP, 2017). They are not chemically bound to the polymers, and can therefore easily leach out, migrate or gas off from the material into the environment, especially when the phthalate containing material are exposed to high temperatures (NRC, 2008).

Direct release into the atmosphere is one of the major pathways for release in the environment, and studies have indicated that atmospheric deposition is a significant source of phthalates to open waters (Xie et al., 2007; AMAP, 2017). Kang et al. (2017) detected phthalates in marine aerosols over the East China Sea, suggesting aerosols with anthropogenic origin as a possible source for marine surface water contamination of phthalates through air-sea transfer. Xie et al. (2007) found a decreasing lateral trend from the Norwegian coast to the high Arctic, suggesting coastal waters near urban areas are more susceptible for phthalate

contamination. Significant higher concentrations of phthalates in coastal waters compared to offshore suggests freshwater input and urban area as sources (Paluselli et al., 2018).

Another important route is release via industrial or municipal wastewater effluents or leaching from landfills. In Nordic countries, various phthalates have been detected in the wastewater effluents. Highest levels observed in municipal wastewater treatments plants are HMW phthalates, but also low concentrations of LMW phthalates are observed (AMAP, 2017). Based on data on release to the environment, the Norwegian environment agency (2018) have calculated that the majority of the phthalate DEHP ends up in natural waters (figure 2.5). Modelling of the phthalates fate in the environment based on their properties suggests that only a small proportion of HMW phthalates will stay in the water compartment, while for the smallest LMW phthalates, DMP and DEP, the majority stay in the water media (Cousins, Mackay & Parkerton, 2013). Determinations throughout the water column have suggested that resuspension from sediments, plastic debris in surface waters and degradation in upper layers play a significant role in phthalate dynamics in coastal waters (Paluselli et al., 2018).

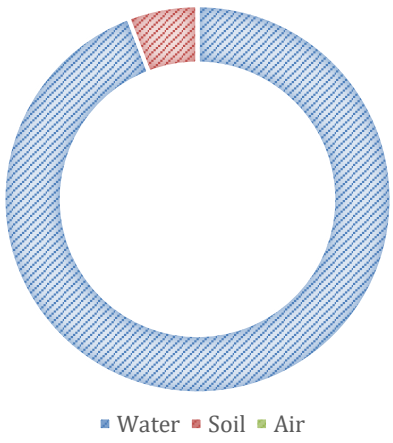

Figure 2.5: DEHP distribution in the environment after release. Data provided by the Norwegian Environment Agency (2018).

2.1.4 Exposure and effects of phthalates

Due to its widespread applications and use, phthalates have become ubiquitous in the environment and are of great concern for human and animal health (Emmanouil et al., 2017). Many studies have found adverse health outcomes of phthalate exposure in mammals. The general exposure route of phthalates for mammals is through ingestion of contaminated food and water, inhalation and absorption (Rocha et al., 2017). Free monoester phthalates, metabolic products of phthalates, are supposed to induce toxicity while parent compounds and glucuronidated metabolites are considered non-toxic (Katsikantami et al., 2016).

Some phthalates are identified as endocrine disruptors, inducing reproductive and development toxicity (AMAP, 2017; Hart et al., 2018). Casarett, Klaassen and Doull (2013) classifies phthalates as environmental antiandrogens, as they seem to lower testosterone levels in mammals. Studies have also shown that phthalates alter the thyroid hormone levels in humans (Casarett, Klaassen & Doull, 2013). These endocrine disruptions can lead to altered age of pubertal maturation, breast cancer, reduced fertility, abnormal reproductive organ development and so on (Casarett, Klaassen & Doull, 2013; Hart et al., 2018). Studies have shown positive correlations between levels of certain phthalates metabolites in human urine and oxidative DNA damage, suggesting that phthalates contribute to oxidative stress (Asimakopoulus et al., 2016; Rocha et al., 2017). Oxidative stress is defined as an imbalance between production of reactive oxygen species and antioxidants, with an overload of reactive oxygen species that can lead to potential damage (Boelsterli, 2007). Oxidative stress contributes not only to direct cell injury, but also affects signal transduction and regulation of gene expression, and act as a trigger for many diseases, including cancer and atherosclerosis (Boelsterli, 2007; Asimakopoulos et al., 2016).

Effects of phthalates on aquatic organisms have been studied on different fish species, molluscs and crustaceans, and reproductive impairment is documented in all of these groups (Oehlmann et al., 2009). Aquatic organisms are likely to be exposed to phthalates because of its widespread occurrence in the aquatic and marine environment, via ingestion or inhalation of phthalate contaminated air or water, food and/or sediments depending in their ecological niche and by direct, indirect or accidental ingestion of plastic debris (Oehlmann et al., 2009; Hart et al., 2018). Reported health outcomes associated with phthalate exposure in fish include among others altered reproductive physiology, metabolism and behaviour (Oehlmann

et al., 2009). As I am aware of, there are no published studies assessing health outcomes of phthalate exposure for marine mammals. Exposure of other endocrine disrupting chemicals, such as polychlorinated biphenyls, in cetacean populations have shown to be linked to increased risk of reproductive failure (Schwacke et al., 2002; Wells et al., 2005).

2.2 Phthalate metabolites

No information about phthalate metabolism is available for harbour porpoises, as I am aware of, but the metabolisms in humans and other mammals are well studied. Phthalates that enter the human body are metabolized and excreted via urine and sweat (Katsikantami et al., 2016). The rate of excretion depends on the molecular weight of the phthalate, where small phthalates is excreted faster than bigger phthalates. Phthalates are relatively quickly metabolized in mammals, with a metabolic pathway that normally follow at least two phases, shown in figure 2.6. Phase I is a hydrolysis reaction, where the dialkyl phthalate esters are hydrolysed to monoalkyl phthalate esters (MPEs), replacing one of the ester substituents with a carboxyl group. Phase II involve a conjugation where the hydrophilic glucuronide conjugate is made, to increase the solubility for a more efficient excretion (Kato et al., 2003; Hu et al., 2016). HMW phthalates often undergo several biotransformations prior to the phase II conjugation, including further oxidation and hydroxylation (Frederiksen, Skakkebaek & Andersson, 2007). For example, for the HMW phthalate DEHP more than 15 metabolites have been identified in mammals, including the oxidized forms mEHHP and mEOHP in addition to its primary metabolite mEHP. Further hydrolysis of the phthalate monoester results in phthalic acid (Peterson & Staples, 2003).

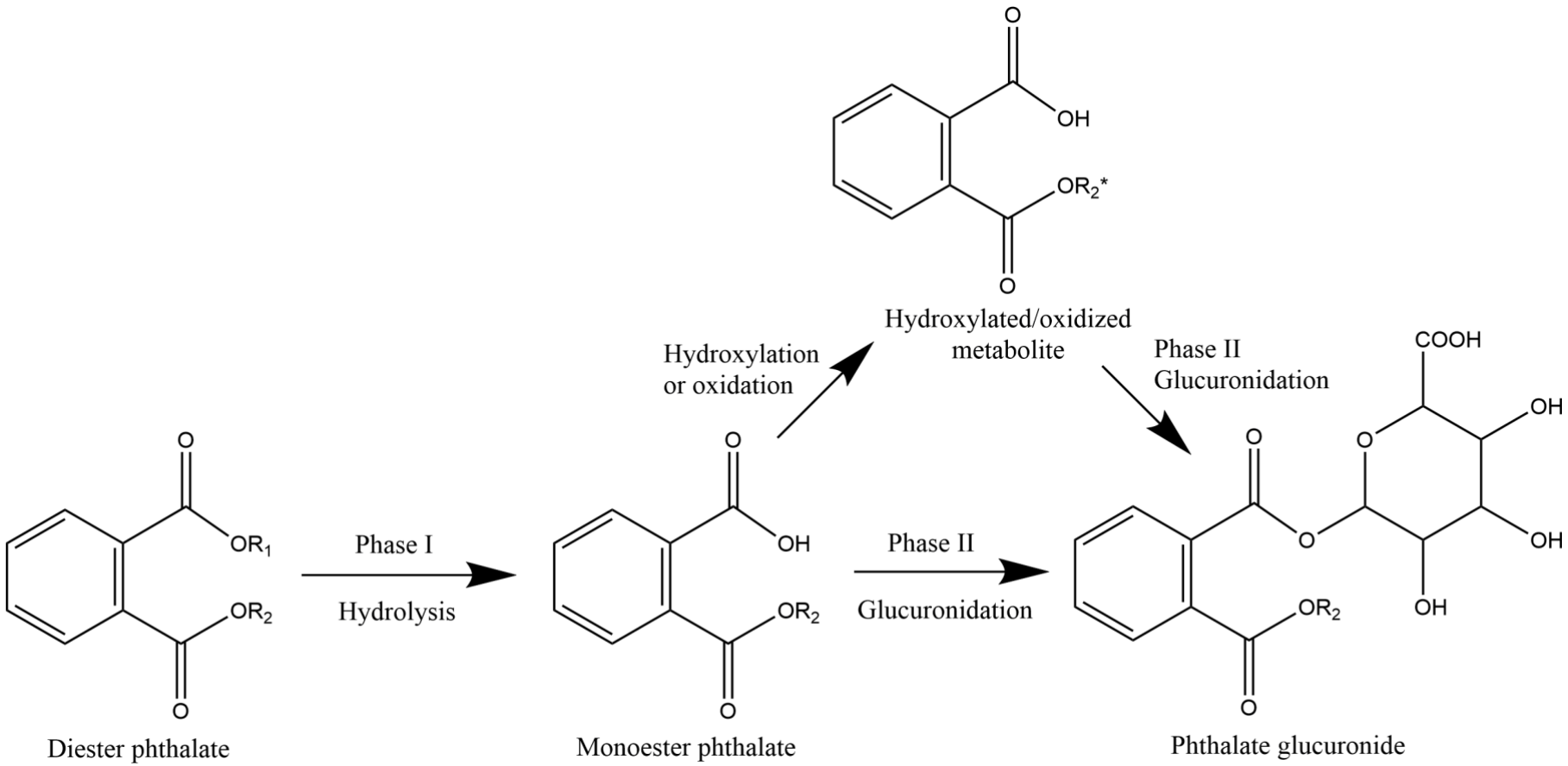

Figure 2.6: General pathway for phthalate metabolism, including hydrolysis in phase I, conjugation with glucuronide in phase II and hydroxylation or oxidation (Reproduced from Frederiksen, Skakkebaek & Andersson, 2007). R_2* is a hydroxylated or oxidized R-group that will follow into the glucuronidated product.

2.2.1 Phthalate metabolites as biomarkers

The phthalates diesters are not considered sufficient indicators of phthalate exposure, due to the relatively quick metabolic break down in the biota (Hu et al., 2016). Phthalates metabolites as biomarkers are considered to be more reliable indicators of phthalates exposure than the parent compounds. This is also because of the high contamination risk when measuring the parent compound (Emmanouil et al., 2017). Analytically, MPEs are normally used as biomarkers, with an enzymatic deconjugation prior to the analysis to get rid of the glucuronide group (Emmanouil et al., 2017). For example, mEP is an effective biomarker used to assess exposure of DEP (Frederiksen, Skakkebaek & Andersson, 2007). Generally, using MPEs as biomarkers after a deconjugation of the glucuronide conjugate are effective for LMW phthalates, but for HMW phthalates the MPEs are quickly further transformed to several metabolic products, where the oxidized metabolites are the main metabolites observed in human urine (Albro et al., 1983; Silva et al., 2005; Silva et al., 2006; Koch et al., 2012). As a result, typically less than 10 % of absorbed dose of HMW phthalates are detected when analysing MPEs in human urine, which leads to an underestimation of phthalates exposure. Phthalic acid (PA), the hydrolysis product of the phthalate monoesters, has been used as an indirect biomarker to assess phthalate exposure. A disadvantage of using PA as a phthalate biomarker is the lack of specificity of the different phthalate compounds (Emmanouil et al., 2017). Therefore, MPEs are preferred when assessing phthalates exposure.

2.3 Study population: Harbour Porpoises (*Phocoena phocoena*)

The harbour porpoise (*Phocoena phocoena*) is a small toothed whale that inhabits coastal waters of the North Pacific, North Atlantic and the Black Sea (Bjørge & Tolley, 2018). The harbour porpoises often reside within an area for a long period of time, but offshore migrations and movements along the coast is observed. Concerns for small cetaceans in northern European waters, especially for harbour porpoises, have arisen due to reported population declines (Hammond et al., 2002). Harbour porpoises are susceptible to incidental catching by the extensive fisheries industry in Norway and are reported as one of the most frequent bycaught mammals (Bjørge, Skern-Mauritzen & Rossman, 2013). Since the harbour porpoises inhabit coastal waters, they are exposed to different negative environmental effects

of the human population, including chemical pollution (Bjørge & Tolley, 2018). Harbour porpoises in the European North Sea are top predators with relatively long life spans and limited capacity for metabolic biotransformation of pollutants compared to other marine mammals (Weijs et al., 2010). Therefore, they are vulnerable for environmental pollutions. For example, different types of persistent organic pollutants are found in tissues of the harbour porpoises in earlier studies (Skaare, 1996; Weijs et al., 2010).

2.3.1 Biological samples

When monitoring phthalate exposure in humans, urine is considered as an important matrix for the determination of phthalates metabolites, because of the high occurrence due to relatively quick metabolism and excretion (Emmanouil et al., 2017). Although urine is a preferred matrix for analysing biotransformed xenobiotics is it not a common matrix used to assess exposure for wildlife (Hart et al., 2018). Phthalates metabolites have been detected in tissues like liver, kidney, muscle, skin and fat in a various group of species (Hu et al., 2016; Valton et al., 2014; Baini et al., 2017). Toxicants absorbed into the body are distributed to different organs, depending on their structure and their properties. The liver has a high capacity for concentrating many chemicals potentially due to active transport into the liver and binding of the toxicants (Casarett, Klaassen & Doull, 2013). Fat has the tendency to store toxicants, but only if they are highly stable and lipophilic. To be able to do a precise determination, different tissue matrices should be screened for the target analytes.

2.4 Data from earlier studies

Phthalates are detected in the environment in air, seawater and biota, with highest concentrations near urban areas (AMAP, 2017). In the biota, phthalates metabolites have been detected in different species in different matrices. Examples are shown in table 2.3, including phthalates metabolites with most available data. Harbour porpoises inhabit the marine environment together with other aquatic organisms and are classified as mammals. Therefore, data from earlier studies from these two groups are relevant to present under this section.

Representing terrestrial mammals, human studies are presented, since their body size is relatively similar to the harbour porpoises.

To date, as I am aware of, exposure of phthalates metabolites has not been assessed before for harbour porpoises. Studies have demonstrated that other marine mammals are exposed to phthalates (table 2.4). Only mEP, mBP, mBzP and mEHP have been measured in cetaceans, in small populations and small number of studies (table 2.3). On the other hand, human studies of urinary phthalates metabolites have shown that humans are subject to an exposure of a broad range of phthalates (table 2.4).

Table 2.3: Mean concentrations of chosen phthalate metabolites in different species, expressed in ng/ml or ng/g dry weight (dw)/lipid basis (l.b). BDL: below detection limit.

Species	Matrix	Unit	mMP	mEP	mIBP	mBP	mBzP	mEHP	mOP	mEOHP	mEHHP	Study	Location
Humans (N=300)	Urine	ng/ml	10.4	278	73.8	113	3.83	36.1	1.66	36.6	53.8	Rocha et al., 2017	Brazil
Humans (N=130)	Urine	ng/ml	14.4	261	46.0	66.5	4.96	133	495	42.8	29.9	Asimakopoulos et al., 2016	Saudi Arabia
Humans (N=145)	Urine	ng/ml	-	74	48	26	6.1	4.7	0.001	10	21	Frederiksen et al., 2013	Denmark
European eel (*Anguilla anguilla*) (N=117)	Muscle	ng/g dw	5.7	33	206	174	2.0	282	82	34	94	Fourgous et al., 2016	France
Roach (*Rutilus rutilus*) (N=4)	Liver	ng/g dw	18.9	263	1610	1500	19.7	237	17.7	7.1	163	Valton et al., 2014	France
American Alligator (*Alligator mississippiensis*) (N=9)	Urine	ng/ml	-	-	-	22.0	6.34	4540	-	-	-	Brock, Bell & Guillette 2016	USA
Basking shark (*Cetorhinus maximus*) (N=6)	Muscle	ng/g l.b.	-	-	-	-	-	84.2	-	-	-	Fossi et al. 2014	Italia
Fin whale (*Balaenoptera physalus*) (N=5)	Blubber	ng/g l.b.	-	-	-	-	-	177	-	-	-	Fossi et al. 2014	Italia
Fin whale (*Balaenoptera physalus*) (N=3)	Skin	ng/g dw	-	-	-	984	32.1	<BDL	-	-	-	Baini et al., 2017	Mediterra-nean sea
Striped dolphin (*Stenella coeruleoalba*) (N=2)	Skin	ng/g dw	-	-	-	<BDL	<BDL	1720	-	-	-	Baini et al., 2017	Mediterra-nean sea
Risso´s dolphin (*Grampus griseus*) (N=1)	Skin	ng/g dw	-	-	-	<BDL	<BDL	464	-	-	-	Baini et al., 2017	Mediterra-nean sea
Bottlenose dolphin (*Tursiops truncatus*) (N=1)	Skin	ng/g dw	-	-	-	780	<BDL	1770	-	-	-	Baini et al., 2017	Mediterra-nean sea
Bottlenose dolphin (*Tursiops truncatus*) (N=17)	Urine	ng/ml	-	11.0	<BDL	<BDL	<BDL	2.3	-	0.3	<BDL	Hart et al., 2018	USA

Table 2.4: Mean concentrations of some phthalates in marine mammals, expressed in ng/g wet weight (ww) or ng/g dry weight (dw). BDL: below detection limit.

Species	Matrix	Unit	DMP	DEP	DBP	BBzP	DEHP	DnOP	DHxP	DnDP	Study	Location
Polar bear (*Urus maritimus*) (N=3)	Liver	ng/g ww	51.5	21.7	12.8	31.0	143*	22.0[a)]	41.3	-	Vorkamp et al., 2004	Greenland
Minke whale (*Balaenoptera acutorostrata*) (N=1)	Liver	ng/g ww	2.5*	15.2	10.7	29.7	86.2	6.7	7.2	-	Vorkamp et al., 2004	Greenland
Pilot whale (*Globicephala melas*) (N=4)	Liver	ng/g ww	2.5*	23.2	15.1	28.0	77.7 [a)]	5.6**	2.5*	-	Vorkamp et al., 2004	Faroe Islands
Ringed seal (*Phoca hispida*) (N=5)	Liver	ng/g ww	3.68**	20.1	7.26**	31.0	117	7.6	13.4	-	Vorkamp et al., 2004	Greenland
Fin whale (*Balaenoptera physalus*) (N=3)	Skin	ng/g dw	-	-		260	7 050	-	<BDL	<BDL	Baini et al., 2017	Mediterranean Sea
Striped dolphin (*Stenella coeruleoalba*) (N=2)	Skin	ng/g dw	-	-		<BDL	21 500	-	<BDL	<BDL	Baini et al., 2017	Mediterranean Sea
Risso´s dolphin (*Grampus griseus*) (N=1)	Skin	ng/g dw	-	-		1630	1 130	-	<BDL	<BDL	Baini et al., 2017	Mediterranean Sea
Bottlenose dolphin (*Tursiops truncatus*) (N=1)	Skin	ng/g dw	-	-		<BDL	26 100	-	<BDL	<BDL	Baini et al., 2017	Mediterranean Sea

* Reported values < LOQ; ** some values involved in average < LOQ; [a)] N=N-1.

2.5 Trace organic analysis

In trace organic analysis the samples contain very low concentrations of the analytes of interest, and physical separation of the analytes from the matrix is often preferred before analysis (Fifield & Haines, 2000). In addition, physical separation can be used to remove interfering components from the extract. Presence of co-extractives can interfere with the determination of the target analytes and can also adversely affect the sensitivity and precision of the analysis due to its contribution to fouling of the chromatographic column and detector (Fifield & Haines, 2000). Trace analysis of phthalates often show too high concentrations because of contamination during sampling, storage and sample prepraration, as they are ubiquitous in both the environment and the laboratory (Emmanouil et al., 2017; Kato et al., 2003). Determination of the phthalate metabolites, rather than the phthalates itself, will give a more precise measurement due to reduction/elimination of possible sources of contamination (Emmanouil et al., 2017).

2.5.1 Sample preparation

Trace analysis of organic contaminants in biological samples is challenging due to several reasons. First, the complexity of the matrix can lead to matrix effects that might exert a negative impact on method validation parameters such as LOD, LOQ, linearity, accuracy and precision. Therefore, it is often necessary to extract out the analytes of interest and remove interfering components. Secondly, organic contaminants or its metabolites in biological samples are often found in low concentrations, so enrichment of the analytes is therefore important (Asimakopoulos, 2014). Sample preparation will ideally transfer only the analytes of interest from the sample matrix into a solvent prior to analysis (Fifield & Haines, 2000).

Solid-liquid extraction

When analysing solid samples, solid-liquid extraction (SLE) can be used to transfer the organic compounds from a solid matrix to a liquid phase (Dean, 2014). Sonication (ultrasonic extraction) uses sound waves to agitate the solid sample immersed into the solvent (Dean, 2014). If the acoustic pressure is high enough, micro-bubbles will form in the liquid, grow,

oscillate quickly and eventually collapse and release large amounts of energy. This creates high temperature and pressure – hotspots able to accelerate chemical reactivity. Bubbles collapsing on the surface of a biological solid matrix will lead to cell membrane rupture, and its content will be released from the cell (Vorobiev & Chemat, 2010).

Solid phase extraction

Solid phase extraction (SPE) is a widely used extraction and clean-up technique, used to extract analytes from a complex sample (Simpson, 2000; Lundanes et al., 2014). In SPE, a specific volume of liquid sample is passed through a cartridge tube packed with a solid material that acts as an extraction agent (Asimakopoulos, 2014). The analyte will selectively be transferred from the solvated sample to the solid phase, either by adsorption to the surface or by penetration of the surface molecules of the solid phase (Fifield & Haines, 2000; Simpson, 2000). After washing out the interferences, the retained components on the solid phase is then transferred to a liquid solvent with a more desirable environment for the components and collected. This process is called elution (Simpson, 2000). The main objectives of SPE are to concentrate the components of interest, to clean up i.e. remove the interfering compounds in the sample before analysis and to exchange solvents (Simpson, 2000).

The solid phase cartridges are often constructed as a funnelled barrel partially filled with a solid porous core, composed of an ideally inert porous support, coated with a liquid phase that provides the chemical surface the analytes are selectively attracted to (Fifield & Haines, 2000). Choice of sorbent is critical, since it determines which components that will adsorb to the solid phase. Nonpolar adsorbents will retain nonpolar components and polar adsorbent will retain polar components (Asimakopoulos, 2014). The general procedure in SPE follow five steps, as shown in figure 2.7. First, the sorbent is 1) activated by condition and 2) rinsed to remove residues remaining after cartridge manufacturing. The next step is 3) to apply the sample, then 4) the sorbent is washed again with a solvent to remove impurities, and finally 5) the analyte is eluted with another solvent with appropriate elution strength (Fifield &Haines, 2000; Lundanes et al., 2014). A vacuum is often applied to assist the transfer of liquids through the cartridges.

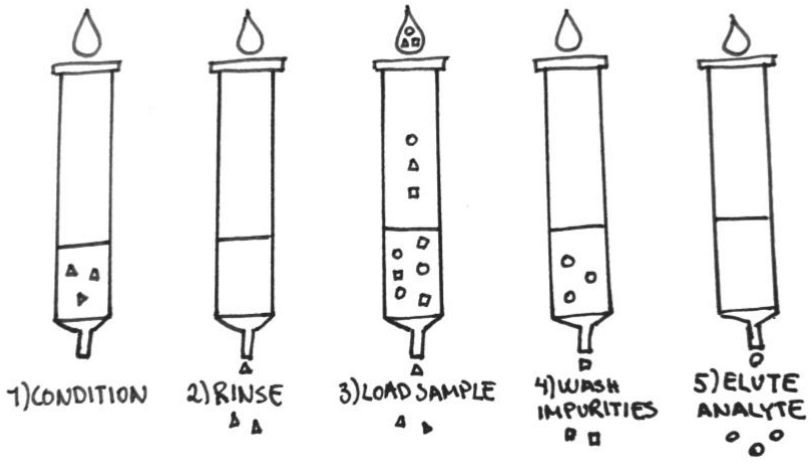

Figure 2.7: General SPE steps.

Enzymatic deconjugation

Phthalates metabolites are conjugated into glucuronides in the liver and intestines in mammals, as observed in humans, dogs, mice, green monkey and guinea pig (Albro et al., 1982; Hanoika et al., 2015). Therefore, an enzymatic deconjugation is necessary to be able to detect monoalkyl phthalate metabolites in the biological samples. An enzyme, which is able catalyse this reaction, is the β-glucuronidase. After incubation with this deconjugating enzyme, the phthalate metabolites will be in the form of monoalkyl phthalate esters.

2.5.2 Analytical technique – LC-MS/MS

Preferred analytical techniques for separation, identification and quantification of phthalate metabolites are liquid chromatography (LC) and gas chromatography (GC) coupled to a mass spectrometer (MS) (Emmanoil et al., 2017). Column chromatography can be used to isolate the individual analytes from a mixture, and in combination with a mass spectrometer (MS) the analytes can be identified and quantified (Fifield & Haines, 2000; Hoffmann & Stroobant, 2007). The separated analytes are introduced one by one regarding their retention time in the chromatographic column to the MS for detection (Hoffmann & Stroobant, 2007). LC-MS is the preferred technique for determining phthalates metabolites from biological matrices, because of its availability and low cost, in addition to its high sensitivity, precision and accuracy (Emmanouil et al., 2017). LC combined with tandem mass spectrometry (LC-MS/MS) for detection of phthalates metabolites present higher sensitivity, resolution and effectiveness compared to other LC-MS techniques.

Liquid chromatography

Liquid chromatography (LC) is a powerful separation method, able to efficiently separate mixtures with many similar compounds. The method can give both qualitative and quantitative information about the compounds in the sample, since each compound has its individual elution time and the peaks area in the chromatogram is proportional to the concentration of the compound (Mayer, 2010). The advanced instrumentation, ultra-performance liquid chromatography (UPLC) can lead to improved analytical resolution and sensitivity (Waters, 2019).

The UPLC instrument include the basic elements presented in figure 2.8. First, the solvents (mobile phase) are mixed and pumped by a high-pressure pump into the injector, where the sample is introduced and dissolved in the solvents. The mobile phase, including the sample, travels further through the column where the separation of the different compounds in the sample occur. The separated compounds are further detected in the detector, and then sent to waste, while signals are transferred to the data acquisition (Mayer, 2010; Lundanes et al., 2014). The signals are recorded as curves called peaks, and the whole entity is known as a chromatogram (Mayer, 2010). The column, the most important part of the LC system, is able to separate the individual components in the sample because different interactions between the different compounds and the stationary phase leads to different retention times in the system (Lundanes et al., 2014). An UPLC column will give increased performance (Waters, 2019). Retention time can be defined as the time between the injection of the sample until the recording of the signal maximum of the component in the detector and can tell us to which extent the compound has interacted with the stationary phase (Mayer, 2010; Lundanes et al., 2014).

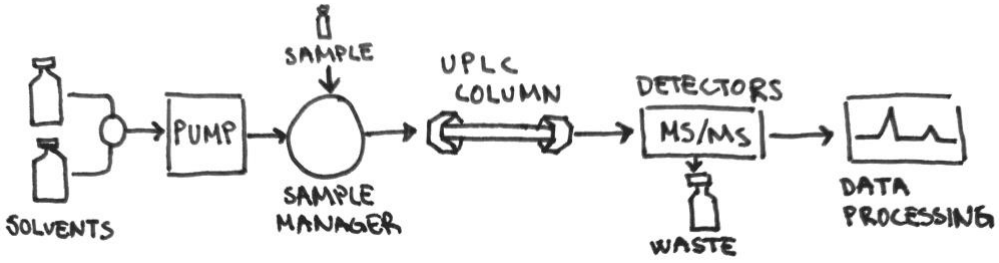

Figure 2.8: Schematic diagram of UPLC instrumentation (reproduced from LaboratoryInfo.com, 2019).

Mass spectrometry

Mass spectrometry (MS) is often used as detector of organic compounds due to its high sensitivity, detection limits, speed and diversity (Fifield & Haines, 2000; Hoffmann & Stroobant, 2007). The sample is introduced into a region in a molecular or atomic state, where it is converted to ions in contact with a high energy electron beam (Fifield & Haines, 2000). The excited ions normally undergo fragmentation to ions of lower masses. In the mass spectrometer, the produced ions are separated according to their mass-to-charge (*m/z*) ratio, and the mass spectrometer shows a plot of ion abundance versus their *m/z* ratio (Hoffmann & Stroobant, 2007). This can be used to identify and quantify the compounds in the applied sample.

A mass spectrometer includes the basic elements shown in figure 2.9. First, the sample is introduced through the sample inlet, often from a combined chromatographic instrumentation. Ions and fragments are produced in the ionization source and are further separated in one or more analysers. After the separation, a detector counts the passing ions and a data processing system will finally produce a mass spectrum (Hoffmann & Stroobant, 2007).

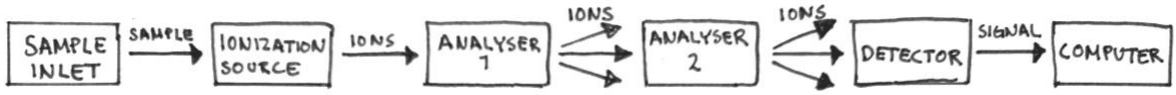

Figure 2.9: Basic diagram for mass spectrometer with two analysers (reproduced from Hoffmann & Stroobant, 2007).

Electrospray ionisation (ESI) can be used as an ionisation source, with its important characteristics – it can produce multiple charged ions from large molecules (Hoffmann & Stroobant, 2007). ESI is produced by the application of a strong electric field to a capillary tube, where a liquid is passing through with a weak flux. To generate positive ions a positive potential is applied to the capillary and to generate negative ions a negative potential is applied (Poole, 2003). This electric field induces an accumulation of charge at the liquid surface at the end of the tube, leading to the formation of highly charged droplets (figure 2.10). A gas is used to spread the droplets in a limited space. These droplets are then passed through either a curtain of heated inert gas or through a heated capillary to evaporate solvents. When the droplets shrink, the charge per unit volume increases and desorption of ions occur form the surface.

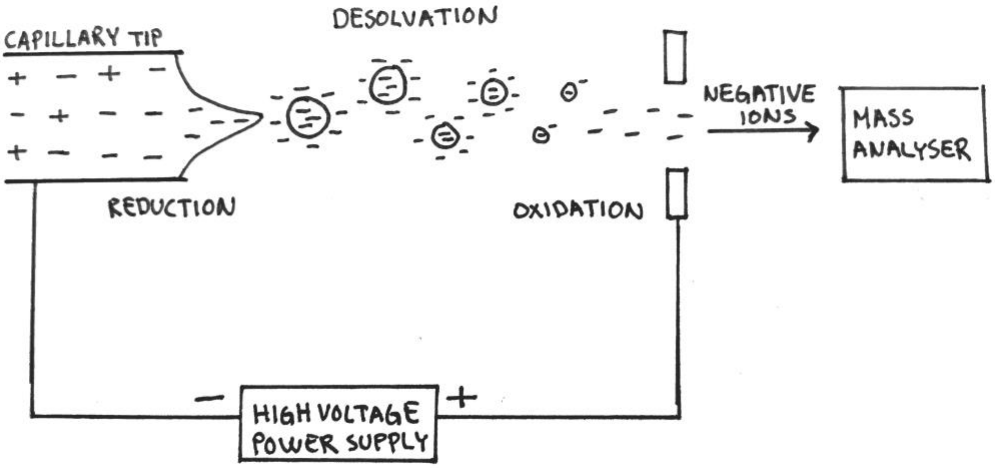

Figure 2.10: Schematic presentation of electrochemical process in negative mode ESI (reproduced from Hoffmann & Stroobant, 2007).

Produced gas phase ions are separated according to their *m/z* ratio by the mass analyser (Hoffmann & Stroobant, 2007). Tandem mass spectrometry (MS/MS) is a popular method involving two stages of mass analysis. Often, the first analyser isolates a precursor ion, followed by a fragmentation, and then the second analyser analyses product ions resulting from the fragmentation of the precursor ion (Hoffmann & Stroobant, 2007). This method allows for high-selectivity and high-sensitivity detection of target compounds in a complex mixture (Hoffmann & Stroobant, 2007). A popular instrumentation used in MS/MS is triple-quadrupole, with high sensitivity and efficiency.

A quadrupole analyser consists of four parallel rods applied with direct-current (dc) and radio-frequency (rf) potentials (Dass, 2007). The quadrupole analyser is able to separate ions by the mass by cycling between negative and positive applied dc potential, making the ions accelerate towards and against the rods leading to different retention in the field depending on their *m/z* ratio. The mass spectrum can be obtained by shifting both dc and rf potentials while keeping their ratio constant, working as a mass filter. As shown in figure 2.11, the triple quadrupole device uses three sequentially arranged quadrupoles to separate and detect the ions and fragments (Dass, 2007). Q_1 operates as a normal mass filter applied with both dc and rf potentials, able to mass-analyse and transmit ions of a specified *m/z* value. These ions are passed over to Q_2 which is operated in an only-rf mode, where the ions undergo a collision-induced dissociation. Here, the precursor ions are excited to higher energy states by collision with inert gas atoms, leading to a fragmentation of the ions. Products formed in Q_2 are passed

over to Q_3, which are scanned to mass-analyse the new fragments by their *m/z* ratio (Dass, 2007).

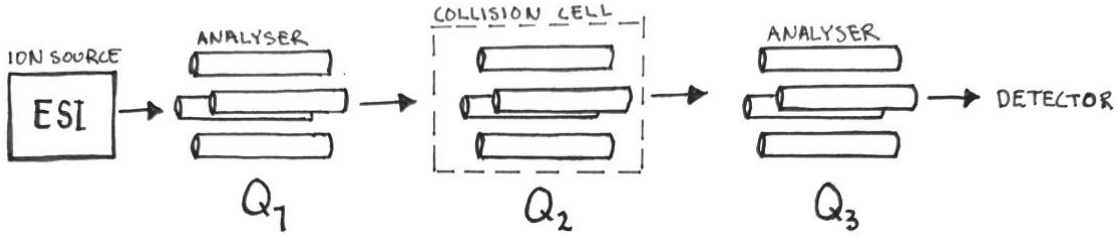

Figure 2.11: Basic elements of the triple-quadrupole mass spectrometer (reproduced from Dass, 2007).

2.6 Quantification and quality assurance

Internal standard method

The internal standard method can ensure a higher reproducibility and accuracy in the method, since all data is referenced to the internal standard (Miller, 2005). A known amount of the internal standard is added into all of the samples and the ratio between the signal of the analyte and the internal standard can be used for quantification (Skoog et al., 2014). The intern standard method can compensate for errors that influence both the target analyte and the internal standard to the same proportion. The method can be used to correct for losses during sample preparation, variations in analysis conditions and matrix effects (Miller, 2005; Van De Steene & Lambert, 2008; Pan et al., 2015). The internal standard should elute close to the analytes of interest, but also be well resolved from them. It should have same or similar physical-chemical properties, be stable during sample preparation and instrumental analysis, not react with components in the sample and be in available in pure form (Miller, 2005; Tan et al., 2012). If the appropriate internal standard is applied, all variations from extraction to detection can be corrected for, including variation in dilution, analyte transfer, adsorption, evaporation, recovery, injection and ion suppression or enhancement (Tan et al., 2012).

Retention time and relative retention time

The retention time (RT) of a compound is defined as the time required for a compound to elute from the chromatographic system, and it depends on the partition between the mobile phase and the stationary phase (Miller, 2005). The RT for a compound can fluctuate between different injections and depend on the applied chromatographic system. Variations in RT can be a result of factors like instability in column temperature and mobile phase flow, column degradation and length differences, and air bubbles in the mobile phase (Asheim, 2018). The relative retention time (RRT) for a compound is referenced to the internal standards retention time. RRT for a compound should be the similar between the runs, since the analyte and internal standard are impacted of the same fluctuations. RRT can be calculated, according to Eq. 2.1, as a ratio between RT of the analyte ($RT_{analyte}$) and RT of an internal standard ($RT_{internal\ standard}$).

$$RRT = \frac{RT_{analyte}}{RT_{internal\ standard}} \tag{2.1}$$

Relative response

The signal intensity for a compound can also vary depending on fluctuations in the chromatographic system and variation in sample preparation. Using the relative response (RR) for quantification of the analyte can overcome e.g. variations in injected volume and in volume measurements during sample preparations (Magee & Herd, 1999). RR is a factor that can be calculated according to Eq. 2.2.

$$RR = \frac{A_{analyte}}{A_{internal\ standard}} \tag{2.2}$$

where $A_{analyte}$ is the peak area of the analyte in the sample, and $A_{internal\ standard}$ is the peak area of the internal standard added in the same sample.

Ion ratio

The ion ratio (IR) is a parameter used for additional confirmation of the target analytes. IR is unique for each analyte in the sample matrix (Asheim, 2018). IR of the compound can be calculated according to Eq. 2.3.

$$IR\ \% = \frac{A_{confirmation\ ion}}{A_{quantification\ ion}} \times\ 100\ \% \qquad (2.3)$$

where $A_{confirmation\ ion}$ is the signal peak area of the confirmation ion, the fragment with smallest peak area, and $A_{quantification\ ion}$ is the peak area of the quantification ion, the fragment with biggest peak area.

Precision

The methods precision gives information about the variability among replicate measurements (Fifield & Haines, 2000). Factors that can lead to variability among the replicates include differences in temperature, shaking times, extraction conditions and flow rates (Prichard, MacKay & Points, 1996). The precision can be evaluated by measuring replicates of the same sample, often with a known amount of the analyte added. The term repeatability applies to variations under constant circumstances, measurements carried out under closely controlled conditions in one place over a short period of time (Fifield & Haines, 2000). Reproducibility refers to measurements performed in different time, place and circumstances, but with ostensible similar method. The methods precision is often expressed in the terms of standard deviation (STD), calculated according to Eq. 2.5. The mean of the data ($\overline{x}$) is defined by equation 2.4.

$$\overline{x} = \sum_i \frac{x_i}{n} \qquad (2.4)$$

where x_i is an individual result and n represent the number of results (Fifield & Haines, 2000).

$$STD = \sqrt{\frac{\sum_i (x_i - \overline{x})^2}{n-1}} \qquad (2.5)$$

where x_i represent individual results, $\overline{x}$ is the experimental mean of the data and n is the number of data. Generally, STD express the distribution of data around its mean, and is an important precision indicator (Fifield & Haines, 2000). STD is often given in the form of relative standard deviation (RSD) calculated by equation 2.6.

$$RSD = \frac{STD}{\overline{x}} \times 100\% \quad (2.6)$$

where *STD* is the standard deviation and $\overline{x}$ represent the mean of the experimental data.

Absolute and relative recovery

The recovery of the method is the fraction of the actual amount of the substance obtained after the procedure (Prichard, MacKay & Points, 1996). The recovery can be used to check the efficiency of the whole procedure or of different stages such as extraction and clean-up. In trace organic analysis it is not only losses of target analytes that should be considered, also gains due to contamination of the samples. Ideally, the recoveries should be better than 95 % but in environmental samples containing low levels of the target analytes this may not be possible. Contamination arising from solvents or elsewhere can be assessed by using a method blank, going through the same steps as the samples. The recovery can be determined by adding a known amount of the analytes into the sample, undertaking the whole procedure. The recovery can be calculated by analysing a sample spiked with the analytes before and after the sample preparation (Eq. 2.7; Eq. 2.8).

$$Absolute\ recovery\ \% = \frac{A_{SP}}{A_{MM}} \times 100\ \% \quad (2.7)$$

where A_{SP} is the area of the analyte in the spiked matrix, spiked prior to the sample preparation, and A_{MM} is the area of analyte in a matrix match sample, spiked after the sample preparation.

$$Relative\ recovery\ \% = \frac{A_{SP}/IS_{SP}}{A_{MM}/IS_{MM}} \times 100\ \% \quad (2.8)$$

where IS_{SP} is the area of internal standard in spiked matrix, spiked before sample preparation, and IS_{MM} is the area of internal standard in a matrix match sample spiked after sample preparation. Relative recovery is a corrected form of the recovery, compensating for losses of analyte during sample preparation (Asheim, 2019).

Instrumental limit of detection and limit of quantification

The lowest amount an analyte in a sample can be detected is called the limit of detection (LOD), but the amount is not necessarily high enough to be determined as an exact value (Miller, 2005). LOD can be specified to fit the purpose, and the limits should be measured by the laboratory itself under accurate conditions of the analysis (Prichard, MacKay & Points, 1996). The lowest amount an analyte in a sample that can be quantitatively determined with suitable precision and accuracy is called the limit of quantification (LOQ). A method to estimate LOQ is to set LOQ as the lowest detected level in the calibration curve following the linearity of the curve (Asimakopoulos et al., 2014). LOD can then be estimated by equation 2.9.

$$LOD = \frac{LOQ}{3} \tag{2.9}$$

Matrix effect

A common problem associated with LC-MS/MS method development is signal suppression or enhancement, which can lead to errors in quantification (Van De Steene & Lambert, 2008; Pan et al. 2015). These effects, called matrix effects (ME), are in most cases considered to be an effect of co-eluting matrix constituents. ME is often observed when analysing environmental samples due to a complex matrix and will influence the reproducibility and accuracy of the method. Matrix compounds entering the ion source at the same time as the analyte may influence the ionization efficiency. ME percentage (ME%) can easily be calculated by comparing the instrumental response of the analyte in a post-extraction spiked sample with the response in a standard solution, expressed by Eq. 2.10 (Asimakopolous, 2014).

$$ME\% = \left(\frac{A_{MM} - A_{RB}}{A_{Std}} - 1\right) \times 100\ \% \tag{2.10}$$

where A_{MM} is the peak area of the analyte in the matrix match sample spiked after sample preparation, A_{RB} is the peak area of reagent blank and A_{Std} is the peak area of the analyte in a standard solvent solution (Asimakopoulos, 2014). ME% < 0 indicates ionization suppression, and ME% > 0 indicates ionization enhancement. Labelled internal standards can be used to

compensate for matrix effects, since the ratio between the peak areas should be constant (Van De Steene & Lambert, 2008; Pan et al., 2015).

Coefficient of determination

A calibration curve can be made out of a series of standard solution prepared with external standards separately from the sample (Skoog et al., 2014). This curve is used to establish the calibration function of the instrument, by plotting the instrument response as a function of the known concentration of the analyte. A linear relationship between instrument response and the concentration of analyte is desirable, especially in the concentration range of the target analyte in the samples. The linearity can be described with the coefficient of determination (R^2), that measure the fraction of variations in the dependent variable. The R^2 value can tell us how much of the variations in the peak area that is explained by the linear model (Skoog et al., 2014). A R^2 value over 0.999 indicate minimal differences in the quality of the analysis, meaning most of the variation within the data is explained by the input values (Meier & Zünd, 2000). The coefficient of determination can be calculated according to equation 2.11 (Skoog et al., 2014).

$$R^2 = 1 - \frac{\sum_{i=0}^{n}[y_i - (b + mx_i)]^2}{\sum_{i=0}^{n}(y_i - \overline{y})^2} \qquad (2.11)$$

where y is the measured response, x is the standard analyte concentration, b is the y-intercept (the value of y where x is zero) and m is the slope of the line. y_i and x_i is the individual pair of data and the $\overline{y}$ is the mean value of y for n calibration points.

2.7 Statistics

Descriptive statistics

When dealing with analytical data, it is important to establish the distribution of the dataset, since the majority of statistical tests assume normal distribution (Fifield & Haines, 2000). Confirmation of normal distribution can be achieved by visual inspection of histograms or by the Shapiro-Wilk test (Shapiro & Wilk, 1965). Means between two groups can be compared by student T-test for normal distributed data, and by a Mann Whitney U-test for non-

distributed data (Bower, 2013). For more than two groups the ANOVA test can be performed to check for differences between groups for normal distributed data, where a Tuckey HSD post-hoc test can be used to find where the significance lies (Meier & Zünd, 2000; Weaver et al., 2017). For non-distributed data a Kruskal Wallis H-test can be used to compare more than two groups, and to find where the significance lies a Games-Howell post hoc test can be applied (Weaver et al., 2017).

Correlation

A correlation analysis can give information on the linear relationship between to variables and how strong it is (Reichenbächer & Einax, 2011). A correlation coefficient, called Pearson correlation (r) can be calculated to quantify the strength of the relationship between the variables. The values range from -1 to 1, where 1 indicate a strong positive correlation, 0 indicate uncorrelated variables and -1 indicate a strong negative correlation (Fifield & Haines, 2000).

Principal component analysis

With a large number of variables, it might be easier to consider a small number of combinations of the data rather than the entire dataset (Crawley, 2013). Principal component analysis (PCA) is a tool used to bring out patterns of similarity in complex datasets, represented as points in a map. This multivariate statistical technique analyses a data set representing observations described by several dependent variables, which generally are inter-correlated (Abdi & Williams, 2010). The goal with the analysis is to express important information from the data set as a set of new orthogonal standardized linear combinations, called principal components. These explain all of the variation in the original data, with a small number of linear combinations of the variables (Crawley, 2013). Correlation between a variable and component in PCA is called a loading (Abdi & Williams, 2010). The variables loadings can be used as coordinates and be plotted as points in the compartment space in a PCA plot. In a PCA biplot, the original variables are shown by arrows indicating relative loadings on the two principal compartments (Crawley, 2013).

Chapter 3

Materials and method

3.1 Sample collection

Liver samples (N=100) for analysis and liver, muscle and blubber samples (N=1) for method development from harbour porpoises (*Phocoena phocoena*) was provided by the Department of Biology, Norwegian university of science and technology (NTNU). The sampling was organised by the Institute of Marine Research. The harbour porpoises had been bycaught in fishery nets along the Norwegian coast, in locations showed in figure B.1 (appendix B). Sampling was carried out during 2016 and 2017 during spring and autumn. Samples were contained in plastic bags and stored in a freezer (-20 °C).

Out of the 100 liver samples, 55 were collected in 2016, including 27 females and 28 males. Among the 45 livers sampled in 2017, 27 were females and 18 males. The majority of the harbour porpoises were bycaught in the coast of northern part of Norway, including Kvænangen (N=8), Lofoten (N=22), Nordkinnhalvøya (N=6), Troms (N=34) and Varangerfjorden (N=9), while the rest were sampled in South and Central Norway (N=21). The weight of the harbour porpoises ranged from 17 to 75 kg, and the lengths ranged from 101 to 173 cm (table 3.1).

When preparing for analysis, 0.1 grams of tissue was weighed out. The tissue was cut with a Teflon knife washed with water and methanol between each sample. Every sample was cut on a surface covered with aluminium foil to avoid contamination. The samples were kept cold pre and post cutting to avoid thawing of the samples and thus enzymatic activity. This was performed by storing the samples on dry ice or in a freezer at -20 °C. Samples were transferred to clean 15 mL polypropylene (PP) tubes.

Table 3.1: Weight and length of harbour porpoises given for different regions and genders. Data is presented as mean of weight (kg) and lengths (cm), RSD (%), minimum and maximum value.

		Weight				Length			
Region	**Gender**	*Mean*	*RSD*	*Min*	*Max*	*Mean*	*RSD*	*Min*	*Max*
Kvænangen	Males	41.3	42.1	23.0	58.0	133	17.1	106	152
	Females	48.9	25.9	34.0	62.5	144	11.4	129	161
Lofoten	Males	37.7	24.0	22.0	50.0	133	11.1	106	152
	Females	43.8	32.2	27.0	71.0	142	12.3	113	169
Nordkinnhalvøya	Males	36.9	12.2	31.0	42.5	132	6.39	123	145
	Females	-	-	-	-	-	-	-	-
Troms	Males	36.2	22.7	22.0	49.0	131	11.8	103	152
	Females	47.8	26.2	30.0	74.0	145	10.1	117	173
Varangerfjorden	Males	47.3	20.7	31.0	59.0	145	10.5	125	158
	Females	50.9	27.2	40.0	66.0	148	5.81	140	162
South and central	Males	30.0	27.2	19.0	41.0	123	13.7	101	143
Norway	Females	39.7	43.2	17.0	67.0	134	15.9	106	163

3.2. Chemicals and materials

Analytical standards for 17 phthalate metabolites (monoethyl phthalate, mEP; monomethyl phthalate, mMP; mono-n-butyl phthalate, mBP; monoisobutyl phthalate, mIBP; mono-n-pentyl phthalate, mPeP; monoisopentyl phthalate, mIPeP; mono-n-hexyl phthalate, mHxP; monocyclohexyl phthalate, mCHP; mono-n-heptyl phthalate, mHpP; monobenzyl phthalate, mBzP; mono-n-octyl phthalate, mOP; mono(2-ethyl-1-hexyl) phthalate, mEHP; mono(2-ethyl-5-oxohexyl) phthalate, mEOHP; mono(2-ethyl-5-hydroxyhexyl) phthalate, mEHHP; mono-n-decyl phthalate, mDP; mono-n-nonyl phthalate, mNP; phthalic acid, PA) were purchased from Chiron AS (Trondheim, Norway). All standards had a concentration of 1000 µg/mL in methanol, except from mBzP (1000 µg/mL in dichloromethane/cyclohexane) and PA (solid, 1 g).

Deuterated internal standards (monoethyl phthalate-3,4,5,6-d_4, mEP-d_4; mono-n-butyl phthalate-3,4,5,6-d_4, mBP-d_4; mono-n-nonyl-phthalate-3,4,5,6-d_4, mNP-d_4) (100 mg) were purchased from Chiron AS (Trondheim, Norway).

β-Glucuronidase from *Helix pomatia* (type HP-2, aqueous solution, ≥ 100,000 units/mL) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (ACS grade) used for washing and orthophosphoric acid (85 %, ACS grade) was purchased from VWR Chemicals (Rue Carnot, Fontenay-sous-Bois, France). Ethyl acetate (for LC), acetonitrile (ACN) (gradient grade for LC), formic acid (98-100 % for LC-MS), sodium dihydrogen phosphate dihydrate (extra pure, DAB) and ammonium acetate (ACS grade) were purchased from Merck (Billerica, MA, USA).

SPE cartridges, ABS Elut-NEXUS 60 mg 3 mL, were obtained from Angilent Technologies, Inc (Folsom, CA, USA). Disposable liners (PTFE) were purchased from Sigma-Aldrich (Steinheim, Germany).

3.3 Sample preparation

3.3.1 Standards

Internal standards

Monoethyl phthalate-3,4,5,6-d_4 (mEP-d_4), mono-n-butyl phthalate-3,4,5,6-d_4 (mBP-d_4) and mono-n-nonyl phthalate-3,4,5,6-d_4 (mNP-d_4) weres used as internal standards (IS). A 1 ppm mix of the three ISs were made through the following procedure. First, each IS was weighted out to ca. 0.010 gram and dissolved in 1 mL acetonitrile (ACN). With calculated volume from the exact weight, 1000 µg was transferred to a glass vial (LC-MS) using an Eppendorf pipette, and ACN was added to a total volume of 1 mL, giving a 1000 ppm solution. A 100 ppm solution was then made by transferring 100 µL 1000 ppm solution of the IS, adding 900 µL ACN. Then, the 1 ppm IS mix was made by adding 10 µL of each 100 ppm IS solution and adding 970 µL ACN. 20 µL 1 ppm IS mix was added in all samples.

Target analytes standards

Spiked samples and matrix match samples were spiked using a 1 ppm target analytes (TA) standard, a mix of the 17 phthalate metabolites (mEP; mMP; mBP; mIBP; mPeP; mIPeP; mHxP; mHpP; mCHP; mBzP; mOP; mEHP; mEOHP; mEHHP; mDP; mNP; PA). 10 µL 100

ppm (in methanol) of each standard were added to a glass vial and 830 µL ACN was added to make a 1 ppm TA mix solution for spiking. In 40 ppb spiked samples 20 µL 1 ppm TA mix was added before extraction. In 25 ppb spiked samples 12.5 µL 1 ppm TA mix was added before extraction. In 10 ppb spiked samples 5 µL 1 ppm TA mix was added before extraction. In 40 ppb, 25 ppb and 10 ppb matrix match samples, 20 µL, 12.5 µL and 5 µL ppb TA mix were added, respectively, after the sample preparation procedure.

3.3.2 Solid-liquid extraction and enzymatic deconjugation

Solid-liquid extraction (SLE) was performed by adding 600 µL 1.0 M ammonium acetate to the samples, followed by 45 min sonication to extract organic compounds from the tissue to the liquid phase. To avoid contamination, plastics foil was wrapped around the caps. The phthalate metabolites where then deconjugated with β-glucuronidase according to Asimakopoulos et al. (2016). This involved incubation of the samples at 37 °C for 12 hours with a buffer of 600 µL ammonium acetate (1.0 M) containing 22 units of β-glucuronidase (2.5 µL β-glucuronidase in 50 mL 1.0 M ammonium acetate). The samples were centrifuged for 5 min, then the supernatants were transferred into new 15 mL PP tubes. To stop enzyme activity, 2 mL phosphate buffer (2 g sodium phosphate monobasic dihydrate dissolved in 100 mL milli-Q water and 1 mL orthophosphatic acid 85 %) was added to each sample. The samples were stored in the freezer (-20 °C) until SPE.

3.3.3 Solid phase extraction and pre-concentration

Solid phase extraction (SPE) was performed to eliminate interferences in the samples after SLE and enzymatic deconjugation. The procedure was extrapolated from Asimakopoulos et al. (2016) and its supportive information. SPE was performed with ABS Elut-NEXUS 60 mg 3 mL cartridges. The cartridges were conditioned with 1.5 mL ACN and rinsed with 1.2 mL phosphate buffer (2 g sodium phosphate monobasic dihydrate dissolved in 100 mL milli-Q water and 1 mL orthophosphatic acid 85 %). Samples were loaded into the cartridges and washed with 2 mL formic acid (1 %) followed by 1.2 mL milli-Q water. The cartridges were then dried under vacuum for ca. 5 min. Elution was performed with 1.2 mL ACN and 1.2 mL

ethyl acetate and the elutes were collected in new 15 mL PP tubes. Samples were stored in the freezer until concentration.

The samples were pre-concentrated using a TurboVap (TurboVap® LV automated evaporation system) to evaporate the solvent. The instrument was washed with methanol before concentration. The samples were evaporated to near dryness in a water bath (milli-Q water) with 30 °C under a gentle nitrogen stream. The solvent was then changed by adding 500 µL acetonitrile:milli-Q water (1:9). Then the samples were centrifuged for 5 minutes and the supernatants were transferred to glass vials ready for analysis. Total volume of the samples was 500 µL. The samples were stored in the freezer (-20 °C) before analysis with LC-MS/MS.

3.4 Analysis

Method for analysis of phthalates metabolites was extrapolated from Asimakopoulus et al. (2016) and its supportive information. Chromatographic separation of phthalate metabolites was performed with a Waters Acquity UPLC combined with Waters Acquity Column Manager, Waters Acquity Sample Manager and Waters Acquity 1 UPLC class Binary Solvent Manager. In method development Kinetex C18 column (30 x 2.1 mm, 1.3 µm, 100Å Phenomenex) was used as separation column, and in analysis of the 100 liver samples Kinetex C18 column (50 x 2.1 mm, 1.3 µm, 100Å Phenomenex) was used. The separation column was serially connected to a Phenomenex guard column (C18). Column temperature was set to 30 °C. The separation was carried out using a gradient elution program with an aquatic phase (milli-Q water with 0.1 % acetic acid) and an organic phase (acetonitrile, ACN, with 0.1 % acetic acid) as binary mobile phase mixture at a flow rate of 0.40 mL/min (table 3.2). The gradient elution began with 5 % (v/v) ACN, increased to 25 % ACN, followed by 40 % ACN, 50 % ACN and at last 90 %, with a time window shown in table 3.2. Then the column was re-equilibrated with 5 % ACN for 1 min. Total run time was 11 minutes.

Table 3.2: Gradient elution program with Kinetex C18 (50 x 21 mm) column, using mobile phase mixture of milli-Q water (0.1 % acetic acid) and acetonitrile (0.1 % acetic acid). Constant flow rate of 0.40 mL/min.

Time [min]	Water [%]	Acetonitrile [%]
Init	95	5
1.0	95	5
1.5	75	25
4.0	75	25
4.5	60	40
6.0	60	40
6.5	50	50
8.0	50	50
8.5	10	90
9.9	10	90
10.0	95	5
11.0	95	5

Identification was performed with the tandem mass spectrometric system Waters Xevo TQ-S, triple quadrupole mass analyser with Zspray ESI in a negative ionization mode. Application of the samples was done by direct infusion. Electrospray ionization (ESI) voltage applied was 3.0 kV. Cone gas (N_2) flow rate was set at 150 L/h. Desolvation gas flow was set to 1000 L/h. Source temperature was set to 150 °C and desolvation temperature to 350 °C. The parent ions and fragmentation ions for the analysis is shown in table 3.3.

Table 3.3: Analyte specific MS/MS parameters with Kinetex C18 (50 x 21 mm) column.

Component	Retention time	Primary transition	Secondary transition
mEP	2.42	193 > 121	193 > 77
mMP	2.08	179 > 107	179 > 77
mBP	4.86	221 > 150	221 > 77
mIBP	4.74	221 > 134	221 > 77
mPeP	5.96	235 > 121	235 > 77
mIPeP	5.75	235 > 85	235 > 77
mHxP	7.00	249 > 121	249 > 99
mCHP	5.82	247 > 97	247 > 77
mHpP	7.59	263 > 113	263 > 77
mBzP	5.27	255 > 183	255 > 77
mOP	8.46	277 > 127	277 > 77
mEHP	8.03	277 > 134	277 > 77
mEOHP	4.97	291 > 143	291 > 121
mEHHP	4.92	293 > 145	293 > 121
mDP	8.95	305 > 261	305 > 77
mNP	8.82	291 > 141	291 > 77
PA	1.87	165 > 121	165 > 77
mEP-d_4	2.38	197 > 125	197 > 81
mBP-d_4	4.81	225 > 81	225 > 71
mNP-d_4	8.79	295 > 141	295 > 81

3.4.1 Calibration curve

Standards used to make the calibration curve was prepared as 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 25 and 50 ppb solutions of the TA mix together with a constant 20 ppb IS mix in all standards. Solvent used was and milli-Q water.

3.5 Data treatment

LC-MS/MS data was acquired with MassLynx and TargetLynx software packages (version 4, Waters, USA). Data processing and statistical calculations was performed using Microsoft Excel. SPSS Statistics was used for some statistical calculations, including Shapiro-Wilk test, Mann Whitney U-test, Kruskal Wallis H-test and Pearson correlation. R was used to plot PCA biplots and correlation heat map. Concentrations were calculated based on the relative areas, using the internal standard with retention time closest to the analyte. Concentrations of the target analytes in reagent blank samples were subtracted from the concentrations in all samples.

Chapter 4

Results and discussion

4.1 Sample matrix

The analytical method described in 3.3 and appendix B was tested analysing non-spiked, pre-extraction spiked and post-extraction spiked triplicates in samples of liver, muscle and blubber during method development. Method validation parameters are presented in chapter 4.2. For evaluating what tissue matrix that was most suitable for determination of phthalate metabolites in harbour porpoises, 14 different phthalate metabolites were analysed. For a total of 7 out of the 14 metabolites, highest target analyte levels were observed in liver as sample matrix (table 4.1).

Table 4.1: Mean concentrations of phthalate metabolites, measured in in triplicates in samples of liver, muscle and blubber, given in ng/g wet weight (RSD%). LOD ranged from 0.033 to 0.067 ppb.

Component	Liver (ng/g ww)	Muscle (ng/g ww)	Blubber (ng/g ww)
mEP	33.0 (9.31)	5.06 (0.144)	<LOD
mMP	4.08 (34.7)*	2.34 (0.041)	2.77 (0.047)
mBP	23.6 (12.8)	21.7 (0.685)	<LOD
mIBP	28.4 (15.0)	17.8 (0.669)	0.468 (0.021)
mPeP	<LOD	<LOD	<LOD
mIPeP	<LOD	<LOD	<LOD
mHxP	0.325 (88.1)	0.524 (0.002)	0.473 (0.002)
mCHP	<LOD	<LOD	<LOD
mBzP	1.20 (7.88)	<LOD	<LOD
mOP	0.185 (8.87)*	0.256 (0.001)	0.722 (0.005)
mEHP	56.5 (7.86)*	51.4 (6.14)	48.2 (6.54)
mEOHP	0.230 (16.0)*	<LOD	<LOD
mEHHP	<LOD	0.204 (0.000)	<LOD
PA	0.932 (216)	1.18 (0.076)	0.532 (0.004)

* (N=2).

The target analytes mPeP, mIPeP and mCHP were detected under the limit of detection in all tissue matrices. The precision of the method varied between the different sample matrices, where generally the RSDs were better in muscle and blubber as sample matrices than for liver. A high variance was observed in the triplicates for PA, mMP and mHxP in liver matrix, otherwise the RSDs showed acceptable precision between the measurements of the triplicates. Since the biggest proportion of the target analytes were detected in highest concentrations in liver as sample matrix, 100 liver samples were picked out for determination of phthalate metabolites in harbour porpoises.

The higher concentrations in liver were most apparent for the smallest phthalate metabolites, while the bigger phthalate metabolites showed more varied occurrence between the different tissue types. Tissue distribution of phthalates metabolites have been observed to vary dependent on type of ester groups and between different species, as observed in different fish species (Hu et al., 2016). In several fish species, highest concentrations for nearly all studied MPEs were observe in the bile. High levels of the most hydrophobic compound investigated, mOP, was observed in the liver, suggesting that liver metabolism is predominant for highly hydrophobic metabolites (Hu et al., 2016). Observations done by McKee et al. (2002) in rats of metabolites of the hydrophobic diisononyl phthalate (DINP) agrees with this trend. In the present study, mOP was found in highest levels in blubber. The smallest, low hydrophobic metabolites have been observed to have low distribution to the liver compared with other tissues such as kidneys and gills (Hu et al., 2016). Similar distribution patterns have been observed for their parent compounds (Adeogun et al., 2015). The present study did not allow for a full mapping of the presence in all tissues of harbour porpoises.

4.2 Quality assurance and method validation

To evaluate the performance of the sample preparation protocol described in chapter 3.3 and instrumental analysis described in 3.4, a method validation was performed based on the following parameters: linearity, ion ratios, matrix effects, recoveries and limits of detections and limits of quantifications. Standard calibration curves were obtained with concentrations ranging from 0.1 to 50 ng/mL with a satisfactory coefficient of determination for all phthalate metabolites for both absolute and relative areas ($R^2 > 0.998$). Contamination was evaluated by

analysing reagent blanks that had followed the whole procedure. During method development (appendix B), analysing spiked and non-spiked triplicates of liver, muscle and blubber samples, high background was observed for many of the target analytes in the reagent blanks (N=9). Some measures were done to prevent contamination during the sample preparation for the analysis of 100 liver samples. In the analysis of 100 livers, high background was observed only for mEHP in reagent blank samples (N=6). Signals in reagent blanks were subtracted from the sample signal when calculating the concentration of all target analytes. Ion ratios for all target analytes (table D.5, appendix D) met the criteria of tolerance presented in the European Commission Decision 2002/657/EC (European Commission, 2002).

4.2.1 Matrix effects

In method development, matrix effects were evaluated by comparing instrumental response of the analytes in post-extraction spiked triplicates of liver, muscle and blubber, with instrumental response in a standard solution (table 4.2).

Table 4.2: Matrix effect (%) for 14 phthalates metabolites in liver, muscle and blubber.

	Matrix effect (%)		
	Liver	*Muscle*	*Blubber*
mEP	-32.1	-32.8	-20.6
mMP	-80.2	-78.0	-35.4
mBP	-24.8	-28.5	-15.1
mIBP	-16.5	-24.8	-7.9
mPeP	-15.7	-31.6	-1.1
mIPeP	-17.2	-36.0	-10.2
mHxP	-34.1	-44.6	-5.6
mCHP	-18.6	-40.8	-13.4
mBzP	-26.9	-54.9	-14.4
mOP	-15.7	-20.1	-8.0
mEHP	-31.5	-28.6	-25.3
mEOHP	-12.3	-17.5	-1.9
mEHHP	-8.9	-14.8	-1.2
PA	-50.8	-4.3	13.7

Calculated matrix effects (ME%) from LC-MS/MS analysis during method development were negative for all phthalate metabolites in all sample matrices, except for PA in blubber (table 4.2). This indicate interference by co-eluents, which have resulted in an ionization suppression (ME% < 0). For PA in blubber, an indication of ionization enhancement was observed. Matrix effects are expected in trace organic analysis in environmental samples, due to low concentrations of the target analytes in addition to complex matrixes containing many possible interfering compounds. In liver, mMP and PA was found to have the most extensive matrix effect. A potential explanation can be interference from co-eluting biological components, like lipids, as they elute first along with the smallest analytes. The matrix effects can be compensated for by quantifying the target analyte using the internal standard method.

4.2.2 Recoveries

The efficiency of the extraction procedures presented in chapter 3.3 was evaluated by comparing the amount of the target analytes obtained in triplicates of pre-extraction spiked liver samples with post-extraction spiked liver samples. Absolute and relative recoveries are shown in table 4.3. The recoveries for mEHP could not be calculated due to high background observed in reagent blanks.

Table 4.3: Mean absolute and relative recoveries (%) for phthalates metabolites in liver samples of harbour porpoises (N=3, 25 ng/mL).

	Absolute recovery (%)	**Relative recovery (%)**
mEP	78.1	123.3
mMP	52.0	82.2
mBP	58.4	141.6
mIBP	83.9	**
mPeP	56.9	108.0
mIPeP	46.6	86.4
mHxP	33.8	**
mHpP	*	**
mCHP	59.6	117.2
mBzP	57.4	93.9
mOP	*	**
mEOHP	71.6	139.6
mEHHP	69.3	135.2
mNP	*	137.1
mDP	*	85.9
PA	*	*

* values << 100 %.
** values >> 100 %.

Absolute and relative recovery for PA was 6.28 % and 12.0 %, respectively. For mHpP, mOP, mNP and mDP the absolute recoveries were 20.2 %, 13.5 %, 10.5 % and 6.8 %, respectively. With low absolute recoveries only a minor fraction of the target analytes will be obtained in the samples after the sample preparation. These compounds were semi-quantified as a quantification cannot be obtained with the employed sample preparation protocol. For mIBP, mHxP, mHpP and mOP the relative recoveries were > 170 %, whereas mHxP stood out with the highest relative recovery of 709 %. High relative recoveries might lead to critical errors in the quantification, leading to an overestimation of the analytes in the samples. They were therefore semi-quantified in the present study.

The absolute recoveries of the phthalate metabolites (table 4.3) were relatively low and variable, indicating loss of sample during sample extraction and/or clean-up. Only mIBP obtained a good recovery. A potential explanation for the low recovery is that the target analytes binds to biological matrices of the sample, for example proteins, leading to low

recoveries (Prichard, MacKay & Points, 1996). Loss of analytes can also be a result of poor extraction efficiency during SLE and SPE or poor enzymatic deconjugation efficiency. The low recoveries were compensated for by calculating the relative recovery, relative to a specific internal standard. Since losses (or gains) were partially corrected for, the relative recoveries were better than the absolute recoveries for the majority of the analytes (table 4.3). Reported relative recoveries ranged from 82.2 to 141.6 %, indicating a good efficiency of the procedure for some of the target analytes. Good recoveries were obtained for mMP, mPeP, mIPeP, mCHP, mBzP and mDP. The recovery for mEP can also be considered acceptable. For the others reported, the recoveries were high (except for PA).

High relative recoveries might be explained by differences in recovery between the target analyte and the internal standard (IS). The IS should be similar to the target analyte and should therefore be affected by the same fluctuations. In theory, the relative recovery should be close to 100 %. In the present study, all target analytes were corrected using only three ISs, correcting with the IS with nearest retention time. They might therefore not be similar enough to the target analytes and may therefore be subjected to other fluctuations than the target analyte itself. Significant differences in extraction recovery for analytes and their deuterated ISs have been observed (Tan et al., 2012). The deuteration can lead to differences in interactions, reaction rates and hydrophobicity. The deuterated IS is often more polar than the analyte and will therefore elute before the analyte in reversed phase LC. A better option would be to use ISs with more similar properties as the target analytes, like ^{13}C internal standards, with target specific ISs (Tan et al., 2012). Good relative recoveries were observed using analyte specific ^{13}C-target specific internal standards in analysis of urinary phthalates metabolites in humans (Asimakopoulos et al., 2016; Rocha et al., 2017).

4.2.3 Precision

The precision of the procedure was evaluated by measuring triplicates of spiked matrices in three different concentrations. SP_{10} and SP_{25}, spiked to a total concentration of 10 ppb and 25 ppb, respectively, were analysed with the 100 liver samples. SP_{40}, spiked to a total concentration of 40 ppb, were analysed during the method development (appendix B). The results, presented as both absolute and relative values, are given in table 4.4.

Table 4.4: Statistics for phthalates metabolites in triplicates of samples spiked prior to sample preparation to concentrations of 10 ppb (SP_{10}), 25 ppb (SP_{25}) and 40 ppb (SP_{40}). Mean and median area, standard deviation (STD) and relative standard deviation (RSD%) for absolute and relative values are presented. The table continues on the next page.

		Absolute values				**Relative values**			
		Mean	*Median*	*STD*	*RSD%*	*Mean*	*Median*	*STD*	*RSD%*
mEP	SP_{10}	4 150	4 050	412	9.94	0.417	0.417	0.022	5.22
	SP_{25}	8 920	8 100	1 990	22.3	0.950	0.953	0.035	3.64
	SP_{40}	11 000	10 900	1 170	10.6	3.05	3.08	0.083	2.72
mMP	SP_{10}	1 070	1 020	84.3	7.91	0.108	0.117	0.018	16.7
	SP_{25}	2 360	2 100	450	19.1	0.252	0.254	0.006	2.39
	SP_{40}	1 061.6	985.5	146.8	13.8	0.293	0.302	0.023	7.71
mBP	SP_{10}	9 530	8 880	1 450	15.3	3.56	1.93	3.17	15.5*
	SP_{25}	17 900	15 500	4 410	24.7	4.06	3.74	1.32	17.1*
	SP_{40}	23 900	24 300	4 900	20.5	1.73	1.72	0.029	1.67
mIBP	SP_{10}	5 780	5 910	270	4.67	2.18	1.07	1.96	3.17*
	SP_{25}	12 200	10 600	4 210	34.5	2.77	2.76	1.08	33.6*
	SP_{40}	15 400	15 700	3 290	21.4	1.12	1.12	0.026	2.37
mPeP	SP_{10}	2 770	2 450	1 250	48.7	1.00	0.730	0.883	60.8*
	SP_{25}	9 260	7 530	3 520	38.0	2.04	2.17	0.560	27.4
	SP_{40}	16 100	15 400	4 500	27.9	1.16	1.12	0.089	7.72
mIPeP	SP_{10}	2 210	1 840	684	30.9	0.770	0.517	0.603	30.3*
	SP_{25}	6 530	6 020	2 600	39.9	1.40	1.52	0.224	16.0
	SP_{40}	12 600	11 800	3 480	27.7	0.901	0.863	0.070	7.79
mHpP	SP_{10}	1 930	1 920	758	39.4	0.427	0.413	0.119	27.8
	SP_{25}	4 940	4 490	1 830	37.1	0.817	0.852	0.134	16.3
mHxP	SP_{10}	2 040	1 920	794	38.8	0.459	0.466	0.140	17.3*
	SP_{25}	5 120	4 100	1 970	38.4	0.861	0.905	0.231	11.7*
	SP_{40}	6 900	6 330	2 140	31.1	0.704	0.861	0.275	39.1
mCHP	SP_{10}	5 060	3 970	2 070	40.8	1.73	1.28	1.33	42.8*
	SP_{25}	11 200	9 790	2 850	25.4	2.57	2.37	0.906	28.2*
	SP_{40}	28 400	26 400	6 210	21.8	2.06	2.13	0.125	16.1
mBzP	SP_{10}	5 830	4 850	2 620	45.0	1.84	1.52	1.15	37.4*
	SP_{25}	12 500	10 000	4 290	34.4	2.79	2.84	0.871	28.2*
	SP_{40}	22 300	22 700	7 100	31.8	1.59	1.65	0.202	12.7
mOP	SP_{10}	1 830	1 930	495	27.0	0.414	0.457	0.083	20.1
	SP_{25}	5 140	4 620	1 940	37.8	0.850	0.893	0.145	17.1

	SP40	15 100	16 100	6 470	42.9	1.39	1.38	0.108	7.79
mEHP	*SP10*	6 860	7 470	2 330	34.0	1.52	1.51	0.293	19.3
	SP25	10 300	8 110	4 040	39.2	1.74	1.83	0.488	28.1
	SP40	11 000	12 400	4 290	38.9	1.03	1.05	0.137	13.3
mEOHP	*SP10*	13 900	11 600	4 350	31.3	4.90	3.26	3.95	32.6*
	SP25	38 200	33 700	9 540	25.0	8.65	8.01	2.64	16.0*
	SP40	56 800	56 700	8 770	15.4	4.14	4.12	0.212	5.11
mEHHP	*SP10*	4 510	4 150	1 380	30.5	1.51	1.04	1.06	22.4*
	SP25	13 400	12 700	2 820	21.0	3.04	2.96	0.853	7.97*
	SP40	24 300	25 500	4 870	20.0	1.76	1.72	0.077	4.38
mNP	*SP10*	1 390	1 500	344	24.7	0.313	0.308	0.041	12.9
	SP25	2 100	2 350	605	28.8	0.350	0.350	0.039	11.2
mDP	*SP10*	683	515	309	45.2	0.151	0.171	0.039	25.8
	SP25	1 450	1 390	606	41.9	0.234	0.240	0.024	10.3
PA	*SP10*	6 070	4 450	2 850	0.852*	0.589	0.507	0.186	7.11*
	SP25	13 000	10 600	5 017	38.7	1.35	1.29	0.221	16.3
	SP40	2 490	2 560	2 430	97.9	0.656	0.642	0.658	100

* (N=2).

The relative standard deviation for absolute values, meaning peak areas not corrected by the internal standards, varied between the different target analytes (table 4.4). The RSDs using absolute values were acceptable for mMP, mEP, mBP and mNP in triplicates of all measured concentrations. The SP_{40} replicates were analysed during the method development, in other conditions and times than for the other triplicates measured, and the precision may not be realistic for the analysis of the 100 liver samples. Generally, a poorer precision was observed for the lowest concentration spiked samples, especially for the biggest phthalate metabolites. This indicates a higher degree of variability among the measurements of the HMW phthalates metabolites than for the LMW phthalates metabolites. A potential explanation for this phenomenon could be that these analytes are subject to interactions that lead to variation between the different samples during the sample preparation. Potential explanations for the low precision can be non-homogenous samples and thus different interactions with the sample matrix, variation in SPE flow rate, temperature variations, differences in deconjugation efficiency and differences in extraction efficiency using sonication.

The triplicates were obtained from the same liver, but each sub-sample were obtained from different parts. In addition, the tissues had different sizes, forms and blood content, due to difficulties in controlling this in frozen samples. An indication of varied blood content could be seen, observed as differences in colours of the solvent after sonication. These variations can lead to differences in analyte interaction with constituents of the biological sample, for example if the amount of proteins varies between the replicates (Prichard, MacKay & Points, 1996). A possible solution to the problem would be to homogenize the whole liver before transferring the smaller portions to vials for sample preparation and analysis. Differences in extraction efficiency and clean-up can also be a possible source of variation between parallels. It is known that in SPE, the solvent flow is critical for the extraction efficiency (Pena-Abaurrea & Ramos, 2011). Particles and other sample constituents can also affect the precision in SPE, for example by analyte break through.

A better precision was expected for the samples after correcting the response with the internal standard method. The RSDs calculated on relative values led to a better precision in the majority of target analytes in the SP_{10} triplicates (table 4.4). A trend where the precision got better the higher the concentration of trace analytes in the sample was observed. In all SP_{40} triplicates, measured during method development, the internal standard compensated precision was acceptable, except for the analytes PA and mHxP. RSDs calculated with relative values for SP_{10} and SP_{25} samples were relatively high for some target analytes. This was expected since all 17 target analytes were corrected with the use of only three internal standards (IS). Different properties between the IS and the target analyte can lead to different interactions and losses during sample preparation, in addition to different matrix effects (Tan et al., 2012). Even a small difference in elution times between IS and target analyte can lead to significant quantification errors due to differences in ion suppression and enhancement. As mentioned in chapter 4.4.2, deuterated internal standards are not always effective in correcting for losses.

For some of the triplicates the RSDs calculated in relative values were close to 100 %, and one of the replicates was removed, leading to a better precision (table 4.4). The high relative recovery of the SP_{10} triplicates was based on the same error for mBP, mIBP, mIPeP, mPeP, mCHP, mBzP, mEOHP and mEHHP, due to the use of the same IS, mBP-d_4. In the same sub-sample, mBP-d_d was detected with a signal over twice the size of the other replicates. A potential explanation can be an error in the amount of the IS added to the sample.

4.2.4 Limits of detection and limits of quantification

The limits of quantification (LOQs) and limits of detection (LODs) for the phthalate metabolites are presented in table 4.5 for the method development and for analysis of 100 liver samples. LOQs for the different analytes ranged from 0.100 ng/mL to 0.500 ng/mL. LODs for the different analytes ranged from 0.033 to 0.167 ng/mL. LODs and LOQs were adequately low for our purpose. Concentrations detected below the LOD was removed from the data set.

Table 4.5: Limit of quantification (LOQ) and limit of detection (LOD) (ng/mL) for target analytes.

	Method development		**Sample analysis**	
	LOQ	*LOD*	*LOQ*	*LOD*
mEP	0.200	0.067	0.10	0.033
mMP	0.100	0.033	0.20	0.067
mBP	0.100	0.033	0.200	0.067
mIBP	0.200	0.067	0.200	0.067
mPeP	0.100	0.033	0.200	0.067
mIPeP	0.100	0.033	0.500	0.167
mHxP	0.100	0.033	0.100	0.033
mHpP	-	-	0.100	0.033
mCHP	0.100	0.033	0.200	0.067
mBzP	0.100	0.033	0.500	0.167
mOP	0.100	0.033	0.100	0.033
mEHP	0.100	0.033	0.100	0.033
mEOHP	0.100	0.033	0.100	0.033
mEHHP	0.100	0.033	0.100	0.033
mNP	-	-	0.100	0.033
mDP	-	-	0.100	0.033
PA	0.100	0.033	0.100	0.033

4.3 Occurrence of phthalate metabolites in livers of 100 harbour porpoises from the Norwegian coast

A total of 14 out of 17 phthalate metabolites were found in livers of harbour porpoises (table 4.6). To my knowledge, this is the first study to report liver concentrations of phthalates metabolites in marine mammals. Seven of their parent compounds have been observed in livers of marine mammals (Vorkamp et al., 2004), but no information is available regarding their metabolites in livers. The metabolites mEP, mBP, mBzP and mEHP have been observed in other tissues of cetaceans, including blubber, skin and urine (table 2.3). The available data regarding phthalate metabolites in cetaceans have a common weakness, all studies have investigated small populations sizes (Fossi et al., 2014; Baini et al., 2017; Hart et al., 2018). In the present study, phthalate metabolites were detected in livers of all of the 100 harbour porpoises. Mean concentrations are presented in figure 4.1. Results and statistics are given in table 4.6. Calculations are based on the group of individuals with target analyte detected over LOD, not the whole population. The metabolites mPeP, mIPeP and mCHP were not detected over LOD, and are therefore not included in figure 4.1 and table 4.6.

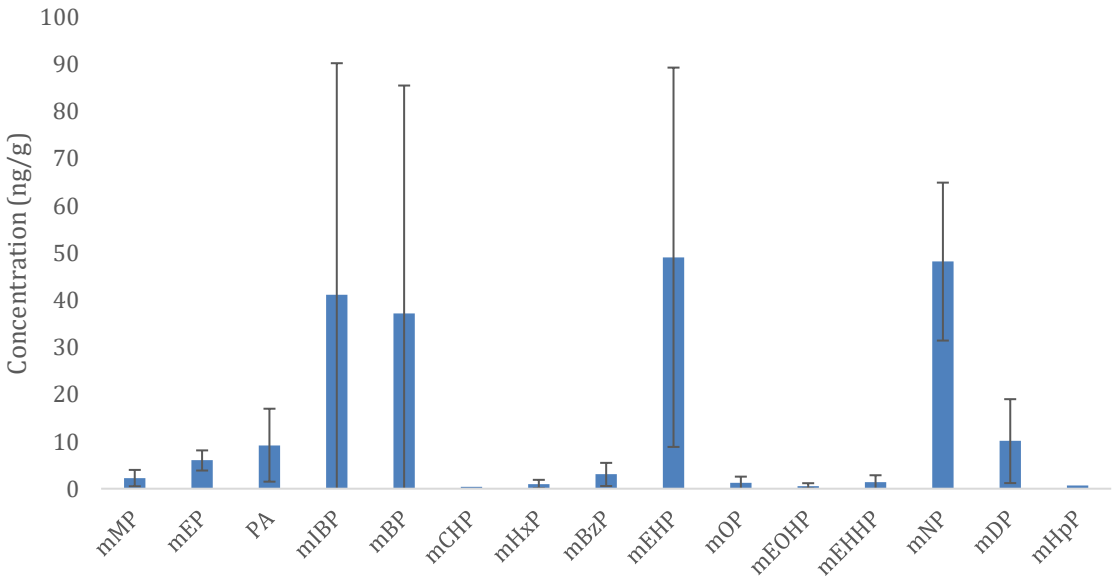

Figure 4.1: Mean concentrations (ng/g ww) of 14 phthalate metabolites, calculated for samples with target analyte found over LOD.

Table 4.6: Statistics calculated for phthalates metabolites (PM) in samples detected > LOD. Concentrations are given in wet weigh (ng/g ww).

PM	*Detection rate (%)*	*Mean (ng/g ww)*	*Median (ng/g ww)*	*Min (ng/g ww)*	*Max (ng/g ww)*	*STD*	*RSD (%)*
mEP	100	5.99	5.67	2.62	17.4	2.13	35.6
mMP	69	2.24	1.72	0.336	8.72	1.74	77.4
mBP	97	37.2	25.2	1.73	299	48.3	130
mIBP	99	41.1	30.6	3.78	419	49.1	120
mHxP *	45	0.984	0.631	0.153	4.64	0.892	90.6
mHpP*	11	0.688	0.452	0.214	1.87	0.514	74.6
mBzP	10	3.02	2.08	0.691	7.29	2.45	81.2
mOP*	21	1.19	0.632	0.192	5.53	1.36	114
mEHP**	100	49.1	39.9	6.85	331	40.2	81.9
mEOHP*	17	0.607	0.363	0.202	2.12	0.555	91.4
mEHHP	27	1.39	1.02	0.167	5.93	1.46	104
mNP*	23	48.1	48.2	24.0	98.9	16.7	34.8
mDP*	3	10.1	12.4	0.253	17.6	8.89	88.2
PA*	85	9.22	7.75	0.214	42.0	7.73	83.8

* Semi-quantified due to poor recoveries.

** High background.

The phthalate metabolites found in highest mean concentrations in liver from harbour porpoises were mIBP, mBP, mNP and mEHP (figure 4.1). mEHP was found in highest levels, detected in the whole population, but results will not be discussed as reliable as reagent blanks showed high contamination and it is not considered a reliable biomarker of DEHP (Koch, Gonzalez-Reche & Angerer, 2003). In the present study, PA was detected in the majority of the harbour porpoises but was semi-quantified due to low recoveries. Since PA is a hydrolysis product of all phthalates it works as a non-specific biomarker and its occurrence therefore indicate that the majority of harbour porpoises have been exposed to phthalates but cannot give any information of which.

Metabolites of LMW phthalates were observed in the majority of the harbour porpoises investigated, while metabolites of HMW were observed in smaller groups of individuals of the population. Studies have shown that phthalates with shorter ester chains, such as DMP, DEP and DBP, are more susceptible to metabolic breakdown than phthalates with longer ester chains, e.g. DCHP, DHxP and DnOP (Jianlong et al., 2000; Chang et al., 2004). The

differences in metabolic breakdown is potentially a result of inhibition of enzymatic degradation by hindering of enzymes from binding to the phthalate, due to the steric effects of the ester side chains (Liang et al., 2008). In the present study, it cannot be proven that a more effective metabolic breakdown of LMW phthalates than HMW phthalates are the reason for higher detection rates of LMW phthalates metabolites in livers of harbour porpoises.

Measured concentrations of phthalate metabolites in livers of harbour porpoises were low compared to observations in livers of roach (table 2.3). A potential explanation is that the roach were exposed to higher levels of phthalates, as freshwater, as they inhabited (Valton et al., 2014), generally contain higher levels than marine and coastal waters (Net et al., 2015). Human studies are often based on urinary concentrations, so it is difficult to make a comparison with liver concentrations determined in this study. Comparing urinary concentration of the bottlenose dolphin determined by Hart et al. (2018) with urinary concentrations reported in various human studies can indicate that cetaceans excrete lower levels of phthalate metabolites than humans through the urine (table 2.3). This is potentially due to lower exposure in the marine environment, since field metabolic rates of harbour porpoises have been estimated to two times higher than for similar-sized humans (Rojano-Doñate et al., 2018).

4.3.1 Occurrence of monomethyl phthalate (mMP)

The metabolite mMP was detected in 69 out of 100 harbour porpoises, with liver concentrations ranging from 0.336 to 8.72 ng/g ww (table 4.6). This indicate that the majority of the harbour porpoises investigated have been exposed to the parent chemical, dimethyl phthalate (DMP). This metabolite has been observed in tissues of marine organisms in previous studies, for example in muscle of European eel in concentrations within the range observed in the present study (Fourgous et al., 2016). As I am aware of, no data of this metabolite in tissue of marine mammals is available. The parent compound DMP on the other hand, has been detected in livers of marine mammals. DMP concentrations ranging from 2.5 to 8.4 ng/g ww were reported in livers of different species of seals and whales in Greenland and Faroe Islands (Vorkamp et al., 2004). The concentrations correspond to the levels of mMP reported in this study of harbour porpoises. LOQs for the parent compounds reported by Vorkamp et al. (2004) were higher than the LOQs of the metabolites measured in the present

study. Assessing phthalate exposure using its metabolites as biomarkers might therefore give a more realistic picture of the real concentrations than the measurement of their parent compounds, since lower levels might be determined (and also lower contamination risk).

4.3.2 Occurrence of monoethyl phthalate (mEP)

The metabolic product of diethyl phthalate (DEP), mEP, was detected in all liver samples, with concentrations ranging from 2.62 to 17.4 ng/g ww (table 4.6). This metabolite was expected to find, since mEP has been observed in human urinary studies in detection rates of 100 % (Frederiksen et al., 2013; Asimakopoulos et al., 2016; Rocha et al., 2017). Higher levels of mEP are reported in tissue of fish, as seen in roach and European eel, than in the present study of harbour porpoises. The concentration differences can potentially be explained by different exposure levels, differences in metabolic rates or differences of the sample matrix. Hart et al. (2018) reports mEP as the most commonly detected metabolite in urine of marine cetaceans. Even though urinary concentration is not directly comparable to tissue concentrations, the results suggests that cetaceans are exposed to DEP. The levels determined in human urine, is much higher compared with concentrations measured in urine of bottlenose dolphin (table 2.3).

Determination of DEP in marine mammals in Greenland and the Faroe Islands have shown liver concentrations ranging from 15.1 to 31.6 ng/g ww for polar bear, minke whale, pilot whale and ringed seal (Vorkamp et al., 2004). The levels of mEP measured in harbour porpoises along the Norwegian coast in this study was relatively low compared to the levels of the parent compound reported by Vorkamp et al. (2004). This can indicate that the marine mammals in Greenland and the Faroe Islands were exposed to higher levels of DEP or that the phthalate and its metabolite have different tissue distributions. Another explanation could be sample contamination of DEP, since the phthalates are abundant in the environment. Anyway, the toxicity is often associated with the metabolites of the phthalates (Katsikantami et al., 2016), so risk assessment will be more realistic based on metabolite concentrations.

4.3.3 Occurrence of mono-n-butyl phthalate (mBP) and monoisobutyl phthalate (mIBP)

The main metabolites of di-n-butyl phthalate (DBP) and diisobutyl phthalate (DIBP), mBP and mIBP (Koch et al., 2012), were detected in the majority of the harbour porpoises (table 4.6). The levels of these metabolites ranged from 3.78 to 419 ng/g ww for mIBP, and from 1.73 to 299 ng/g ww for mBP. It is important to have in mind that the relative recoveries of these metabolites were high, especially for mIBP, which can result in an overestimation. Reported concentrations of mIBP were semi-quantified and cannot be discussed with certainty. A high significant correlation was observed between these two metabolites ($r=0.871$ at $p < 0.01$), suggesting similar sources and exposure routs. High correlations between the two isomers have also been observed in earlier studies (Fourgous et al., 2016; Rocha et al., 2017). Their parent compounds, DBP and DIBP, have very similar application properties, and may therefore be used as a mixture of both isomers in the applications (Maag et al., 2010). The data obtained from ECHA suggests that DIBP is used in lower amounts than DBP (table 2.3), but the concentrations determined in this study suggests the opposite. As mentioned above, a high relative recovery of mIBP might lead to an overestimation of the concentrations. Data on use in Norway on the other hand reveal a potentially higher use of DIBP than DBP from 2010 to 2017 (figure 2.4). Occurrence of these metabolites indicate a source of DBP and DIBP exposing the marine mammals inhabiting the Norwegian coast.

It should also be mentioned that mBP can be a metabolic product of butylbenzyl phthalate (BBzP), but this is not expected since human studies of BBzP have shown that only a minor fraction of the metabolic products of this compound is excreted as mBP (Anderson et al., 2001). In addition, no correlation was observed between mBzP and mBP, indicating that mBP observed in this study was a metabolic product of DBP and not BBzP.

Comparing the results with other studies, similar trends with higher concentrations of mIBP than mBP have been observed in two human studies, European eel and roach (table 2.3). Only one of the isomers, mBP, have been found in cetaceans. Mean concentrations of mBP in dw skin samples of fin whale and bottlenose dolphin in the Mediterranean Sea were much higher than observed in this study (Baini et al., 2017). This could indicate higher exposure of cetaceans in the Mediterranean Sea than in the Norwegian coast, or species differences between the investigated species and harbour porpoises. Another potential explanation can be

differences between the different sample matrices, so a real comparison is not possible based on the information provided. It is also necessary to consider the number of individuals in the studies, since individual differences might be crucial. In the study of fin whale and bottlenose dolphins only 1 and 3 individuals, respectively, were investigated compared to 100 harbour porpoises in this study. In the present study, a high variance of liver concentrations between the different individuals in the population investigated were observed. Levels of DBP measured in marine mammals in Greenland and Faroe Islands were lower than mBP levels measured in the present study (table 2.4).

4.3.4 Occurrence of mono-n-pentyl phthalate (mPeP) and monoisopentyl phthalate (mIPeP)

The isomers mPeP and mIPeP, metabolites of di-n-pentyl (DnPeP) phthalate and diisopentyl phthalate (DIPeP), were not found in livers of harbour porpoises. Occurrence of these phthalates have not been extensively established, but two earlier studies have reported mIPeP in human urine in high detection rates (> 98 %) while mPeP was found in only one out of total 194 individuals investigated, at very low concentration, in the same studies (Rocha et al., 2017; Souza et al., 2018). Based on results from the present study and the observations in human urine, limited exposure of DnPeP might be suggested. The SPIN data base and ECHAs chemical search did not report any use or manufacturing of the phthalate, agreeing with this suggestion (SPIN, n.d.; ECHA, 2019). It might also be suggested that mPeP is not the main metabolite in humans and harbour porpoises, as observed in rats after administration of DnPeP where a hydroxylated metabolite stood out as the main metabolite in levels over 4 times higher than mPeP (Silva et al., 2011). Both studies investigating these metabolites in human urine were performed on a Brazilian population (Rocha et al., 2017; Souza et al., 2018). Comparing with the results of mIPeP in this study, potential exposure differences of DIPeP can be suggested, with limited exposure of DIPeP for harbour porpoises in the Norwegian coast compared to the human population in Brazil.

4.3.5 Occurrence of monocyclohexyl phthalate (mCHP)

The metabolite mCHP was not found in livers of harbour porpoises from the Norwegian coast. Occurrence of this metabolite have been established in urine of humans and alligators in relatively low detection rates and/or concentrations (Asimakopoulos et al., 2016; Brock, Bell & Guillette, 2016; Rocha et al., 2017). Our data suggest limited exposure of the parent phthalate, dicyclohexyl phthalate (DCHP) for marine mammals inhabiting the Norwegian coast. No information about the efficiency of mCHP as a biomarker of DCHP exposure of marine mammals is available, so a potentially more effective biomarker might be established in future studies.

4.3.6 Occurrence of monobenzyl phthalate (mBzP)

As mentioned above, mBP can be a metabolic product of benzyl butyl phthalate (BBzP), but the major metabolic product in humans is observed to be mBzP (Anderson et al., 2001). An assumption can be made that mBzP is the main metabolite in harbour porpoises as well. The assumed major metabolite of BBzP was detected in 10 out of 100 liver samples, with concentrations ranging from 0.691 to 7.29 ng/g ww (table 4.6). This indicate that there is a source of BBzP exposing marine mammals along the Norwegian coast, assuming that the harbour porpoises do not move long distances. Data from the SPIN database have showed that BBzP use in Norway has decreased since 2000 but still has been in use (1-2 tonnes/year) the most recent years (figure 2.4). A 10 times higher mean concentration of mBzP have been observed in skin of fin whale in the Mediterranean Sea (Baini et al., 2017), compared to mean liver concentration in the present study. This may suggest higher exposure in the Mediterranean Sean than in the coast of Norway, or that mBzP can found in higher levels in skin compared to liver. Higher levels have also been observed in liver of roach, indicating that differences might be due to different exposure levels or species variations (Valton et al., 2014). Muscle dw concentrations of mBzP of European eel (table 2.3) were observed in concentration ranges similar to this study.

The parent compound BBzP have been observed in livers of marine mammals in Greenland and Faroe Islands in levels corresponding to the mBzP level in skin of fin whale in the Mediterranean Sea (Vorkamp et al., 2004; Baini et al., 2017). Baini et al. (2017) measured

both phthalate and metabolite concentrations in skin of fin whale, and the results showed that the concentrations of BBzP were over 8 times greater than for mBzP. The higher level of the parent compound is potentially a result of contamination of the sample matrix or low efficiency of metabolic breakdown of the compound. Assuming a similar ratio between liver BBzP and liver mBzP, an estimated concentration of mBzP in pilot whales from Faroe Island would be close to mean concentration of mBzP in livers of harbour porpoises measured in this study.

4.3.7 Occurence of mono-n-hexyl phthalate (mHxP)

The phthalate metabolite mHxP were semi-quantified and showed a detection rate of 45 % (table 4.6). The relative recovery of this metabolite was very high and might have led to an overestimation. In human urinary studies, mHxP have been detected in < 13 % of the populations with median concentrations < 1.00 ng/ml (Asimakopoulos et al., 2016; Rocha et al., 2017). It is difficult to compare liver and urinary concentrations, but relatively low levels of mHxP have been measured in both human urine and now in livers of harbour porpoises. Comparing the detection rate, a higher percent of the harbour porpoise population was exposed compared to humans in the two study populations. In livers of marine mammals, the parent compound di-n-hexyl phthalate (DHxP) have been observed (Vorkamp et al., 2004). Reported DHxP levels for three out of the four species investigated were found in higher levels than the metabolite studied in this work. A high risk of contamination is associated with analysis of phthalates and might explain the differences. Baini et al. (2017) did not detect DHxP over the detection limit in cetacean skin samples from the Mediterranean Sea.

4.3.8 Occurrence of mono-n-heptyl phthalate (mHpP)

The metabolite mHpP were semi-quantified and detected in 11 % of the harbour porpoises (table 4.6). Information of use, manufacturing and import of its parent compound di-n-heptyl phthalate (DHpP) were not available on neither the SPIN database or ECHAs search for chemicals. Only two of the studies presented in table 2.3 included mHpP, where mHpP was detected in human urine in three out of 130 individuals in Saudi Arabia and not found in the population investigated in Brazil (Asimakopoulos et al., 2016; Rocha et al., 2017). Mean

urinary concentration of the three individuals reported was 0.37 ng/ml (Asimakopoulos et al., 2016), whereas the semi-quantified liver concentrations of harbour porpoises ranged from 0.214 to 1.87 ng/g ww. The findings of mHpP in the present study, indicates that marine mammals might be exposed to DHpP in the Norwegian coast.

4.3.9 Occurrence of mono-n-octyl phthalate (mOP)

The metabolite of di-n-octyl phthalate (DnOP), mOP, was semi-quantified and detected in 21 % of the harbour porpoise population (table 4.6). Other oxidative metabolic products of the parent compound have been observed (Silva et al., 2005; Calafat at al., 2006) but were not analysed in this work. In earlier studies of urinary concentrations in human populations, mOP have been detected in under 14 % of the populations investigated (Frederiksen at al., 2013; Asimakopoulos et al., 2016; Rocha et al., 2017). The higher detection rate in the present study is potentially a result of different exposure levels or a result of different metabolic pathways between humans and the harbour porpoises. The oxidative metabolite of DnOP, mono-(3-carboxypropyl) phthalate (mCPP) have been found in considerably higher concentrations than mOP in rats after administration of DnOP, suggesting that mOP may be a poor biomarker of DnOP (Silva et al., 2005; Calafat et al., 2006). Frederiksen et al. (2013) also found higher urinary concentration of mCPP than mOP in humans. Importantly, mCPP is not a specific metabolite of DnOP (Frederiksen et al., 2013).

The higher detection rate in harbour porpoises livers than in human urine suggest a potential for the hydrophobic mOP to be distributed to the liver, as observed in different fish species (Hu et al., 2016). Silva et al. (2015) observed metabolism of DnOP to mOP and following formation to oxidised forms of mOP in liver of rats, indicating that metabolism of DnOP occurs in the liver. Valton et al. (2014) found mOP in highest levels in liver of roach, compared to the other tissues investigated. These findings might support that the more hydrophobic metabolites are metabolised in the liver. The levels of mOP in livers of roach were higher than for harbour porpoises, and levels measured in muscle samples from European eel have shown even higher levels (table 2.3). Importantly, these determinations were performed on dry weight samples rather than wet weight, so a comparison with wet weight samples might not be valid.

DnOP has been observed in livers of marine mammals in Greenland/Faroe Islands, with mean concentrations higher than mOP measured in the present study (table 2.3). Contamination of the samples when measuring parent compounds is a potential explanation. In addition, other metabolic products than mOP might be predominant in liver of harbour porpoises. Valton et al. (2014) reported a much higher concentration of the parent compound than the mOP in the liver of roach, while Hu et al. (2016) reported the opposite in tissues of a group of wild marine organisms. These comparisons indicate differences between different species and tissues regarding DnOP and mOP ratios.

4.3.10 Occurrence of mono-n-nonyl phthalate (mNP)

In the present study, the metabolite mNP have been found in marine mammals for the first time, as I am aware of. In 23 out of the 100 harbour porpoises, mNP was detected and semi-quantified with concentrations ranging up to 98.9 ng/g ww. No usage or manufacturing data was found for the parent compound, di-n-nonyl phthalate (DnNP) in the SPIN database or ECHAs chemical search. A potential source might be from applications of the isomeric form, diisononyl phthalate (DINP), which is used in high amounts, where DnNP might serve as an impurity from the manufacturing. The present study cannot provide any estimation of exposure of DnNP since no information about the efficiency of mNP as a biomarker for DnNP in harbour porpoises is available. Many different metabolites have been observed for the isomer DINP (Silva et al., 2006). Therefore, the corresponding linear chained DnNP might potentially have other metabolic products in addition to mNP. The majority of DINP metabolites in livers of rats have been observed as oxidation products of the monoester (McKee et al., 2002). Using mNP as a biomarker of DnNP could potentially lead to an underestimation of the exposure if the metabolic pathways are similar between the two isomers. To conclude, our results indicate that DnNP is an environmental pollutant in the Norwegian coast with the potential to enter the biota.

4.3.11 Occurrence of mono-n-decyl phthalate (mDP)

In 3 % of the investigated population, mDP was detected over LOD. The methods recovery of mDP was low, indicating a risk of analyte loss during sample preparation. To my knowledge, the present study is the first to report occurrence of this metabolite in marine mammals. Baini et al. (2017) measured the parent compound, di-n-decyl phthalate (DnDP), in skin matrix of three cetacean species in the Mediterranan Sea but all measurements were below the detection limit. mDP have been observed in urine of human children in Brazil with a detection rate close to observations in this study (Rocha et al., 2017). No data of use or manufacturing of DnDP was found in the SPIN database and ECHAs chemical search. DnDP is potentially an impurity of the isomer diisodecyl phthalate (DIDP) which is used in high amounts (figure 2.4). Metabolites of the isomer DIDP in rats include the hydrolytic monoester along with many oxidised metabolic products, whereas the monoester has been detected as a minor metabolite (Kato et al., 2007). DnDP might therefore have a variety of metabolic products, and mDP may be a poor biomarker of DnDP exposure. Our findings indicate a risk of DnDP exposure in the Norwegian coast.

4.3.12 Occurence of di(2-ethyl-1-hexyl) phthalate (DEHP) metabolites

Three metabolic products of DEHP were measured in this study, including mEHP, mEOHP and mEHHP. Based on the concentrations measured in this study, mEHP seem to be the main metabolite of DEHP in livers of harbour porpoises. Earlier studies have also observed high levels of mEHP in tissues of cetaceans (table 2.3) On the other hand, mEHP had high background levels indicating contamination of the samples. The MPE mEHP is an environmental contaminant itself, since it can be formed by abiotic processes (Heudorf, Mersch-Sundermann & Angerer, 2007). It could therefore not be discriminated between mEHP as a metabolic product or contamination. The other metabolites measured, mEHHP and mEOHP, were detected in a minority of the samples, in 27 and 17 individuals respectively (table 4.6). The metabolite mEHHP was found in higher concentration and occurrence than mEOHP, suggesting that mEHHP is a more extensive metabolic product of DEHP than mEOHP in harbour porpoises. Corresponding ratios have been found in liver of roach, muscle of European Eel and human urine (table 2.3). The levels of mEHHP and mEHOP observed in other studies have been higher than the levels observed in liver of

harbour porpoises in the present study (table 2.3). Hart et al. (2018) did not detect mEHHP over the detection limits in urine of bottlenose dolphins, and mEOHP was detected in low concentrations. Anyway, a comparison with other sample matrices might not be valid due to heterogenous distribution between tissues and urine. The data for mEHHP and mEOHP showed a moderate correlation, but not significant, so a common origin cannot be proven.

Exposure of DEHP is often estimated based on the sum of its metabolites, which in this case would involve the three metabolites measured. Frederiksen et al. (2013) included an additional metabolite measured in human urine. Rocha et al. (2017) and Asimakopoulos et al. (2016) included two additional metabolites and excluded mEHP from the calculation of urinary DEHP in humans. A calculated sum of DEHP metabolites in this study gave a mean concentration of 51.1 ng/g ww. As mentioned above, mEHP in samples was suspected to arise from contamination and should therefore be excluded in the calculations. The sum of DEHP in this study was therefore calculated to 2.00 ng/g ww. Lack of the additional metabolites would potentially lead to an underestimation of DEHP, which was evident when comparing with other studies. The sum of DEHP calculated without excluding mEHP was comparable with measurements of the parent compound in cetaceans in Greenland and Faroe Islands, but much lower than the levels detected in the Mediterranean Sea (table 2.4).

A general problem associated with the estimation of DEHP exposure using its metabolites as biomarkers, is that DEHP have many different metabolic products (Albro et al., 1982; Frederiksen et al., 2013). Many polar metabolic products have been observed in rats and humans (Albro et al., 1982; Albro et al., 1983; Frederiksen et al., 2013). Variation is observed regarding the ratios of the metabolic products of DEHP in different species. For example, a study of the DEHP metabolite distribution in different species showed that mEHHP accounts for 38.2 % of urinary metabolites in green monkey and only 3.4 % in guinea pig (Albro et al., 1982). Therefore, with no information about the metabolic products of DEHP and their ratios in harbour porpoises, an estimation of DEHP exposure was difficult to establish based on the quantifications in this study.

4.4 Exposure patterns

Exposure patterns have been investigated based on the levels of phthalates metabolites found in livers of harbour porpoises. The investigation was performed by analysing variance between the different groups of the population, correlation tests and PCA biplots. Differences between different sampling years and genders within the population were investigated for the most commonly detected phthalates metabolites in this study (N<60). The metabolites mEHP and PA were excluded due to the high background and low recoveries, respectively. The population was grouped by years, genders, and regions of sampling. A correlation between weights and lengths of the harbour porpoises and phthalate metabolites levels was also investigated.

4.4.1 Year differences

A concentration difference for the phthalate metabolites with highest detection rate was observed between the two sampling years, 2016 and 2017 (figure 4.2).

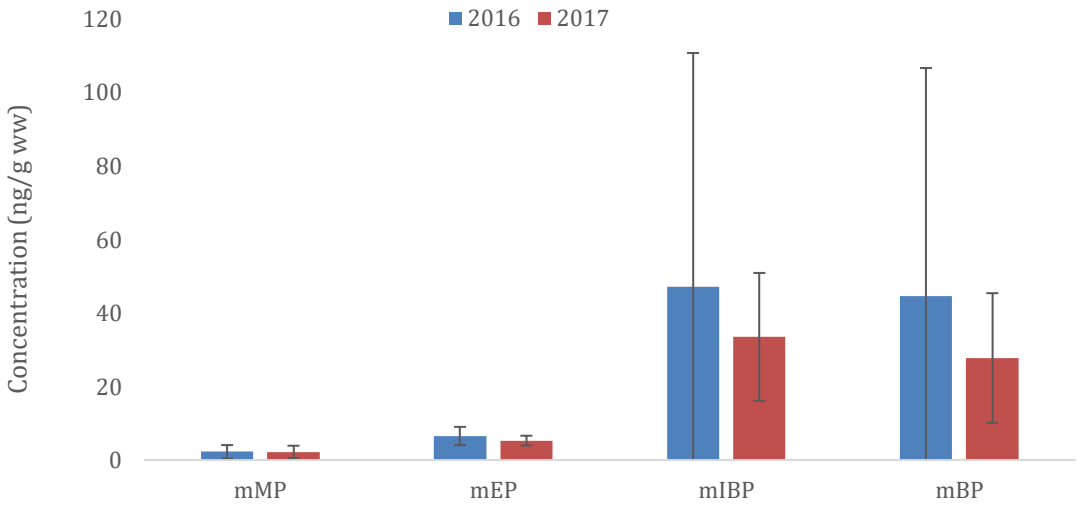

Figure 4.2: Liver concentrations (ng/g ww) of some phthalate metabolites divided in to years of sampling.

The mean concentrations were higher for all of the investigated phthalate metabolites in 2016, but only significantly higher for mEP ($p<0.05$). The PCA biplot of data sorted by year (figure G.1, appendix G) agreed with this trend to some extent. The higher levels of mEP in 2016 was

contradictory to the reported amounts of use in Norway, since usage data from the SPIN database showed that the registered use of DEP was greater in 2017 than in any other years since 2000. The trend of higher liver concentrations in 2016 may indicate that the harbour porpoises along the Norwegian coast were exposed to higher levels of phthalates during 2016 than 2017, but can only be proven statistically for DEP.

4.4.2 Gender differences

Between the two genders in the harbour porpoise population, a concentration difference was observed (table 4.3).

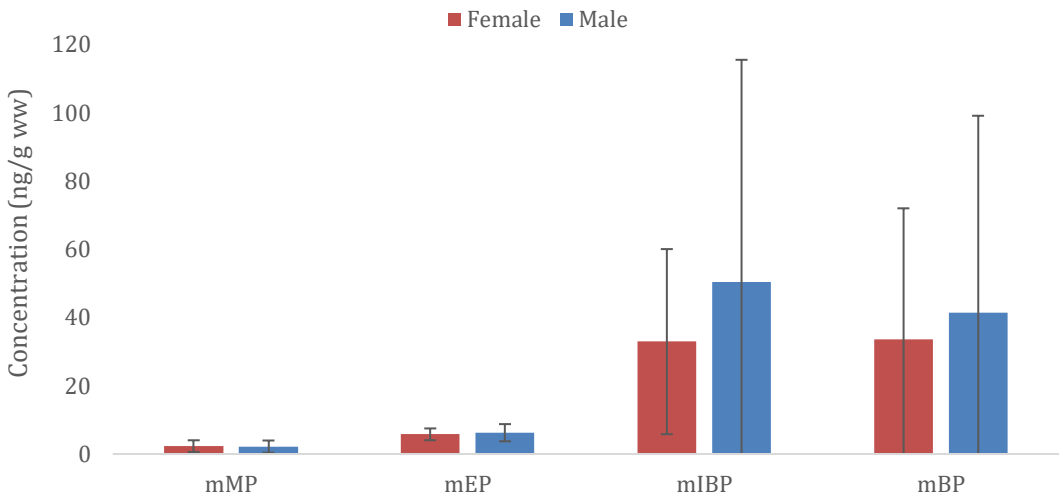

Figure 4.3: Liver concentrations (ng/g ww) of some phthalate metabolites in females and males.

The mean concentrations of mEP, mIBP and mBP were observed to be higher for males than females. The PCA biplot (figure G.2, appendix G) did not show any obvious differences between the groups. To a small extent, the plots representing males were distributed towards the compartment space where the phthalates metabolites showed a positive loading on Dim1, while the points representing females were distributed towards the compartment space where the body size showed a negative loading on Dim1. This might be a result of the greater weights and lengths of the females compared to males (table 3.1). Regarding mMP, the mean concentrations were observed to be higher for females than males. A Mann-Whitney U-test

showed no significant differences between the two genders for any of these phthalate metabolites, so no gender differences can be proven in this study.

4.4.3 Regional exposure patterns

None of the 14 phthalate metabolites found showed any significant differences among the different regions. The PCA biplot (figure G.3, appendix G) showed no obvious distribution between the plots for the different regions, except that all points for Nordkinnhalvøya were observed to group together away from the phthalate metabolites loadings. A trend was observed with increasing concentrations for some of the metabolites from the most northern region, Nordkinnhalvøya, to the most southern region, South and Central (figure 4.4). This concentration gradient along the Norwegian coast cannot be proven since no significant variations between the different regions were found. Regional differences in levels of the phthalate metabolites found at highest detection rates are presented in figure 4.5.

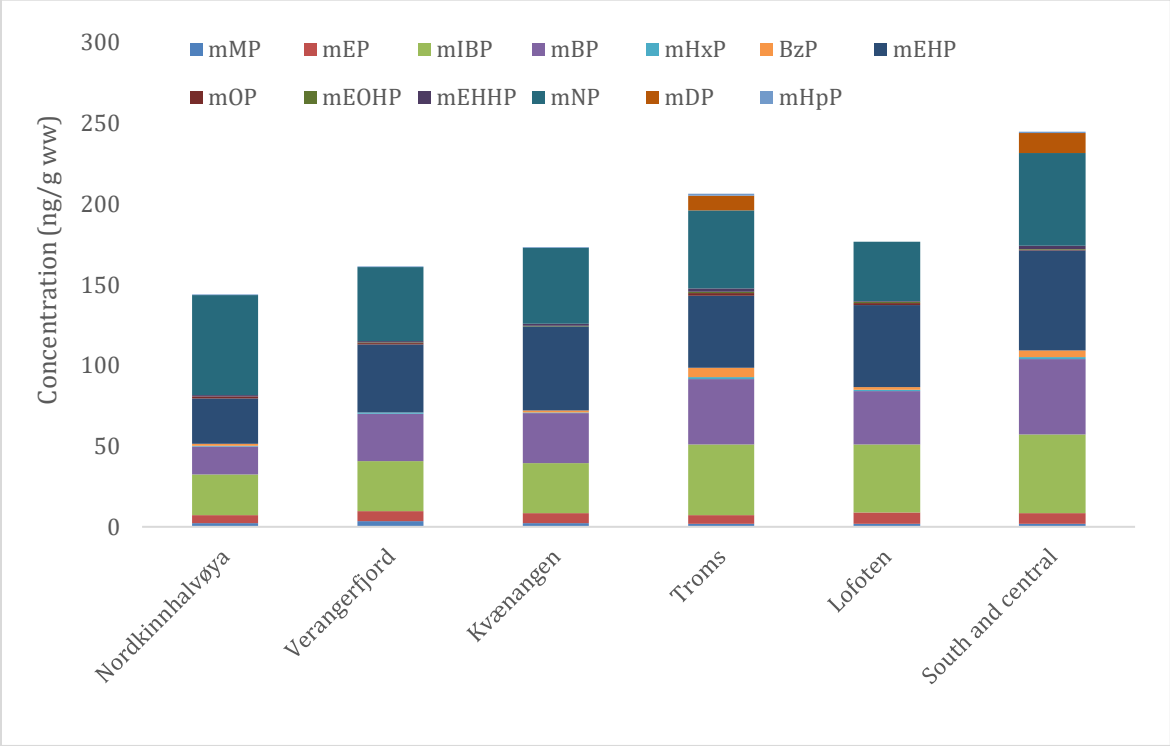

Figure 4.4: Stacked mean liver concentrations (ng/g ww) of all detected phthalates metabolites (>LOD) except PA in the different sampling regions.

In figure 4.4, data for mEHP were included despite the high background. Since the mEHP levels followed the trend, the metabolite might potentially be a marker of DEHP exposure levels in the environment. Interestingly, mEHP were measured in lowest levels in Nordkinnhalvøya, where the two other metabolites of DEHP, mEOHP and mEHHP, had the lowest detection rate. Higher levels were observed in Tromsø than in Lofoten, potentially due to a smaller civilisation in the latter region. This trend might suggest that the exposure levels of marine mammals along the Norwegian coast are dependent on the human civilisation in the regions they inhabit.

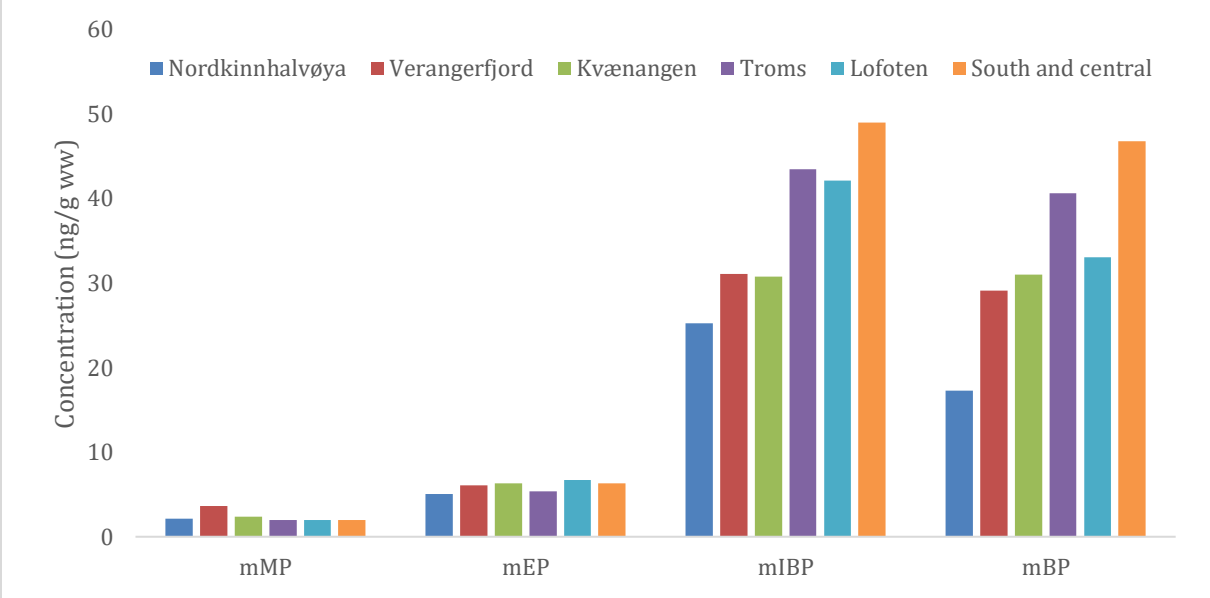

Figure 4.5: Liver concentrations (ng/g ww) of some phthalate metabolites in the different sampling regions.

Regarding the two smallest metabolites, no obvious trend was observed from north to south, while the trend with increasing concentration toward south of the Norwegian coast was observed for mBP and mIBP (figure 4.5). The suggested trend cannot be proven due to the non-significant differences between the regions.

4.4.4 Correlations between phthalate metabolites and body size

According to the PCA biplots (appendix G), a negative correlation was observed between the body weights and lengths of the harbour porpoises and the metabolites mNP and mDP. The

correlation heat map (figure H.1, appendix H) showed that the correlations was non-significant. A correlation analysis showed a small non-significant negative correlation between mNP and both weights and lengths of the harbour porpoises, while for the three individuals with mDP concentrations measured over LOD a moderate non-significant positive correlation was found with the size of the harbour porpoises. The only phthalate metabolite that showed a significant correlation with the weights and lengths of the harbour porpoises was the semi-quantified PA, with a small negative correlation ($r = -0.290$, $p < 0.01$). To conclude, no correlation between the harbour porpoises body size and phthalates metabolite levels in liver can be proven in this study.

Chapter 5

Conclusions

Determination of occurrence and quantification of several phthalate metabolites in livers of harbour porpoises using LC-MS/MS was achieved. Liver was found to be the most suitable tissue matrix for analysis compared to muscle and blubber, since liver was observed to have the highest content of most of the target analytes. The bioanalytical method performance characteristics were demonstrated, including linearity, ion ratios, recoveries, matrix effects and limits of detection (LODs). The linearity and ion ratios were acceptable for all target analytes. LODs for all target analytes were adequately low for our purpose. High background was observed for mono(2-ethyl-1-hexyl) phthalate (mEHP), indicating contamination, and consequently no conclusion could be drawn from its data. The sample preparation protocol showed satisfactory extraction efficiency for several of the target analytes. The recoveries were poor for some of the phthalate metabolites (e.g. phthalic acid, PA), and were semi-quantified. Only three deuterated internal standards were used to compensate for losses during sample preparation and matrix effects. A suggestion for further work would be to obtain ^{13}C-target specific internal standards.

Phthalate metabolites were found in all individuals of the investigated population of harbour porpoises from the Norwegian coast. A total of 14 out of 17 phthalate metabolites were detected over LOD, whereas highest detection rates were observed for monoethyl phthalate (mEP; 100 %), monoisobutyl phthalate (mIBP, 99 %), mono-n-butyl phthalate (mBP, 97 %) and monomethyl phthalate (mMP, 69 %). The highest mean concentrations were found for mono-n-nonyl phthalate (mNP, 48.1 ng/g ww), mIBP (41.1 ng/g ww) and mBP (37.2 ng/g ww). No significant associations were found between the body size or sampling year and concentrations of phthalates metabolites. Significantly higher mEP concentrations were observed in males compared to females. A possible trend of increasing phthalate exposure from north to south was observed. Further studies should be conducted to evaluate this trend. To conclude, this is the first study to document phthalates metabolites concentrations in harbour porpoises, suggesting a novel approach in assessing phthalates exposures in marine mammals by measuring their metabolites and not parent compounds (as has been performed thus far).

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Appendix A

Molecular structures of target analytes and internal standards

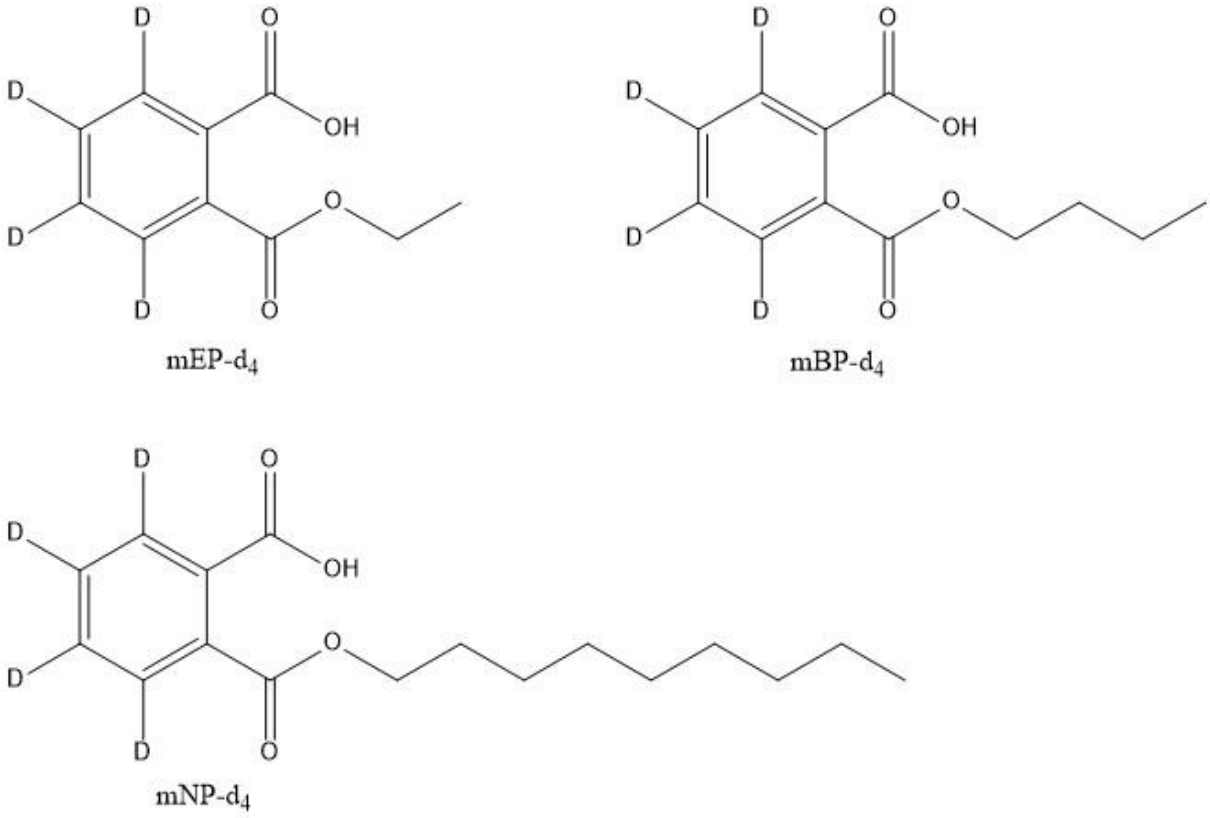

Figure A.1: Molecular structures of deuterated internal standards used in this study.

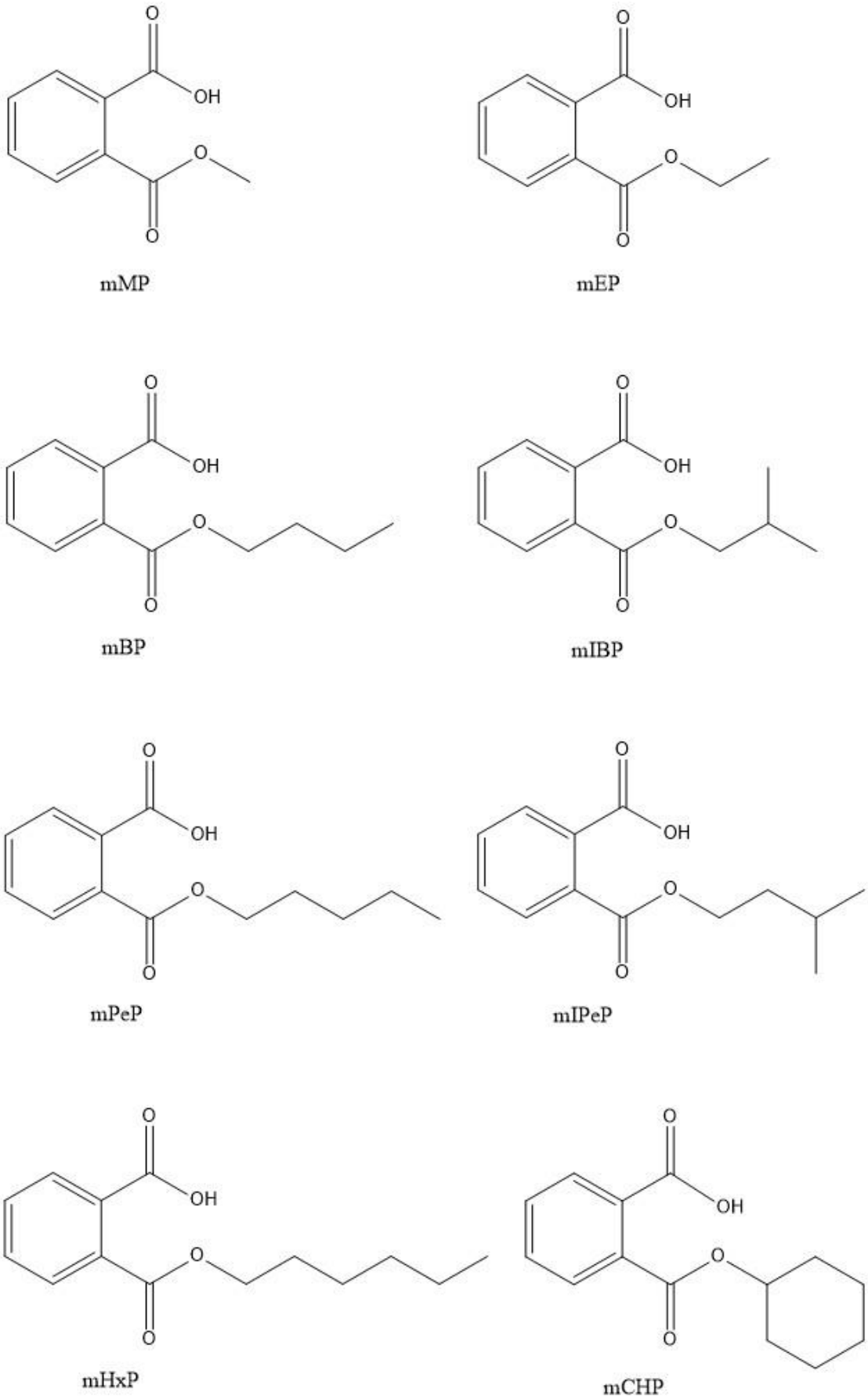

Figure A.2: Molecular structures of target analytes in this study. Part 1.

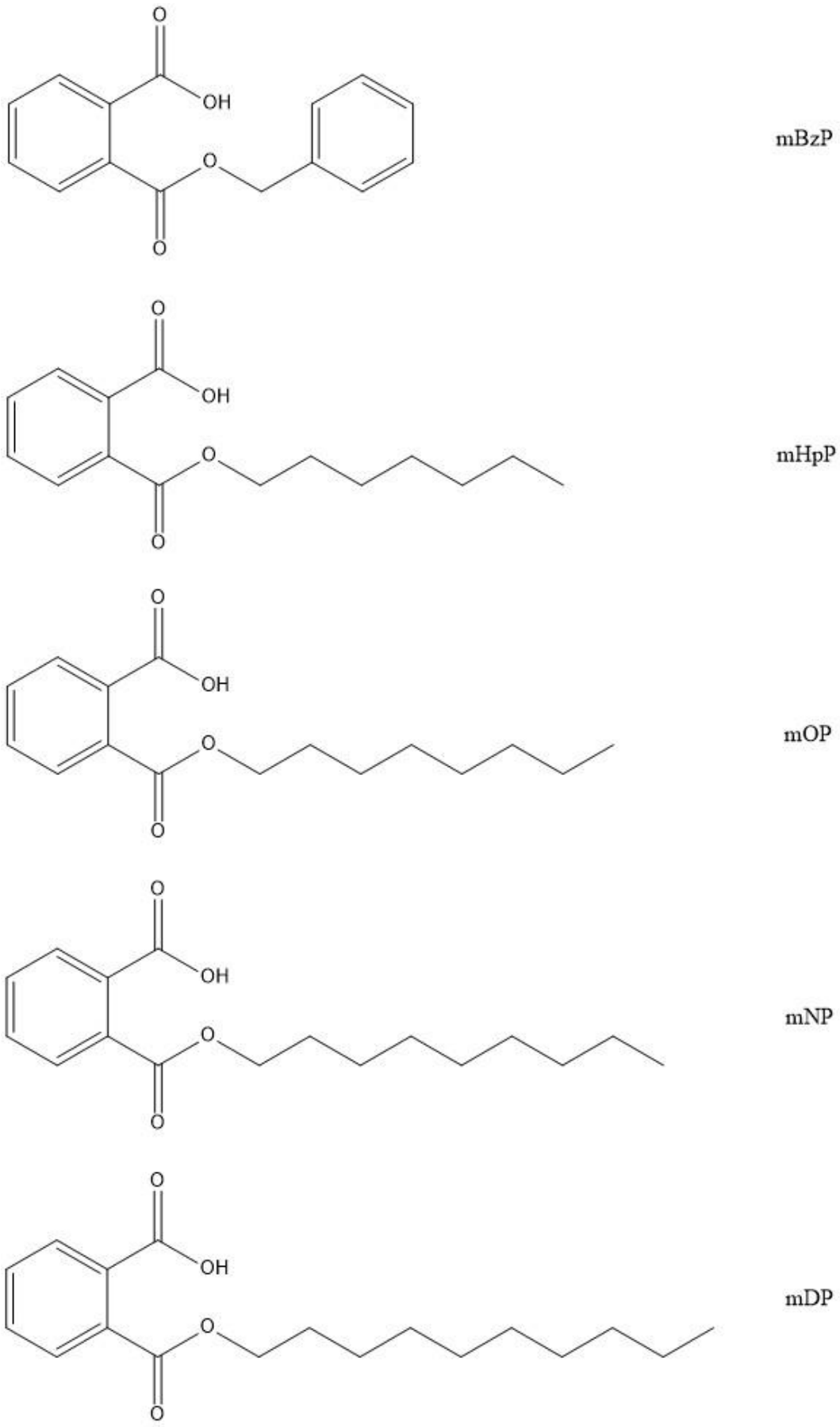

Figure A.2: Molecular structures of target analytes in this study. Part 2.

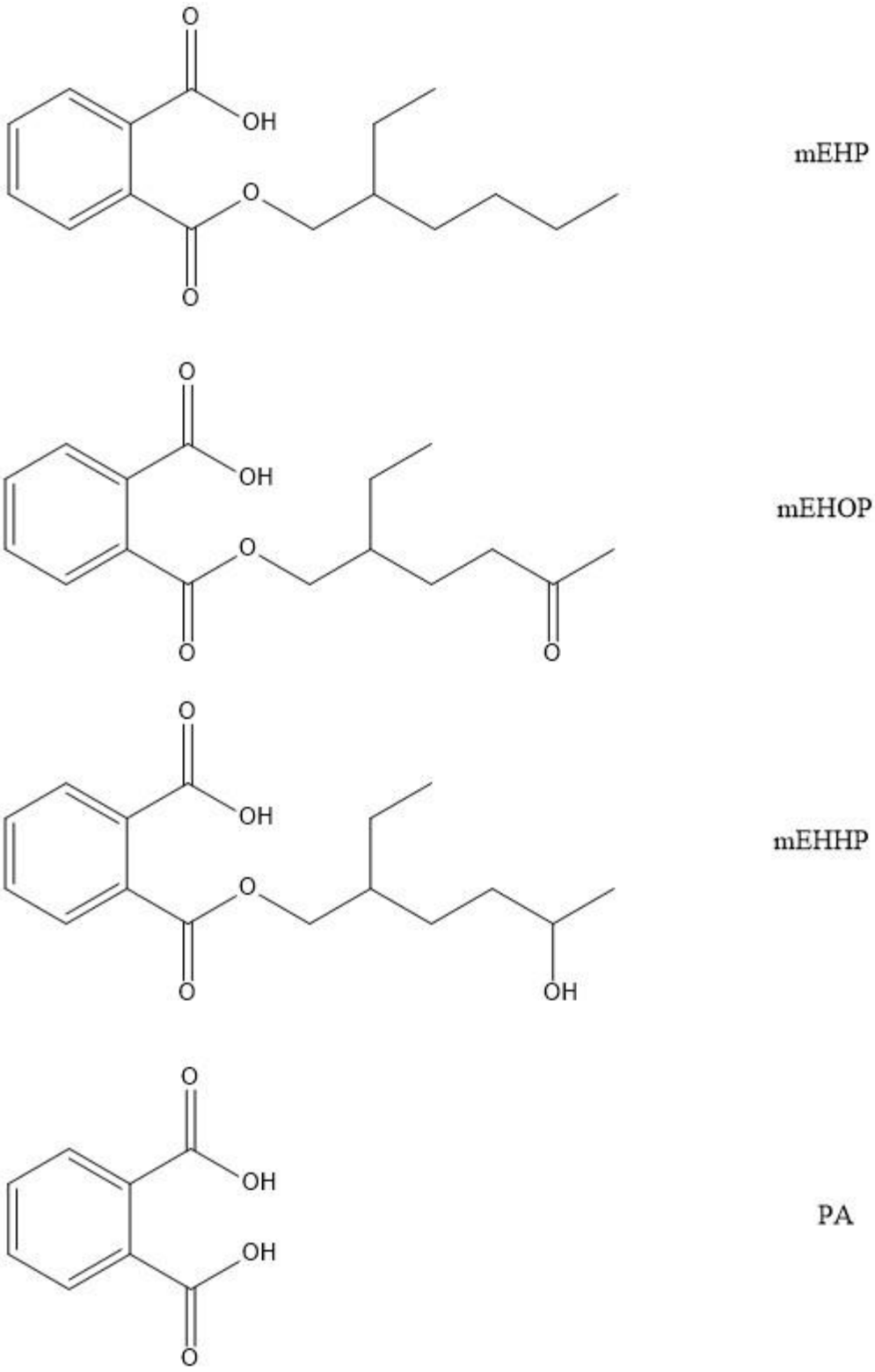

Figure A.2: Molecular structures of target analytes in this study. Part 3.

Appendix B

Conditions for method testing

Differences from methodology presented in 3. Experimental in the method test is presented beneath as points. Due to observations of contamination of samples in the method test, some measures was done to reduce contamination in the analysis of 100 liver samples from harbour porpoises.

- 0.1 to 0.2 grams of tissue was weighed out for analysis.
- Tissues used in the analysis were thawed prior to the sample preparation.
- Spiking was performed with a TA mix containing 14 phthalates metabolites: mEP, mMP, mBP, mIBP, mPeP, mIPeP, mHxP, mBzP, mCHP, mOP, mEHP, mEHHP, mEOHP and PA. 20 µL 1 ppm TA mix was added to the samples.
- No foil was wrapped around the PP tubes during sonication.
- Digestion with β-glucuronidase was done in an own, not an incubator.
- Samples were stored in the refrigerator instead of the freezer between the different stages in sample preparation and between sample preparation and analysis.
- The TurboVap was not cleaned with methanol before concentration.
- UPLC column was change due to problems, in method test a Kinetex C18 (30 x 21 mm) column was used.

Table B.1: Weight (g) of harbour porpoise tissue in sub-samples for method testing. All sub-samples were obtained from the same individual. Internal standards (IS) were added in each sample (40 ppb).

	Sub-sample weight (g)		
Sample	*Liver*	*Muscle*	*Blubber*
Sample 1, IS only	0.1653	0.1051	0.1385
Sample 2, IS only	0.1854	0.1182	0.1191
Sample 2, IS only	0.1831	0.1711	0.1407
Spike 1, 40 ppb	0.1471	0.1796	0.1447
Spike 2, 40 ppb	0.1750	0.1900	0.1714
Spike 3, 40 ppb	0.1821	0.1450	0.1370
Matrix Match 1, 40 ppb	0.1553	0.1881	0.1510
Matrix Match 2, 40 ppb	0.1955	0.1166	0.1100
Matrix Match 3, 40 ppb	0.1830	0.1287	0.1562
Total number of samples:	9 + 3 reagent blanks (no tissue added)	9 + 3 reagent blanks (no tissue added)	9 + 3 reagent blanks (no tissue added)

Table B.2: Analyte specific MS/MS parameters with Kinetex C18 (30 x 21 mm) column used in method development including primary and secondary transitions, retention times (RT) and relative retention times (RRT; internal standard used to calculate RRT for the analyte).

Compound	RT	RRT (IS)	Primary transition	Secondary transition
mEP	1.60	1.01 (mEP-d_4)	193 > 121	193 > 77*
mMP	1.36	0.861 (mEP-d_4)	179 > 107*	179 > 77
mBP	3.35	1.02 (mBP-d_4)	221 > 150	221 > 77*
mIBP	3.17	0.958 (mBP-d_4)	221 > 134*	221 > 77
mPeP	4.71	1.42 (mBP-d_4)	235 > 121	235 > 77*
mIPeP	4.56	1.38 (mBP-d_4)	235 > 85	235 > 77*
mHxP	6.02	0.76 (mNP-d_4)	249 > 121	249 > 99*
mCHP	4.48	1.36 (mBP-d_4)	247 > 97	247 > 77*
mBzP	4.18	1.27 (mBP-d_4)	255 > 183	255 > 177*
mHpP	8.80	0.861 (mNP-d_4)	263 > 133*	263 > 77
mOP	7.17	0.901 (mNP-d_4)	277 > 127*	277 > 77
mEHP	6.87	0.863 (mNP-d_4)	277 > 134*	277 > 77
mEOHP	3.66	1.11 (mBP-d_4)	291 > 143*	291 > 121
mEHHP	3.51	1.06 (mBP-d_4)	293 > 145*	293 > 121
PA	1.17	0.730 (mEP-d_4)	165 > 121	165 > 77*
mEP-d_4	1.59		197 > 81	-
mBP-d_4	3.31		225 > 81	225 > 71
mNP-d_4	7.96		295 > 141	295 > 81

* Quantification ion

Table B.3: Gradient elution program with Kinetex C18 (30 x 21 mm) column for method development, using mobile phase mixture of milli-Q water (water) and acetonitrile, both acidified with 0.1 % acetic acid. Constant flow rate of 0.40 mL/min. Total run time 10 min.

Time [min]	Water [%]	Acetonitrile [%]
Init	95	5
0.5	95	5
1.0	75	25
3.5	75	25
4.0	60	40
5.5	60	40
6.0	50	50
7.5	50	50
7.8	10	90
8.9	10	90
9.0	95	5
10.0	95	5

Appendix C

Sample information - liver samples from 100 harbour porpoises

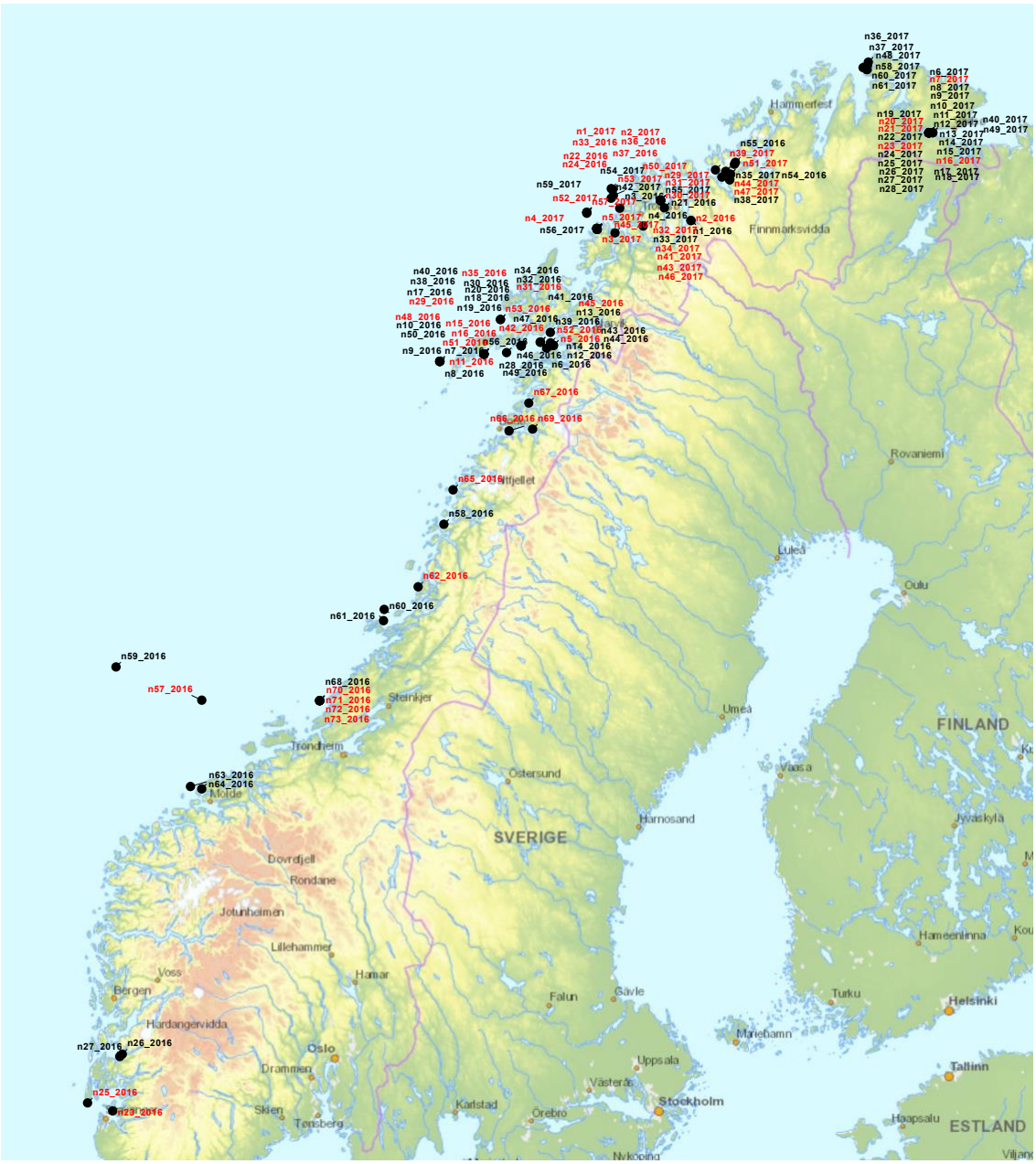

Figure C.1: sample locations, samples given as nID_year. See table C.2 for sample ID and more information.

Table C.1: Sample information, including sample ID, year and month of sampling, latitude and longitude of sampling location, harbour porpoise weight (kg) and length (cm), region of sampling and mass of weighed out liver sample (g).

ID	Year	Month	Latitude	Longitude	Weight (kg)	Length (cm)	Gender	Region	Sample weight (g)
1	2016	10	69,55	20,60	49	152	Male	Troms	0.1414
2	2016	9	69,55	20,60	39	140	Female	Troms	0.1218
3	2016	9	69,75	18,28	41	141	Male	Troms	0.0968
4	2016	9	69,53	19,02	22	103	Male	Troms	0.1101
5	2016	10	68,20	15,00	27	113	Female	Lofoten	0.1269
6	2016	10	68,17	15,79	35	133	Male	Lofoten	0.0924
7	2016	10	68,00	12,50	39	133	Male	Lofoten	0.0953
8	2016	9	68,00	12,50	33	127	Male	Lofoten	0.1361
9	2016	9	68,00	12,50	22	106	Male	Lofoten	0.1166
10	2016	9	68,00	12,50	47	149	Male	Lofoten	0.0965
11	2016	9	68,00	12,50	58	162	Female	Lofoten	0.1036
13	2016	10	68,20	15,00	26	117	Male	Lofoten	0.0945
14	2016	10	68,23	15,91	42	144	Male	Lofoten	0.1235
17	2016	10	68,10	13,85	50	152	Male	Lofoten	0.1324
20	2016	10	68,10	13,85	27	113	Male	Lofoten	0.1100
21	2016	10	69,72	19,76	27	115	Male	Troms	0.1207
22	2016	10	69,53	17,49	38	137	Female	Troms	0.1049
23	2016	10	59,07	5,83	43	140	Female	South and Central	0.1397
24	2016	10	69,53	17,49	68	156	Female	Troms	0.1021
25	2016	9	59,12	5,25	41	154	Female	South and Central	0.0937
26	2016	9	59,73	5,87	27	116	Male	South and Central	0.1043
27	2016	10	59,70	5,82	19	101	Male	South and Central	0.1181
29	2016	9	68,10	13,85	71	169	Female	Lofoten	0.1359
31	2016	10	68,10	13,85	39	140	Female	Lofoten	0.1319
32	2016	10	68,10	13,85	34	127	Male	Lofoten	0.0920
33	2016	9	69,53	17,49	34	131	Female	Troms	0.1002
36	2016	10	69,53	17,49	48	147	Female	Troms	0.1027
37	2016	9	69,53	17,49	49	154	Female	Troms	0.0901
39	2016	10	68,35	15,91	49	146	Male	Lofoten	0.1156
42	2016	10	68,20	15,00	40	137	Female	Lofoten	0.0999
45	2016	9	68,20	16,00	33	135	Female	Lofoten	0.0904

ID	Year	Month	Latitude	Longitude	Weight (kg)	Length (cm)	Gender	Region	Sample weight (g)
47	2016	9	68,20	16,00	42	135	Male	Lofoten	0.0970
48	2016	9	68,50	14,35	42	136	Female	Lofoten	0.1212
49	2016	10	68,20	15,00	44	149	Male	Lofoten	0.1143
51	2016	10	68,10	13,85	52	157	Female	Lofoten	0.0942
53	2016	9	68,50	14,35	32	131	Female	Lofoten	0.1398
54	2016	9	70,16	22,28	23	106	Male	Kvænangen	0.0949
55	2016	10	70,14	22,24	30	123	Male	Kvænangen	0.0958
57	2016	9	63,88	6,63	52	149	Female	South and Central	0.1132
58	2016	9	66,13	12,80	33	132	Male	South and Central	0.1188
59	2016	10	64,11	4,28	41	142	Male	South and Central	0.1354
60	2016	10	65,12	11,25	40	143	Male	South and Central	0.0951
61	2016	9	64,99	11,25	36	137	Male	South and Central	0.1174
62	2016	9	65,40	12,15	31	121	Female	South and Central	0.1121
63	2016	9	62,88	6,63	24	106	Male	South and Central	0.1073
64	2016	10	62,87	6,92	20	103	Male	South and Central	0.1363
65	2016	10	66,53	13,03	64	154	Female	South and Central	0.0966
66	2016	10	67,22	14,64	58	157	Female	South and Central	0.1247
67	2016	10	67,54	15,23	67	163	Female	South and Central	0.1219
68	2016	9	64,02	9,70	30	125	Male	South and Central	0.1236
69	2016	10	67,24	15,34	25	107	Female	South and Central	0.0955
70	2016	9	64,02	9,70	28	129	Female	South and Central	0.0970
71	2016	9	64,02	9,70	17	107	Female	South and Central	0.1233
72	2016	9	64,02	9,70	22	106	Female	South and Central	0.1492
73	2016	9	64,02	9,70	28	123	Female	South and Central	0.1146
1	2017	2	69,52	17,50	47	145	Female	Troms	0.1297
2	2017	2	69,52	17,50	44	144	Female	Troms	0.1102
3	2017	2	69,52	17,50	37	128	Female	Troms	0.1376
4	2017	2	69,52	17,50	61	157	Female	Troms	0.1113
5	2017	2	69,52	17,50	30	117	Female	Troms	0.1200
7	2017	3	70,11	28,90	66	162	Female	Varangerfjorden	0.1001
10	2017	3	70,11	28,90	57	158	Male	Varangerfjorden	0.0951
15	2017	4	70,11	28,90	42	142	Male	Varangerfjorden	0.1240
16	2017	3	70,11	28,90	40	140	Female	Varangerfjorden	0.0956

ID	Year	Month	Latitude	Longitude	Weight (kg)	Length (cm)	Gender	Region	Sample weight (g)
20	2017	3	70,11	28,90	47,5	146	Female	Varangerfjorden	0.0907
21	2017	3	70,11	28,90	57	151	Female	Varangerfjorden	0.1200
23	2017	4	70,11	28,90	44	143	Female	Varangerfjorden	0.1101
24	2017	3	70,11	28,90	59	156	Male	Varangerfjorden	0.1036
26	2017	3	70,11	28,90	31	125	Male	Varangerfjorden	0.1120
28	2017	4	70,11	28,90	49	146	Male	Verangerfjorden	*
29	2017	3	69,81	19,65	34	128	Female	Troms	0.1251
30	2017	3	69,81	19,65	33	133	Female	Troms	0.1148
31	2017	3	69,81	19,65	53	161	Female	Troms	0.1433
32	2017	3	69,81	19,65	37	127	Female	Troms	0.1022
33	2017	3	69,81	19,65	30,5	115	Male	Troms	0.1160
34	2017	3	69,81	19,65	57,5	168	Female	Troms	0.0989
35	2017	3	70,03	22,07	58	152	Male	Kvænangen	0.1100
36	2017	3	71,05	27,38	42,5	145	Male	Nordkinnhalvøya	0.1190
37	2017	3	70,97	27,28	32	123	Male	Nordkinnhalvøya	0.1055
38	2017	3	70,03	22,07	54	152	Male	Kvænangen	0.1134
39	2017	3	70,10	21,58	43,5	131	Female	Kvænangen	0.1480
41	2017	4	69,81	19,65	66	157	Female	Troms	0.1000
42	2017	3	69,87	18,01	35,5	131	Male	Troms	0.0935
43	2017	4	69,81	19,65	74	173	Female	Troms	0.1464
44	2017	3	69,97	22,02	62,5	161	Female	Kvænangen	0.0954
45	2017	3	69,47	18,08	48,5	142	Female	Troms	0.1028
46	2017	4	69,81	19,65	32	126	Female	Troms	0.1090
47	2017	3	69,97	22,02	34	129	Female	Kvænangen	0.1360
48	2017	3	71,01	27,32	40	131	Male	Nordkinnhalvøya	0.1423
50	2017	3	70,01	21,77	60,5	161	Female	Troms	0.0985
51	2017	4	70,07	21,92	55,5	155	Female	Kvænangen	0.0975
52	2017	3	69,71	17,18	50,5	146	Female	Troms	0.0949
53	2017	3	69,97	18,08	46	142	Female	Troms	0.0903
54	2017	3	69,98	18,03	36	138	Male	Troms	0.1181
55	2017	4	69,81	19,65	46	145	Male	Troms	0.1070
56	2017	3	69,72	17,18	36	128	Male	Troms	0.1250
57	2017	3	69,72	17,18	59	156	Female	Troms	0.1084

ID	Year	Month	Latitude	Longitude	Weight (kg)	Length (cm)	Gender	Region	Sample weight (g)
58	2017	3	71,00	27,16	38	135	Male	Nordkinnhalvøya	0.0903
59	2017	3	69,90	18,09	39	137	Male	Troms	0.1067
60	2017	3	70,97	27,28	31	123	Male	Nordkinnhalvøya	0.1409
61	2017	3	70,97	27,28	38	136	Male	Nordkinnhalvøya	0.1200

* Individual used in method development (liver, muscle and blubber weights in appendix B).

Appendix D

Conditions for analysis of 100 liver samples from harbour porpoises

Table D.1: Masses (g) of standards and internal standards weighed out for stock solution preparations.

Sample	**Weight (g)**
mEP-d_4	0.0118
mBP-d_4	0.0124
mNP--d_4	0.0104
PA	0.1088

Table D.2: Weights (g) of harbour porpoise tissue samples in mix of samples for triplicates used for method. Samples picked out for use in the mix was performed by random number generator.

Sample ID, year	Weight (g)
51, 2016	0.1100
54, 2017	0.1010
10, 2017	0.1049
56, 2017	0.1004
33, 2017	0.1114
2, 2017	0.1058
55, 2016	0.1237
29, 2016	0.1080
54, 2016	0.1266
22, 2016	0.0982
47, 2016	0.1373
23, 2017	0.1084
9, 2016	0.1107
61, 2016	0.1012
1, 2017	0.1220
Total number of samples included in the mix:	15

Table D.3: Weights (g) of harbour porpoise tissue from sample mix used as triplicates for method validation. Internal standards were added to all samples (40 ppb).

Sample	Weight (g)
Sample 1, IS only	0.0976
Sample 2, IS only	0.1091
Sample 2, IS only	0.1056
Spike 1, 10 ppb	0.0932
Spike 2, 10 ppb	0.1088
Spike 3, 10 ppb	0.1038
Spike 1, 25 ppb	0.0933
Spike 2, 25 ppb	0.0929
Spike 3, 25 ppb	0,1066
Matrix Match 1, 10 ppb	0.1375
Matrix Match 2, 10 ppb	0.0948
Matrix Match 1, 25ppb	0.1057
Matrix Match 2, 25 ppb	0.1172
Total number of samples:	13 + 6 reagent blank (no tissue added)

Table D.4: Target analytes, internal standards, analyte specific MS/MS parameters with Kinetex C18 (50 x 21 mm) column including primary and secondary transitions, retention times (RT) and relative retention times (RRT (IS)) in analysis of 100 liver samples.

Compound	**RT (min)**	**RRT (IS)**	**Primary transition**	**Secondary transition**
mEP	2.42	1.01 (mEP-d_4)	193 > 121	193 > 77*
mMP	2.08	0.862 (mEP-d_4)	179 > 107	179 > 77*
mBP	4.86	1.01 (mBP-d_4)	221 > 150	221 > 77*
mIBP	4.74	0.981 (mBP-d_4)	221 > 134	221 > 77*
mPeP	5.96	1.23 (mBP-d_4)	235 > 121	235 > 77*
mIPeP	5.75	1.92 (mBP-d_4)	235 > 85	235 > 77*
mHxP	7.00	0.795 (mNP-d_4)	249 > 121	249 > 99*
mCHP	5.82	1.17 (mBP-d_4)	247 > 97	247 > 77*
mHpP	7.59	0.861 (mNP-d_4)	263 > 113*	263 > 77
mBzP	5.27	1.09 (mBP-d_4)	255 > 183	255 > 177*
mOP	8.46	0.961 (mNP-d_4)	277 > 127*	277 > 77
mEHP	8.03	0.911 (mNP-d_4)	277 > 134*	277 > 77
mEOHP	4.97	1.03 (mBP-d_4)	291 > 143*	291 > 121
mEHHP	4.92	1.02 (mBP-d_4)	293 > 145*	293 > 121
mDP	8.95	1.02 (mNP-d_4)	305 > 261	305 > 77*
mNP	8.82	1.00 (mNP-d_4)	291 > 141*	291 > 77
PA	1.87	0.8** (mEP-d_4)	165 > 121	165 > 77*
mEP-d_4	2.39		197 > 125	197 > 81
mBP-d_4	4.82		225 > 81	225 > 71
mNP-d_4	8.80		295 > 141	295 > 81

* Quantification ion.

Table D.5: Mean ion ratio % (RSD%) based on the 6 highest calibration points.

	Ion ratios % (RSD%)	Quantification ion	Confirmation ion
mEP	54.5 (2.84)	193 > 77	193 > 121
mMP	89.8 (2.64)[a)]	179 > 77	179 > 107
mBP	0.892 (152)	221 > 77	221 > 150
mIBP	70.2 (22.8)	221 > 77	221 > 134
mPeP	14.3 (15.7)[b)]	235 > 77	235 > 121
mIPeP	67.4 (20.6)	235 > 77	235 > 85
mHxP	32.5 (3.66)	249 > 99	249 > 121
mHpP	99.4 (12.2)	263 > 113	263 > 77
mCHP	79.4 (11.7)	247 > 77	247 > 97
mBzP	68.4 (9.29)	255 > 177	255 > 183
mEHP	41.9 (2.28)	277 > 134	277 > 77
mOP	77.2 (5.92)	277 > 127	277 > 77
mEOHP	98.9 (18.7)	291 > 143	291 > 121
mEHHP	83.1 (15.5)[b)]	293 > 145	293 > 121
mNP	87.2 (14.3)	291 > 141	291 > 77
mDP	71.4 (14.7)	305 > 77	305 > 261
PA	5.39 (30.8)	165 > 77	165 > 121

[a)] N=3, [b)] N=5

Calibration curves

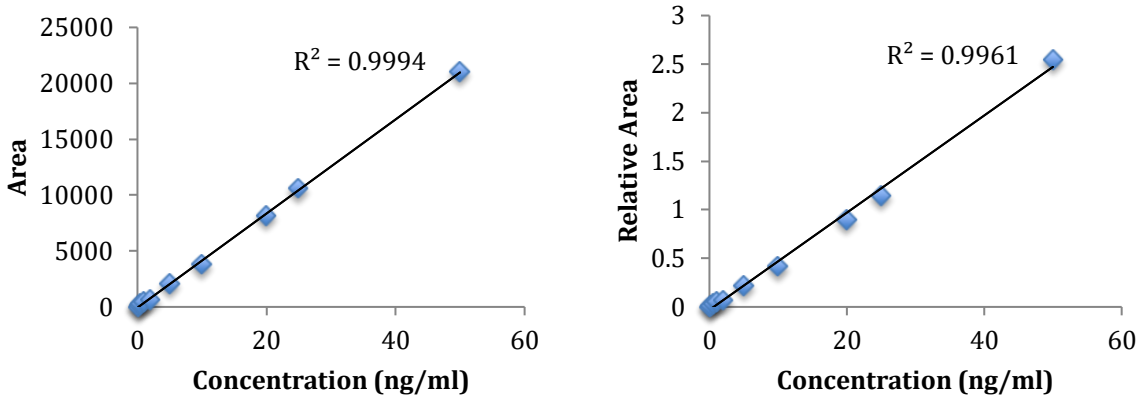

Figure D.1: Calibration curves for mMP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mEP-d_4.

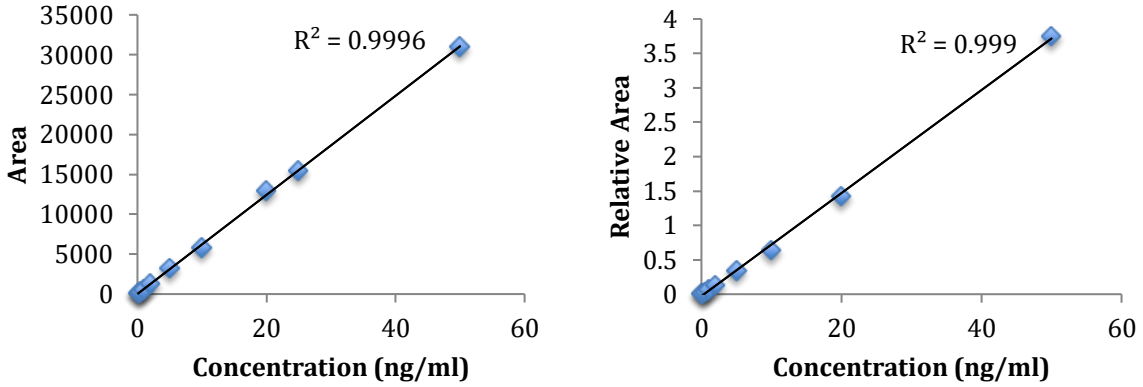

Figure D.2: Calibration curves for mEP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mEP-d_4.

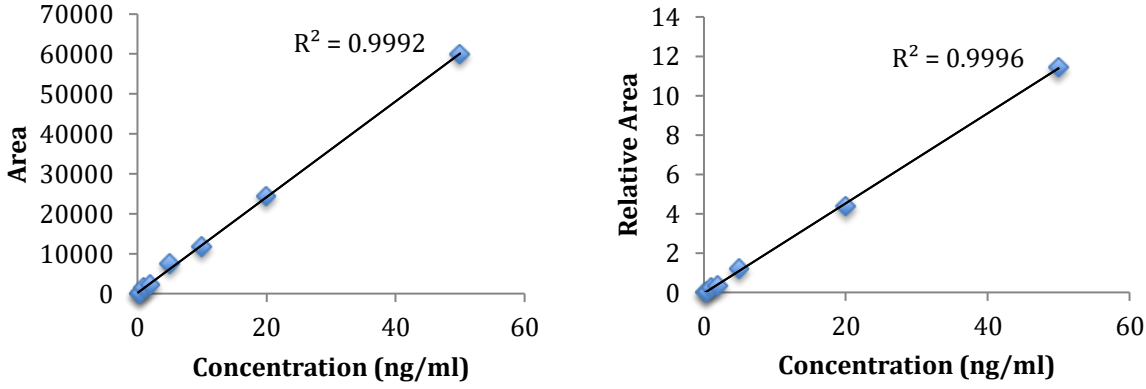

Figure D.3: Calibration curves for mBP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

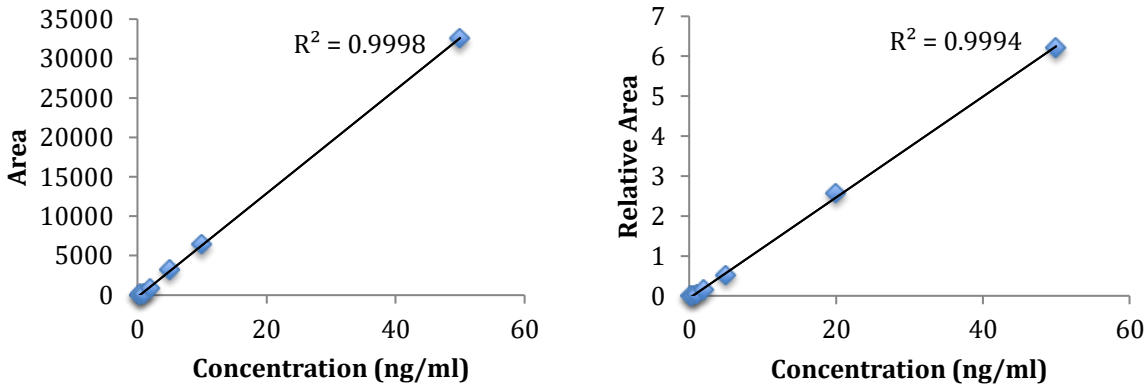

Figure D.4: Calibration curves for mIBP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

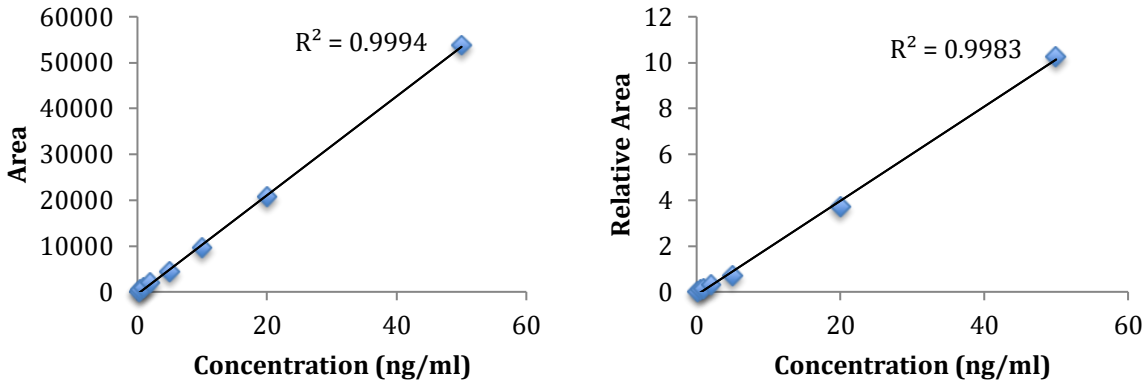

Figure D.5: Calibration curves for mPeP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

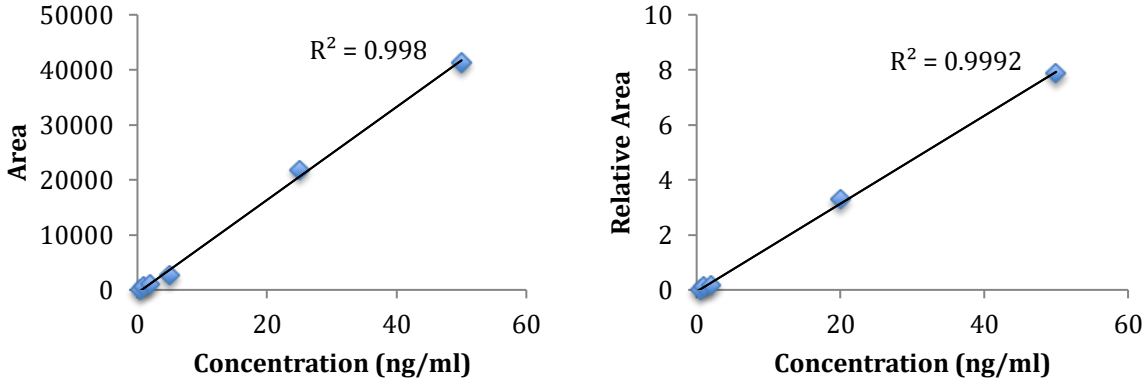

Figure D.6: Calibration curves for mIPeP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

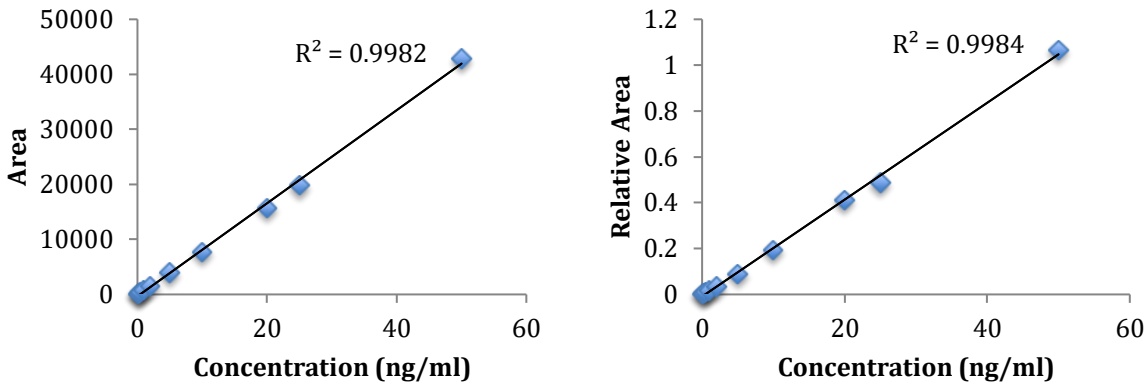

Figure D.7: Calibration curves for mHxP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mNP-d_4.

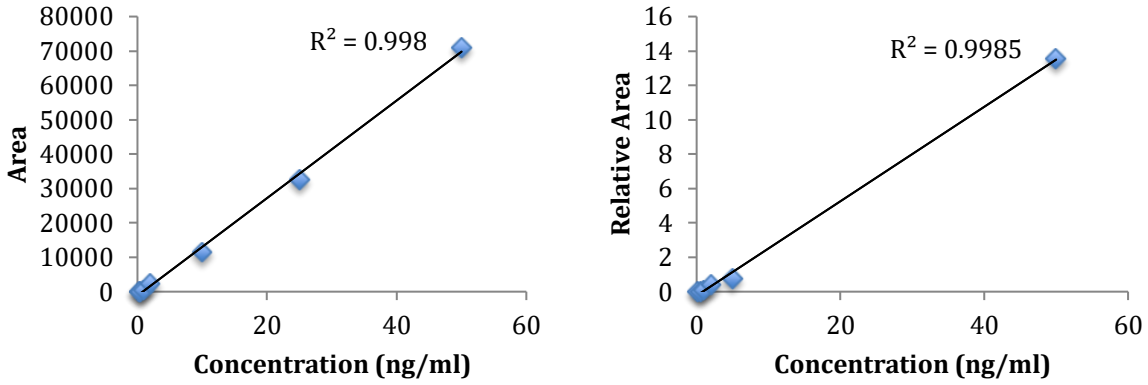

Figure D.8: Calibration curves for mCHP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

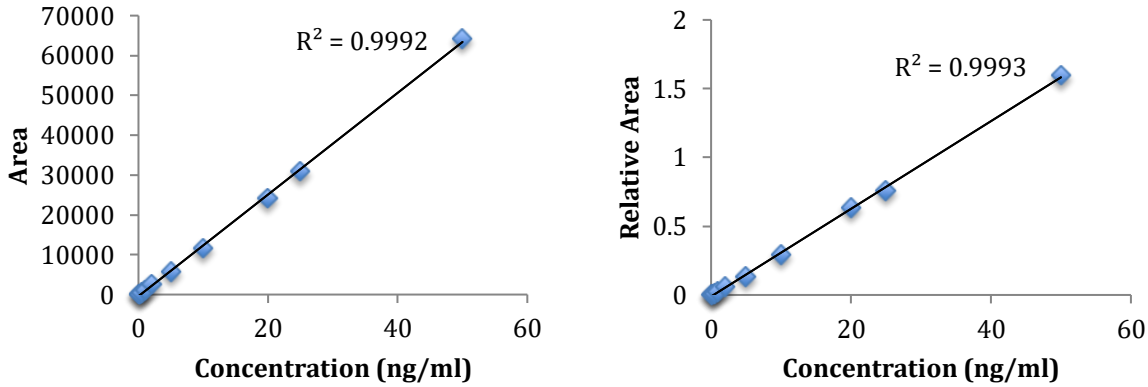

Figure D.9: Calibration curves for mHpP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mNP-d_4.

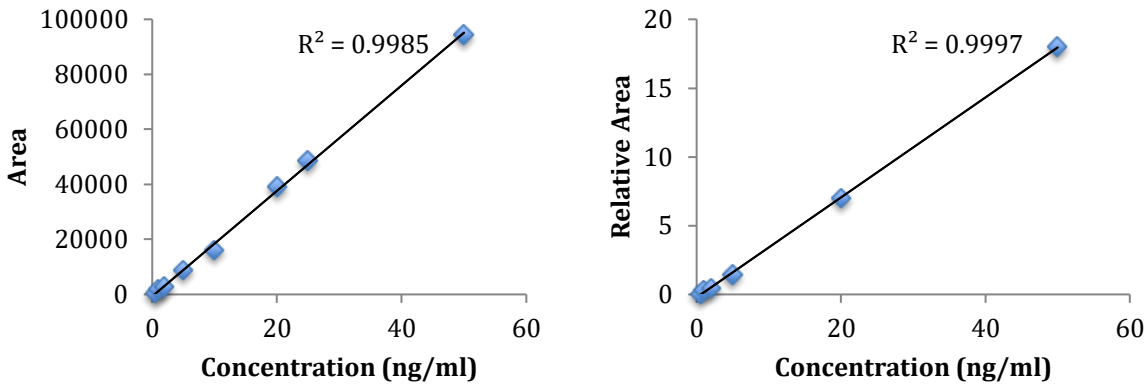

Figure D.10: Calibration curves for mBzP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

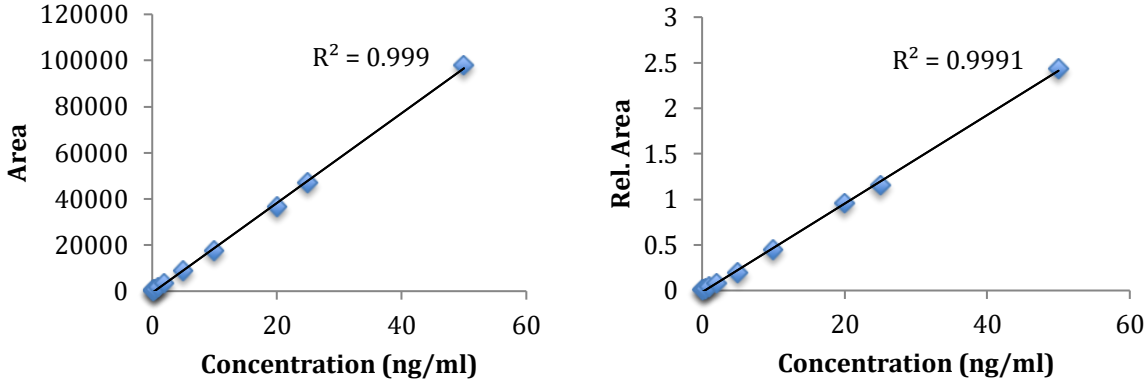

Figure D.11: Calibration curves for mOP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mNP-d_4.

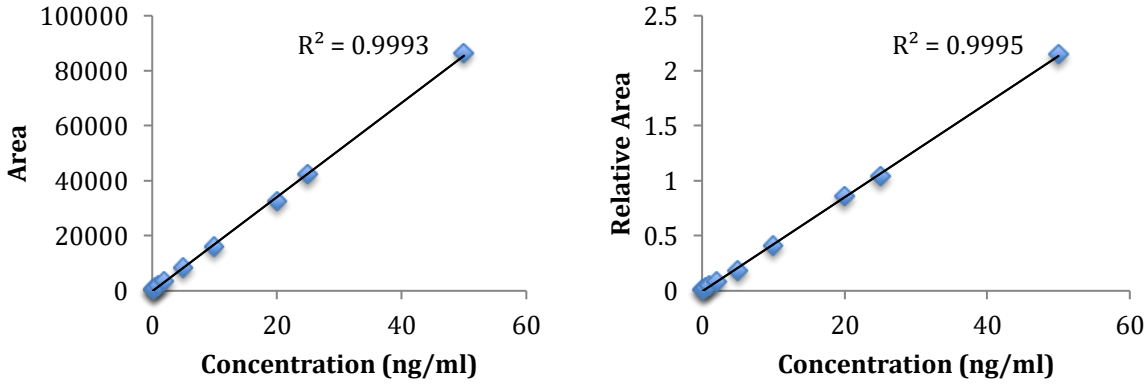

Figure D.12: Calibration curves for mEHP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mNP-d_4.

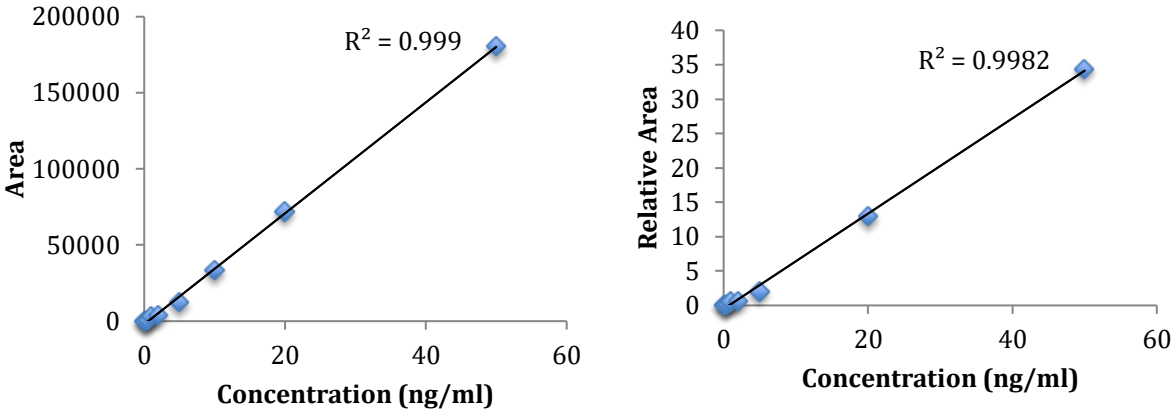

Figure D.13: Calibration curves for mEHOP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

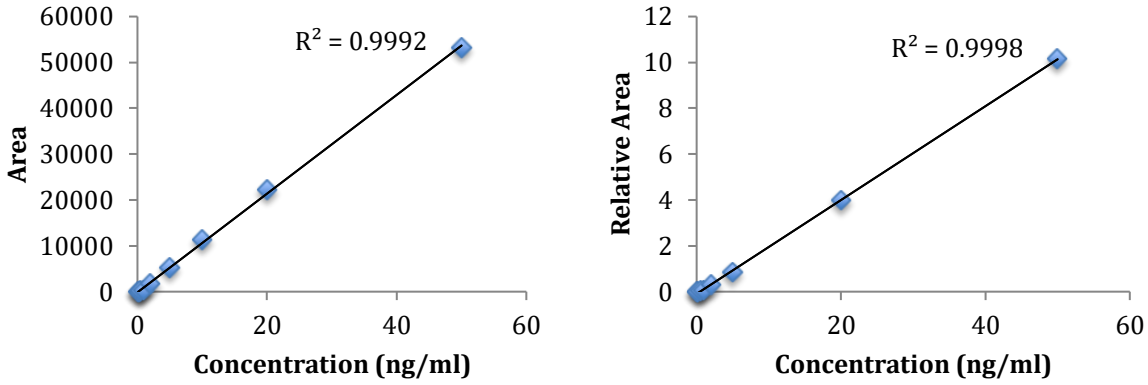

Figure D.14: Calibration curves for mEHHP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mBP-d_4.

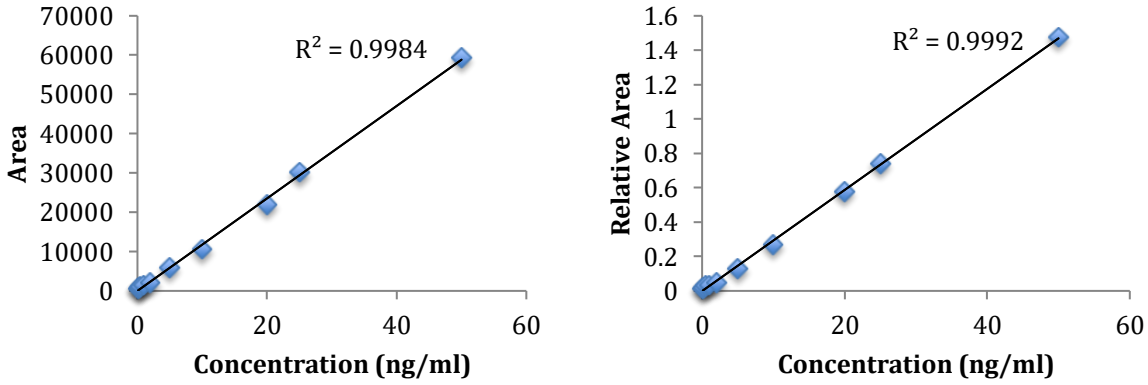

Figure D.15: Calibration curves for mNP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mNP-d_4.

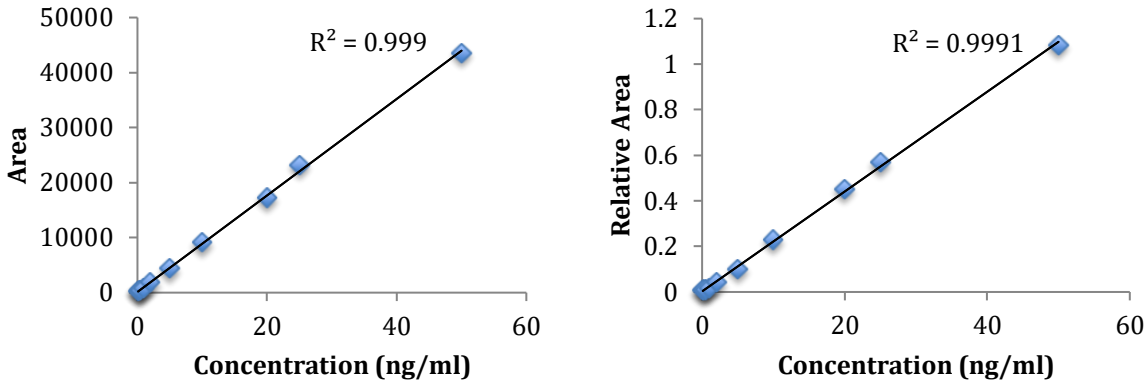

Figure D.16: Calibration curves for mDP based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mNP-d_4.

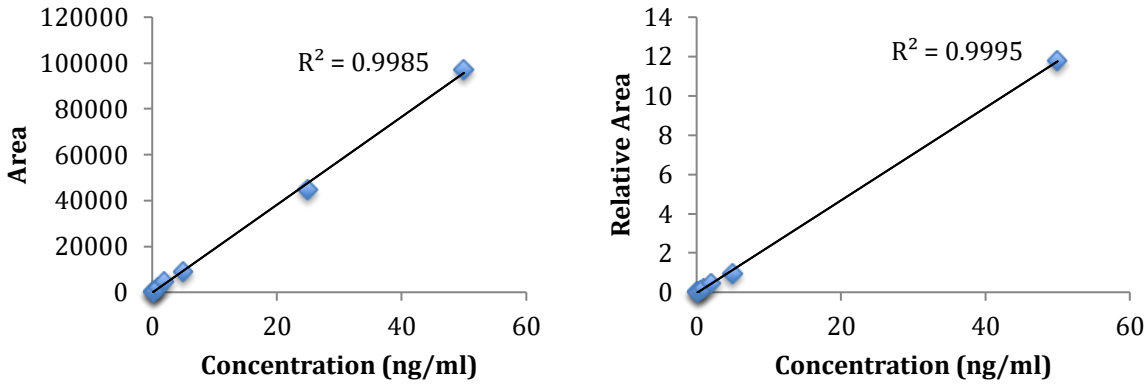

Figure D.17: Calibration curves for PA based on absolute and relative areas, respectively. Internal standard used for calculating relative area was mEP-d_4.

Appendix E

Chromatograms

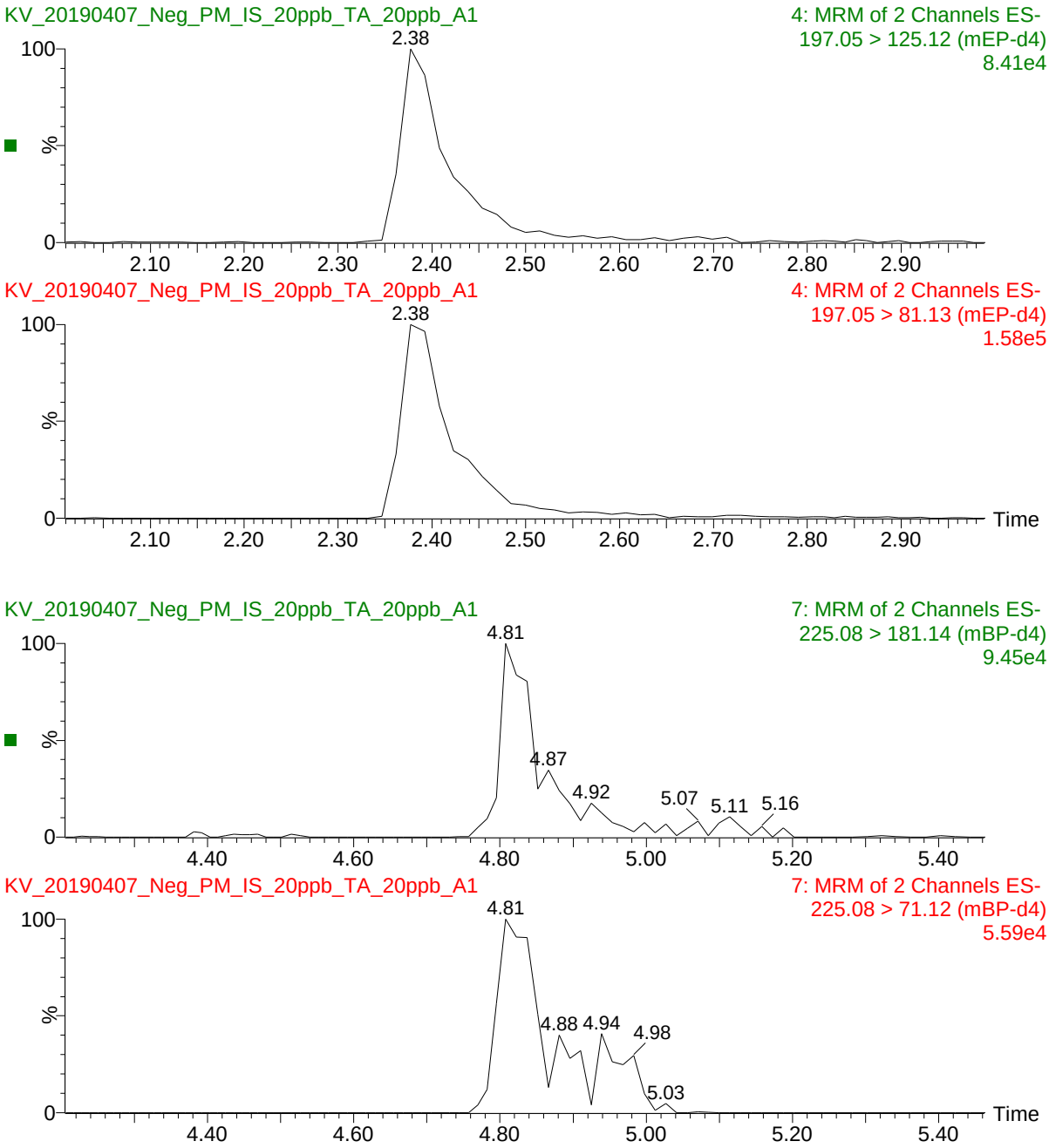

Figure E.1: MRM chromatograms for deuterated internal standards in 20 ppb calibration curve standard (primary and secondary transition). Part 1.

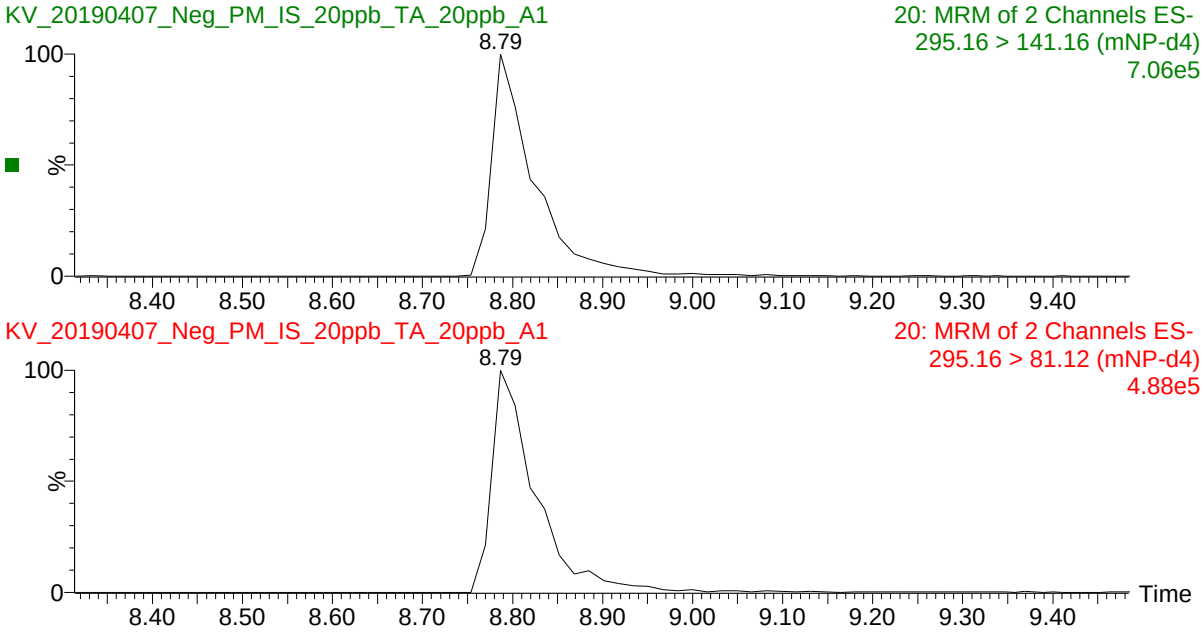

Figure E.1: MRM chromatograms for deuterated internal standards in 20 ppb calibration curve standard (primary and secondary transition). Part 2.

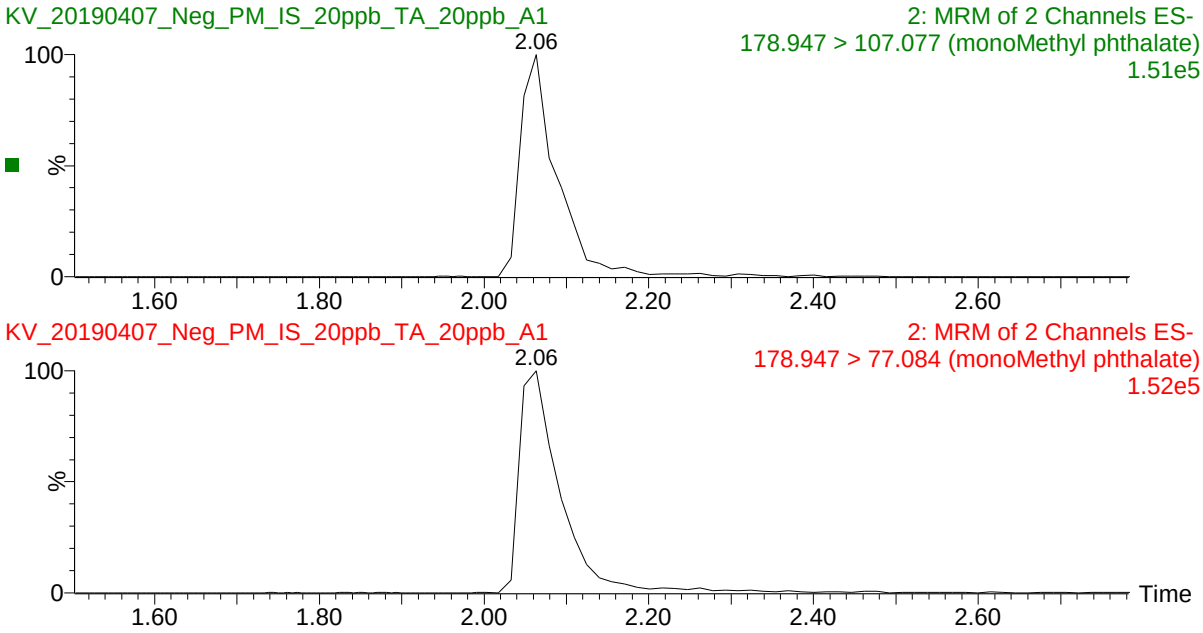

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 1.

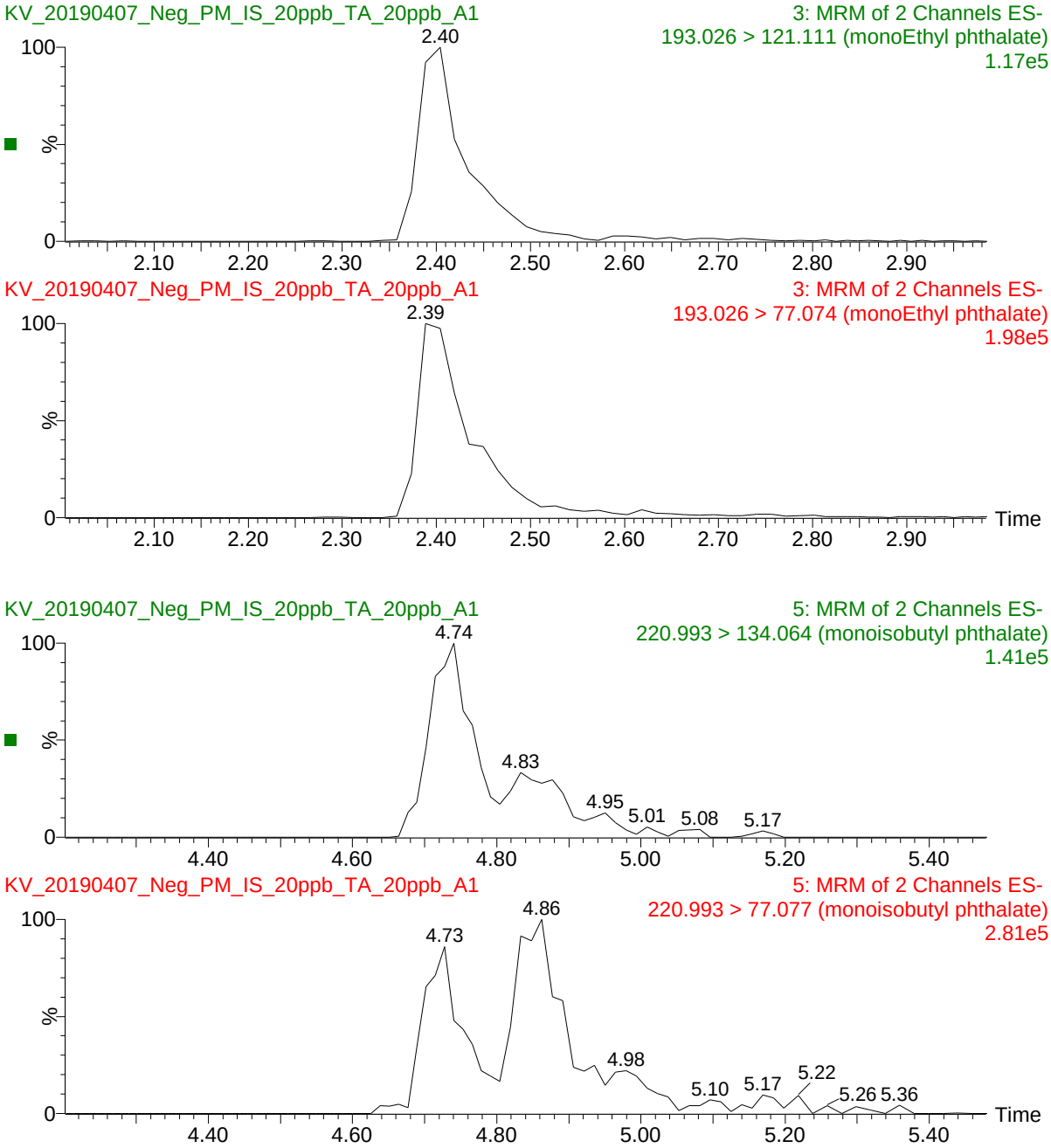

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 2.

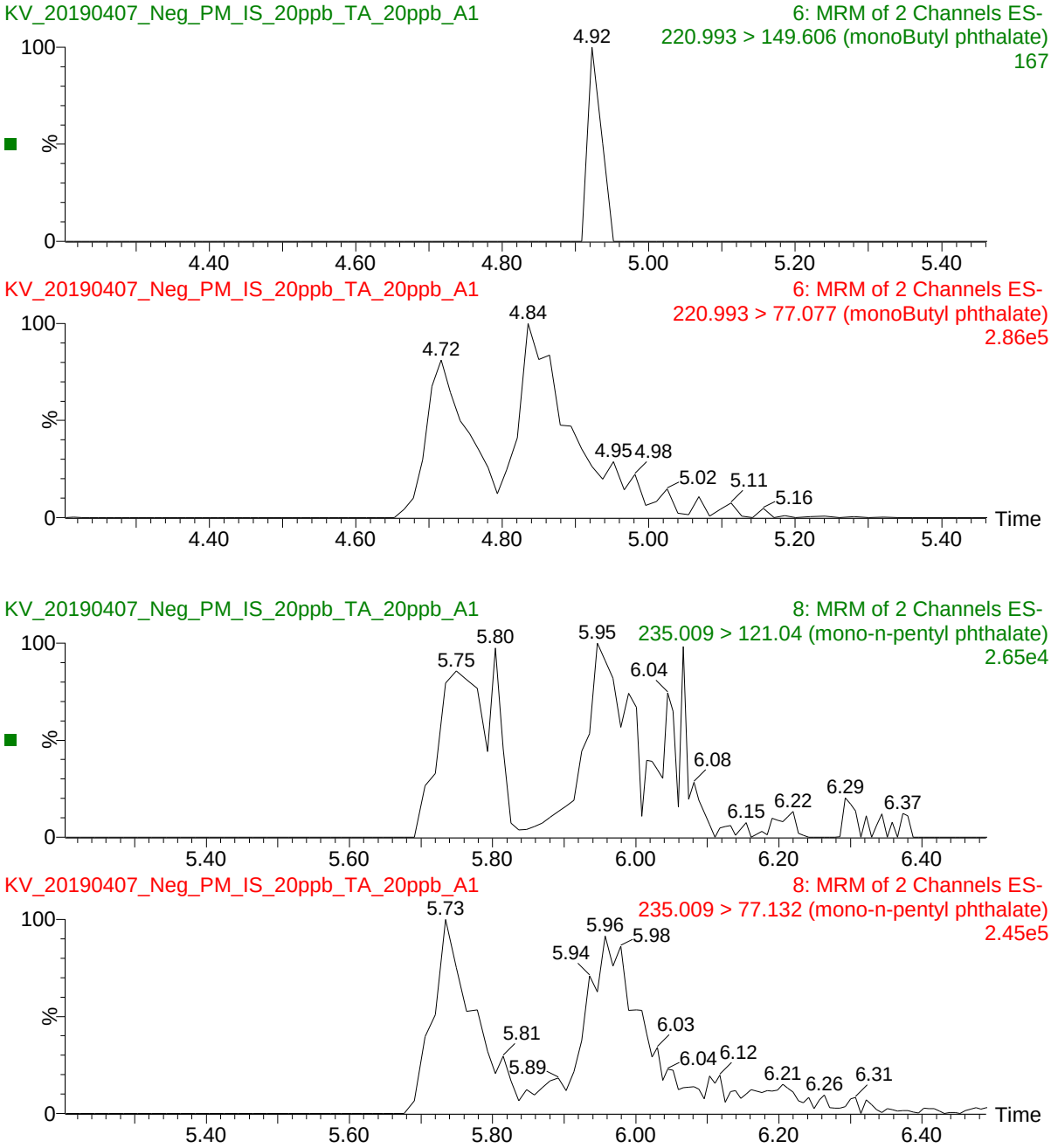

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 3.

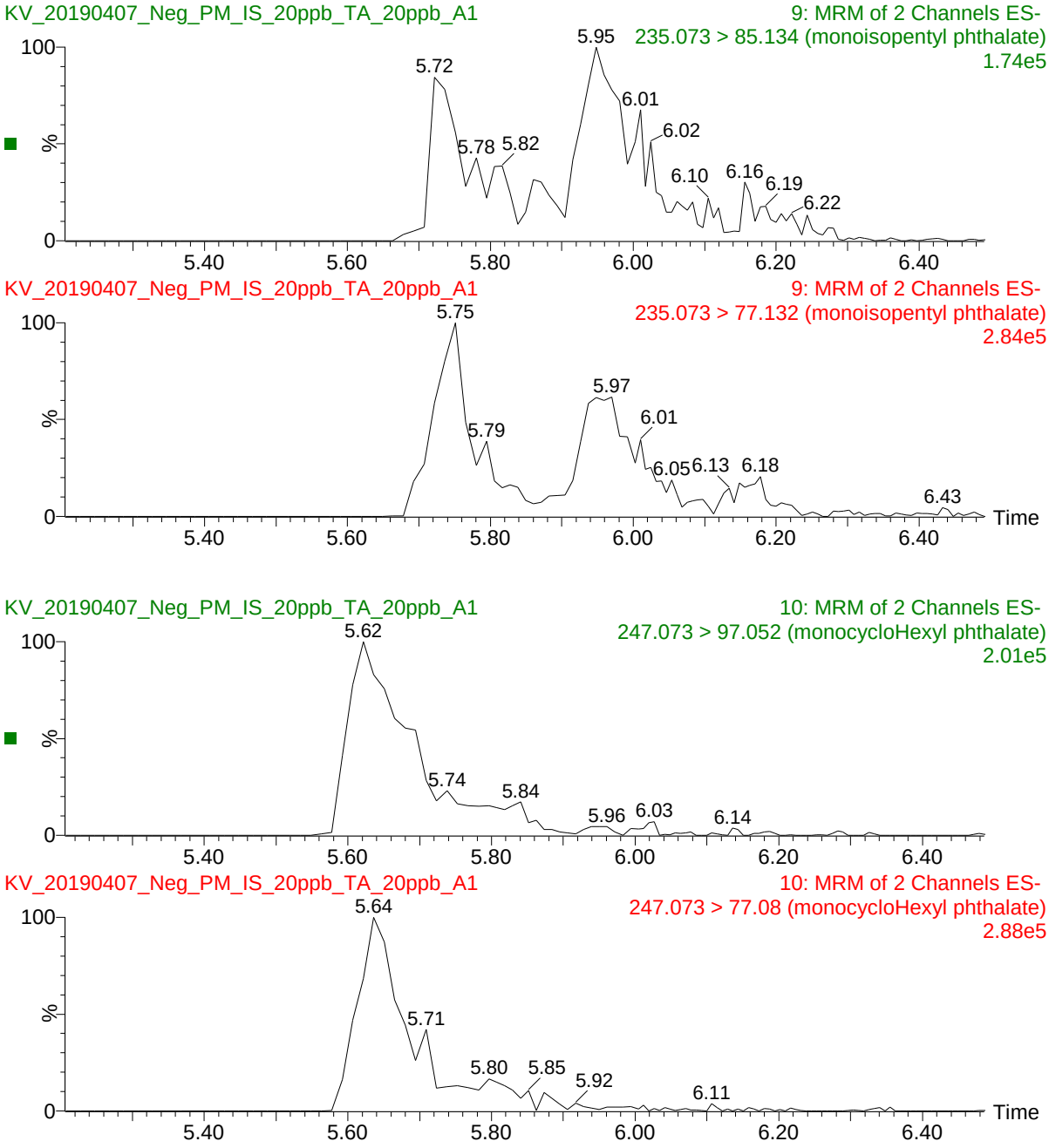

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 4.

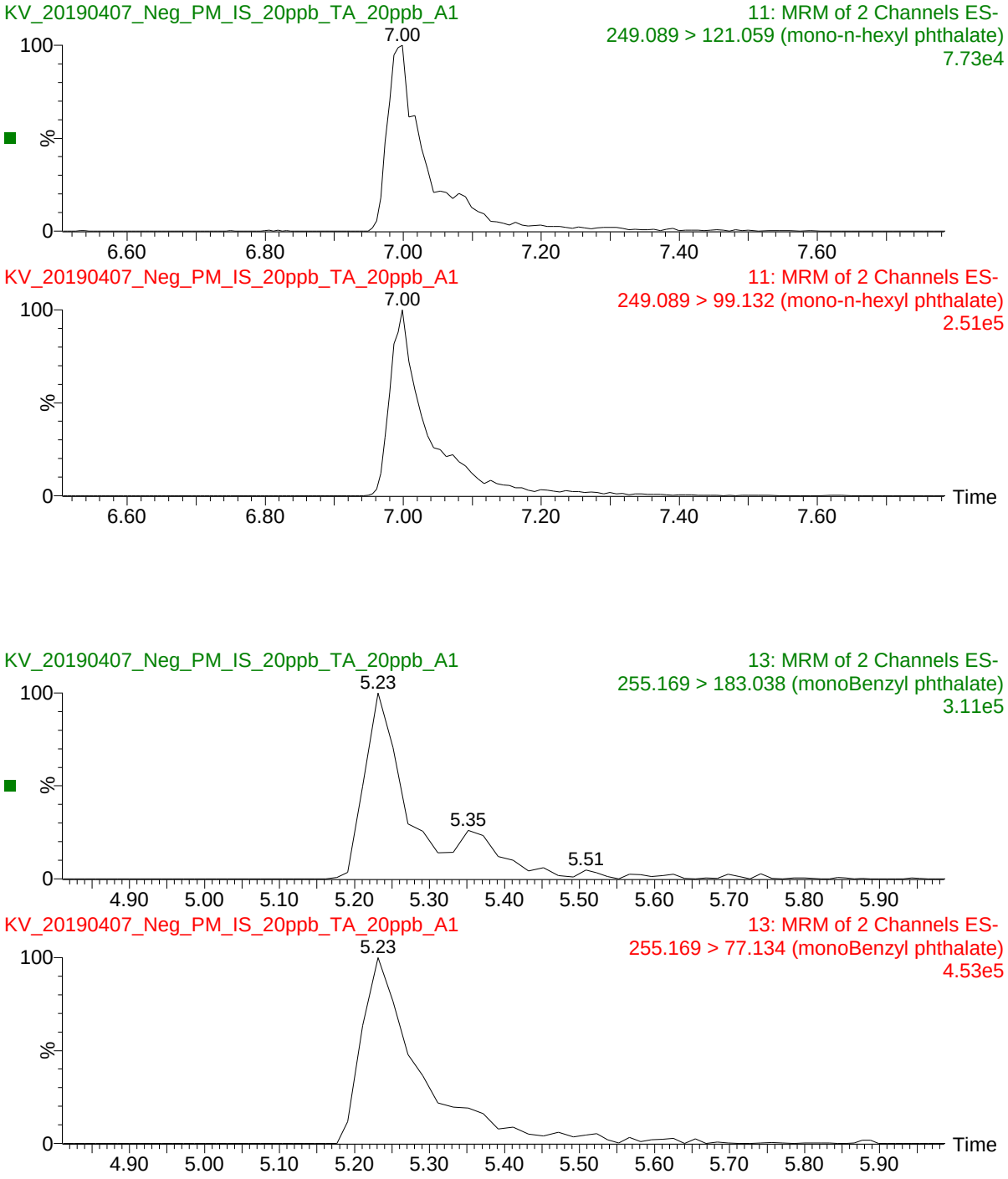

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 5.

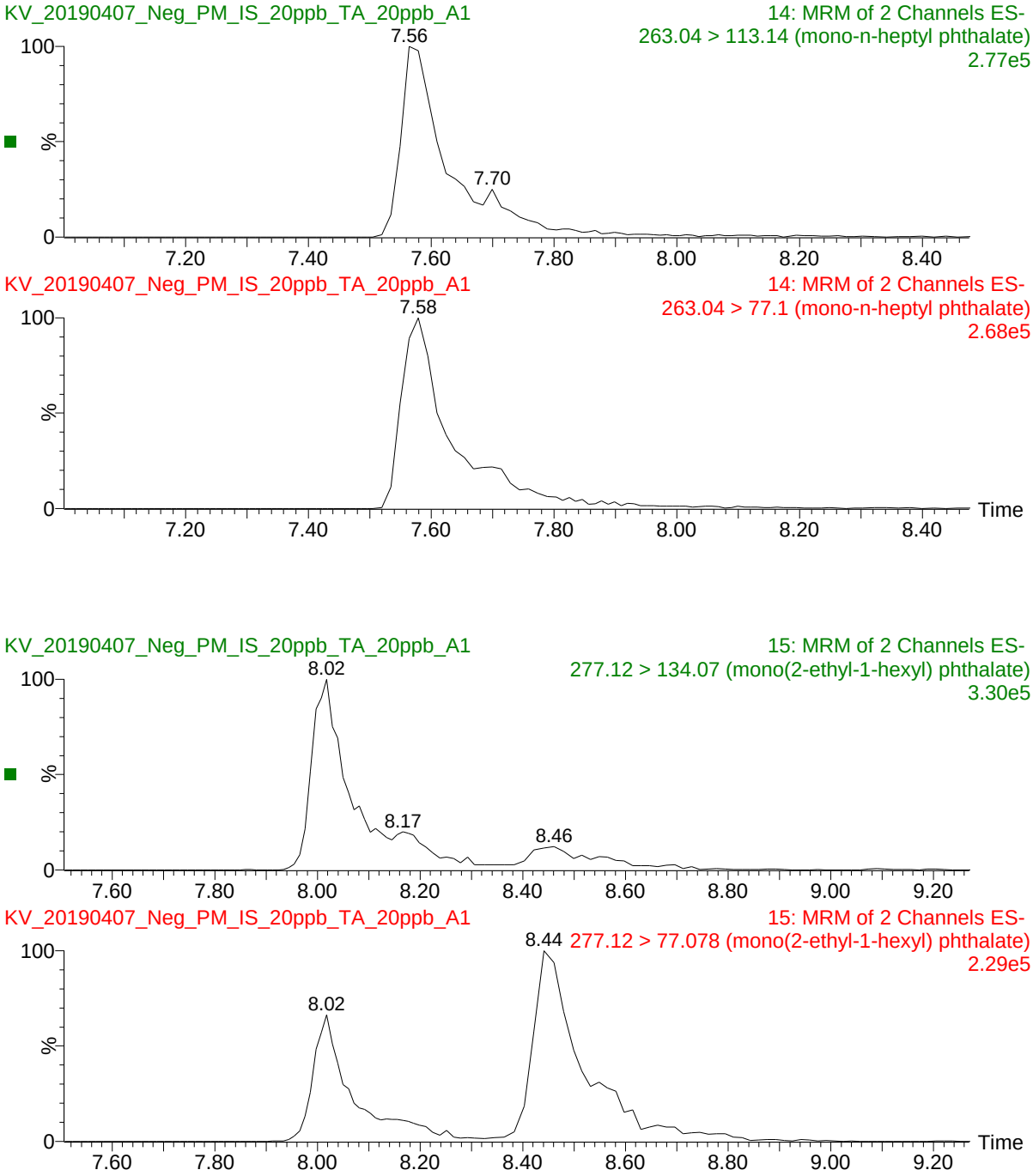

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 6.

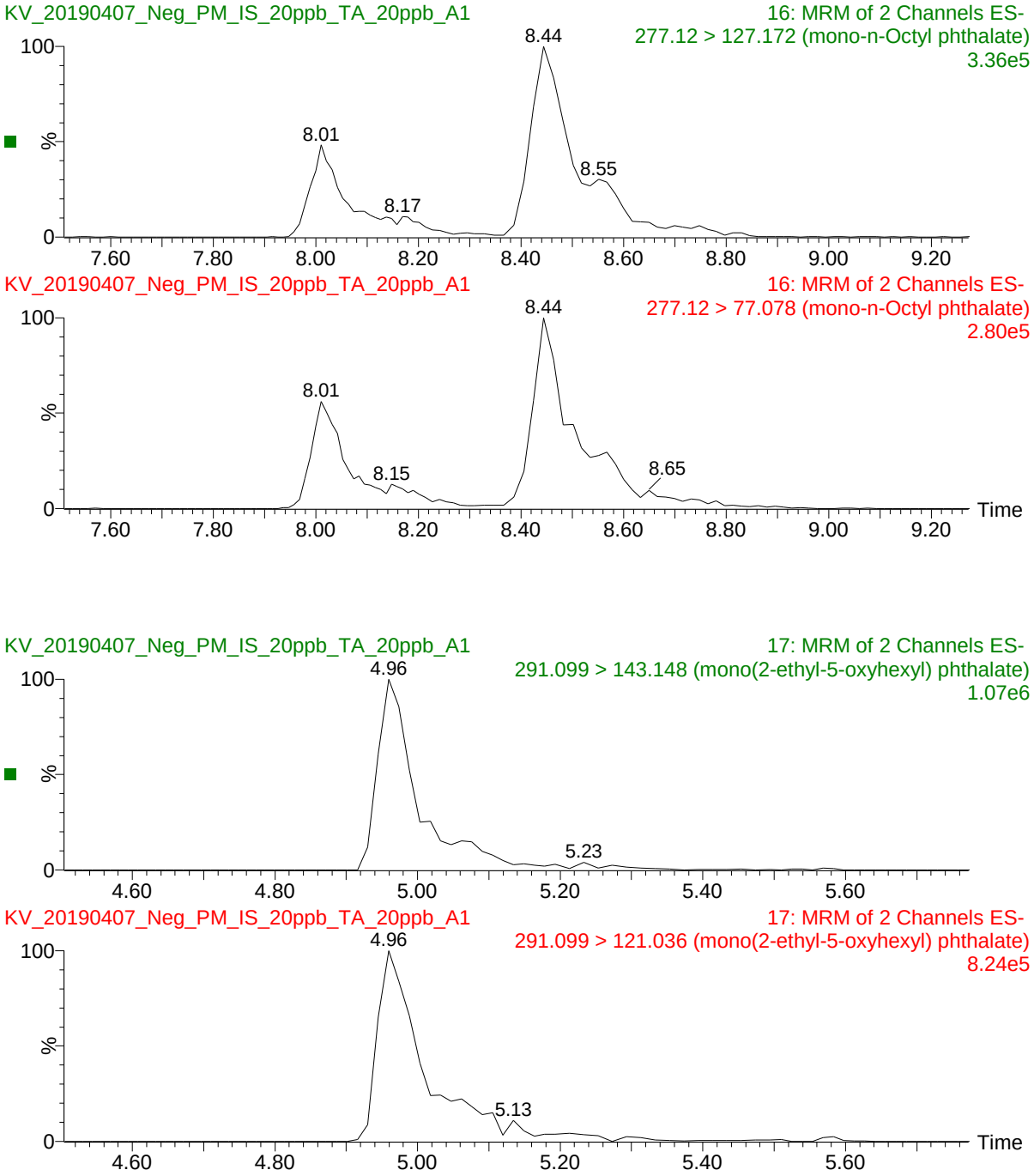

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 7.

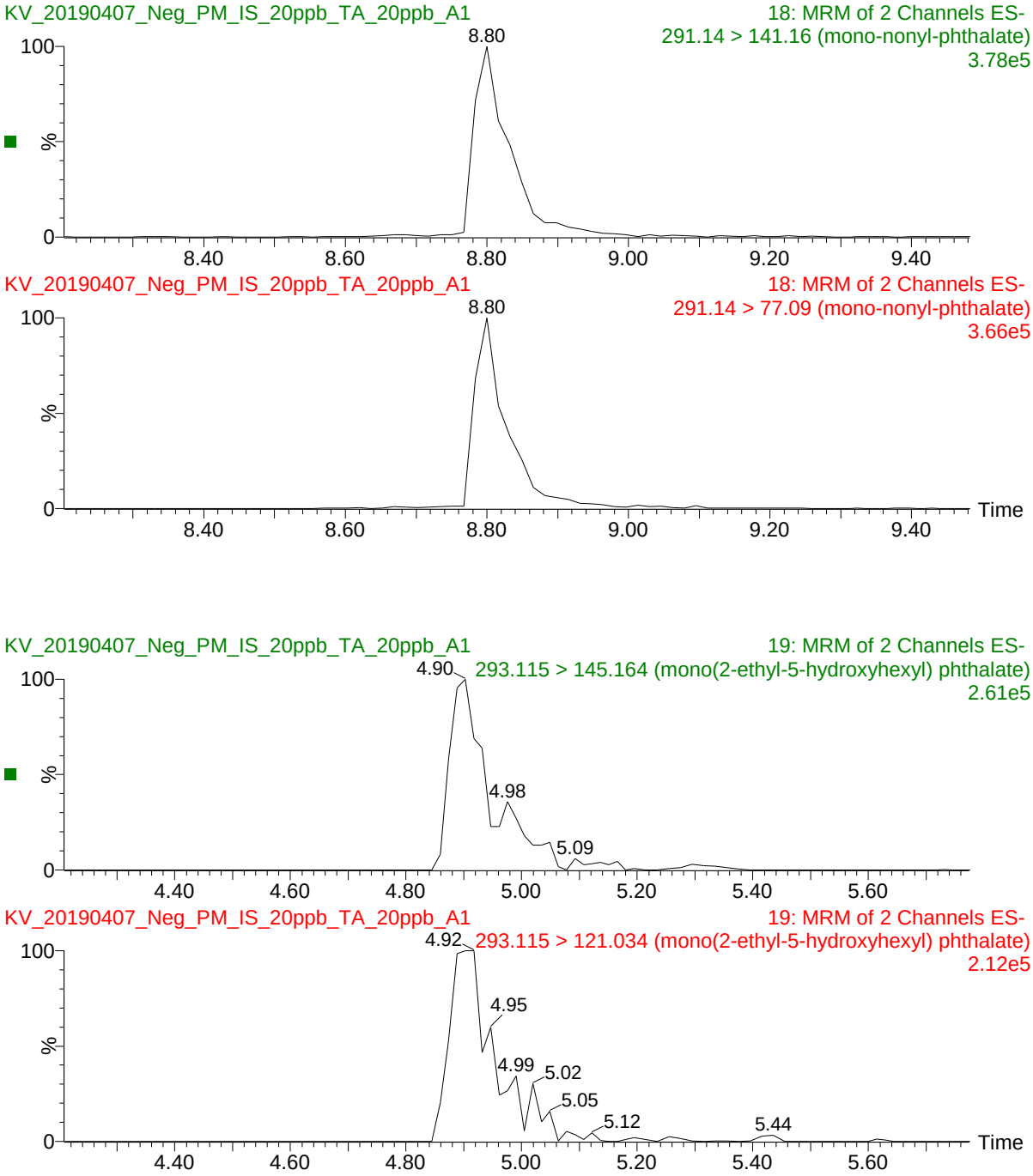

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 8.

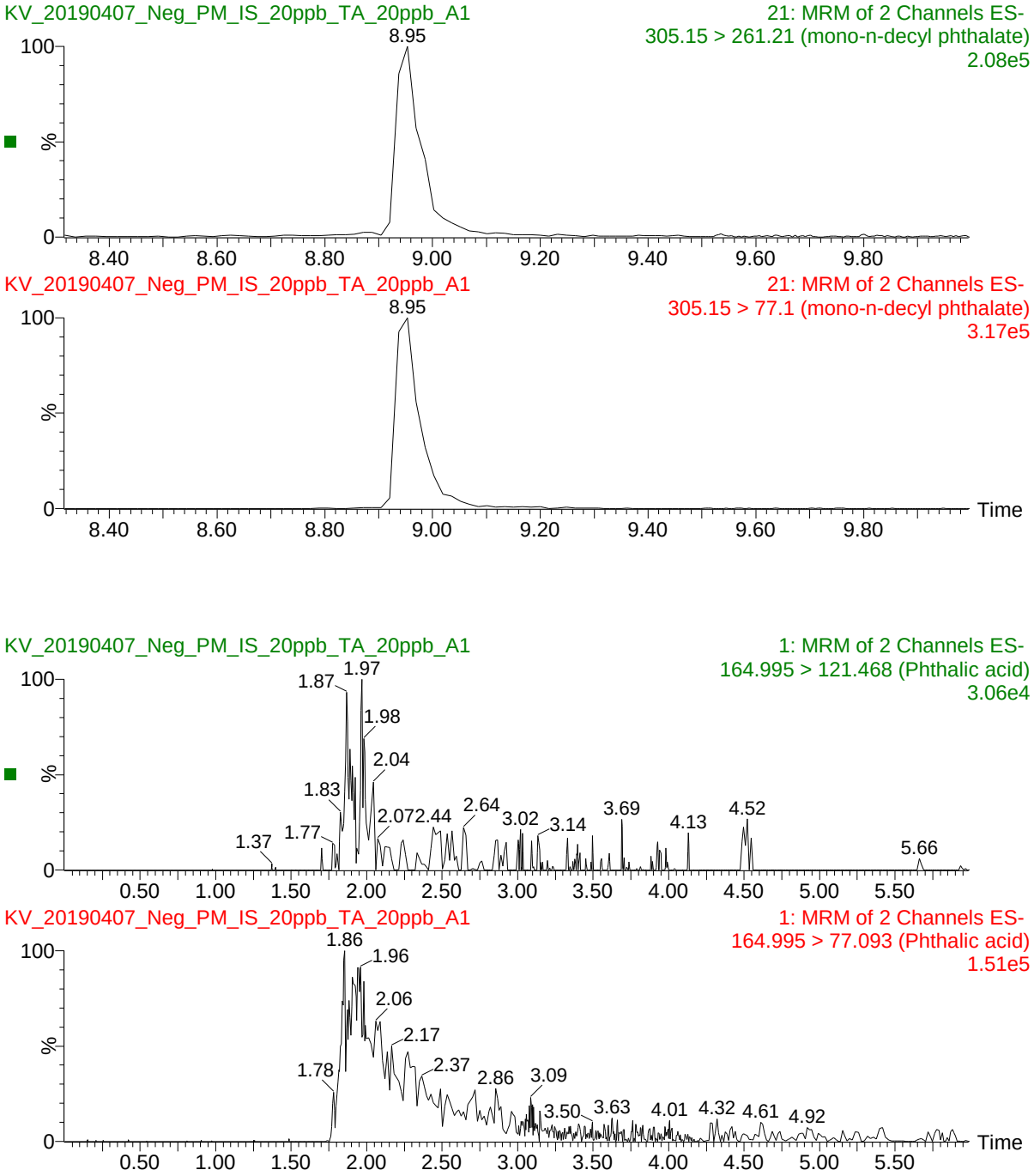

Figure E.2: MRM chromatograms for 17 phthalates metabolites in 20 ppb calibration curve standard (primary and secondary transition). Part 9.

Appendix F Results

Table F.1: Calculated concentrations (ng/g ww) of all measured phthalates metabolites in 100 liver samples from harbour porpoises. Metabolites not detected over LOD are not presented in the table (mPeP, mIPeP and mCHP). The table continues over the next pages.

ID	Year	mMP	mEP	PA*	mIBP*	mBP	mHxP*	mBzP	mEHP**	mOP*	mEOHP	mEHHP	mNP*	mDP*	mHpP*
1	2016	1,559	6,894	10,430	182,786	265,097			39,379			4,665			
2	2016		4,490	4,554	172,758	186,299			23,537						
3	2016	6,528	2,856	0,885	38,620	22,307		6,713	13,423	0,678		1,617			
4	2016		7,366	10,855	30,474	33,701			26,257	0,464					
5	2016	1,122	7,619	7,508	21,819	25,587	0,545		32,702						
6	2016	7,688	17,433	20,043	162,743	150,389			39,444						
7	2016	0,473	6,683	12,806	89,285	39,634	0,273		78,280		2,120				
8	2016	1,072	7,189		46,721	19,181	0,546		46,624	1,598					
9	2016		6,156	5,519	43,552	23,628	1,368		69,519			0,226			
10	2016		6,946	9,828	21,193	47,680	0,300	2,688	31,136				30,419		
11	2016	3,620	5,936	10,996	18,617	15,186			69,154			1,390			
13	2016		8,607	19,684	62,263	61,411		0,924	58,266		0,413				
14	2016	0,693	5,741	8,131	30,627	32,872			106,746				55,655		
17	2016	1,402	3,856	4,527	52,630	20,791		0,691	29,920				36,432		
20	2016	1,995	6,066	6,426	41,834	25,864	4,641		68,735		0,492				
21	2016		7,545	3,915	18,522	4,103	0,319		39,060						
22	2016		6,397	15,459	33,608	54,088			64,530						
23	2016		4,646	3,011	32,198	20,409			40,443						

ID	Year	mMP	mEP	PA*	mIBP*	mBP	mHxP*	mBzP	mEHP**	mOP*	mEOHP	mEHHP	mNP*	mDP*	mHpP*
24	2016	0,809	7,012	11,059	3,793	1,726	1,176		41,795						
25	2016	1,161	6,795	2,278	32,946	41,967			112,799		0,202				0,820
26	2016	0,591	5,940		31,730	22,880			42,655			1,818			
27	2016	5,830	7,583	6,992	36,951	25,165	0,963		68,605			0,638			
29	2016	0,844	4,206	0,214	7,907	6,085			26,477						
31	2016		3,824		50,615	62,503	0,153		25,669	0,216			29,190		
32	2016	2,113	8,911	3,042	66,015	24,156	1,323		85,554			0,530			
33	2016		4,625	2,116	28,900	17,430			25,369		0,228				
36	2016	3,355	4,672		63,720	63,947			32,774	1,374					
37	2016	2,452	6,672	1,078	14,612	11,730	3,114		36,038	0,550					
39	2016	2,205	4,239	1,691	16,005	5,513	2,004		38,698						
42	2016	2,041	7,418		20,945	16,154	0,865		43,915						
45	2016		8,600		67,867	52,711	0,444		45,534						
47	2016		7,464	12,156	51,545	30,884			21,719						
48	2016		4,485		21,862	10,012	1,544		40,171		0,329	0,167			
49	2016		4,682	0,249	6,547	13,899			24,700			0,365			
51	2016	1,131	5,344	0,577	10,960	30,443			23,851		0,792		32,952		
53	2016	1,230	6,395	12,648	15,557	12,924			114,301			0,174			
54	2016	3,647	13,337	41,967	8,357		0,996	1,117	65,740		0,486	2,107			0,215
55	2016	4,944	7,375	22,015	39,204	39,085			108,006						0,871
57	2016	4,193	12,301	8,871	30,564	12,458	0,345		40,931	0,192	0,263			12,406	
58	2016	0,413	7,040	20,407	13,580	4,866	0,414		42,248		0,331		41,136		

ID	Year	mMP	mEP	PA*	mIBP*	mBP	mHxP*	mBzP	mEHP**	mOP*	mEOHP	mEHHP	mNP*	mDP*	mHpP*
59	2016		6,431	10,554	32,106	42,563			21,138						
60	2016		4,162	15,988	32,128	40,987			15,258						
61	2016	3,145	6,484	11,201	419,148	299,331			37,334				58,701		
62	2016	0,584	7,644	4,834	91,368	215,379			33,083						0,259
63	2016	1,089	5,276	19,099	55,890	37,043	0,282		49,165			5,928			
64	2016	1,556	7,380	13,075	33,673	27,406	0,557		105,770		0,311	1,252	27,955		
65	2016		7,151	12,770	4,346	22,782			15,923						
66	2016	0,374	4,572	4,112	8,852	8,972	1,433		330,837						
67	2016	2,889	7,016	1,333	25,360	33,663	1,462		19,290						
68	2016	1,717	2,620	3,670	12,304	6,180			23,440						
69	2016	2,211	9,663	22,144	12,054	6,806			19,283				98,862		
70	2016		6,046	8,897	24,024	14,803	0,941		139,398						0,704
71	2016		5,069	21,344	12,428	23,367	1,925	4,460	73,968	0,586		1,481	61,786		
72	2016		3,937		59,646	62,647			14,914						
73	2016		5,504	16,010	27,128	12,449	0,304		54,761			0,302			
1	2017	1,345	4,233	8,724	29,639	43,605			22,674						
2	2017		6,346	11,755	20,042	22,585			18,058						
3	2017	0,336	4,417	15,780	49,941	55,866	2,676		45,979	0,632		0,260			1,874
4	2017		4,537	3,293	25,622	16,968			22,355	0,374					
5	2017	1,008	4,115	2,866	43,523	52,764			6,852	0,483			23,981	0,253	
7	2017	3,600	8,144	7,941	15,211	29,210	1,831		86,864			0,190			
10	2017	3,791	7,662	6,076	62,500	51,983			17,503						

ID	Year	mMP	mEP	PA*	mIBP*	mBP	mHxP*	mBzP	mEHP**	mOP*	mEOHP	mEHHP	mNP*	mDP*	mHpP*
15	2017	1,745	4,938	1,476	30,294	29,823	0,519		46,921	0,280		1,164	43,249		
16	2017	6,370	5,753	11,299	15,776	3,238	0,733		62,017	1,386		1,850	48,169		
20	2017	8,722	5,620	20,893	14,278				12,961						0,452
21	2017	2,944	4,240	7,871	15,170	11,278			17,693						
23	2017	2,226	4,334	2,412	11,511	11,963			17,740	0,799		0,444			
24	2017	1,983	8,733	1,904	58,393	47,272			39,724						
26	2017	1,378	4,950		56,394	47,882	1,590		76,528		0,241				
29	2017	1,707	3,641	6,622	29,901	36,142	1,179		116,425	4,189		2,196	37,608		
30	2017		4,819	7,313	27,760	47,890	0,205		42,185			4,121			
31	2017	1,400	3,989	7,874	3,783	14,800	1,060		70,590						
32	2017		5,165	16,219	55,947	51,865	0,631	3,745	110,367			0,283	48,118	17,580	0,428
33	2017	0,750	3,379	3,413	10,974	32,149			42,991		0,275				
34	2017	2,092	6,442	10,920	37,036	49,455			52,859	2,362					
35	2017		4,295	7,689	22,014	5,702	0,733		34,507			0,986			0,442
36	2017	2,037	6,138	8,048	22,838	7,101	0,284		47,627			1,022			0,214
37	2017		4,475	4,221	46,668	27,982			20,439						
38	2017	0,598	4,816	2,814	65,223	58,764	0,449	1,089	57,139			0,354			
39	2017	0,809	5,209		13,671	8,777			32,570				38,703		
41	2017	3,498	7,989	24,708	34,797	13,777			68,937		0,363				
42	2017	1,205	5,317	2,385	49,308	41,973			58,415				51,505		
43	2017	1,609	3,870	3,965					18,001						
44	2017		5,239	2,423	31,407	21,624	0,221		26,457				53,227		

ID	Year	mMP	mEP	PA*	mIBP*	mBP	mHxP*	mBzP	mEHP**	mOP*	mEOHP	mEHHP	mNP*	mDP*	mHpP*
45	2017	2,119	5,064		44,480	47,823	0,507	7,290	37,311				73,245		
46	2017	3,027	4,066	16,336	48,745	16,207	0,332		30,904				48,331		
47	2017		3,845	4,299	19,617	25,362			46,354				49,307		
48	2017	3,722	4,788	2,921	17,314	12,824	0,353	1,472	53,151						
50	2017	1,973	3,988	11,279	44,603	5,821			117,468				56,545		
51	2017	1,713	6,252	11,968	46,769	57,653	0,374		45,569						
52	2017		7,536	0,761	46,098	4,270	0,312		34,763		1,494	2,113			
53	2017	2,155	6,606	7,749	37,391	24,788			51,874		1,486				
54	2017	1,332	4,424		56,094	40,891			22,345	0,318					
55	2017		4,717	5,981	29,105	9,353			66,586						
56	2017	0,501	5,643	7,065	4,305	6,988			33,518	0,789	0,495				
57	2017	4,252	7,935	1,559	67,824	27,602	2,055		59,486	5,534					1,292
58	2017	1,674	5,690	36,125	17,746	13,473			23,488	0,513			61,948		
59	2017	1,277	5,829		48,471	16,821			27,026						
60	2017	1,827	5,002		24,724	33,485			16,451	1,712					
61	2017	1,387	4,078		22,070	8,857			7,041						

*: Semi-quantified.

**: High background

Appendix G Principal components analysis (PCA) biplots

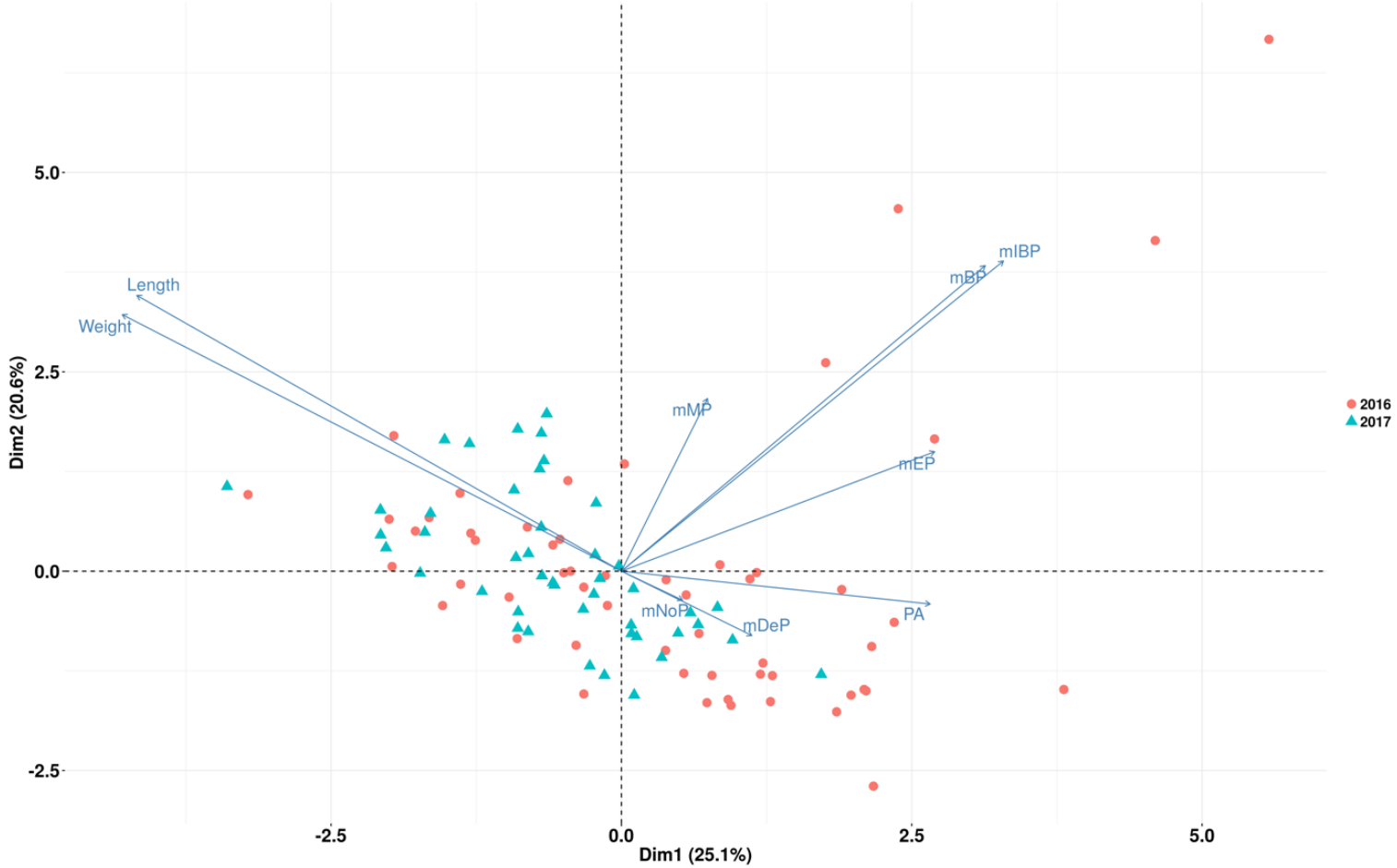

Figure G.1: PCA biplot of harbour porpoise weight and length, and selected analytes (mMP, mBP, mIBP, mEP, PA, mDP (mDeP) and mNP (mNoP)). Grouped in sampling years (2016 and 2017).

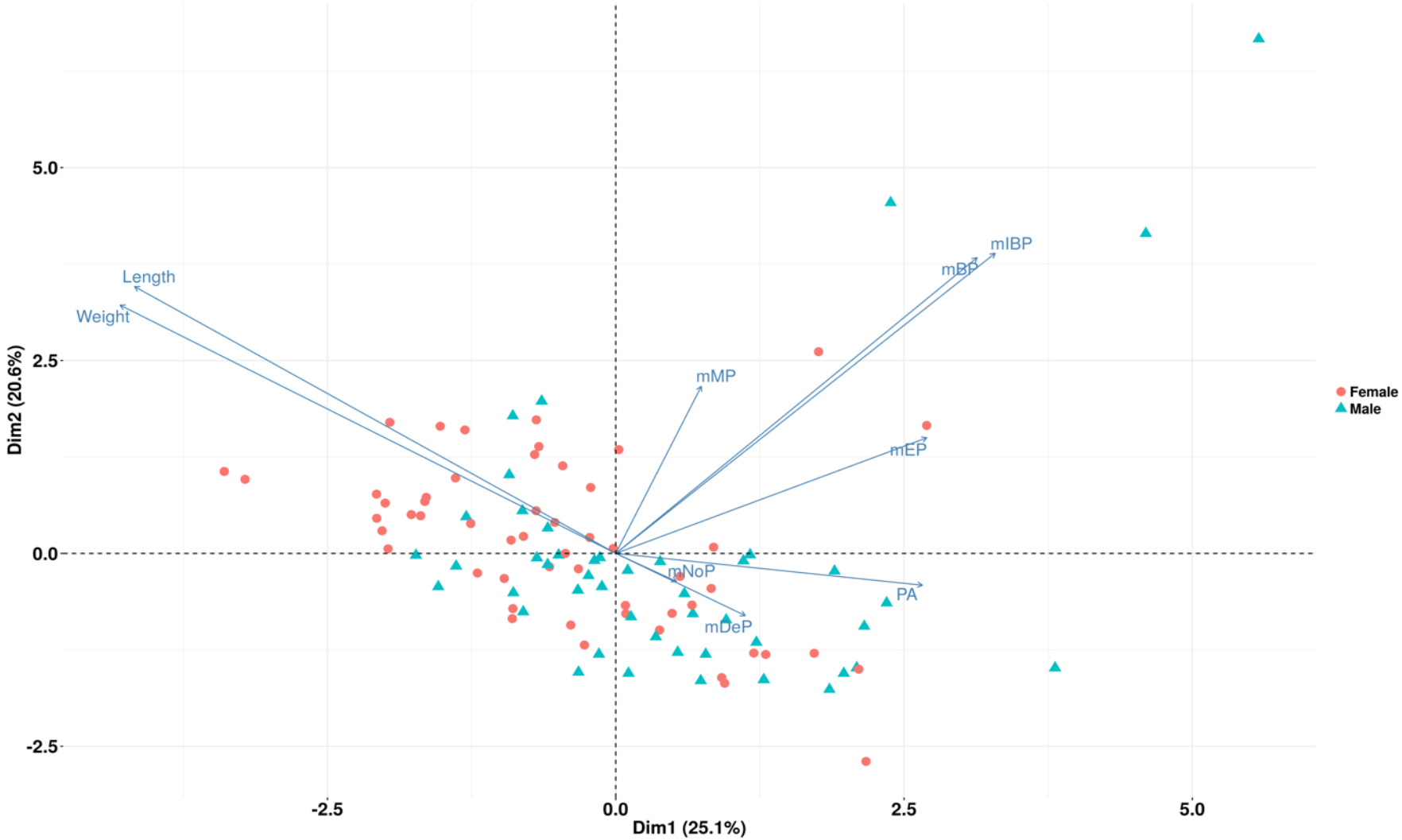

Figure G.2: PCA biplot of harbour porpoise weight and length, and selected analytes (mMP, mBP, mIBP, mEP, PA, mDP (mDeP) and mNP (mNoP)). Grouped out from gender.

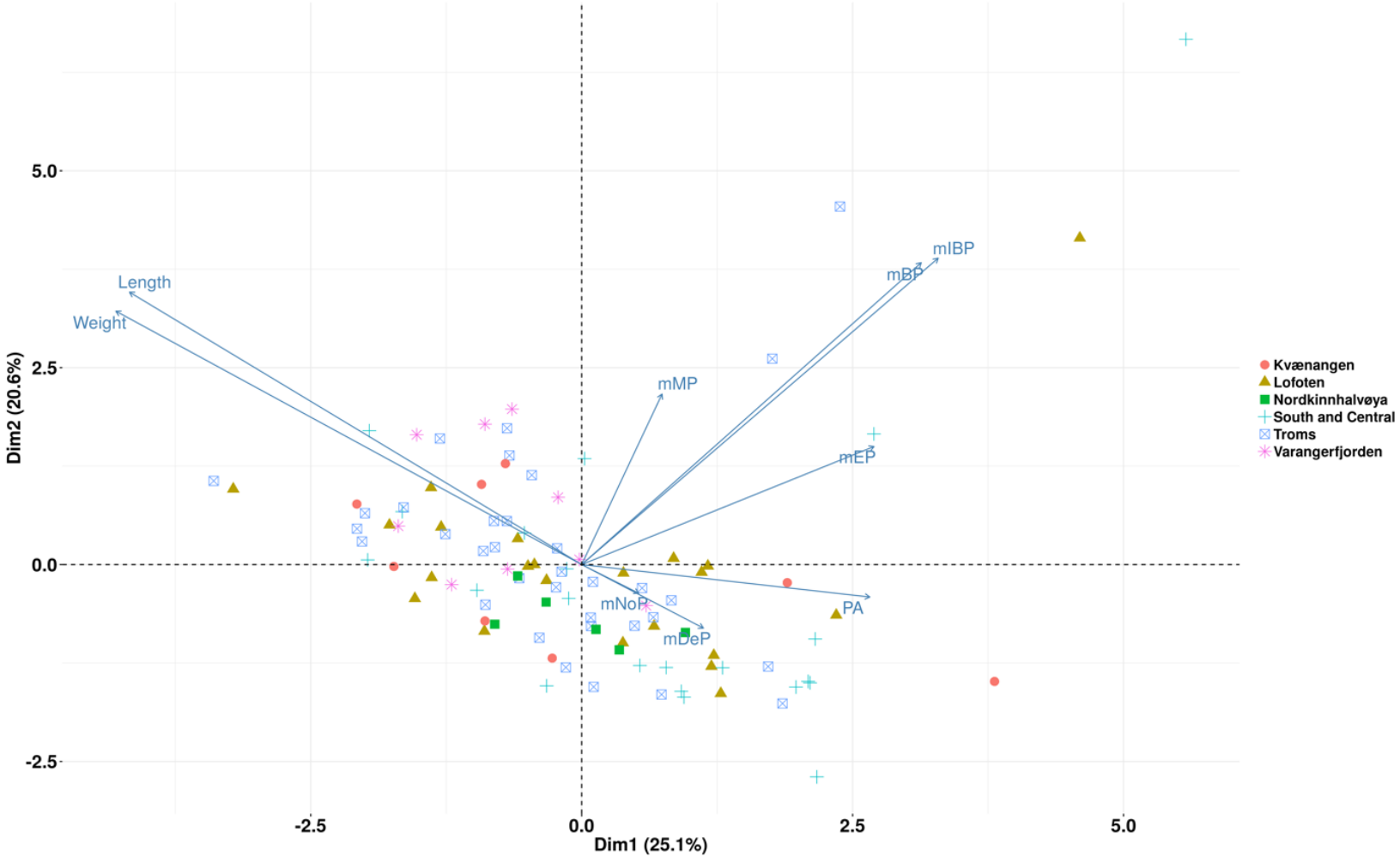

Figure G.3: PCA biplot of harbour porpoise weight and length, and selected phthalates metabolites (mMP, mBP, mIBP, mEP, PA, mDP (mDeP) and mNP (mNoP)). Grouped in different sampling regions.

Appendix H

Correlations

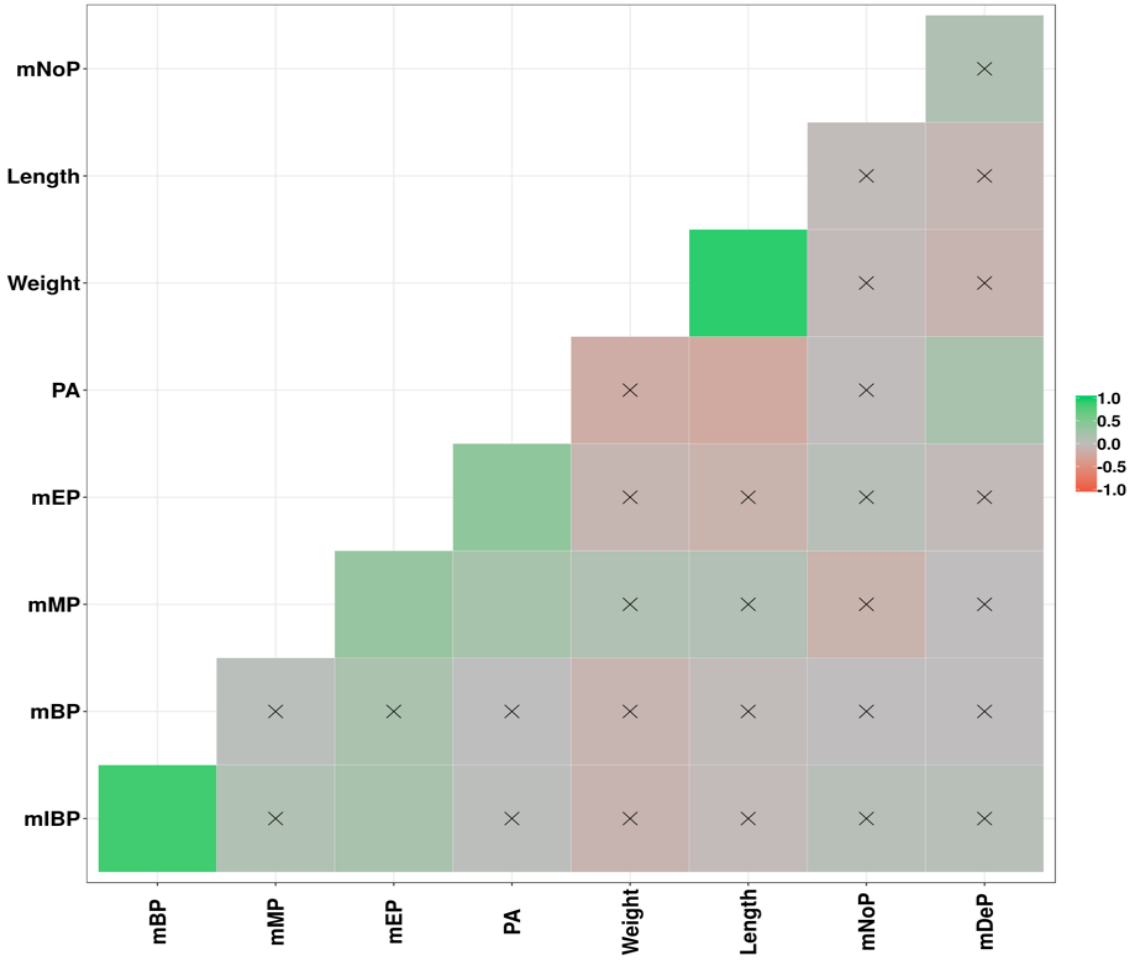

Figure H.1: Correlations plot of selected phthalates metabolites (mMP, mBP, mIBP, mEP, PA, mDP (mDeP) and mNP (mNoP)). Colour codes: green = positively correlated, red = negatively correlated.

Table H.1: Pearson correlations for phthalates metabolites measured in samples > LOD and harbour porpoise weight and length.

	Weight	Length	mMP	mEP	PA	mIBP	mBP	mHxP	mBzP	mEHP	mOP	mEOHP	mEHHP	mNP	mDP	mHpP
Weight	1	.943**	.038	-.087	-.290**	-.090	-.096	.022	.050	-.033	.269	.250	-.141	-.141	.519	.230
Length		1	.048	-.126	-.326**	-.025	-.027	-.026	.071	-.009	.220	.203	-.123	-.152	.513	.216
mMP			1	.376**	.224	.121	.075	.011	.470	-.128	.149	-.222	-.086	.227	1.00**	-.247
mEP				1	0.411**	.188	.203	-.047	-.424	.055	.088	-.001	.038	.436	.340	-.315
PA					1	.023	.061	-.124	-.193	.058	-.183	-.134	.216	.498*	.959	-.247
mIBP						1	.871**	-.096	-.079	-.064	.330	.536*	.436*	.099	.279	.270
mBP							1	-.074	-.111	-.097	.060	.005	.475*	.027	-.245	-.181
mHxP								1	.188	.149	.166	-.281	-.241	.340	1.00**	.956**
mBzP									1	-.205	1.00**	1.00**	.162	.892*	[c]	1.00**
mEHP										1	.600**	-.062	-.191	-.022	.912	.057
mOP											1	1.00**	.615	-.097	-1.00**	-1.00**
mEOHP												1	.597	-.101	[c]	-1.00**
mEHHP													1	-.099	[c]	-.587
mNP														1	1.00**	[c]
mDP															1	[c]
mHpP																1

** Correlation is significant at the 0.01 level (2-tailed); * Correlation is significant at the 0.05 level (2-tailed); [c] Could not be computed since N=1.

May Britt Rian

Determination of Occurrence of 17 Phthalate Metabolites in Harbour Porpoises from the Norwegian Coast

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