Bachelor's thesis

NTNU
Norwegian University of Science and Technology
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Department of Materials Science and Engineering

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Development of Sample Preparation Techniques for Analysis of Environmental Toxins in Fish Tissue by GCxGC-HRMS

Utvikling av opparbeidningsmetoder for analyse av miljøgifter i fiskevev ved bruk av GCxGC-HRMS

Bachelor's thesis in Chemical Engineering
Supervisor: Lene Østby
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Summary of Bachelor's Thesis

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Preface

This thesis marks the completion of a bachelor's degree in chemical engineering at the Norwegian University of Science and Technology (NTNU), specifically within the Institute of Material Science (IMA). Carried out in collaboration with SINTEF Ocean during the spring semester of 2024. All laboratory work was conducted at SINTEF Ocean laboratories.

The primary objective of this thesis was to develop and evaluate sample preparation methods for fish tissue analysis, aiming to detect environmental toxins through chromatographic techniques. The work in this thesis is a part of two separate SINTEF Ocean projects, AQUAvit and ToxiGen.

We extend our appreciation to our internal supervisor at NTNU, Lene Østby, whose guidance and support were useful throughout this thesis. Additionally, we are grateful to our external supervisors at SINTEF Ocean, Lisbet Sørensen and Mari Egeness Creese, for their assistance and prompt responses to our endless questions.

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Furthermore, all uncredited images in this thesis were either self-illustrated or produced by the authors.

We hereby declare that this thesis is independently developed and in compliance with the examination regulations of The Norwegian University of Science and Technology.

Norwegian University of Science and Technology
Trondheim, May 2024

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Abstract

Efficient environmental monitoring of marine species hinges on careful sample preparation methods to navigate the complexities of tissue matrices and observe toxin spread. This study test out diverse techniques for preparing fish tissues, focusing on homogenization, extraction, and clean-up. Through a comprehensive literature review, a modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method and solvent extraction coupled with gel permeation chromatography (GPC) or NH_2 clean-up were selected for experimentation. Collaboration with SINTEF Ocean within the framework of the ToxiGen and AQUAvit projects facilitated this research.

The investigation unveiled critical insights into optimizing homogenization, where addition of solvents (dichloromethane:*n*-Hexane and acetonitrile) before and salt (NaCl and Na_2SO_4) after homogenization proved most efficient, with acetonitrile demonstrating superior tissue breakdown capabilities. Extraction methodologies diverged; the QuEChERS method involved a single step, whereas GPC solvent extraction necessitated three steps employing ultrasonication. The importance of the amount of analytes extracted using these methods needed to be considered along with the following clean-up step.

Clean-up procedures played an important role in eliminating interfering biotic compounds. Differentiating between sample tissues, NH_2-fractionation turned out as suitable for polar cod organ tissues, by excelling at lipid removal while preserving nonpolar target compounds. For salmon muscle tissue, the selection between GPC and QuEChERS was dependent upon several factors including time consumption and cost-effectiveness. QuEChERS demonstrated efficiency and profitability, while GPC excelled in analyte detection.

Noteworthy adjustments in the QuEChERS clean-up method, particularly the amount of the sorbent Z-Sep from 50 mg to 200 mg, resulted in significant reductions in biological compounds such as cholesterol (-49.1 %), 2-oleoyl glycerol (-99.0 %), niacinamide (-80.9 %), and C18 unsaturated fatty acids (-88.9 %), although with an increase in contamination by 93.6 %. Chromatographic analysis outlined method differences; GPC exhibited residual lipids, while QuEChERS presented lower peak intensity and fewer identified compounds, suggesting unique characteristics in effectiveness.

Sammendrag

Effektiv miljøovervåkning av marine arter avhenger av effektive prøveopparbeidingsmetoder for å håndtere kompleksiteten i vevsmatriser og observere spredningen av giftstoffer. Denne oppgaven utforsker ulike teknikker for opparbeiding av fiskevev, med særlig fokus på viktige trinn som homogenisering, ekstraksjon og opprensing. Etter en grundig litteraturgjennomgang ble en modifisert Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) metode og løsemiddelekstraksjon kombinert med gel permeasjonskromatografi (GPC) eller NH_2-opprensing valgt til utprøving. Samarbeidet med SINTEF Ocean innenfor prosjektrammene til ToxiGen og AQUA-vit har gjort denne oppgaven mulig.

Arbeidet ga økt innsikt i optimalisering av homogenisering, hvor tilsats av løsningsmidlene diklorometan:*n*-heksan eller acetonitril før, samt salt som NaCl og Na_2SO_4 etter homogenisering, viste seg å være mest effektivt. Acetonitril var særlig egnet til vevsnedbrytning. Metodene for ekstraksjon varierte; QuEChERS-metoden bestod av kun ett trinn, og DCM:Hex løsemiddelekstraksjonen bestod av tre trinn med bruk av ultralydbad. For en sammenlikning, må mengden analytter ekstrahert ved brukt av de ulike metodene ses i sammenheng med de påfølgende opprensningstrinnene.

Opprensningsmetodene spilte en viktig rolle i fjerningen av forstyrrende biologiske forbindelser. Ved separasjon av ekstraktet, viste NH_2-fraksjonering seg egnet for organer fra polar torsk ved å effektivt fjerne lipider samtidig som den bevarte ikkepolare analytter. For muskelvev fra laks var valget mellom GPC og QuEChERS avhengig av flere faktorer, inkludert tidsforbruk og kostnadseffektivitet. GPC utmerket seg i analyttdeteksjon, mens QuEChERS viste seg å være effektiv og økonomisk.

Endringer i opprensningsmetoden til QuEChERS, hvor mengden Z-Sep sorbent ble justert fra 50 mg til 200 mg, resulterte i en betydelig reduksjon av biologiske komponenter som kolesterol (-49,1 %), 2-oleoyl glyserol (-99,0 %), niacinamid (-80,9 %) og C18 umettede fettsyrer (-88,9 %), til tross for en økning i forurensning på 93,6 %. Kromatografisk analyse avdekket forskjeller i metodene: GPC viste en høyere andel lipider, mens QuEChERS hadde lavere intensitet på toppene og færre identifiserte forbindelser, noe som indikerer unike egenskaper med hensyn til effektivitet.

Contents

List of Abbreviations

ACN	Acetonitrile
AH	Aromatic Hydrocarbons
APCI	Atmospheric pressure chemical ionization
APPI	Atmopheric pressure photoionization
CEC	Contaminants of emerging concern
DAD	Diode array detector
DC	Direct current
DCM	Dichloromethane
dSPE	Dispersive solid-phase extraction
EI	Electron impact ionization
EIC	Extracted-ion chromatogram
ESI	Electron spray ionization
GC	Gas Chromatography
GC-MS	Gas chromatography coupled with mass spectrometry
GCxGC	Two-dimensional gas chromatography
GF/F	Glass fiber filter
GLC	Gas-Liquid Chromatography
GPC	Gel permeation chromatography
GSC	Gas Absorption Chromatography
HRMS	High resolution mass spectrometry
LC	Liquid Chromatography
m/z	Mass-charge ratio
MS	Mass spectrometry
NTS	Non-targeted screening
OCP	Organochlorine pesticide
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
QA	Quality Assurance
QC	Quality Control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe

RF	Radio frequency
RIS	Recovery internal standards
SEC	Size-exclusion chromatography
SIS	Surrogate internal standards
SOP	Standard operating procedure
SPE	Solid Phase Extraction
TIC	Total ion chromatogram
ToF	Time-of-Flight

1 Introduction

In today's society there is a significant increase in the consumption and production of chemicals. These chemicals carry the potential risk of deviating from their intended use or disposal paths, and end up in the environment [1]. When these chemicals accumulate in ecosystems, they may pose substantial environmental hazards and health risks to humans and wildlife [1].

Oceans cover over 70 % of the Earth's surface, and within marine ecosystems, a large number of species are exposed to accumulation of pollutants [2]. Marine species are often used as an indicator to assess the degree of pollution, as runoff from land, rivers and various marine activities results in the accumulation of pollutants and waste in marine environments, serving as precipitation zones for these contaminants [3]. Toxins can accumulate in tissue, and pose a health risk to the species and consumers from upper trophic levels [4]. One of Norways' main industries are the use of natural marine resources [5]. It is crucial to adopt a sustainable approach towards utilizing these resources.

The continuous introduction of new chemicals, coupled with a shifting regulatory focus from single compounds to effects of mixtures, makes the current target screening processes limited in their efficacy [1]. Target screening, the traditional screening approach, focuses on predetermined compounds [6]. It is desirable to develop efficient analysis methods for non-target screening, to identify trace compounds without prior knowledge [7]. Analysis of biotic tissue faces a lot of difficulties due to the complexity of the matrix, which makes extraction and analysis of emerging pollutants difficult [8]. These challenges makes the development and reliability of screening procedures difficult.

There are two research projects associated with this bachelor thesis. AQUAvit is a SINTEF-project funded by RACE (Research at ACE) related to the transfer of environmental toxins from fish feed pellets to tissue of farmed salmon. ToxiGen is a Norwegian Research Council funded project led by the University of Tromsø, which studies the effects of oil pollution on reproduction in polar fish species.

AQUAvit

The AQUAvit-project aims to assess chemical contamination in fish feed and the transfer to the farmed salmon with the application of a novel analytical monitoring platform.

Over 90 % of feed ingredients used in the salmon industry in Norway are imported [9]. Subsequently, there is an uncertainty about the ingredient quality from some of the sources [9]. Chemical pollutants in the feed can transfer to the salmon and the surrounding ecosystem, and further to consumers.

ToxiGen

The ToxiGen-project researches how pollution, with focus on oil spills effects the reproductive success, subsequent fitness and the survival of future generations of polar cod. Current approach models for impact assessment does not take into consideration that the impacts can be transferred through several generations and that in early life stages the sensitivity of the species may be increased.

1.1 Research Question and Objectives

Given the escalating utilization and development of new chemicals, coupled with potential accumulation in marine ecosystems, the need for developing innovative approaches to efficiently analyze and identify trace compounds in biotic tissue is crucial. The research in this thesis is motivated by different issues:

- Development of a standardized sample preparation method, including homogenization, extraction and clean-up, for each individual tissue composition (liver, gonad, brain and muscle).
- Assessing chemical contamination in fish feed and how/if it transfers to farmed salmon. - *AQUAvit project*
- Investigating the effects of oil pollution on the reproductive success and health of polar cod by characterizing which petroleum hydrocarbons accumulate in different parts of the fish (gonad, liver, brain). - *ToxiGen project*

Based on the issues presented, the research question for this thesis is:

How can innovative methodologies be developed to enhance the efficiency of sample preparation techniques for analysing different fish tissues, with a focus on understanding the impacts of increased chemical pollutants on specific marine ecosystems?

Using the research question and background motivation, the objectives of this thesis are:

- Conduct a comprehensive literature search to identify existing methodologies and weaknesses in approaches of sample preparation and analysis of marine tissue.
- Optimize the homogenization procedure to break down the tissues thoroughly while minimizing degradation of analytes.
- Refine the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method for sample extraction and purification.
- Compare the performance of the QuEChERS method with other clean-up methods, evaluating efficiency, matrix interference and suitability for different tissue types.
- Employ two-dimensional gas chromatography analysis and develop data processing methods to detect and identify target compounds.

2 Theory

2.1 Environmental Toxins

The emergence of environmental toxins presents a growing concern, driven by the production and widespread use of numerous new chemicals, which can result in more chemicals entering the ecosystems [1]. Exposure to these chemicals can potentially be harmful, and affect ecosystems and human health [4]. The increased production of new chemicals exceeds the capacity of current risk assessment technologies and monitoring [10]. Environmental toxins is a broad category, where two main classes are relevant to this thesis: contaminants of emerging concern (CECs) and legacy contaminants.

2.1.1 Contaminants of Emerging Concern

The increased engagement in anthropogenic activities such as agriculture and industrial operations leads to more discharged pollutants into the environment. Water and sediments works as accumulation zones for these contaminants [11]. While sediments is known as an accumulation zone for legacy contaminants, the concentration of CECs in water may increase [11].

Contaminants of emerging concern (CEC) is a group of pollutants that have raised alarms recently for their ecological and human health risks [12]. CECs are often characterized by widespread environmental distribution, a diverse range of unforeseen hazards and uncertain baseline levels [13]. However, there is currently no routine monitoring, and their fate, behavior, and toxicological effects have not yet been explicitly studied [14]. Due to the complexity of pathways, targets, sources, and effects of CECs, there has been recent advancement in the development of various technologies for removing or treating these contaminants [15].

CECs represent a broad category of chemical compounds, such as novel flame retardants (NFR), pharmaceuticals and personal-care products, nanomaterials, endocrine-disrupting compounds, plasticizers, organometallics, perfluorinated compounds and perfluoroalkyl substances [16, 17].

CECs are not necessarily new in the environment and some can stay there for a long time. They are compounds that inherently possess possible problematic properties, such as toxicity, persistence, or both [13]. Some CECs are inherently persistent, while others undergo biotransformation, which leads to the formation of metabolites and by-products that may be difficult to degrade [18].

It is possible that these contaminants can accumulate in biota, and therefore pose a health risk to human and wildlife [13]. While some aspects of CEC bioaccumulation are well understood, limited data exists for others [14]. Some CEC persist in sediments, resulting in bioaccumulation of CECs in benethic organisms which is eaten by fish, which can result in accumulation up the food-web [10]. Factors that influence how CECs bioaccumulate are chemical properties, specific organisms, and environmental conditions [1].

2.1.2 Legacy Contaminants

Some of the main groups under legacy contaminants are: polychlorinated biphenyls, polybrominated diphenyl ethers, aromatic hydrocarbons. These groups are discussed in the following chapters.

2.1.3 Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) were widely used in the 1930s and 1940s in different industrial applications, such as hydraulic fluids, oil for transformers, dialectrics in transformers, lubricant for pumps and turbines and heat exchange fluids [19]. These endocrine-distrupting chemicals are stable organic molecules that have entered the environment both with legal and illegal use and disposal [19]. They are relevant due to their presence in the environment despite the ban of these compounds in Europe since 1985 [20].

In recent years it has been revealed that PCBs still have a presence in the environment [21]. The primary route PCBs enter the human body is through the ingestion of contaminated foods, such as seafood and dairy products [21]. PCBs pose a health risk to animals because of their ability to affect reproductive functions [22]. Exposure to these chemicals can increase the risk of developing diabetes, cardiovascular diseases, liver diseases and cancer [23].

The chemical structure of PCB is shown in *Figure 1.* The structure consists of two aromatic rings, where the hydrogen atoms can be replaced by one to ten chlorine atoms.

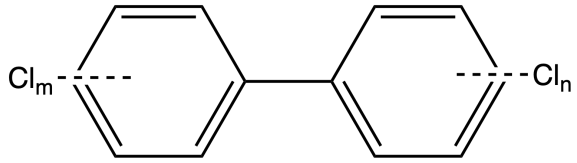


Figure 1: Chemical structure of PCB

2.1.4 Polybrominated Diphenyl Ethers

Polybrominated diphenyl ethers (PBDE), primarily used as flame retardants, are synthetic chemicals. These substances demonstrate environmental persistence, meaning they resist degradation, and can accumulate in the tissues of living organisms, and bioaccumulate in the food chain [24, 25].

PBDE consist of two phenyl-rings connected by an ether-bridge. Each ring can accommodate one to five bromine atoms [26]. Due to different positions and number of bromine atoms attached, 209 different congeners are possible [26]. The chemical structure of PBDE is depicted in *Figure 2.*

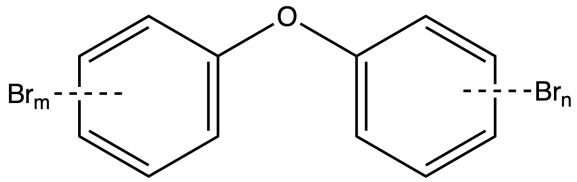


Figure 2: Chemical structure of PBDE

PBDEs can expose toxic effects in aquatic biota, similar to the PCBs. The endocrine disrupting properties of the chemicals can predispose marine mammals, fish and their offspring [3].

2.1.5 Aromatic Hydrocarbons

Aromatic hydrocarbons (AH) are hazardous organic compounds found in the atmosphere [27]. AHs consists of mono-aromatic hydrocarbons, including BTEX (collective term for benzene, toluene, ethylbenzene, xylene), additional alkyl-substituted benzene compounds and poly-aromatic hydrocarbons (PAHs) [28].

Even though AHs consist of aromatic rings, some can consist of cyclic rings connected to the aromatic rings [27]. Alkyl-substituted benzene can have different arrangements of the alkyl groups, relative to the benzene ring. Alkyl branched benzene have alkyl groups attached in a branched manner, while linear benzene have straight chain alkyl groups [27, 28]. Both are organic compounds, and are used as components in detergent [27].

2.1.6 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) originate from various sources, both natural and anthropogenic, and are aromatic compounds composed of two or several benzene rings [29]. PAHs are widespread environmental pollutants, known for their adverse biological effects, including toxicity, mutagenicity and carcinogenicity [29]. The structures of twelve commonly studied PAHs are shown in *Figure 3*.

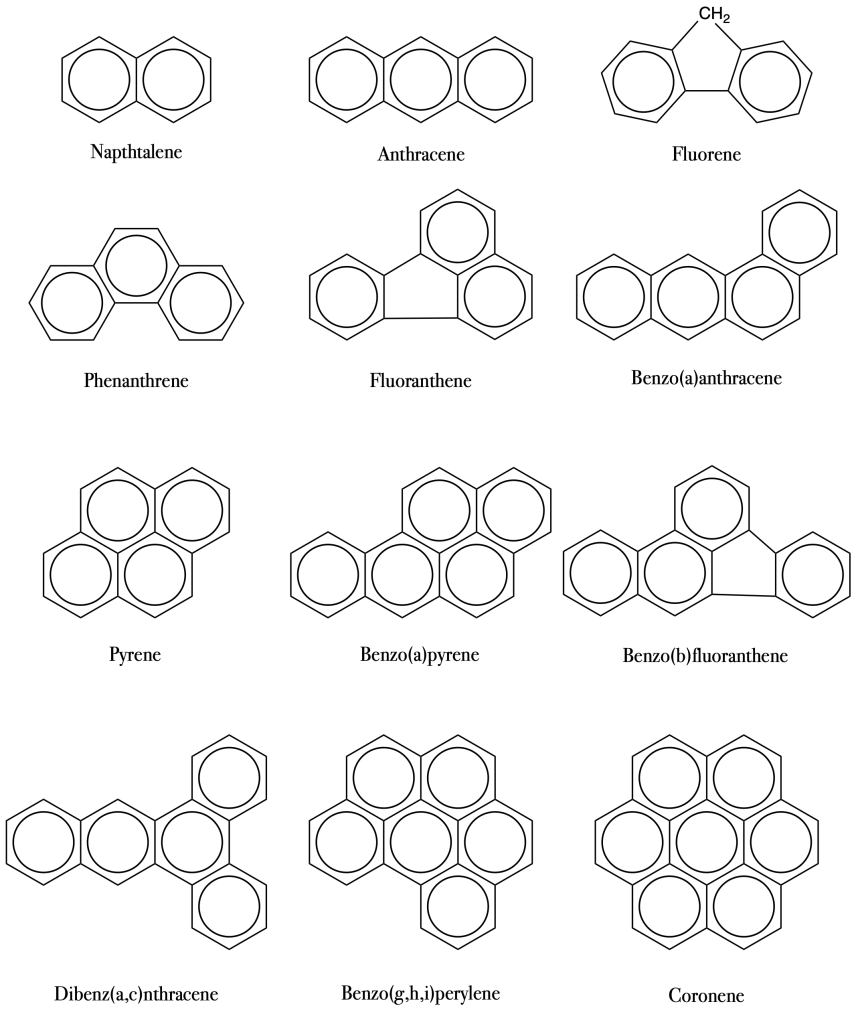


Figure 3: Chemical structure of common PAHs

Some of PAHs natural sources are forest fires, oil sieves, volcanic eruptions and exudates from plant material [29]. Anthropogenic sources can be the burning of fossil fuels, wood burning, coal tar and industrial lubricants [29]. PAHs hardly degrade naturally in the environment, which makes them prone to accumulation [29].

PAHs have been included as priority pollutants to be monitored by the United States Environmental Protection Agency (US EPA) and the European Union (EU), due to their carcinogenic and mutagenic properties [30]. In living organisms, PAHs can undergo biotransformation into more toxic metabolites, exacerbating their potential impact on ecosystems and animal health [30].

Alkylated PAHs, known as alkyl-PAHs can be more persistent and toxic than their parent compounds [31]. Even though some can be more toxic, there has not been enough research on them to support including them in the regular measurements [32].

In aquatic environments, PAHs pose a significant threat to fish, by accumulating in certain organs of fish, specifically the liver [33]. However, PAHs are also toxic for the gonads, which is the reproductive organ in fish [34]. This can cause reproductive dysfunction in some fish species [34]. The carcinogenic properties have raised concerns about the potential long-term impact on fish populations and the overall health of aquatic ecosystems.

2.1.7 Pesticides

Pesticides are a group of chemicals designed to control organisms that are considered harmful [35]. Pesticides can be classified under both CEC and legacy contaminants. Although pesticides are commonly used in agriculture to control and protect crops, research shows that a considerable amount of these chemicals does not reach their intended pest targets [36]. Instead, they often end up in the surrounding environments, posing risks to both the ecosystems and human health. The classification of pesticides are based on their chemical structures. The most common are carbamates, dithiocarbamates, organochlorines, synthetic pyrethroids, organophosphorous compounds, thiocarbamates and phenoxyacetates [37]. While controlling pests can indeed enhance food production, it is crucial to remember that pesticides are recognized as environmental pollutants [38].

Pesticides enter marine environments through various pathways [39]. This includes surface runoff from agricultural fields and the use of them in non-agricultural areas. Biocides are the class of pesticides that are used for non-agricultural areas, such as wood preservatives and anti-fouling agents on boats and underwater structure, that also contribute to pesticide pollution in aquatic environments [37, 39].

Pesticide exposure can cause two kinds of toxic effects; chronic and acute. The acute effects can include nausea, headache or other serious effects, and most severely; death. Chronic health effects can occur when individuals are continuously or repeatedly exposed [37]. Pesticides have been identified as endocrine disruptors, which means that they can effect the normal function of the endocrine system of both humans and wildlife [40]. These compounds can join the food webs and the concentration can increase with each trophic level. The accumulation of these compounds can cause endocrine, metabolic and reproductive disorders in fish [40].

2.1.8 Pharmaceuticals

Pharmaceuticals can have a significant impact on marine environments, both from the use of medicine in fish farming industries, and from pharmaceuticals gone astray from other purposes [41]. Antimicrobial compounds are used to treat infections in farmed salmon [42]. These chemicals can move into the wild marine environment, and have a negative impact on marine biodiversity as a result of antibiotic-resistant bacteria or antibiotic resistance genes [42].

However, not all pharmaceuticals present in aquatic environment are a result of the use of them in fish farming. The excessive use of medicine globally, and pharmaceuticals resistance to traditional sewage treatment systems, has led to these compounds becoming a serious environmental problem [43]. Pharmaceuticals are increasingly being detected in marine and coastal environments, with growing indications of their impact [44]. Either direct through bioaccumulation up the food chain to toxic levels, or indirect through the loss of species that is particularly sensitive to pharmaceuticals [44].

2.2 Test Species

Polar cod and farmed salmon are the two fish species this thesis will focus on. The tissues chosen for testing are brain, gonad and liver from polar cod and muscle tissue from farmed salmon.

2.2.1 Farmed Salmon

Since the 1970s, the fish-farming industry in Norway has had a significant growth [45]. and is one of the main industries. Norway is the worlds largest exporter of farmed salmon, and it is one of the country's main industries [45]. Over the last decades the production of farmed salmon has replaced the wild salmon in markets. Fish-farming exposes a risk to other aquatic wildlife due to the spread of diseases, escapees and pollution of the surrounding environment [45].

Figure 4 shows a shoal of farmed salmon and *Figure 5* shows the setting of a fish farm on the Norwegian coast.

Figure 4: Farmed salmon shoal [46]

Figure 5: Norwegian fish farm [47]

Farm-raised salmon are fed dry feed in the form of pellets. In 2020 this consisted of over 60 % plant-based protein and oils, around 22 % marine ingredients, the rest is composed of carbohydrate sources and micro ingredients [9]. As much as 92 % of these ingredients are imported and only 8 % are of Norwegian origin. In 2020 over 3,400 tonnes of imported ingredients were of undefined origin [9]. This leads to an uncertainty about the quality of the ingredients from some of the sources.

Another important aspect regarding the transfer of chemicals to farmed salmon involves the use of pharmaceuticals. Antibiotics, treatments against salmon lice and intestinal worms, sedatives and anesthetics are mostly used [48]. Florfenikol and oxolinic acid are the most used antibiotics for fish [48]. Imidacloprid, azamethiphos, hydrogen peroxide, diflubenzuron and teflubenzuron are common pharmaceuticals

used for the removal of lice [48]. To get rid of intestinal worms the drug praziquantel is mainly used [48]. Isoegenol in lower doses are used as a sedative for fish, in higher doses it works as an anesthetic [48]. Benzocaine and tricaine mesylate are other common sedative and anestethic drugs. The drug is used when handling de-licing and vaccinating of fish [48]. The use of pharmaceuticals in open-water fish farms makes them prone to distribution to the surrounding ecosystems.

2.2.2 Polar Cod

The polar cod (*Boreogadus saida*) is one of the most abundant fish in the Arctic [49], where cold, sub-zero water temperatures occur. It is a pelagic or semipelagic fish, that lives in the open water masses, or towards the seafloor. The species produces eggs that are epipelagic and float towards surface areas, preferably under the Arctic ice [50]. In order to develop successfully the eggs are dependent on the ice for protection from harsh weather and prey. Polar cod plays a vital role in the Arctic ecosystem, serving as a crucial component in the food chain for various marine birds, marine mammals, and other fish species [49]. It is considered a high-energy prey for the upper trophic levels, and provides an essential link between organisms of lower and higher trophic levels [51].

The polar cod is a small species, with a length generally around 20 cm, and a maximum length of 30 cm [51]. It is a relatively short lived fish, with a maximum life of 7 years [51]. *Figure 6* shows an image of polar cod.

Figure 6: Polar Cod *(Boreogadus saida)* [52]

The distribution of polar cod extends around the sub-Arctic shelf seas near the northern coasts of Norway and Russia [49], an area experiencing increasing human activity. Drilling and production sites for oil and gas exploitation are expanding in these areas, exposing them to accidental release of petroleum compounds [49]. Due to the vital role in the ecosystems, the polar cod has been used in exposure studies of petroleum compounds on fish in the Arctic marine ecosystem [53]. Exposure to petroleum compounds can have an effect on the physiological processes, such as reduced somatic growth, depressed metabolism and alteration in lipid metabolism and phospholipid levels in muscle and liver [53]. The reproductive period is a sensitive stage in the life cycle of polar cod [53]. The gonadal development is fueled by energy reserves that provides the basis for reproduction. The consequence of exposure to petroleum compounds may be a reduction in lipid energy reserves in exposed fish.

2.2.3 Biotic Tissue Composition

Biotic tissue are complex matrices, containing elevated levels of lipids, proteins and other complex biological compounds. The complex nature of biotic tissue complicates the extraction and analysis of toxins, potentially hindering the detection of contaminants [4]. Tissue composition depends on the sample, for instance, muscle tissue usually have a lower content of lipids compared to other biotic tissues, such as liver [54]. In fish the major constituent of the fish muscle is water, which accounts for 70-80 % of the fillet weight. The protein content ranges from 17 % to 22 %, while lipids are the third major constituent [55]. 0.7 % to 0.8 % are phospholipids, which are structural lipids containing cholesterol [55]. The lipid content between different species of fish varies, and fish is therefor often classified by their fat content. Lean fish has fat less than 5%, while fatty fish has a fat content of 5 % or higher [55]. Another difference between fatty and lean fish is where fatty acids are stored. Lean fish often accumulate the fatty acids in organs like liver and gonads, while in fatty fish species the fatty acids accumulate in the muscle tissue [56].

Aquatic biotic tissue is especially vulnerable to the accumulation of pollutants due to their environmental placement. Pollutants originated from landmasses end up in the ocean through precipitation and weathering, resulting in both free-floating and accumulated pollutants in aquatic environments [1]. Some dissolve in water and are directly absorbed by organisms through gills, ingestion or skin. [1]. Over time, these pollutants can bioaccumulate, posing significant risk to aquatic ecosystems.

2.3 Sample Preparation

Prior to extraction of analytes homogenization of tissue is necessary. This process releases the interstitial and intracellular analytes [57]. The aim is to acquire a homogeneous suspension of cell constituents by cell disruption [57]. Common homogenization methods utilize cutting, centrifugal precipitation, tissue grinder and chemical/enzymatic solubilization [57].

To induce phase separation after homogenization, salt is added to the samples. This salting-out effect influences analyte partition, due to the polarity of water and affinity for the salts that causes an aqueous layer separate from other compounds and the solvent [58]. This happens because the salt additive promotes partitioning of analytes into the organic solvent layer. Drying salts have the function of binding water in the samples, and to bind a significant fraction the amount of salt has to exceed the saturation concentration. The concentration of salts directly effects the polarity of the solvent, due to the water percentage in the organic layer [58]. Magnesium sulphate as a drying salt can improve recoveries in the QuEChERS method [58].

The next step after homogenization is extraction of analytes, which plays a crucial role in analytical chemistry by isolating target compounds from complex samples. The extraction is followed by sample clean-up to remove interfering matrices, before instrumental analysis.

2.4 Solvent Extraction

Solvent extraction, or solid-liquid partitioning, is a method that relies on the separation of components in a mixture according to their solubility in a solvent [59]. It is a versatile technique applicable to various technologies, including hydrometallurgy, waste treatment, material preparation and effluent purification [59, 60]. The objective is to utilize a liquid solvent for dissolving particular molecules or a group of compounds within the solute, extracting them from the liquid sample material. Afterwards, the solvent is separated from the solute to increase the concentration of the solute [60].

Four main categories of solvent extractants are used: acidic, chelating cation, solvating and anion exchanging reagents [59]. The phase transformation of the analytes can rely on chemical principles that entail electrostatic interactions between the ionic

substrate and the extractant [59]. While electrostatic interactions play a significant role, solvent extraction involves other mechanisms as well. These include hydrogen bonding, dipole-dipole interactions, and steric effects [61].

The sample is prepared by dissolving the sample containing target analytes and interfering matrix in an adequate solvent. The choice of solvent is based on how effectively it can separate the target analytes from the interfering substances based on solubilities [62]. The choice of solvent is critical for further steps. Solvents have different affinities for for specific analytes and interfering compounds. Therefore, the collection of the analyte fraction will depend on which solvent system is employed.

Solvents have varying chemical properties which affects the efficiency, selectivity and safety in the extraction process [63]. The classification of solvents are often based on their polarity and solubility with water [64]. Commonly used solvents in extraction processes are dichloromethane (DCM), acetonitrile (ACN), hexane, chloroform and ethyl acetate. Chemical properties of DCM, ACN and *n*-Hexane are shown in *Table 1.*

Solvent	**DCM**	**ACN**	***n*-Hexane**
Polarity	Moderately polar	Polar	Non-polar
Boiling point [°C]	40.0	81.6	69.0

Table 1: Chemical properties of common solvents used in solvent extraction

Dichloromethane is an extensively used solvent in extraction procedures. DCM is moderately polar and has a high solubility for non polar compounds, and moderately solubility for polar compounds [64].

n-Hexane is a non-polar linear hydrocarbon used as a solvent [65]. The non-polar characteristics makes it efficient for the extraction of non-polar analytes [65]. The most common areas of use are the food industry for the extraction of vegetable oils, fats, fragrances, flavors and bioactive ingredients [65].

ACN is another versatile solvent, because of the ability to efficiently isolate a wide range of pesticides, both polar and nonpolar, without extracting undesired matrix compounds [63]. For pesticide analyses it provides high selectivity. ACN is easily separated from water by the addition of salt followed by centrifugation. This allows for efficient removal of residual water compared to other solvents [63]. ACN is also less toxic than DCM and therefore extraction procedures involving ACN can be considered more environmental friendly [63].

To further enhance the extraction of analytes, solvents with different solubilities can be combined. The solvents must be miscible [64]. DCM combined with *n*-Hexane (1:1, v:v) combines the polar and nonpolar properties of the two solvents, allowing for dissolving of a wider range of analytes [64].

2.5 Clean-Up Methods

Clean-up methods aims to remove interference and impurities in samples prior to analysis. Various techniques are used, and they are crucial for enhancing the sensitivity and accuracy of analytical measurements by eliminating the unwanted substances.

2.5.1 Gel Permeation Chromatography

Gel permeation chromatography (GPC) is a separation technique based on separating molecules according to their size, also known as size-exclusion chromatography (SEC). The sorbent in the column and the pore size determines the quality of the extract. The solid structure of the column and the arrangement of porous particles enable larger analytes to travel alongside the mobile phase between particles, while smaller molecules navigate through the column particles. This configuration leads to shorter retention times for larger molecules [2, 66, 67]. Since biotic molecules are larger than most contaminants, GPC can be utilized for the clean-up of biotic tissue samples.

The chromatographic separation capacity of GPC is comparatively low compared to other chromatographic methods [68]. Therefore, GPC is commonly employed for the elimination of lipids, proteins, and natural resins from samples [68]. Gel permeation can use different types of packings, including hydrophobic packing for separating organic non-polar species, hydrophilic packing for polar compounds, ionic exchange packing for separating ions based on their charge, and affinity packing for selective separation based on interactions [69]. The choice of packing depends on the specific application and the column material.

Instrumental designs typically consist of several key components shown in *Figure 7*. These include a solvent delivery system, a sample injection device, a set of detectors, and a data acquisition and handling system [70].

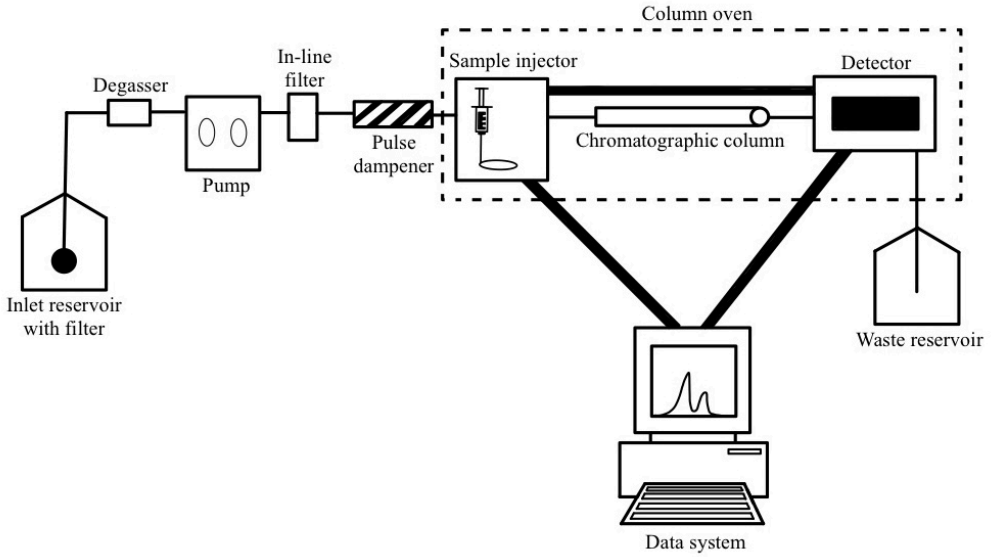


Figure 7: General setup of SEC [70]

2.5.2 Liquid Chromatography

Liquid chromatography (LC) is extensively used in analysis of environmental samples for identification and quantification of chemicals [71]. LC techniques can also be used as a sample clean up or purification of samples containing high levels of interfering substances that are of no interest, such as lipids, proteins, sugars and salts [71].

In LC a liquid solvent serves as the mobile phase, which is passed through a column packed with small particles. The resolution and efficiency of the separation in LC are affected by column dimensions and particle size of the packed column. Typically, the internal diameter is around 4.6 mm and length varying from 30 to 250 mm [72]. The particle size typically utilised are from 2 to 5 µm [72].

The components are separated in the column due to interactions with the stationary phase [67]. To increase the separation capacity by enhancing plate number, additional columns can be connected in series, keeping back-pressure limitations in mind.

The column used in LC offers different separation mechanisms based on the properties of the stationary phase. To obtain different properties for the stationary phase, the silica can be bonded with functional groups [67]. For the separation of nonpolar compounds, such as PAHs, pesticides and microplastics, a silica-based stationary phase is bonded with aminopropyl functional groups [67]. Polar compounds interacts with the amino groups through hydrogen bonding, dipole-dipole interactions and other polar interactions. Amin-columns are commonly used in pharmaceutical, environmental and biochemical analyses [67].

There are many different detectors that can be used for LC, such as the UV-detector, light scattering detectors, refractive index detectors and fluorescence detectors [67]. Diode array detector (DAD) is a common detector used in LC that sends a white light through the sample cell, which is then split into different wavelengths by using a holographic grating [67]. The absorbance of the sample at these wavelengths are measured in the focal plane by an array of photodiodes (diode array) [67].

A scheme of DAD is shown in *Figure 8*, including a deuterium lamp, slit, flow cell, grating and diode array.

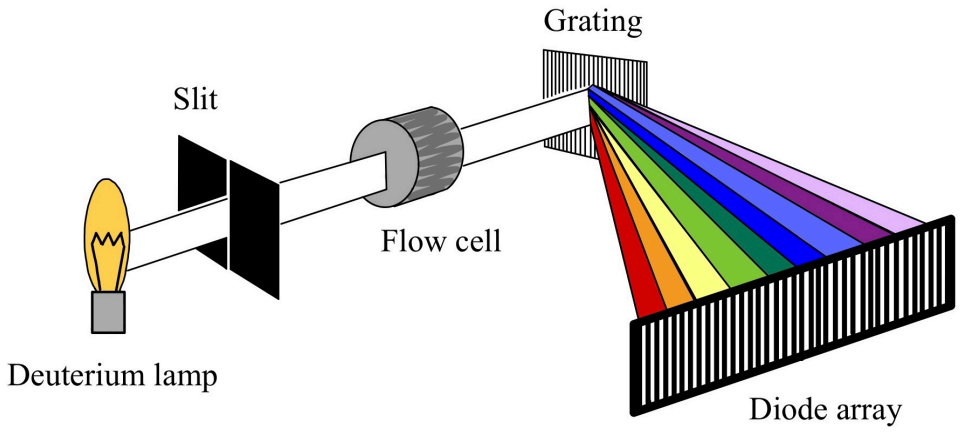


Figure 8: Diode array detector

Since the DAD utilizes polychromatic light, it enables the simultaneous measurement of multiple wavelengths. The wavelengths measured converts into an electrical signal that corresponds to concentration of the sample at a specific time point [67]. This generates a significant volume of data, which requires a computer for processing.

The structure and composition of molecules makes them have unique energy levels that absorb light at a specific wavelength [69]. *Table* 2 shows different absorption wavelengths for selected compounds in cyclohexane measured using Agilent 8453 UV-Vis Spectrophotometer [73]. Information about the analyte can then be acquired by measuring the emitted radiation as it returns to ground state [69].

Table 2: Maximum absorbance for different compounds in cyclohexane [73]

Compound	**Wavelength [nm]**
Naphthalene	275
Pyrene	241
Phenanthrene	252
Anthracene	356.25
Perylene	435.75
Phenol	270.75
Benzothiazole	217

By analyzing the absorbance data of the sample at different wavelengths the analytes can be identified and quantified. DAD provides spectral information allowing for sample characterization.

2.5.3 Dispersive Solid-Phase Extraction

Solid phase extraction (SPE) is a common sample preparation technique using a solid sorbent [74]. Areas of use includes sample clean-up, purification and concentration of target analytes. Dispersive solid phase extraction (dSPE) is a type of SPE, where the sorbent is dispersed directly into the sample matrix or extract [74].

As a clean-up technique dSPE has been used on a wide range of compounds, especially complex matrices [75]. It has significant capacity for eliminating matrix interferences, such as the presence of lipids or proteins in biological samples, sediment components in environmental samples, and co-eluting compounds in complex matrices or chemical additives [75]. Additionally, the impact of matrix effect is reduced which has gained this method wide acceptance.

This approach relies on dispersing a sorbent within liquid samples for the extraction, isolation, and purification of various analytes from complex matrices [76]. There are different methods based on the previous extraction of the sample. One method uses

a tube filled with a sorbent, this can be porous particles or a polymerized monolith [67]. The interactions between the analytes and sorbent are used to extract them from complex samples.

The most common sorbents used in dSPE are silica embedded with different functional groups, such as ethylsilane, aminopropyl, Supelclean PSA, Supelclean C_{18} [76]. Usually these are applied for extraction, preconcentration and clean-up for compounds over a wide range of different pH or elimination of sample interference [76]. For sample clean-up of more complex matrices, sorbents like Z-Sep or Z-Sep+ have been employed [76].

2.6 QuEChERS Method

2.6.1 Extraction

The Quick, Easy, Cheap, Effective, Rugged and Safe method (QuEChERS), developed in 2003 by *Anastassiades et al.* [77], is a widely used extraction method. It was originally intended to extract pesticides from vegetables and fruit, but today it is used for various matrices and analytes. For extraction it is considered a reliable alternative to extract a large range of analytes from different chemical families, and for a large number of matrices [78].

The first step involves an extraction approach based on the dispersion of salts (salting-out) and an equilibrium between an organic layer and an aqueous layer [78]. The second step involving dispersive solid-phase extraction (dSPE) cleans up the matrix interfering substances using porous sorbents and salts [78].

The method is cheap and offers flexibility. Modifications can still provide high recoveries of the sample. For the extraction DCM, ACN, acetone or ethyl acetate are the most commonly used solvents, because of their miscibility with water and are typical mobile phases employed in liquid chromatography [30]. Among these, ACN stands out due to higher selectivity. ACN effectively extracts pesticides with a broader range of polarities, due to interactions with both the stationary and mobile phases [30]. Common salts used to induce phase separation are: $MgSO_4$, $NaNO_3$, NaCl, $MgCl_2$, Na_2SO_4 and LiCl. The use of salt with drying characteristics improves recoveries of polar compounds [30].

2.6.2 Clean-Up

As mentioned in *Chapter 2.5.3*, dSPE is used as a clean-up procedure for samples, and is employed for the QuEChERS method.

The most common way is to separate the extracted organic phase and mix it with $MgSO_4$ and a sorbent [79]. The purpose of this step is to remove water and undesired co-extractives. After agitation, centrifugation and filtration the supernatant can be analysed directly with gas or liquid chromatography [79].

2.7 Analytical Methods

Advanced instruments for molecular analysis are used as tools to detect chemical compounds. The components of a mixture can be separated, identified and quantified.

2.7.1 Gas Chromatography

Gas chromatography (GC) is a method for analysing mixtures of volatile components with the purpose of obtaining information of the molecular composition. A gas chromatograph typically includes a carrier gas system, injector, gas chromatographic column, detector, and data processing unit [67]. The carrier gas employed is an inert gas with minimal adsorption capabilities, such as hydrogen, helium, or nitrogen [80], and serves as the mobile phase. The gas should be of high purity and must not react with the sample components or the stationary phase.

Separation is obtained based on two principles; adsorption and partition chromatography. Adsorption chromatography occurs when the analytes in the mobile phase have different adsorptivity to the solid phase [67]. The separation occurs in the column, situated within the column oven. The liquid mixture is injected and heated until vaporization. The vapor is then carried by the mobile phase to the column inlet where the compounds are slowed down by their interaction with the stationary phase, forming a narrow band of analytes at the head/inlet end of the column. The temperature program and the carrier gas drives the separation through the column [67].

Separation by GC can be performed with open tubular columns or packed columns [67]. For a packed column the stationary phase can consist of filling particles or a liquid stationary phase that is carried by a particle matrix. The length of a packed column differs between 2 to 3 m [67]. Open tubular columns ranges with an internal diameter of 0.1 to 0.5 mm and lengths of 10 to 100 m [67]. The material in an open tubular column is usually fused silica with a polyimide coating [67].

GC separation can be obtained with gas absorption chromatography (GSC), where the stationary phase is an absorbent, typically active carbon, molecular sieves or porous polymers [67]. In gas-liquid chromatography (GLC) the liquid stationary phase is either a thin film or a phase distributed directly on solid porous particles [67]. GLC nonpolar stationary phase will have little or no interactions with sample components, and separates solely based on boiling point [67]. If a polar stationary phase is used, separation is obtained according to both polarity and boiling point [67].

The chemical properties of the compounds defines how long the interactions in the column will last before detection. To avoid condensation of eluted components, the detector is heated to at least 20 °C above the highest used column temperature [80].

GC can be used in both qualitative and quantitative analyses. It fits over a wide range of applications mostly used to determine the composition of complex samples, such as trace determination in pollution and forensics, essential oils, food and beverage characterisation and petroleum [67].

2.7.2 Two-Dimensional Gas Chromatography

To effectively separate the hundreds to several thousands of different chemical compounds present in complex samples, relying on a single column often proves insufficient. To achieve a better separation two columns can be coupled together [67].

Two dimensional gas chromatography (GCxGC) is a multi-dimensional analysis that combines two distinct separation steps. It provides a significant improvement from traditional one-dimensional GC, by increasing the ability to separate chemical compounds [81]. This includes multidimensional ordering of chemical properties, separation capacity and an increase in signal-to-noise ratio [81].

The system is based on two chromatographic columns, where the phases have distinct separation mechanisms, the *first* and *second* dimension columns. Typically, the first column measures 15-30 meters, while the second column is only 1-2 meters long [82]. These are connected in tandem by a modulator interface [83]. This setup enables the detailed detection of complex samples at a molecular level, allowing for the observation of analyte composition in a straightforward and structured manner [83].

The modulator plays a central role in the process and is the key to ensuring the success of the GCxGC process. Regardless of the design, a modulator must fulfill three criteria: (i) continuously accumulate or trap small consecutive fractions of the effluent from the first column during the first-dimensional separation process; (ii) refocus the trapped fractions either temporally or spatially; and (iii) inject the refocused fractions as narrow pulses onto the second-dimensional column [82].

There are essentially two primary categories into which modulators can be categorized, thermal and valve-based modulators. Thermal modulators employ temperature control, such as crygogenic and hot air pulses, to capture analytes from the first column and subsequently release them for separation in the second column [84]. Valve-based modulators utilize gas flow control to regulate and isolate segments from the first column and redirecting these portions via injection for further separation [85].

The first column primarly separates based on boiling point [67]. Typically, the second columns characteristics lean towards either polar or shape-selective properties, which significantly influence the necessary orthogonal separation criteria. In this setup, separation spans from 1 to 10 seconds, in contrast to the first dimension separation, which lasts from 45 up to 120 minutes [82]. As a result, this process is conducted under nearly isothermal conditions. Since the second GC column is shorter and thinner than the first, it works quickly at a steady temperature. It separates compounds based on their properties, creating narrow peaks with brief retention periods [86]. Further description of the data processing and visualisation of peaks are described in *Chapter 2.7.5.*

A simple overview of a two dimensional GC is shown in *Figure 9*, including an injector, first and second dimension GC-column, modulator and detector.

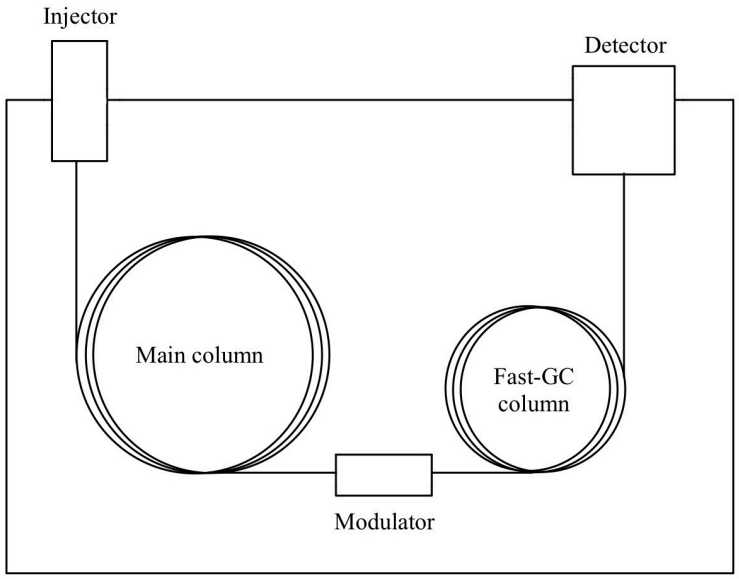


Figure 9: Overview of a comprehensive gas chromatography apparatus

2.7.3 Mass Spectrometry

Mass spectrometry (MS) is a key analytical tool in chemical, pharmaceutical and medical industries [67]. It aims to generate ions and then separate them based on their *mass-to-charge-ratio (m/z)*. The mass spectrometer is often employed as a detector in GC [67].

A mass spectrometer consists of an ionization source, a mass analyzer and a detector. The MS is a mass-sensitive detector, where the signal is dependent on analyte concentration and the flow rate of the mobile phase. Additionally if a split is used, it is also dependent on the split ratio in the chromatographic system [67].

The sample is injected into the ionisation source, and then converted to a gaseous phase through ionization of electrons, photons, ions or molecules [67]. A simplification of a mass analyser is depicted in *Figure 10*.

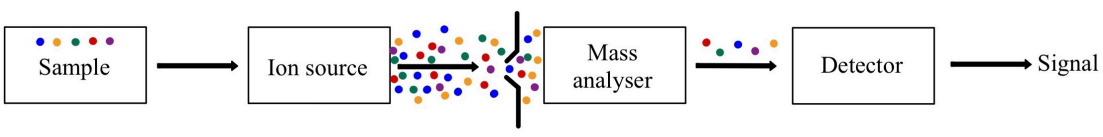


Figure 10: Mass analyser overview

The sequence consist of four steps: ionization, acceleration, deflection and detection [87, 88]. In the initial ionization stage, the molecules loose one or more electrons, resulting in a formation of cations. This will happen to any substance, regardless if it typically forms negative ions (e.g. chlorine), or never form ions at all (e.g. argon). This process is known as electron impact ionization (EI), and is the default ionization method for GC. However, ionization methods differ for LC. Common ionization techniques used are electron spray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) [89]. These methods are compatible with LC, and allows for a wide range of analysis, including those compounds that may not efficiently ionize under EI conditions.

After ionization, the ions enters the acceleration stage where they are uniformly accelerated to attain identical kinetic energy levels [88]. In the deflection stage, the ions experience deflection due to a magnetic field, with the degree of deflection correlating to their masses [87]. Ions with a lower mass undergo greater deflection. The extent of deflection is affected by the ion charge - the higher charge, the greater deflection. This paragraph has described the quadrupole mass analyzer, but there are different types available. For example Time-of-Flight, Magnetic Sector and Electrostatic Sector mass analysers, with different limitations and advantages, making them suitable for different applications. The final stage involves electric detection of the ion beam that passes through the apparatus, completing the MS process. After the detection a mass spectrum is constructed. MS provides information of chemical structure, which can be used for identification in addition to quantification of compounds.

2.7.4 High Resolution Mass Spectrometry

High resolution mass spectrometry (HRMS) is an analytical method utilized for accurately and precisely determining the *mass-to-charge ratio* of ions [90]. Some examples of HRMS instruments are Orbitrap and Time-of-Flight (ToF). Typically, these devices accurately measure the precise mass of analytes without fragmentation [90]. However, they can also incorporate a quadrupole, which introduces the possibility of fragmentation, and thereby enhancing the methods' selectivity [90].

ToF mass spectrometers separate ions based on their flight-time through a field-free drift tube [67]. The ToF consists of two plates and a light source with a large difference in potential. The ions enter the ToF between two plates, and a light source accelerate the ions. Due to pulses of high potential difference the ions will

get similar kinetic energy moving toward the drift tube. When they enter only the kinetic energy will determine the movement. Because of this, the velocity is directly proportional to their *m/z-ratio*. Heavy ions will have a longer flight time than lighter ions, this time difference separates the large from the small ions and the spectrum is recorded. The ToF-MS can consist of a single-stage ion source with linear field-free drift region or two-stage ion sources combined with ion reflectors and field-free drift regions [91].

Figure 11 illustrates a quadrupole and ToF in a GC-MS. Ions first enter the quadrupole, and then they are pulsed up the drift-free flight tube at high rates by the accelerator before reaching the detector.

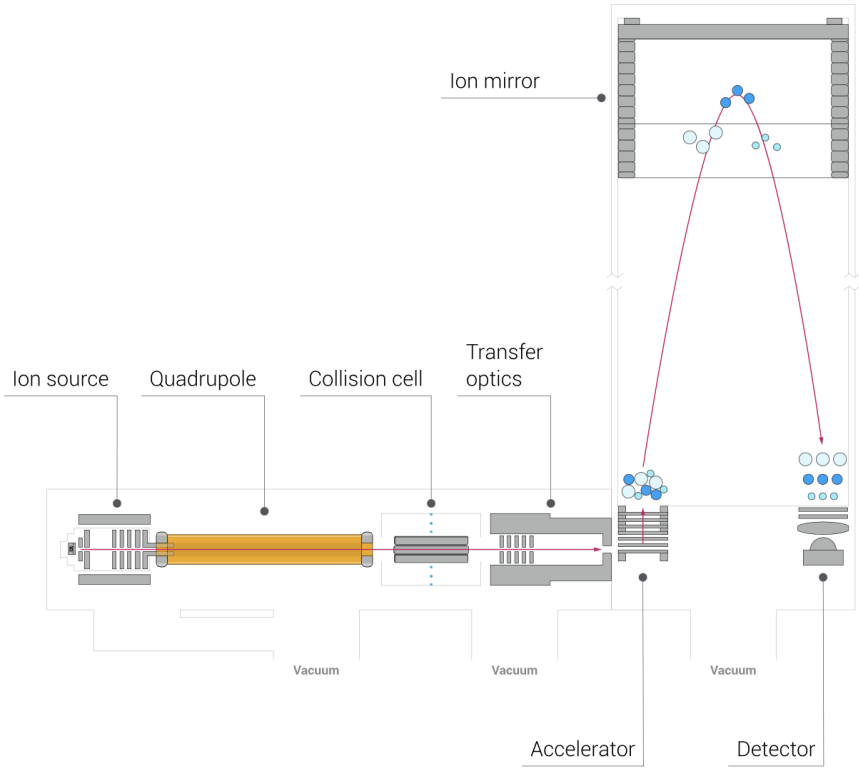


Figure 11: Quadrupole and ToF in a GC-MS [92]

ToF mass analyzers, known for their high mass resolution and wide mass range, along with their compatibility with other techniques, offer significant advantages [91]. In environmental analysis, they prove particularly valuable for resolving intricate mixtures [91]. Overall, ToF analyzers present efficiency and versatility across a spectrum of applications.

Quadrupole mass analysers have ions entering an oscillating electric field. This electric field is created by four identical parallel rods. The two pairs of parallel rods are connected electrically [67]. A certain direct current (DC) and radio frequency (RF) are applied to one pair of the parallel rods, and opposite DC and RF are applied to the other pair. This creates an electric field between the rods.

Ions enter this field and start to oscillate. Only ions of a specific *m/z-ratio* can pass through the quadrupole at specific DC and RF values. When controlling the applied values, whole mass spectra can be obtained full scan mode. An illustration of a quadrupole with ions is depicted in *Figure 12*.

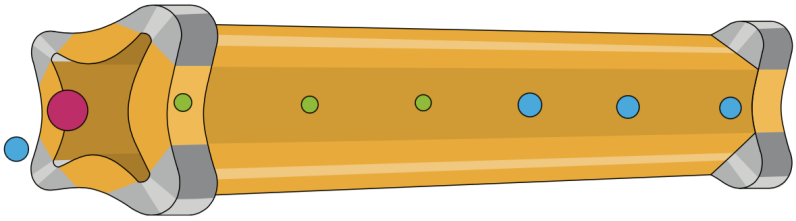


Figure 12: Quadrupole with ions [92]

Quadrupole and Time-of-Flight can be combined in a mass spectrometer, leveraging two distinct set of scan types for data acquisition, which allows it to detect trace level compounds [93].

2.7.5 Data Processing from GCxGC

GCxGC results are a set of rapid second-dimension chromatograms, as shown in *Step 1* in *Figure 13*. These are placed together resulting in a two-dimensional matrix with one axis representing the retention time of the first column, and the other axis representing the retention time from the second column [82]. This process is called transformation and is shown in *Step 2* in *Figure 13*.

The last step of data processing is shown in *Step 3* in *Figure 13*, here the data gets visualized, usually by utilizing specialized software.

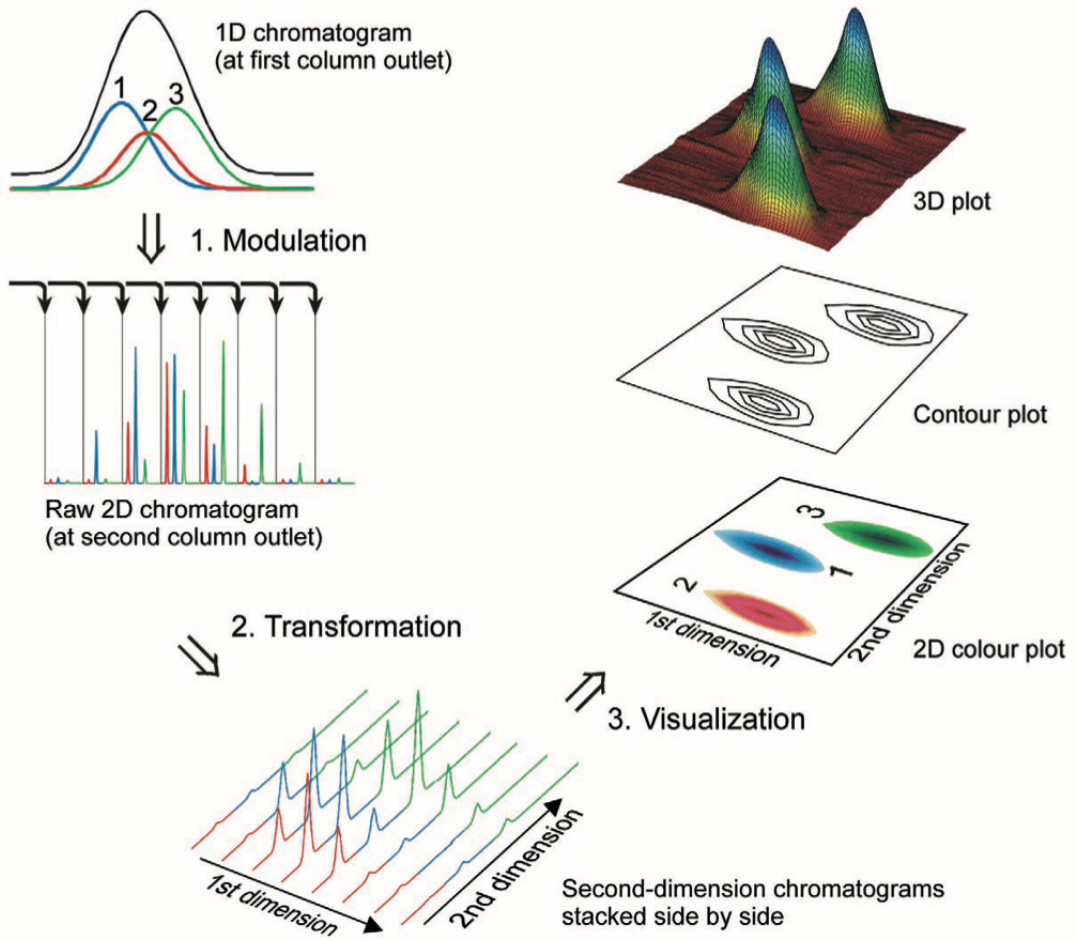


Figure 13: Creating and displaying a GCxGC chromatogram [82]

Since GCxGC is often coupled with (HR)MS the data processing becomes more complex than described earlier. This includes visualization of total ion chromatograms (TIC), which provides an overview of all the ions detected at various mass-to-charge ratios, over time. Unlike extracted-ion chromatogram (EIC), which concentrates on specific *m/z values*, TICs cover the full range of detected ions [94]. This provides a more comprehensive perspective on the sample composition. As a result, the data files become significantly larger due to increased amounts of information gathered. This makes the use of powerful software programs like *GC-Image*® or in-house scripts necessary for both targeted and non-targeted analysis of complex sample matrices.

2.8 Quality Assurance

To ensure the quality of analytical methods, the quality of results and the performance quality of tools and instruments needs to be evaluated continuously [69]. Quality assurance (QA) and quality control (QC) plays crucial roles in ensuring consistency in data over time and that results obtained by different laboratories are comparable [95]. The aim of QA is to ensure that the development and maintenance of the overall process meets the requirements of the objective, while QC evaluates and verify the quality of individual measurements [96].

QA often involves the use of standard operational procedures (SOP), regular instrument calibration and method validation [69]. Key aspects of QC are the use of internal standards, blank samples and spike samples, and in addition use several sample replicates [69]. Internal standards are used to check recovery for the analysis, blank samples are used to check for false positives, and spike samples are used to check for false negatives.

2.8.1 Internal Standards

Internal standards are chemical reference substances added at consistent concentrations to all sample parallels. This calibration technique is widely utilized in spectroscopy and chromatography [97].

Surrogate internal standard (SIS) and recovery internal standard (RIS) are two different types of standards that are commonly used in analytical chemistry. SIS are structurally similar to the primary analyte, but not already present in the sample, typically a deuterated, or 13C-isotope variant of the primary analyte [98]. SIS is added to the sample prior to sample preparation. The function of SIS is to serve as a control measure that evaluates the efficiency of sample preparation and analysis. By measuring the recovery of the standard throughout the analytical process, it is possible to assess the reliability and accuracy of the analytical method [97]. In contrast, RIS are added after sample preparations, but before the analysis. This standard has a lot of the same functions as SIS, however it focuses more on sample recovery. By measuring the concentration of RIS and comparing it to the known concentration, it is possible to determine the extent to which the primary analyte has been recovered. The information gathered from the addition of RIS is crucial for safeguarding the reliability of analytical results [97].

2.8.2 Checking for False Positives using Blank Samples

When analyzing a large number of samples, the use of blank samples are helpful to ensure the quality of the analysis. Blank samples contains only the solvent or matrix, without any analytes. Their purpose is to identify and quantify background contamination or interference [99]. By treating equal to the other samples, they can help determine if contamination is due to method errors or already present in the real samples.

Different types of blanks are used for various purposes. These are; method blank, field blank and reagent blank. Method blanks are made using the same steps as the real samples but without any analyte [100]. It checks for contamination from chemicals, equipment, and the surroundings. Field blanks are collected from the sampling site, without being exposed to the sample matrix [100]. This assesses contamination from sample collection procedures and transportation. Reagent blank is prepared using the same reagents without any sample, and detects any impurities in the reagent [100].

2.8.3 Checking for False Negatives Using Spike Samples

Spike samples play a crucial role in analytical procedures by assessing the recovery of an analyte and validating the accuracy of the method used. Known amounts of analyte are intentionally added to the sample, or blank matrix [101]. Then, both the spiked and normal sample undergo analysis. The percentage recovery is then calculated, comparing the measured concentration after spiking to the expected concentration. Optimal recovery is close to 100 %, but acceptable recoveries are >65 % [101]. Other values may signal issues such as matrix effects, instrument malfunction, or method errors. Spike samples are widely used in environmental, pharmaceutical, and food analyses [101].

2.9 Analytical Screening Methods

Detecting chemical substances within a sample can be achieved through a variety of methods, which often depend on the extent of prior information available. Screening methods offer a valuable way to precisely detect known substances and uncover novel compounds within the sample matrix, particularly when applied to biotic tissue to identify toxic compounds.

2.9.1 Targeted Screening

Targeted screening involves using a mass spectrometer to identify trace levels of known compounds in complex solutions. This strategy relies on analyzing a set of reference standards, which serve as a basis for identifying and quantifying the target compounds [6]. After detection, concentrations are measured by combining internal standards and calibration curves based on the responses of reference standards. This method promotes precise quantification of target compounds, which is crucial for various applications [6].

This method is frequently used in environmental monitoring, to detect potential pollutants in samples. It is also utilized to ensure food safety by detecting pesticide residue, food additives, or contaminants, as well as in pharmaceutical analysis for assessing drug quality and safety [6, 102].

Targeted screening methods face limitations due to the availability and expense of analytical standards. As a result, the detection of emerging contaminants in the environment might be costly and challenging to achieve [103]. Many will therefore choose suspect or non-targeted screening for these approaches.

2.9.2 Suspect Screening

Suspect screening detects chemical features using HRMS and compares this to a database of known contaminants [11]. This screening method can uncover a wide range of chemicals present in environmental matrices, such as air, soil, water, sediment and biota samples, based on a predefined suspect lists [104]. The list includes exact mass and isotope information of the chemicals, and can be used to identify potential matches [105].

The key applications of suspect screening is environmental monitoring by identifying emerging contaminants, pollutants, and potential hazards in natural ecosystems, and research about human exposure to specific chemicals by analyzing human biospecimens [104, 106].

2.9.3 Non-Targeted Screening

Unlike targeted screening, non-targeted screening (NTS) begins without any prior information about the compounds to be detected [7].

Figure 14 shows a typical workflow for a non-targeted screening analysis. The method often include sampling and extraction in combination with either LC or GC coupled with HRMS [107]. Sophisticated data processing tools are frequently utilized along with comparisons to mass spectral libraries to help identification during data analysis.

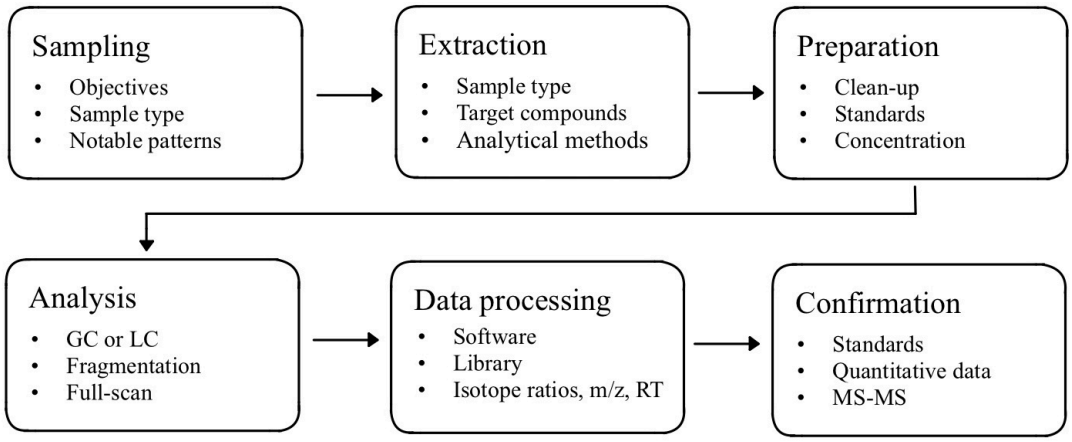


Figure 14: Non-targeted screening workflow

The interest in non-targeted screening has surged in recent years due to advancements within HRMS technology [4]. The technology behind HRMS offers several advantages, including superior resolution, precise mass accuracy, and broad mass ranges. Consequently, HRMS excels in identifying peaks with subtle mass variations, making it suitable for detecting and identifying various substances and compounds [4]. Quadrupole Time-of-Flight is the most commonly used HRMS technique for both targeted and non-targeted screening purposes [4].

2.9.4 Comparison of Screening Methods

The three screening methods - targeted, suspect, and non-targeted - offer distinct advantages and limitations. Targeted screening requires reference standards for comparison, enabling precise quantification and confirmation [11]. On the other hand, suspect screening and non-targeted screening eliminate the need for reference standards.

Suspect screening matches sample contaminants to a predetermined list during data analysis, while non-target screening utilizes spectral data to identify compounds without prior information [105]. These latter methods leverage HRMS and computational tools to explore a wide array of chemicals within samples [106]. In contrast, traditional targeted analysis methods are designed for quantifying only a limited number of specific chemicals [106].

Without available standards, suspect and non-targeted screening may have limitations in detecting contaminants. Compound identification can be uncertain, and information regarding concentration is therefore semi-quantitative [105]. Despite this, suspect and non-targeted screening play crucial roles in monitoring strategies [108].

Each screening method has its own approach to achieving goals such as accurate identification, quantification, or the discovery of new compounds.

3 Experimental

This chapter offers a detailed description of the experimental procedures carried out during the course of this work.

3.1 Materials and Equipment

All of the materials and equipment presented in *Table 3* were utilized in the various experimental methods.

Table 3: Materials and equipment used in the experiments

Method	**Materials**	**Equipment**
Homogenization	• Acetonitrile (Honeywell, CAS: 75-05-8) • Methanol (Sigma-Aldrich, CAS: 67-56-1) • DCM:*n*-Hexane (1:1, v/v)	• Mettler Toledo XPE205 Analytical Weight • 12 mL Kimax vials • IKA T10 Ultra-Turrax Homogenizer • IKA T10 S10N-8G Stainless steel knife
QuEChERS extraction	• Acetonitrile (Honeywell, CAS: 75-05-8) • NaCl (Sigma-Aldrich, CAS: 7647-14-5) • Na_2SO_4 (Sigma-Aldrich, CAS: 7757-82-6)	• Mettler Toledo XPE205 Analytical Weight • Vortex Minishaker MS2 • Centrifuge Eppendorf 5804 R • Glasspipettes
Solvent extraction for GPC/NH_2	• Dichloromethane (Rathburn, CAS: 75-09-2) • *n*-Hexane (Sigma-Aldrich, CAS: 110-54-3) • DCM:*n*-Hexane (1:1, v/v) • Na_2SO_4 (Sigma-Aldrich, CAS: 7757-82-6) • Surrogate internal standards – SIS-PAH – SIS-phenol • Recovery internal standards – RIS-PAH	• Mettler Toledo XPE205 Analytical Weight • Vortex Minishaker MS2 • GC vials • Volumetric syringes • Stuart SBH130D/3 Heat block N_2 evaporator • Glasspipettes • Bandelin Sonorex Ultrasonication bath • Centrifuge Eppendorf 5804 R
dSPE clean-up	• Z-Sep (Sigma-Aldrich, CAS: 55418-U) • $MgSO_4$ (VWR, CAS: 7487-88-9) • Acetonitrile (Honeywell, CAS: 75-05-8)	• Centrifuge Eppendorf 5804 R • Mettler Toledo XPE205 Analytical Weight • Vortex Minishaker MS2 • GC vials • 12 mL Kimax vials • Filter flask with glass connector • Glass fiber filter (0.25 µm) • Porcelain Buchner funnel • Glasspipettes

GPC clean-up	• Dichloromethane (Rathburn, CAS: 75-09-2) • Isopropanol (Honeywell, CAS: 67-63-0)	• HPLC-GPC instrumentation • Collection tubes for GPC
NH_2-fractionation	• Dichloromethane (Rathburn, CAS: 75-09-2) • *n*-Hexane (Sigma-Aldrich, CAS: 110-54-3)	• Agilent 1260 HPLC • 1260 FC-AS Fraction collector • Agilent 1200 series G1315 DAD
Analysis on GCxGC-HRMS	• Helium gas (6.0 purity)	• Agilent Technologies 7250 Accurate-Mass Q-ToF GC/MS • Zoex ZX2 cryogenic modulator • 7693 Autoampler

3.2 Collection of Samples

ToxiGen

ToxiGen is a collaborative project with University of Tromsø (UiT) where SINTEF is a project partner. UiT has conducted the exposure experiment following the method in *Strople et al.* [109]. The polar cod species were exposed to water-soluble fractions of crude oil during the spawning process. The organ samples were sent to SINTEF Ocean ready for sample preparation and analysis.

There is two main types of polar cod samples within this project, control samples and high exposed samples. Due to limited number of samples, only 7 control samples and 10 high exposed samples were analysed for this thesis. The control samples contain an environmentally relevant level of crude oil, while high samples contain a higher concentration for measure. An overall overview of the ToxiGen sample replicates are presented in *Table 4.* All the ToxiGen samples were prepared by DCM:Hex solvent extraction and NH_2-fractionation clean-up before analysis on GCxGC.

Table 4: Overview of ToxiGen sample replicates

Tissue type	**Sample type**	**Replicates**
Gonad	Control	2
	High	4
Liver	Control	3
	High	4
Brain	Control	2
	High	2
Spike	*N/A*	1
Blank	*N/A*	2

An overview of all samples form ToxiGen prepared in relevance of this thesis is presented in *Appendix A*.

AQUAvit

AQUAvit samples of fish feed and salmon were collected from Rataren (ACE), a fish farming site operated by a commercial farming operator, directly linked to SINTEF Ocean. When filleting the fish, two samples from the muscle were taken; one from the lipid-rich part and one from the leaner part. The feed was analysed to compare the findings in these samples with the fish muscle samples, to see how much of the potential toxins from the feed that transfers to the muscle tissue.

In total, 68 different AQUAvit samples was prepared and analysed by GCxGC, with 34 prepared using QuEChERS and 34 prepared using DCM:Hex/GPC. *Table 5* shows an overview of the AQUAvit sample replicates for both methods; QuEChERS and DCM:Hex/GPC.

Table 5: Overview of AQUAvit sample replicates from QuEChERS and DCM:Hex-GPC

Sample type	**Replicates**	
	QuEChERS	DCM:Hex/GPC
Salmon muscle tissue	20	20
Feed pellets	3	3
Spikes	3	3
Blanks	8	8

A detailed overview of all samples produced associated with the AQUAvit project is presented in *Appendix B*, with information regarding sample type and sample ID.

3.3 Pre-Experimental Procedures

A few preparatory steps were done before conducting experiments, aimed at ensuring precision, reproducibility, and safety in the research procedures:

- All of the glassware was baked at 450 °C for 3 hours. The only exception to this was volumetric equipment, such as graduated pipettes e.g.
- The chemicals used in powder form were baked following the same procedure as the glassware, except for the sorbent Z-Sep.

- The inside of the extractor hood was cleaned and underlay paper was changed each day.
- The glassware used were marked with their respective contents. All vials were at all times marked with the LIMS-ID, sample ID and variable specifications.
- Risk assessment of all laboratory work correlated with this thesis was completed and presented in *Appendix C*.

3.4 Method Testing

This chapter serves as an introduction, describing initial trial and error processes for various methods found in the preliminary literature search. It provides an account of what was tested before the final methods were established.

3.4.1 Homogenization Procedure

For the initial testing of the homogenization method, frozen store-bought salmon was used. The fish was thawed, and 1 g of salmon tissue was added to Kimax vials. The Ultra-Turrax system was set up on an appropriate stand in an extractor hood. Seven different compositions of solvent and salt were added to each of the vials. DCM:Hex (1:1, v/v) combined with Na_2SO_4 and ACN combined with Na_2SO_4 and NaCl, following the procedures. An overview of chemicals added in each salmon test sample, numbered from 1 to 7 is presented in *Table 6*.

Table 6: Sample composition of homogenization test samples using different amounts of solvent and salt

Salmon sample number	**Solvent and amount**	**Salt**
1	*N/A*	*N/A*
2	DCM:*n*-Hexane, 1 mL	*N/A*
3	DCM:*n*-Hexane, 2, mL	*N/A*
4	DCM:*n*-Hexane, 2 mL	Na_2SO_4
5	Acetonitrile, 1 mL	*N/A*
6	Acetonitrile, 2 mL	*N/A*
7	Acetonitrile, 2 mL	Na_2SO_4 and NaCl

Each sample was homogenized with the Ultra-Turrax at speed settings varying from 3 to 6, for approximately 1-1.5 minutes, depending on the composition of the sample.

The final method established after this method testing is described in *Chapter 3.5*.

3.4.2 QuEChERS Extraction and dSPE Clean-Up

For the initial testing of the entire QuEChERS method, including QuEChERS extraction and dSPE clean-up, frozen store-bought salmon was used. After homogenization with ACN, following the method in *Chapter 3.5*, different amounts of a salt mixture containing NaCl and Na_2SO_4, and 2 mL of ACN were added to the Kimax vials.

The low-temperature fat precipitation step was tested by storing the samples in the freezer for 4 hours and overnight, as well as without freezing to observe the effect.

In the dSPE clean-up step, the addition of the sorbent Z-Sep was tested for different amounts. The difference between baked and unbaked $MgSO_4$ was also evaluated. The contents of each sample, numbered from 1 to 5, are shown in *Table 7*.

Table 7: Salt and sorbent composition of five different dSPE test samples

Salmon sample number	**$MgSO_4$ (mg)**	**Z-Sep (mg)**
1	150 (Not baked)	50
2	150 (Baked)	50
3	150 (Baked)	100
4	150 (Baked)	150
5	150 (Baked)	200

The filtration step was initially tested by folding a GF/F filter in a funnel to observe if gravitational forces would filter the extract and collect a decent amount in a vial underneath. Subsequently, the same procedure was tested using a Buchner funnel connected to a vacuum filter flask.

The final method established for QuEChERS extraction and the dSPE clean-up is described in *Chapter 3.8.1* and *Chapter 3.8.2*, respectively.

3.5 Homogenization

Details about the chemicals and equipment used for the homogenization procedure are described in *Table 3*.

Samples from different tissues including muscle tissue from salmon, and liver-, gonad- and brain tissue from polar cod were weighed into 12 mL Kimax vials. All 40 salmon AQUAvit samples were approximately 1 g. The ToxiGen samples varied more in weight because of the density and size of the various organs. Six samples each of gonad, liver or brain from different polar cod individuals were pooled into one Kimax vial for each tissue, varying from under 1 g to 5 g. For homogenization, the Ultra-Turrax blade S10N-8G was used, as seen in *Figure 15*.

Salmon feed from AQUAvit was crushed with a mortar and pestle before samples of approximately 1 g were weighted into Kimax vials for further homogenization.

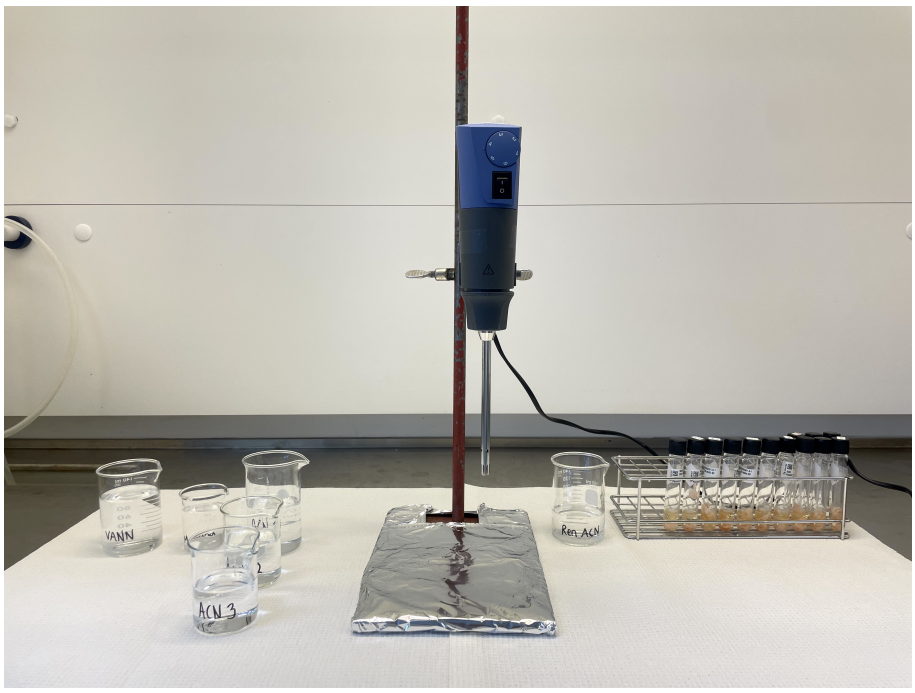


Figure 15: Homogenization setup including Utra-Turrax system, samples and solvent beakers

Before using the Ultra-Turrax, 2 mL of either ACN or DCM:*n*-Hexane (1:1, v/v), solvent was added to each vial using a graduated pipette. ACN was used for the QuEChERS method, while DCM:*n*-Hexane was used for solvent extraction and GPC clean-up. The samples were then homogenized at Ultra-Turrax speed settings ranging from 4 to 5 for about 1 minute, or until the tissue reached a consistent texture.

Between each sample, the Ultra-Turrax blade was washed. The blade was rinsed in three different beakers with the appropriate solvent until there was no more visible residue in the last beaker. It was then washed in methanol to dissolve the solvent, followed by rinsing in water to dissolve the methanol, before removing the blade from the Ultra-Turrax and thoroughly washing it with water. Before homogenizing the next sample, the blade was rinsed with the appropriate solvent.

3.6 Solvent Extraction for GPC/NH_2

Details about the chemicals and equipment used in the solvent extraction for GPC are described in *Table 3*.

A laboratory blank sample for each sample set was prepared in an empty 12 mL Kimax vial with a Teflon-lined cap. The blank was treated as a normal sample throughout the entire procedure.

After homogenization with DCM:*n*-Hexane, as described in *Chapter 3.5*, 100 µL of SIS was added to the samples. Then, approximately 30 mg of baked Na_2SO_4 was added to each vial, and the vials were vortexed. An additional 2 mL of DCM:*n*-Hexane was added, resulting in a total of 4 mL in the vials, and vortexed again.

The samples were placed in an ultrasonication bath at room temperature for 30 minutes. Afterward, the samples were vortexed again and centrifuged for 5 minutes at 2,000 rpm. The supernatant was transferred to a new preweighed Kimax vial using a baked glass pipette, ensuring that no residue was transferred with the sample.

Another 2 mL of DCM:*n*-Hexane was added to the vials containing the tissue and briefly vortexed. The samples were placed in ultrasonication bath again, followed by centrifuging, and transfer of supernatant. This step was repeated until a total of 8 mL of solvent had been added (4 + 2 + 2 mL).

The samples were then evaporated using a heat block evaporator at 40 °C and a gentle flow of N_2 until only 0.5 mL of the sample remained. Care was taken to ensure that the samples did not go dry. The evaporation setup is depicted in *Figure 16*.

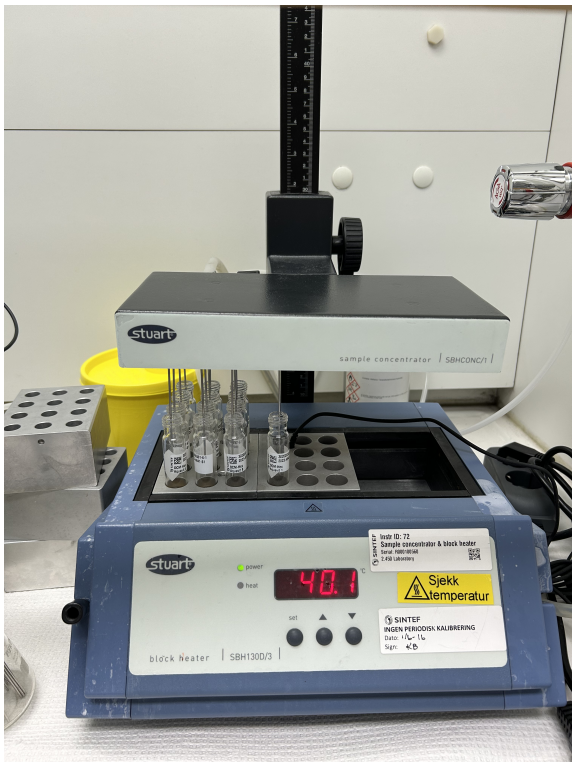


Figure 16: Evaporation setup using heat block at 40 °C and with N_2-gas stream for removal of solvent (DCM:Hex)

The evaporated samples were transferred into GC-vials using three portions of DCM:*n*-Hexane to ensure proper transfer of residual sample material in the Kimax vials. The volume was then adjusted to 1 mL using the heat block at 40 °C and a gentle flow of N_2 again. The samples were stored in the refrigerator until GPC/NH_2 clean-up.

3.7 Clean-up

3.7.1 GPC

Details about the chemicals and equipment used for the GPC clean-up are described in *Table 3*. This method was executed by laboratory engineers at SINTEF Ocean. A summary of the procedure is given in this chapter.

Samples were adjusted to room temperature and filtered if necessary before clean-up. 5 mL of hexane was added to collection vials and placed in the GPC collection chamber.

For the GPC procedure, the SOP given by SINTEF Ocean was followed provided in *Appendix D* of this thesis. The samples were collected during the elution time of 6-10 minutes (lipid fraction) and at 10-16 minutes (analytical fraction). Between each sample, a blank injection of DCM:*n*-Hexane (1:1, v/v) was run according to the SOP. Six GPC laboratory blank injections were run, with SIS-standards added, spaced out between the real samples.

After completing the clean-up, analyte fractions (10-16 minutes) were transferred to Kimax vials, rinsing out the collection vials using three portions of DCM:*n*-Hexane. The samples were evaporated to 0.5 mL using the Turbovap, set at 40 °C, and a gentle flow of N_2, ensuring they did not go dry. The evaporated sample was then transferred to GC vials, cleaning with three portions of solvent, and adjusting the volume to 0.4 mL using the evaporator. Finally, 100 µL RIS-PAH was added to each GC vial, so final sample volume was 0.5 mL.

The lipid fractions (6-10 minutes) were transferred to pre-weighted Kimax vials, before being fully evaporated. After evaporation the samples were set to cool down to room temperature, before weighing the vials containing the lipid content.

3.7.2 NH_2-Column Fractionation

Details about the equipment used in the NH_2-column fractionation are described in *Table 3*. This method was executed by laboratory engineers at SINTEF Ocean. A summary of the procedure is given in this chapter.

500 µL of each sample was injected into an Agilent 1260 HPLC. The system was equipped with a 1260 FC-AS fraction collector and an Agilent 1200 G1315 diode array detector (DAD). The system was set up with two Waters µBondapak NH_2 10 µm 3.9 mm x 300 mm columns in series.

The column was first eluted with *n*-Hexane for 8.8 minutes with a flow of 1 mL/min then followed by a flow of 2 mL/min for 0-30 minutes. The analyte fraction was collected between 8 and 30 minutes for further analysis. After this the column was backflushed with 2 mL/min DCM for 30-40 minutes. Test samples were used to identify cut-off for the fraction collection.

3.8 QuEChERS

3.8.1 QuEChERS Extraction

Details about the chemicals and equipment used in the QuEChERS procedure are described in *Table 3*.

As laboratory blank sample, 2 mL of ACN was added to Kimax vials and treated the same as the normal samples throughout the entire procedure.

After homogenization with ACN, as described in *Chapter 3.5*, the samples were fortified with 100 µL of SIS using a syringe. The syringe was cleaned with DCM before and after use, as well as between each sample.

A salt mixture, consisting of approximately 250 mg of NaCl and 1 g of Na_2SO_4, was added to each vial and vortexed. An additional 2 mL of ACN was added and vortexed again. The vials were then centrifuged at 2,000 rpm for 10 minutes.

The supernatant was transferred to new 12 mL Kimax vials using baked glass pipettes. A low-temperature fat precipitation step was then performed by storing the vials below -20 °C for a minimum of 4 hours, preferably overnight.

3.8.2 dSPE

Details about the chemicals and equipment used for the dSPE clean-up are described in *Table 3*.

After the extraction, described in *Chapter 3.8.1*, the sample supernatants were vaporized to 1 mL in the Kimax vials, using the heat block, set at 79 °C and a gentle flow of N_2. 150 mg of $MgSO_4$ and 200 mg of Z-Sep were added to each sample vial. Afterward the samples were vortexed for 30 seconds and shaken vigorously for 1 minute. Further the samples were centrifuged at 3,700 rpm for 15 minutes. The supernatant was then filtered through 0.25 µm GF/F in a Buchner funnel connected to a vacuum filter flask, into a Kimax vial. The setup is shown in *Figure 17*.

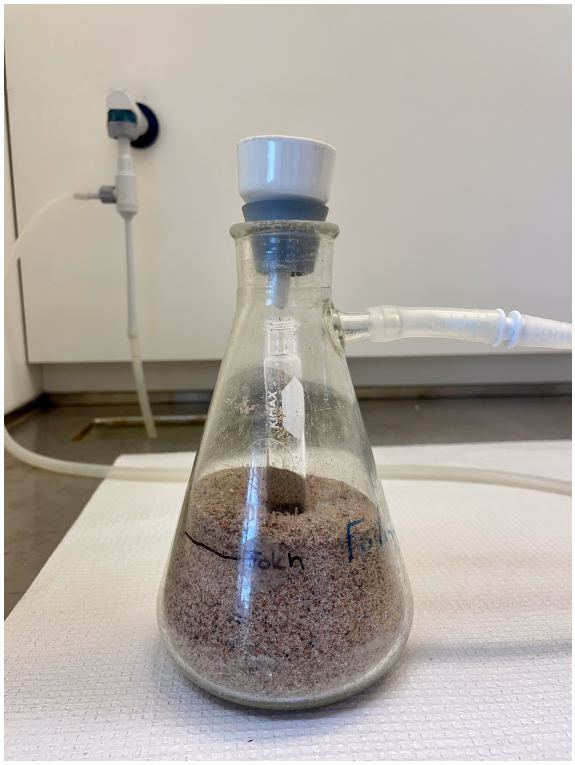


Figure 17: Filtration of supernatant from dSPE and QuEChERS into Kimax vial including Buchner funnel and vacuum filter flask

After filtration the samples were transferred to GC vials, evaporated down to 0.4 mL with the heat block set at 79 °C and a gentle flow of N_2 gas. 100 µL of RIS-PAH was added to each vial and vortexed to ensure completely homogeneous solution. The vials were placed in the refrigerator until analysis.

3.9 GC-MS

Details about the equipment used in the GC-MS experimental procedure are described in *Table 3*. This method was executed by laboratory engineers at SINTEF Ocean. A description of the procedure is given in this chapter.

GC-MS was used for screening of fractions from the GPC clean-up, and analysis of test samples cleaned with dSPE. The samples were analyzed using an Agilent 7890A GC coupled to an Agilent 5975C MS. 1 µL of the samples were injected at 300 °C in a pulsed split-less mode.

Separation was achieved using an Agilent HP-1MS UI column, featuring dimensions of 30 m in length, an internal diameter of 0.25 mm, and a film thickness of 0.25 µm.

Helium (purity 6.0) was used as the carrier gas, and was held at a constant flow of 1.1 mL/min. The temperature program set for the column oven was 42 °C for 1.6 minutes, followed by a temperature gradient of 10 °C per minute until 320 °C, where it was held constant for 20 minutes. The transfer line temperature was set to 300 °C, the quadrupole temperature was 150 °C and the ion source temperature was 230 °C. The ion source was operated in full scan mode with a mass-to-charge ratio range of 50 to 500 and an electron energy of 70 electron volts (eV).

3.10 GCxGC-HRMS

Details about the equipment used in the GCxGC-GToF-MS analysis are described in *Table 3*. This method was executed by researchers at SINTEF Ocean. A description of the procedure is given in this chapter.

Comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-MS) analyses were conducted using a setup consisting of an Agilent 7890B GC coupled with an Agilent 7250 quadrupole Time-of-Flight mass spectrometer, which was interfaced with a Zoex ZX2 cryogenic modulator.

The primary column was a Zebron ZB-1plus (30 m × 0.25 mm × 0.25 µm), followed by a secondary BPX50 column (1.0 m × 0.25 mm × 0.25 µm). The columns were interfaced by a 1 m × 0.25 mm deactivated fused silica modulation loop. The carrier gas was high purity helium, which was held at a constant flow of 1.1 mL/min.

1 µL of the samples were injected at 250 °C under splitless conditions. The oven temperature was programmed to start at 60 °C with a 1-minute hold, followed by a temperature gradient of 5 °C/min to reach 300 °C (maintained for 10 minutes). The hot jet temperature started at 70 °C, held for 1 minute, then ramped at a rate of 7 °C/min until reaching 360 °C (held for 10 minutes). The modulation time was set to 6 seconds with a pulse length of 350 ms.

The method included a transfer line temperature of 300 °C, ion source temperature at 200 °C, and quadrupole temperature maintained at 150 °C. The electron ionization (EI) source operated at 70 eV. The mass spectrometer was set to scan 50 times per second (Hz), covering a mass range from 50 to 650 m/z.

3.11 Processing and Interpretation of Data

The software *GC Image®* version 2.7 was used for visualisation, analyzing and interpretation of data for both projects.

3.11.1 ToxiGen Data

A template to match potentially detected compounds in the ToxiGen samples was made using OilMix and standard ToxMix, the contents of the ToxMix standard is presented in *Appendix E*. The two dimensional GC chromatograms of OilMix and standard ToxMix are shown in *Figure 18* and *Figure 19*.

Initially the retention time for n-alkanes and other compounds present in standard ToxMix were noted. The detected compounds were annotated in a template. The chromatogram of OilMix was then used to detect additional compounds, such as cyclic aromatics. The compounds were detected using the GC Image tool SIC-view (Single Ion Chromatogram) to filter compounds based on their known molecular mass. CAS numbers and retention times from analyses preformed by *Julie Metzger* at SINTEF Ocean [110] on known compounds were used to match them with potential compounds detected in the chromatogram by the MS-tool.

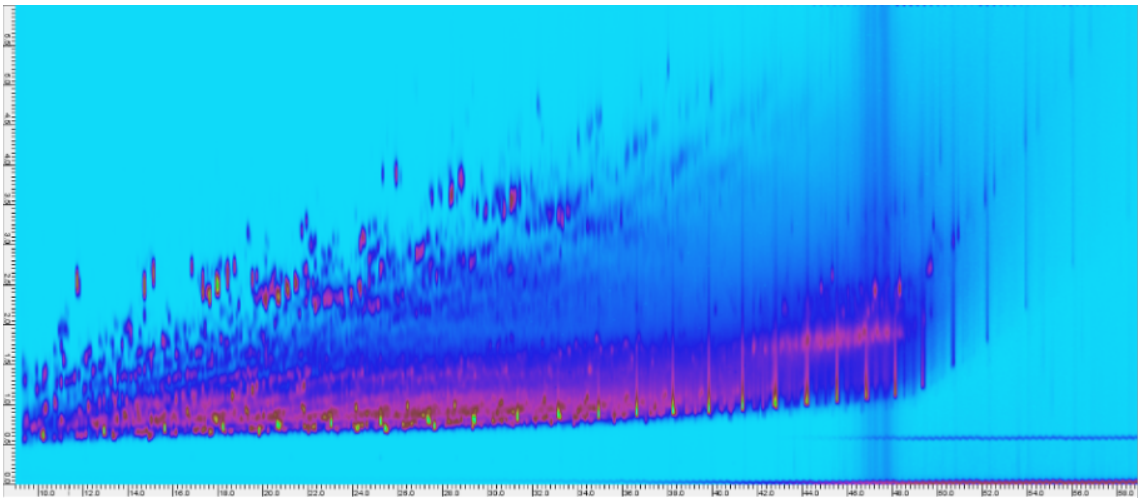


Figure 18: Total ion chromatogram of OilMix

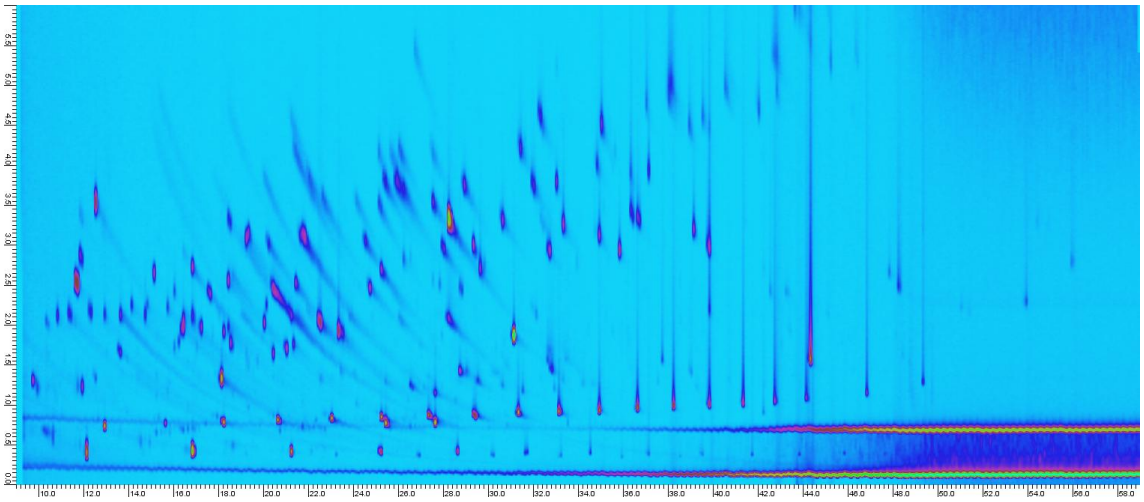


Figure 19: Total ion chromatogram of ToxMix standard

After the detection of compounds, the template was divided into five different compound groups. Saturates/non-cyclic, cyclic/monoaromatics, diaromatics, triaromatics and polyaromatics. For a better overview, each of the groups were given a distinct color. *Table 8* shows the groups and their given colors. To exclude column bleeding and miscellaneous system interference, two graphic groups in black were marked around those areas.

Table 8: Groups marked in GC-image template and their colors

Group	**Color**
Saturates/non-cyclic	Turqouise
Cyclic/monoaromatic	Dark blue
Diaromatics	Green
Triaromatics	Yellow
Polyaromatics	Purple
Column bleed and debris	Black

3.11.2 AQUAvit Data

Chromatogram data acquired from the AQUAvit extracts from the QuEChERS method and DCM:*n*-Hex/GPC workflow were compared qualitatively and evaluated on the number of peaks detected from the ToxMix standard that were also detected in the sample.

3.12 Quality Assurance

3.12.1 Calibration and Standardization

The analytical weight (Mettler Toledo XPE205) was calibrated daily using precision weights. This routine helped maintain the accuracy and reliability of the measurements, as even a slight deviation in balance calibration can lead to significant errors in sample weights.

Additionally, SINTEF Ocean have established procedures for the calibration of all laboratory equipment. This includes all analytical instruments, and other tools such as syringes and pipettes. Because of this, all equipment used in the analyses are operating at optimal performance levels and providing accurate results.

3.12.2 Internal Standards

Three different internal standards were used in the experiments to assure quality. SIS-PAH and SIS-Phenol as surrogate internal standards, and RIS-PAH as recovery internal standard. Mid-level concentrations of the standards were used to account for detector sensitivity and adequate concentration for detection. The contents of each of these standards are listed in *Table* 9.

Table 9: Chemical content of internal standards; SIS-PAH, SIS-Phenol and RIS:PAH

Compound	**Concentration [µg/mL]**
SIS-PAH	
Naphthalene-$d8$	2.522
Phenanthrene-$d10$	0.480
Perylene-$d12$	0.500
Chrysene-$d12$	0.508
SIS-Phenol	
Phenol-$d6$	25.334
p-Cresol-$d8$	1.042
4-n-Propylphenol-$d12$	1.374
RIS-PAH	
Acenaphtene-$d10$	1.000
Fluorene-$d10$	1.000

3.12.3 Reproducibility and Replicates

To ensure repoductibility in the findings, comprehensive analyses were conducted on a variety of samples. This included analysing 20 samples each using the different sample preparation methods for the AQUAvit samples. 20 samples were prepared using the modified QuEChERS method, and 20 samples were prepared using DCM:Hex (1:1, v/v) extraction followed by GPC clean-up. This resulted in a total of 40 salmon tissue samples. By doing this, any potential variations in results caused by factors such as sample preparation or instrument performance could be identified. Additionally, to minimize the potential for variability, 10 samples underwent sample preparation procedures simultaneously each day in the laboratory.

The feed samples from AQUAvit, and spike samples from both projects were analyzed in triplicates, meaning three replicates of each sample type were prepared and analysed on GCxGC-(HR)MS. This technique is common in quality assurance and allow for the assessment of variability within the sample set.

It is important to note that due to the limited availability of sample material from ToxiGen, only a few sets of pooled organs were analysed per method.

3.12.4 Quality Control Samples - Spike Samples

Spike samples were prepared to assess the accuracy and precision of the analytical methods and the recovery of the analytes. The samples consisted of the addition of 50 µL of SpikeMix 1 and SpikeMix 2. The contents of these are listed in *Appendix F*.

Each spike sample underwent the same preparation process as the real samples. However, the spike mix was added after clean-up, but before the final GCxGC analysis. This allows for evaluation of instrument performance in presence of sample matrix, using known quantities of SpikeMix 1 and SpikeMix 2.

An important factor is that the spike samples were handled in another laboratory than the real samples. This precautionary measure was taken to prevent any potential contamination of other samples during the addition of spike mixes.

3.12.5 Blank Samples

For each series of 10 samples from AQUAvit, 3 blank samples containing only solvent and internal standards were made. Resulting in 6 blank samples with DCM:*n*-Hexane and 6 with ACN - in total 12 blank samples.

For each series of feed and spike sample triplicates, one blank sample was prepared without containing tissue. Resulting in a total 4 blanks.

The blank samples were treated as normal samples. SIS and RIS standards were added to these aswell.

3.13 Contamination Control and Checks

3.13.1 Blank Checks

As mentioned in *Chapter 2.8.2*, blank samples acts as a baseline measurement, to identify and quantify background contamination or interference [99]. It is desirable that the analysis of the blank samples only includes peaks from compounds in the added standards.

The blank samples were continuously monitored for visible contamination or discrepancies. Any findings were written down in the laboratory journal and taken to account when looking at the results. Comparison of various blank samples and identification of peaks were preformed post GCxGC analysis. Chromatograms from the blank samples were thoroughly examined. The detailed outcomes are presented and discussed in *Chapter 4.9*.

3.13.2 Carryover Effects

To minimize potential carryover effects in the GCxGC analyses, a clean DCM sample was injected and run after every 10 samples. DCM effectively flush out any residual compounds from the injection system and column, and ensures that samples are analyzed without interference. Additionally, the DCM ensures that the instrument works correctly. If the clean DCM sample chromatogram is not normal, it might indicate something wrong with the instrumentation.

3.13.3 Instrument Cleaning and Maintenance

Cleaning and maintenance of instruments are important to ensure accurate and reliable data. At SINTEF Ocean, established protocols ensure the cleaning and maintenance. Instruments are cleaned between sample sets to prevent cross-contamination. Routine maintenance, such as minor adjustments and changing small parts, are preformed by laboratory engineers. Additionally, maintenance and control check-ups are carried out by technical engineers specifically trained and authorised to perform maintenance and repairs by the instrument manufacturer to fix any larger issues.

4 Results and Discussion

4.1 Preliminary Literature Search

The literature search for methods began with two articles; *NORMAN* [54] and *Hajeb et al.* [4] suggested by SINTEF supervisors. Both articles gave an overall overview of various sample preparation techniques. However, no method were directly established from these articles. Further literature search were based on the articles sources, especially *Table 4* in *Hajeb et al.*, which presented different methods with their original source [4].

Information about the analytical instrument to be used for the analysis was given in advance.

Additional methods for sample preparation were found searching various articles, based on a reference review: *Baduel et al.* [111], *Shapozhnikova et al.* [112], *Kalachova et al.* [113], *Dubocq et al.* [114], *Rebryk and Haglund* [115], *Forsberg et al.* [116], *Norli et al.* [77], and *Jensen et al.* [117]. An overview of the potential methods discovered in these papers, including the extraction, clean-up methods and target compounds, are presented in *Table 10*.

Table 10: Overview of potential methods for sample preparation from literature

Literature source	**Extraction**	**Clean-up**	**Target compounds**
Baduel et al. [111]	QuEChERS	dSPE: 150 mg $MgSO_4$, 50 mg Z-Sep	PCBs, PBDEs, PCPs, PAHs, OCPs, pesticides
Shapozhnikova et al. [112]	QuEChERS with MeCN extraction	dSPE + 150 mg $MgSO_4$ + C18 + PSA / Z-Sep / Z-Sep Plus	PCBs, PBDEs, PAHs
Kalachova et al. [113]	QuEChERS	10 mg BDE 37 + ^{13}C-CB 77 followed by dSPE	PCBs, PBDEs, PAHs
Dubocq et al. [114]	Ultrasonication extraction	Multilayer silica	PCBs, PBDEs, pesticides
Rebryk and Haglund [115]	*Jensen et al.* [117]	GPC	PCBs, PBDEs, OCPs
Forsberg et al. [116]	QuEChERS + modified QuEChERS	dSPE	PAHs
Norli et al. [77]	Matrix solid-phase dispersion	Matrix solid-phase dispersion	PCBs, OCPs

The selection of methods for this study was a result of collaborative discussions with supervisors, considering factors such as the nature of the target compounds and the availability of necessary chemicals and equipment.

After careful considerations, it was determined that the QuEChERS extraction method, coupled with the dSPE clean-up procedure outlined by *Baduel et al.* [111], would be most suitable. This decision was motivated by several reasons. Firstly, the desire to explore alternative methodologies not commonly utilized by SINTEF Ocean. Additionally, multiple research articles indicated promising results with the QuEChERS method, or described methods quite similar to this. Also this approach required minimal additional purchases for the method in the form of chemicals and equipment.

Additionally, the GPC clean-up method was used alongside a solvent extraction method developed at SINTEF Ocean. The decision to explore GPC clean-up was based on insights from the work of *Rebryk and Haglund* [115]. GPC is a commonly employed method at SINTEF Ocean, known for the methods efficacy in yielding good results, which influenced the choice.

The clean-up method following the QuEChERS extraction is a part of the total QuEChERS method is dispersive solid-phase extraction (dSPE). The selection of solvent and composition was guided by theoretical considerations aimed at optimizing results: a mixture of Z-Sep and $MgSO_4$ was chosen for testing because it resulted in the highest purity in the samples after clean-up as described by *Baduel et al.* [111].

4.2 Sources of Error

During the experimental procedures, several factors emerged as potential sources of error. These factors are described in this chapter to give context and insights before presenting the experimental results.

In the gonad control sample from ToxiGen, the septa of the GC vial accidentally dislodged and fell into the sample before the testing of NH_2-fractionation as a clean-up method. As a result of this, no usable data could be obtained from this particular sample.

During the GPC clean-up of the real samples, the machine malfunctioned due to excessive pressure and an overheated sampling arm. As a consequence, sample AQUAvit 12 (ID: 2023-4851-S1-S2) was inadvertently diluted with 500 µL of DCM:*n*-Hexane.

Another common error occurring in trace analyses, is contamination. Contamination in solvents or reagents can introduce background noise, affecting the sensitivity and reliability of the measurements. As a result of this, unwanted signals or interference in the GCxGC chromatograms might appear. In addition, contamination of the sample could occur from other objects in the laboratory during sample handling or preparation. Even minor contact with contaminants can affect the accuracy and reliability of the analysis.

Lastly, phenols are highly volatile compounds, and their evaporation can contribute to fluctuations in measurements, posing a challenge to obtaining consistent and reliable data. When the sample is contaminated with phenols, it can be challenging to effectively remove them.

4.3 Optimization of Homogenization Conditions

There was no clear description of the homogenization process found in the literature. *Baduel et al.* described the method to the following extent: "Fish muscle samples were homogenized using a kitchen blender and stored at -20 °C." [111]. In addition, the *NORMAN* article only states, "Samples need to be homogenized before extraction." [54]. This meant that the homogenization method needed to be tested and optimized without any prior information regarding the use of solvent and salt.

4.3.1 Sample Composition

It was observed that the homogenization differed between various tissue samples. Liver tissue was the easiest to homogenize, brain and muscle were more challenging, and gonad was the most challenging. During homogenization the gonad turned stiff and glue-like, and an additional 1 mL of solvent was added during homogenization, but it did not have a significant effect, due to the gonad and solvent having difficulty mixing.

There were notable differences in sample sizes observed among the ToxiGen tissues. Each sample consisted of 6 organs (liver, gonad or brain) from different polar cod individuals pooled together to ensure a sample size large enough for sample preparation, Specifically, the total amount of brain tissue weighed approximately 1 g, while the total gonad weight was approximately 5 g. Consequently, varying amounts of solvent were required for each sample type. Between the samples of same tissue type, there was observed some color differences. The liver samples color varied between slightly orange/pink and green, and the gonad tissue varied between light pink and light orange. Given the difference in colors present within the samples, it may have been advantageous to separate out the specimens with the most extreme color variations. By doing so, a more homogeneous sample set could have been obtained, which could potentially reduce variability in subsequent analyses.

Similarly, in the salmon muscle tissue obtained from the AQUAvit project, distinctions were noted. A comparison was made between the lipid-rich side of the muscle and the leaner part. *Figure 20* shows the difference between fatty tissue sample (left) and lean tissue sample (right). The lean tissue is comparably smaller and has a visible tougher structure. These differences were evident in the homogenization process. The lean sample demanded higher speed, with speed settings at approximately 5, and a longer duration, about 2-3 minutes, with the Ultra-Turrax due to tougher composition. As a result, lean samples might have accidentally underwent a more thorough homogenization and therefore extracted more analytes than from the fatty tissue.

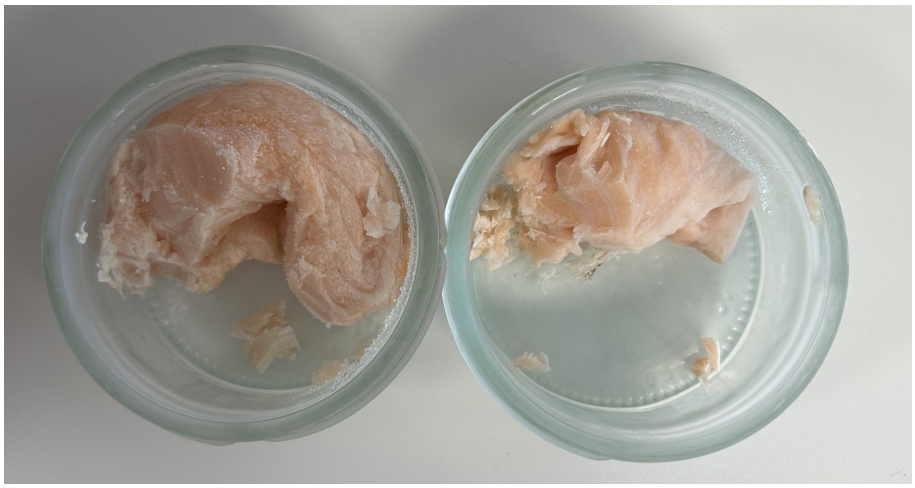


Figure 20: Difference between fatty (left) and lean salmon (right) tissue

Feed pellets for the AQUAvit project were analysed to compare them with the salmon samples. The feed samples went through the same sample preparations as the tissue. Due to the size of the pellets being to large for the Kimax vials, they were initially crushed into finer grains using a pestle and mortar. After pestling, the feed samples were homogenized with solvent using the Ultra-Turrax. The pellets were the easiest to homogenize across all sample types, because of the grainy composition and the high fat content. This made the homogenization process easy. *Figure 21* shows the fish feed pellets before and after pestling.

(a) Dry feed pellets (b) Feed after grinding with a pestle

Figure 21: Feed pellets before and after pestling

4.3.2 Solvent

The use of solvent during the homogenization process gave significantly different results. It became clear early on that the fish tissue needed addition of solvent before homogenization. The reason for this could be that solvents contributes to the break down of cellular membranes and structures within the cell, making homogenization more effective [57]. Before homogenization, about 2 mL or enough to cover the tissue, of solvent were added to the samples. Some tissue types demanded more solvent due to sample size and consistency.

The difference between the chosen solvents DCM:Hex (1:1, v/v) and ACN were made clear during initial testing. ACN made the homogenization easier, and DCM:Hex made the tissue lumpy and sticky. This stickiness resulted in tissue sticking to the knife blade and sample vial, which made the cleaning process between samples difficult. As a result, more sample waste occurred when using DCM:Hex as solvent.

Additionally, during the initial testing, another solvent was mistakenly used. DCM was utilized for homogenization of store-bought salmon samples. However, no differences between this approach and the use of DCM:Hex were noted.

4.3.3 Salt

Addition of salt before or after homogenization were initially tested on store-bought salmon samples. It was observed that addition of salt before homogenization did not have a beneficial effect; instead, it made the tissue harder to homogenize, prolonging the process. This could probably be due to salt coming in the way of the knife blade. These results were the same for both solvent-systems; ACN and DCM:Hex. However, there was a difference between the two salt-systems. In the ACN samples the salts NaCl and Na_2SO_4 were added, following the QuEChERS extraction method, and in the DCM:Hex samples, only Na_2SO_4 was added, according to the method for solvent extraction for GPC/NH_2. This resulted in more visible salt in the ACN samples, and therefore more difficulty in getting the samples fully homogeneous after salt addition.

This initial testing was only performed on the store-bought salmon, and consequently, there are no results regarding how the different tissue types reacted to the addition of salts before or after homogenization.

Additionally, there was no indication that adding salt before or after made any difference in the extraction process or the drying effect of the salt. After these observation the addition of salt after homogenization was used as the preferred method on the remaining samples.

4.4 Optimization of GPC and NH_2 Clean-Up Conditions

4.4.1 GPC Clean-Up

A series of test samples were run to determine the efficiency of GPC clean-up for the different tissues from ToxiGen and AQUAvit. The test samples included liver, gonad and brain from non-exposed polar cod for the ToxiGen project, and store-bought salmon fillet for the AQUAvit project. The aim of GPC clean-up is to separate the target analytes from the sample matrix. The purpose of this is to ensure minimal contribution from the biological matrix, which could compromise the analysis.

The UV-trace chromatograms presented in this chapter presents the samples monitored at six different wavelengths, where various analytes are absorbed at each wavelength due to their structure and composition [69]. The different wavelengths monitored for GPC clean-up are listed in *Table 11*, including the colors of the peaks at each wavelength in the chromatograms.

Table 11: Wavelengths monitored for GPC clean-up and their respective colors

Wavelength (nm)	**Peak color in GPC chromatograms**
200	Dark yellow
210	Blue
254	Red
280	Green
300	Purple
360	Pink

After testing, the decision was made to collect the analyte fraction between 10 and 16 minutes. In this time interval the analytes and the matrix were best separated. The analyte mixture (ID: 2024-1058) chromatogram depicted in *Figure 22* exhibits a distinct analyte peak between 10 and 14 minutes. Extending the interval to 16 minutes ensured that all analytes were collected.

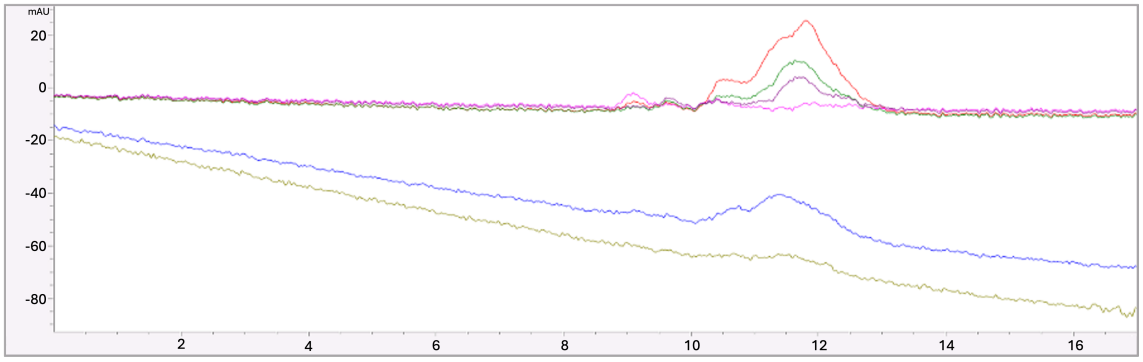


Figure 22: GPC UV-trace chromatogram of analyte mixture (ID: 2024-1058) monitored at different wavelengths with different peak colors (dark yellow: 200 nm, blue: 210 nm, red: 254 nm, green: 280 nm, purple: 300 nm, pink: 360 nm)

The same approach was employed to determine the time interval for the lipid fraction. Earlier tests runs by SINTEF Ocean have made it clear that the lipid fraction appear between 6 and 10 minutes. Lipids can be collected in this time interval, with minimal exclusion of analytes. The following sections focuses on the lipid fraction to ensure minimal contribution from fatty matrices, which would compromise the analyses of extract. The UV-trace chromatograms of the lipid fractions presented in this section is shown to demonstrate the applicability of the GPC method to separate the analytes from the sample matrix.

Liver Tissue from Polar Cod

The organ tissues from ToxiGen responded differently with GPC clean-up. The unexposed liver control sample (ID: 2024-672), yielded promising results. The peak is clear, appearing almost within the designated interval. However, the cutoff is slightly after 10 minutes, but not significantly enough to warrant discarding this method for liver samples. The GPC chromatogram of the liver sample is shown *Figure 23*.

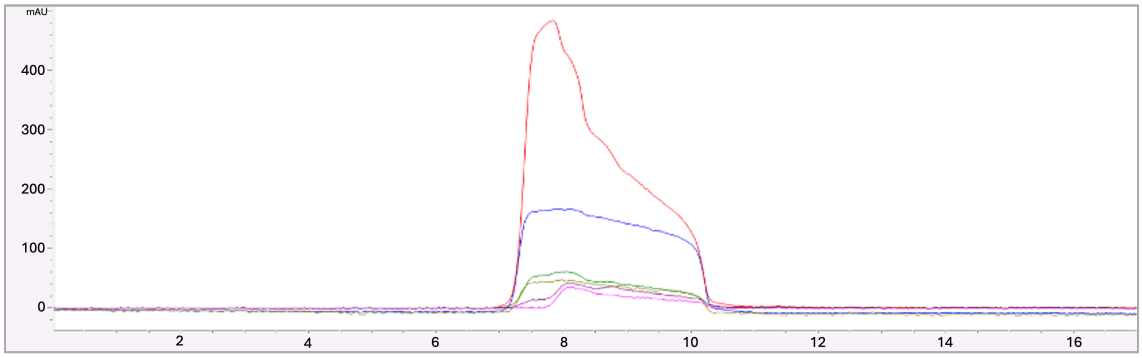


Figure 23: GPC UV-trace chromatogram of liver tissue (ID: 2024-672) monitored at different wavelengths with different peak colors (dark yellow: 200 nm, blue: 210 nm, red: 254 nm, green: 280 nm, purple: 300 nm, pink: 360 nm)

Gonad Tissue from Polar Cod

Further, the gonad sample (ID: 2024-668) chromatogram is depicted in *Figure 24*. This sample could possibly have been analysed further without clean-up due to the low lipid content in the gonads [118]. The peaks' heights on the vertical axis are remarkably low (approximately 35 mAU) compared to the height of the peaks in the liver samples (approximately 500 mAU). As a result of the low concentration, it is challenging to determine the exact location of the cutoff, but it may suggest a cutoff after the desirable 10 minute mark.

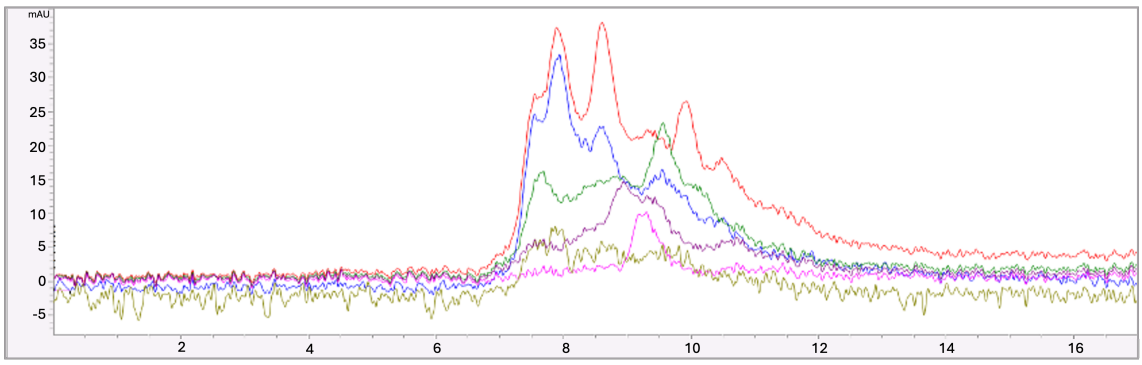


Figure 24: GPC UV-trace chromatogram of gonad tissue (ID: 2024-668) monitored at different wavelengths with different peak colors (dark yellow: 200 nm, blue: 210 nm, red: 254 nm, green: 280 nm, purple: 300 nm, pink: 360 nm)

Brain Tissue from Polar Cod

The last tissue from the ToxiGen project is brain tissue, with the brain control sample (ID: 2024-664) chromatogram depicted in *Figure 25*. The first peak looks promising due to the retention time, but the presence of a second unknown peak between 10-12 minutes suggests that GPC clean-up is not sufficient for this tissue.

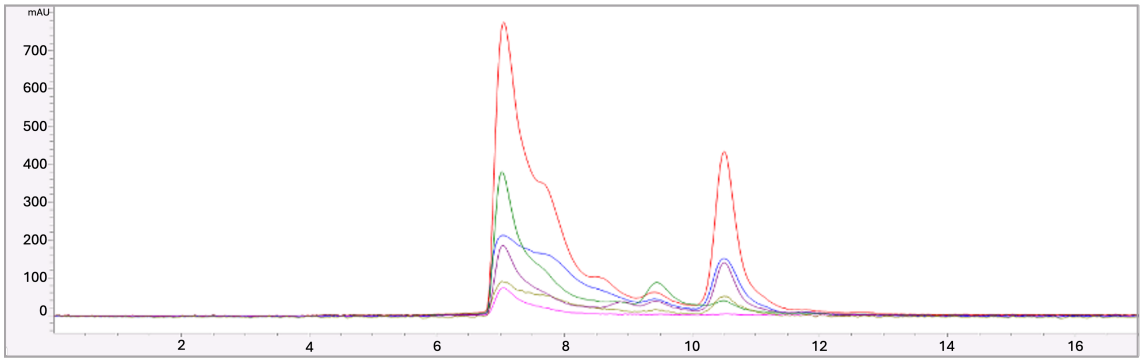


Figure 25: GPC UV-trace chromatogram of brain tissue (ID: 2024-664) monitored at different wavelengths with different peak colors (dark yellow: 200 nm, blue: 210 nm, red: 254 nm, green: 280 nm, purple: 300 nm, pink: 360 nm)

SINTEF Oil-mix

The oil utilized in the ToxiGen exposure experiment with polar cod test species consists of the water-soluble fraction of crude oil sourced from the Norwegian continental shelf. OilMix (ID: 2024-1062) is a standard consisting of oil components, and is representative for the same type of aromatic hydrocarbons that the ToxiGen project aims to study. The chromatogram depicted in *Figure 26* supports the argument that GPC clean-up is an ineffective method for the ToxiGen test samples. Within the lipid fraction for GPC clean-up (6-10 minutes), numerous oil components are observed, as evidenced by the peaks in the chromatogram.

If this clean-up method were employed for biotic samples, these oil components would be removed along with the lipids, leading to significant loss of target compounds. This challenge is caused by co-elution of long-chain alyklated benzenes and similar PAHs with the lipid fraction before the 10 minute mark (*Personal communication* [119]). Consequently, there's a risk of losing important analytes for the ToxiGen projects aims, due to the presence of oil components within the lipid fraction.

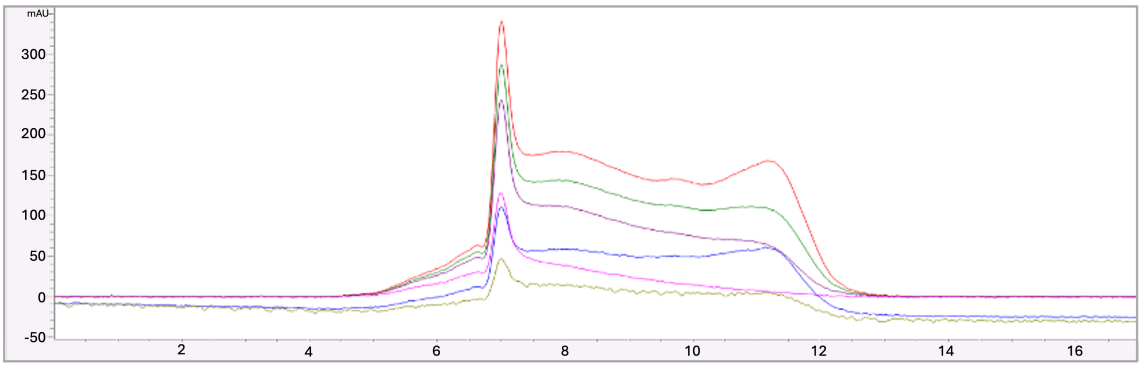


Figure 26: GPC UV-trace chromatogram of OilMix (ID: 2024-1062) monitored at different wavelengths with different peak colors (dark yellow: 200 nm, blue: 210 nm, red: 254 nm, green: 280 nm, purple: 300 nm, pink: 360 nm)

Salmon Tissue

The chromatogram from the GPC clean-up of salmon tissue is presented in *Figure 27*. There is a noticeable peak within the lipid fraction of the chromatogram. This peak serve as an indication of successful removal of interfering lipids, which leads to an enhancement of the analytical accuracy of target compounds within the sample. This suggest that GPC clean-up is effective for salmon tissue.

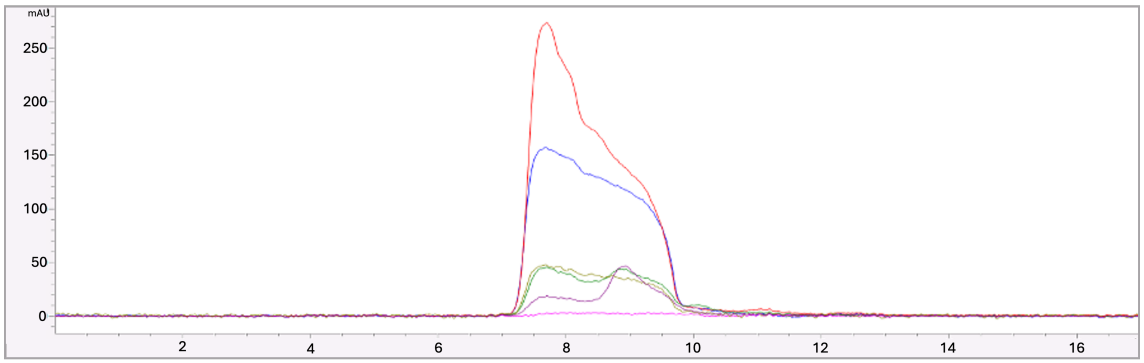


Figure 27: GPC UV-trace chromatogram of salmon sample monitored at different wavelengths with different peak colors (dark yellow: 200 nm, blue: 210 nm, red: 254 nm, green: 280 nm, purple: 300 nm, pink: 360 nm)

When examining the chromatograms of the ToxiGen tissue samples, it became evident that the GPC clean-up method did not achieve the desired outcomes. The decision to exclusively employ GPC clean-up for the AQUAvit samples was driven by the methods performance relative to the specific requirements of each sample type.

In the fractions collected after GPC clean-up, color was observed in both the analyte fractions and lipid fraction of feed samples. This may be due to incomplete removal of lipids within the analyte fraction. *Figure 28* shows the collected fractions from sample AQUAvit Feed 1 (ID: 2024-1573-S1), where the first fraction pertains to lipids, while the subsequent two corresponds to the analyte fractions.

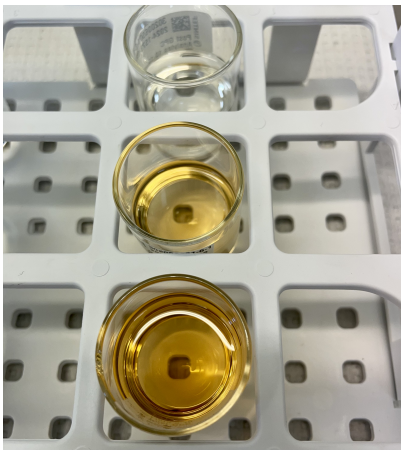


Figure 28: GPC lipid fraction and analyte fractions collected from AQUAvit Feed sample 1 (ID: 2024-1573-S1)

4.4.2 NH_2-Column Fractionation

To assess the efficiency of NH_2-fractionation for sample clean-up a test series of samples were used. For ToxiGen, the test samples consisted of control and exposed liver, brain and gonad, and for AQUAvit the test sample was store-bought salmon. Additionally, spike samples, containing OilMix and analyte mixture was tested.

Each sample is monitored at seven different wavelengths during NH_2-fractionation process. These wavelengths and their respective peak color in the chromatograms are summarized in *Table 12*.

Table 12: Wavelengths monitored for NH_2 fractionation with corresponding peak colour

Wavelength (nm)	**Peak colour in NH_2 chromatograms**
200	Blue
210	Dark yellow
230	Pink
254	Red
280	Green
300	Dark green
360	Purple

Between 8 to 30 minutes, only non-polar analytes fractionated through the column, as depicted in the analyte mixture chromatogram (ID: 2024-1060) in *Figure 29*. The polar compounds eluated at 35 minutes, indicated by the peak. The distinct cutoffs observed at 8.8 minutes is due to the *n*-Hexane flow rate increasing, and the cutoff at 35 minutes is due to transition of the mobile phase from *n*-Hexane to DCM, followed by backflushing for 30 to 40 minutes. These cutoffs are present in all of the chromatograms since this procedure is standardized for all samples.

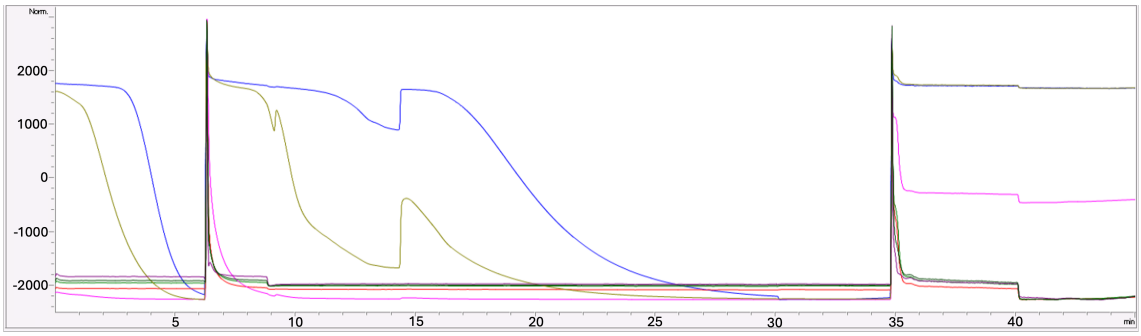


Figure 29: NH_2 UV-trace chromatogram of analyte mixture (ID: 2024-1060) monitored at different wavelengths with different peak colors (blue: 200 nm, dark yellow: 210 nm, pink: 230 nm, red: 254 nm, green: 280 nm, dark green: 300 nm, purple: 360 nm)

ToxiGen - Control Liver Tissue from Polar Cod

GC-MS analysis of the fractions revealed that aromatic hydrocarbons from oil eluted between 8 and 30 minutes. Non-polar compounds from the spike mix also eluted within this time interval, while the more polar compounds from the spike mix eluted at 35 minutes. ToxiGen aims to study the non-polar analytes to see the effect of oil exposure and uptake in polar cod. Therefore, NH_2-column fractionation was efficient for clean-up of ToxiGen samples. The chromatogram is depicted in *Figure 30*,

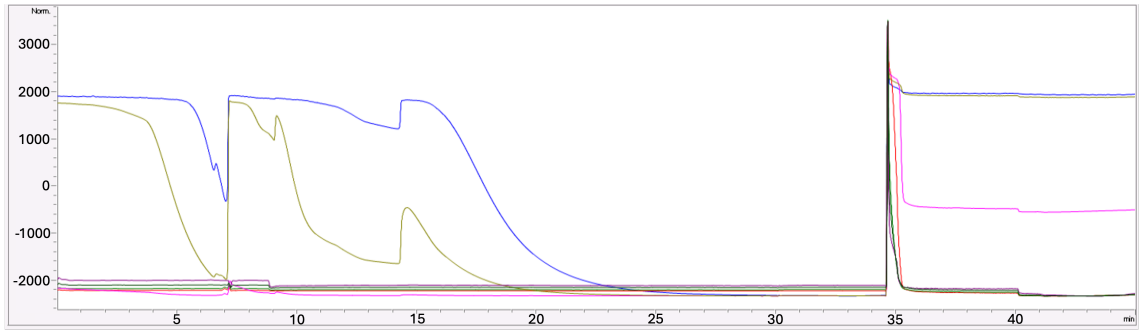


Figure 30: NH_2 UV-trace chromatogram liver control sample monitored at different wavelengths with different peak colors (blue: 200 nm, dark yellow: 210 nm, pink: 230 nm, red: 254 nm, green: 280 nm, dark green: 300 nm, purple: 360 nm)

AQUAvit - Samlon Muscle Tissue

The AQUAvit salmon tissue sample chromatogram from NH_2 fractionation is presented in *Figure 31*. By first glance, this chromatogram looks almost identical to the liver control in *Figure 30*. However, this clean-up method is not sufficient for the salmon samples due to the different aims of the AQUAvit and Toxigen projects. ToxiGen aims to separate and analyse non-polar hydrocarbon from oil, and AQUAvit aims for both non-polar and semi-polar compounds. The semi-polar compounds are not present within the analyte interval (8-30 minutes), and therefore lost during this clean-up procedure. Due to the properties of the amin-column [66], NH_2-fractionation was not empolyed as a clean-up method for AQUAvit samples.

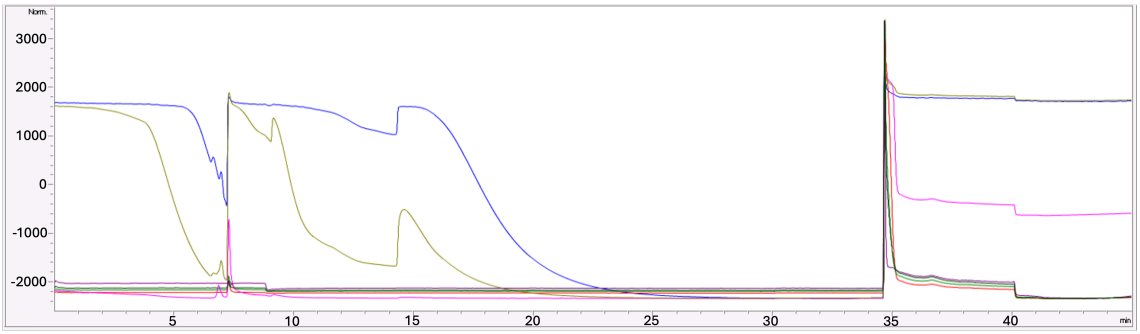


Figure 31: NH_2 UV-trace chromatogram of salmon sample monitored at different wavelengths with different peak colors (blue: 200 nm, dark yellow: 210 nm, pink: 230 nm, red: 254 nm, green: 280 nm, dark green: 300 nm, purple: 360 nm)

For each of the tissues, biogenic substances exclusively eluted after 30 minutes, indicating that the method was highly effective in removing these, but still unsuitable for AQUAvit due to the interest in more polar analytes as well. It was determined that the use of NH_2-column fractionation for sample clean-up was only efficient for ToxiGen polar cod samples. These findings emphasize the necessity of aligning analytical methodologies with project-specific goals.

4.5 Optimization of QuEChERS Method

4.5.1 Extraction

QuEChERS extraction was optimized based on the amount of salts. In the original method plan developed by *Baduel et al.* [111], 1 g of Na_2SO_4 and 4 g of NaCl per 10 gram fish tissue were to be added [111]. Due to modification in sample size from 10 g to 1 g, the amount of salt was consequently modified. It was determined to use 200 mg Na_2SO_4 and 1 g NaCl for 1 g tissue samples.

The centrifugation step was extended from 10 to 15 minutes due to visible particles in the supernatant. Following this modification, all visible particles were precipitated. The extraction process could be optimized even more by repeating the extraction step up to three times, and test the use of ultrasonication bath in between the extraction steps.

4.5.2 dSPE Clean-Up

During the initial method testing, different amounts of the sorbent Z-Sep was added to the samples during clean-up to detect potential differences in results. Four amounts ranging from 50 mg to 200 mg, with a 50 mg interval, were added during dSPE clean-up, using store-bought salmon. Since salmon was the test species here, the relevant project for this clean-up method is AQUAvit. The samples were analysed by GC-MS in full scan. The peaks of six different compounds were identified and peak volume were integrated, with one of them being contamination from the Z-Sep.

Figure 32 shows an overlay of the total ion chromatograms for 50 mg (green) and 200 mg (blue) Z-Sep with niacinamide, tricane, contamination, C18 unsaturated fatty acids, 2-oleoyl qlycerol and cholesterol peaks to illustrate the differences in peak area.

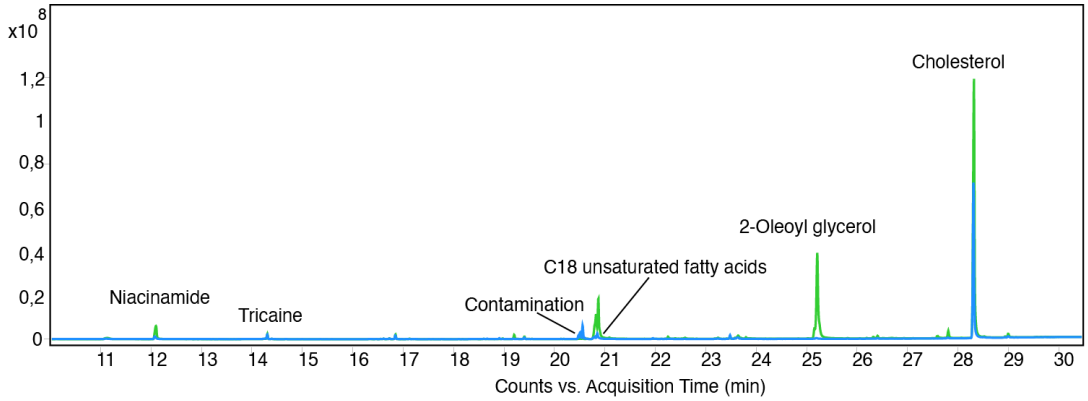


Figure 32: Chromatogram of 50 mg (green) and 200 mg (blue) added Z-Sep, with named peaks: niacinamide, tricaine, C18 unsaturated fatty acids, contamination, 2-oleoyl glycerol and cholesterol

The difference in peak area between 50 mg and 200 mg added Z-Sep is discussed in this chapter. *Table* 13 shows the integrated peak area and percentage change of the six identified compounds. The most significant reduction happened to 2-oleyl glycerol and the most significant increase was the contamination from Z-Sep.

Table 13: Peak area difference between 50 mg and 200 mg added Z-Sep for cholesterol, 2-Oleoyl glycerol, Niacinamide, Tricane, C18 unsaturated fatty acids and contamination from Z-Sep

Compound	**Integrated peak area**		**Difference [%]**
	50 mg Z-Sep	**200 mg Z-Sep**	
Cholesterol	238963214	121529252	−49.1
2-Oleoyl Glycerol	110031046	1104049	−99.0
Niacinamide	15802787	3018599	−80.9
Tricaine	2912329	2669830	+9.1
C18 unsaturated fatty acids	71364335	7889511	−88.9
Contamination	1012860	15700490	+93.6

An interesting peak to compare the difference between 50 mg and 200 mg Z-Sep is cholesterol. This sterol has a retention time of around 28 minutes and play a unique role in animal cells, where it is an important component of cell membranes and a precursor of steroid hormones and vitamin D [120, 121]. Due to the significance of cholesterol, cells have developed intricate systems to effectively control the levels

and distribution of sterols within the cells [120]. This poses a challenge for the removal of cholesterol compared to other lipids. The addition of an additional 150 mg of Z-Sep resulted in a 49.1 % decrease in peak area of cholesterol observed in *Figure 33*. This finding underscores the efficiency of Z-Sep in removing lipids and suggests a great potential as a promising clean-up approach for biotic tissues.

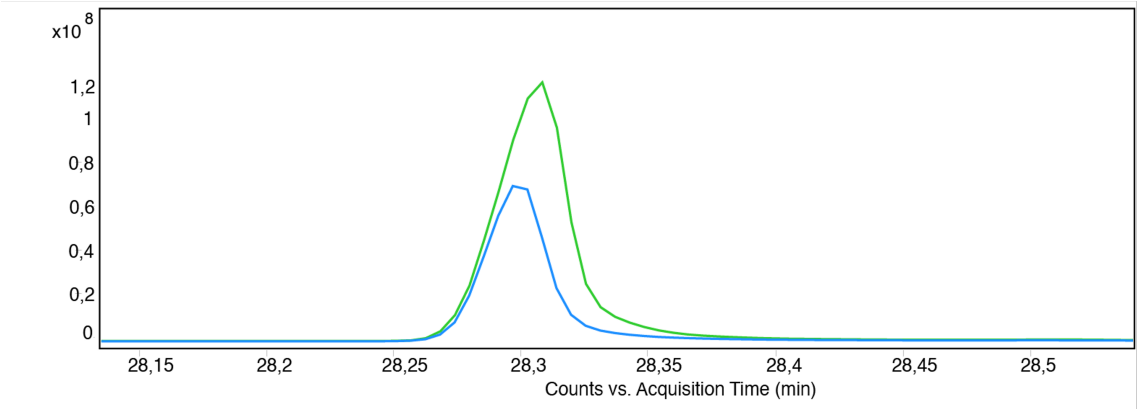


Figure 33: Total ion chromatogram of salmon tissue. Zoom of cholesterol peak at 50mg (green) and 200 mg (blue) Z-Sep

The niacinamide peak was significantly reduced after the addition of 200 mg Z-Sep compared to 50 mg. Niacinamide, also known as vitamin B3, is commonly used in skincare and other personal care products [122]. It can derive from an abundance of sources when detected in fish tissue. *Figure 34* shows a comparison of the niacinamide peaks at 50 mg and 200 mg Z-Sep. The peak area at 200 mg Z-Sep was reduced by 80.9 % compared to the peak area of 50 mg Z-Sep .

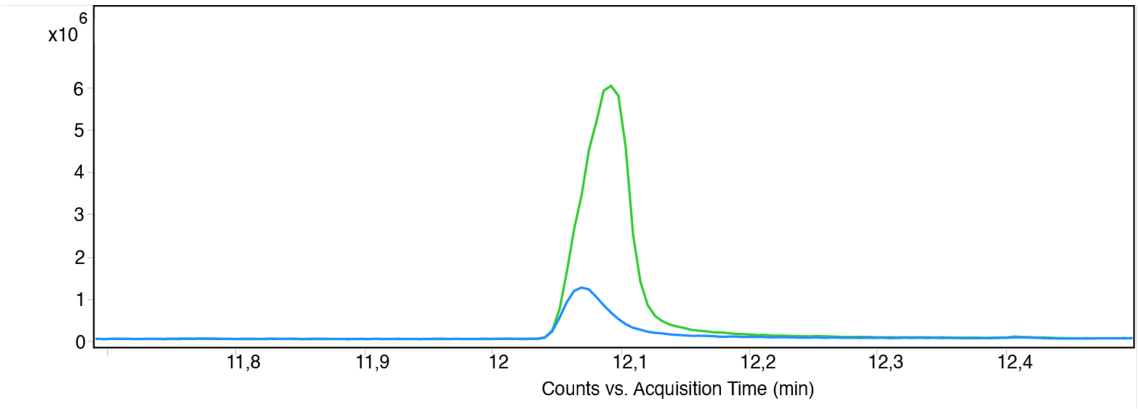


Figure 34: Total ion chromatogram of salmon tissue. Zoom of niacinamide peak at 50mg (green) and 200 mg (blue) Z-Sep

The peaks identified as 2-Oleoyl Glycerol also differed significantly from 50 mg to 200 mg of Z-Sep. It was observed that at a concentration of 200 mg Z-Sep, indicated by the blue line in the chromatogram shown in *Figure 35*, the peak corresponding to this compound was nearly eliminated. The line appears almost indistinguishable from the baseline, compared to the green peak (50 mg). The peak had a total area reduction of 99.0 %.

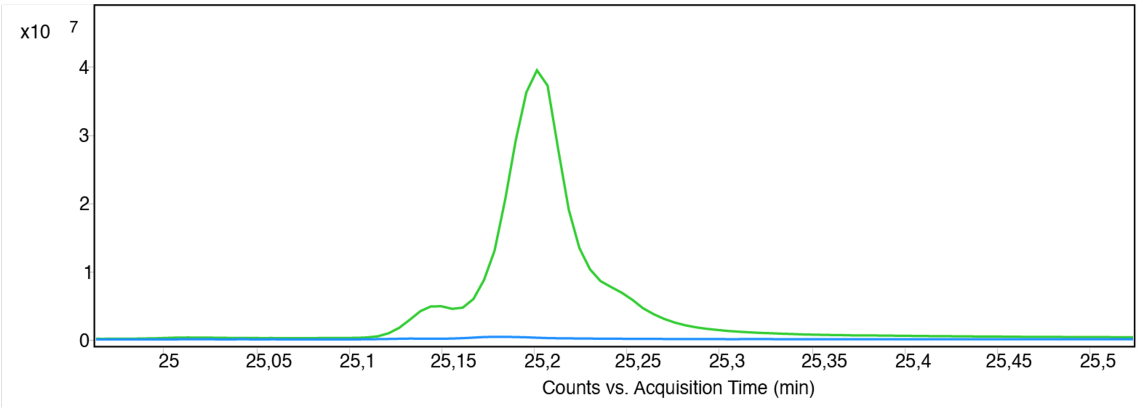


Figure 35: Total ion chromatogram of salmon tissue. Zoom of 2-oleoyl glycerol peak at 50 mg (green) and 200 mg (blue) Z-Sep

Another detected compound in the samples was tricaine, commonly used as a sedative or anesthetic when handling fish [48]. It was observed a slight increase in this compound when the amount of Z-Sep was increased from 50 mg to 200 mg, as shown in *Figure 36*. The total increase of peak area was 9.1 %. It appears that Z-Sep do not have an effect in the removal of tricaine. The different amounts in the sample can come from different amounts of tricane used when handling fish.

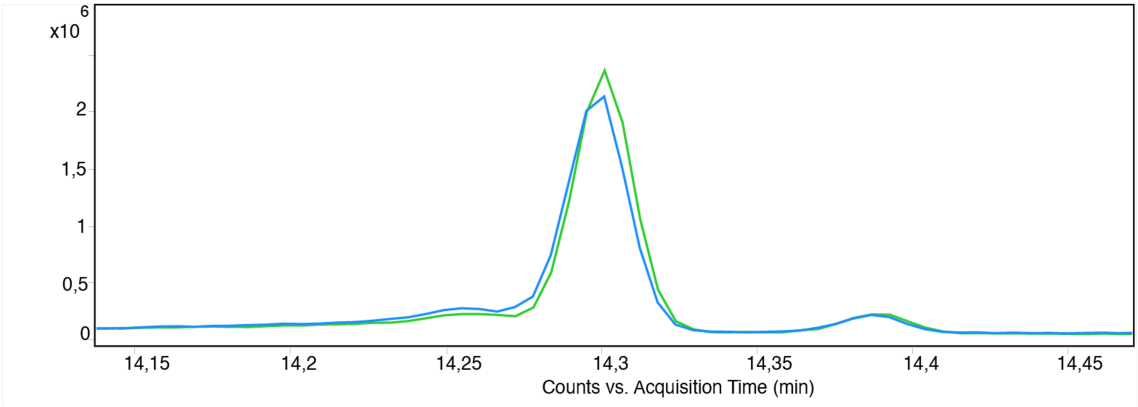


Figure 36: Total ion chromatogram of salmon tissue. Zoom of tricane peak at 50mg (green) and 200 mg (blue) Z-Sep

From the analysis on GC-MS it was observed that the percentage of C18 unsaturated fatty acids was significantly reduced by adding additional 150 mg of Z-Sep, as indicated by the peaks towards the right side in the chromatogram in *Figure 37*. The reduction of peak area was 88.9 %. The reduction of unsaturated fatty acids is helpful for further analysis on comprehensive GC to reduce noise and minimise lipid deposits, and accurately detect compounds [123].

However, an increase of Z-Sep in the samples led to an increase in contamination, as indicated by peaks towards the left in the chromatogram in *Figure 37*. Total peak area of the contamination increased by 93.6 % after addition of 200 mg Z-Sep. The presence of this contamination raises concerns regarding the potential impact on subsequent results.

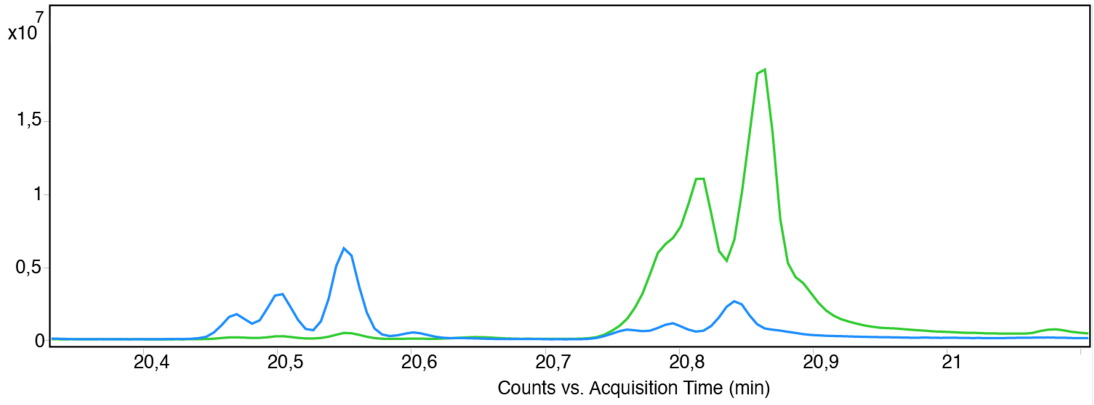


Figure 37: Total ion chromatogram of salmon tissue. Zoom of C18 unsaturated fatty acid peak (right) and contamination peaks (left) at 50mg (green) and 200 mg (blue) Z-Sep

The results of this method testing reveal the notable efficacy of Z-Sep in lipid removal from biological samples. The comparison between 50 mg and 200 mg of Z-Sep showed substantial improvements in lipid removal with higher sorbent dosage. This suggests that increasing the amount of Z-Sep enhances the capacity to absorb lipids, and therefore better purification of the sample matrix. Z-Sep has shown great efficiency as a clean-up sorbent for biota samples in other studies as well, in *Han et al.* [124] and *Rejczak et al.* [125] Z-Sep removed co-extracting lipids and adsorbed the majority of fatty non-polar interference. In addition, another version of Z-Sep is utilized in many studies called Z-Sep+, which is a hybrid material made of zirconia and C18. *Oliveira et al.* [126] had great results while employing Z-Sep+ to remove cholesterol and other fatty acids. However, this enhancement comes with a downside of increased contamination levels in the salmon samples, which warrants careful consideration in dosage selection. Surprisingly, none of the articles [124–126] described any type of contamination occurring due to the addition of Z-Sep.

Despite this contamination present in the samples, the observed reduction in areas of peaks, such as 2-oleoyl glycerol and cholesterol, underscores the potential of Z-Sep to enhance analytical sensitivity by minimizing matrix interference. This is particularly crucial in trace analysis where accurate quantification is important. Even though the contamination is present in the samples, the effect was seen as negligible in comparison to the enhanced efficiency in sample clean-up.

The decision around the amount of Z-Sep was based on these findings, and 200 mg was the final amount used for the clean-up of real samples.

4.6 Comparison of Extraction Methods

The two extraction methods varied in several aspects. The time used for each of the extraction methods are equal, due to the long step of freeze precipitation used in the QuEChERS extraction. During the process it was observed that the use of an ultrasonication bath dissolved the tissue further into the solvent, possibly extracting more target compounds. The solvent extraction in QuEChERS consisted of only one step.

The effect of ultrasonic energy makes several processes happen in a sample [127]. These include agitation, shock waves, pressure, vibration, cavitation, compression and the formation of radicals [127]. The energy from these processes causes compression and expansion. This phenomena causes cycles of air bubbles to form and collapse, and these mechanisms allows for solvent penetration further into the matrix. This facilitates for an enhanced mass transfer of analytes into the solvent, and makes it more likely that solvent extraction combined with ultrasonication can extract more target analytes.

The initial method testing for solvent extraction was preformed for 6 salmon samples using ACN, and 6 salmon samples using DCM:Hex. The sample extracts were fully evaporated and weighed. Extract weight percentage was calculated as a fraction of the salmon sample wet weight, and presented in *Appendix G*. The average extract weight percentage and standard deviation for ACN samples were 0.9($\pm$0.2) % and for DCM:*n*-Hexane samples were 16.6($\pm$3.1) %. The 15 % increase in weight percentage of the extract from the use of DCM:Hex compared to ACN, indicates differences in their extraction efficiencies. Using DCM:Hex as the solvent appears to extract a larger quantity of biogenic material from salmon samples compared to using ACN. This is potentially due to the differences in extraction methods or the solvents

chemical properties. These results also indicates that DCM:Hex samples need more rigorous clean-up step to remove unwanted compounds, such as GPC. With an extract weight percentage at only 0.9(±0.2) % for ACN samples suggest that certain analytes may not have been extracted from the sample.

In addition to the efficiency of analyte extraction, the differences between DCM:Hex solvent extraction and QuEChERS extraction extend to impact on the samples integrity. DCM:Hex solvent extraction, with multiple steps involving centrifugation and ultrasonication, may dispose the samples to greater stress, which can potentially lead to alteration of the target analytes or matrix. The number of steps also increase the risk of loosing analytes on the way. On the other hand, QuEChERS extraction, have a milder approach, an may preserve the samples integrity, particularly for sensitive analytes of matrices prone to degradation.

The choice between these extraction methods may also rely on favors such as cost and environmental considerations. Solvent extraction typically requires large volumes of organic solvents, which can be costly and raise environmental concerns regarding disposal. In contrast, QuEChERS extraction uses smaller amounts of solvent and generates less waste. This approach align better with green chemistry principles [128] and can potentially reduce overall costs.

4.7 Comparison of Clean-Up Methods

4.7.1 Comparison of GPC Clean-Up and NH_2 Fractionation

Upon examining the chromatograms from both GPC clean-up and NH_2 fractionation, as presented in *Chapter 4.4*, several intriguing findings emerge.

The primary objective is to determine the most suitable method for the removal of lipids and other impurities, and target compound separation across various tissue types. The two relevant projects, AQUAvit and ToxiGen, involves different species and tissues, making it an opportunity to identify different methods for each project. Due to the different aims of the projects, different methods were selected that aligned with the aims. ToxiGen aims to detect non-polar hydrocarbons from oil, and AQUAvit aims to identify semi and non-polar compounds from fish feed.

The results revealed distinct performance differences across tissue types for both techniques. Initially, GPC appeared promising as a clean-up method, but the effectiveness varied significantly based on the tissue that was analysed. For the brain, gonad and liver samples from the ToxiGen projects, GPC gave unsatisfactory results. In contrast, NH_2 fractionation demonstrated remarkable efficiency for these samples. For the AQUAvit samples, consisting of salmon muscle samples, GPC clean-up became the preferred choice due to the effectiveness of removing lipids and unwanted compounds.

NH_2 fractionation operates on the principle of separating compounds based on the properties of the stationary phase [66]. For separation of nonploar analytes like PAHs and pesticides, which are target compounds for both projects, the polar components in the sample binds to the amino groups in the NH_2-column [66]. Resulting in only the nonpolar components separating efficiently. This method proves efficient in removing lipids (which are polar) in samples with high lipid content and complex matrices. Given the ability of NH_2 fractionation clean-up to separate out nonpolar compounds, the results align with the aim of the ToxiGen project, but is not suitable for the AQUAvit project, which is also interested in isolating semi-polar analytes.

On the other hand, GPC is based on the principle of size separation, and not based on the polarities of the compounds [64]. Consequently, GPC is efficient at removing large interference's such as lipids. Especially in samples with less complex matrices with low lipid content. Based on this, GPC will also separate the semi-polar compounds as well as the nonpolar. This aligns with the AQUAvit project.

This trial underscores the importance of tailoring sample preparation methods to the specific sample composition.

4.7.2 Comparison of dSPE and GPC Clean-Up Procedures

To compare clean-up methods, time consumption and cost are relevant factors. The GPC instrument is expensive, and has a long clean-up time per sample. QuEChERS on the other hand, is quicker, containing only a straightforward manual filtration clean-up. The equipment used for the dSPE clean-up is standard laboratory equipment consisting of a funnel, vacuum flask and collection vials, with an additional purchase of sorbent Z-Sep.

A thesis comparing clean-up methods for toxicologic testing of animal liver tissue for analysis on GC-MS done by *J. Noguera* in 2021, had results that leaned towards the QuEChERS and dSPE method compared to GPC clean-up method [129]. Both in terms of cost and time efficiency, as well as the success in removing lipids. The results from the thesis of *Noguera* observed a larger cholesterol peak after GPC clean-up compared to the peak after dSPE clean-up. These findings suggest that dSPE may be the preferred method, offering a balance of cost-effectiveness and lipid removal efficiency [129].

However, if dSPE and GPC is compared with regards to the extraction methods preformed before clean-up. GPC solvent extraction has the opportunity to extract more analytes by employing the strong organic solvents: DCM:Hex, and subsequently using GPC for sample clean-up.

Despite the interesting findings from *Noguera* [129], the efficiency in removing lipids and unwanted compounds while retaining analytes in fish tissue is best assessed through a review of chromatograms obtained from each method. Therefore, the final selection of the most suitable methods for various tissues, irrespective of cost and time consumption, will be based on the GCxGC results, presented and discussed further in *Chapter 4.8*.

4.8 GCxGC Data

In this chapter, total ion chromatograms obtained from GCxGC analysis of ToxiGen and AQUAvit samples are presented. The distribution of compounds appear in two dimensions. The first dimensional separation occurs along the horizontal axis and separates components according to boiling point. Components with low boiling point will elute first, and therefore appear to the left in the chromatogram. The second dimensional separation appears vertically, and separates components mainly based on polarity. The least polar components eluates first.

In *Figure 38* the total ion chromatogram of OilMix are shown, to illustrate an example.

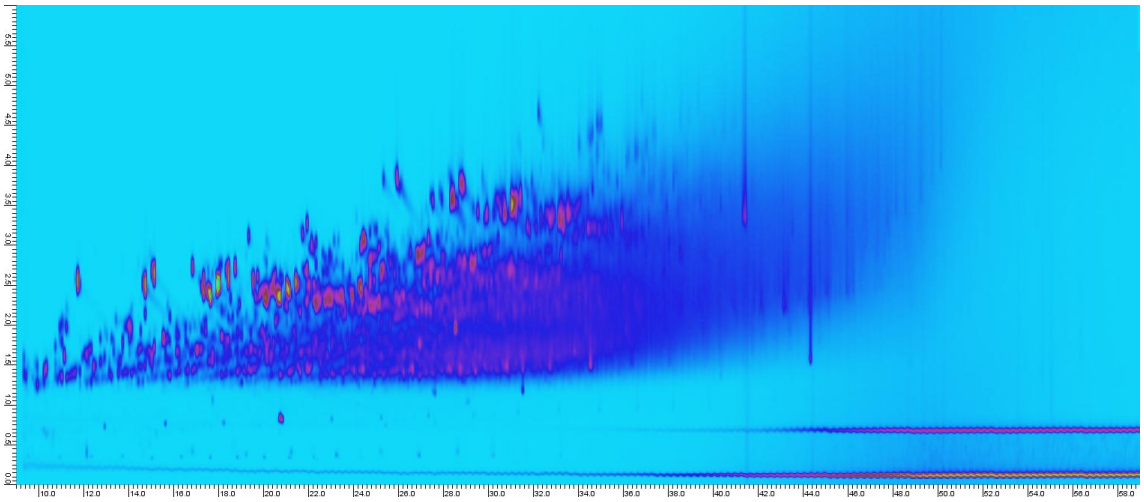


Figure 38: Example of a two dimensional GC chromatogram, using OilMix to illustrate

The two horizontal lines at the bottom of the chromatogram are column bleeding and shows up in all of the total ion chromatograms. Vertical or horizontal lines appearing in other parts of the chromatogram are column overload. They usually happen in the second column because larger molecules may not fully elute from the second dimension column during one modulation cycle.

The colour intensity of compounds appearing in the GCxGC chromatograms correlates to the concentration. *Figure 39* shows the colorization legend relevant for all GCxGC chromatograms in this thesis. Some of the chromatograms have the legend on their right side; however, not all do. Therefore, it is initially presented to give context to the colors in the chromatograms.

Figure 39: Colour intensity in GCxGC chromatograms

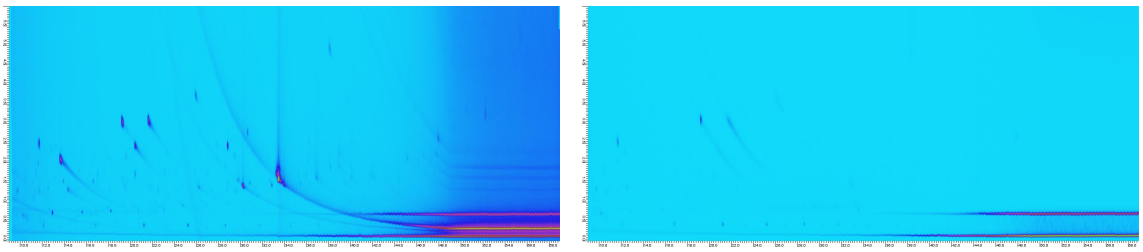
4.9 QC and QA of Methodology using Blank and Spike Samples

The blank samples were spiked with the same surrogate and recovery internal standards that were added to real samples, and were used to detect contamination or systematic errors in sample preparations [99]. After analysis it is observed that variations between the blank samples occur among blanks prepared using the same method and between the different methods, giving an indication of background contamination and variation of this inter (and intra) day.

Spike samples offer valuable insights into the recovery rates of target analytes within the sample matrix [101]. Analyzing the chromatograms to determine the extent to which spike mixtures are detected within the spike sample provides critical information on the method's efficiency.

4.9.1 Blank Checks

Figure 40a shows sample Blank 2 (ID: 2024-1037) from AQUAvit-project prepared using DCM:Hex/GPC. In *Figure 40b* the chromatogram from AQUAvit Blank 5 (ID: 2024-1040) is shown, this was prepared following the QuEChERS procedure. The visual components appear clearer in the chromatogram in *Figure 40a*. In Blank 2 the overload of the GC columns is greater than in Blank 5, where there is no observable overload.



(a) Chromatogram from Blank 2 (ID: 2024-1037), prepared using DCM:Hex/GPC

(b) Chromatogram from Blank 5 (ID: 2024-1040), prepared using QuEChERS

Figure 40: Chromatograms from Blank 2 and Blank 5 prepared using different methods

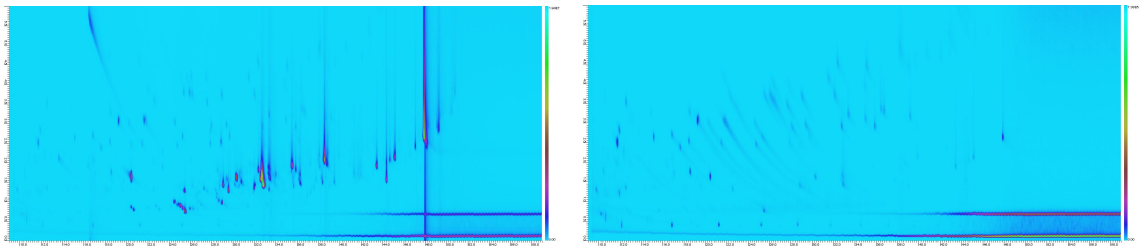
The internal standards, SIS and RIS, are added to the blank samples, as well as the real samples. The contents of SIS and RIS, are presented in *Table 9*. Based on the visual appearance of the peaks in the chromatograms, not all of the compounds from the standard are present. Especially in the blank from QuEChERS, Blank 5 in *Figure 40b*, only a few components are observable. The detected compounds in

these two samples differ, where Blank 2 had 33 detected compounds and Blank 5 only had 26 detected compounds, as presented in *Appendix H*. The 'missing' components from the internal standards could have been detected in the chromatograms by adjusting the color map or scale. However, the color map was set identically for all chromatograms to ensure equality in visual assessments and comparisons.

4.9.2 Spike Checks

For spike checks, AQUAvit lacks chromatograms featuring only SpikeMix 1 and SpikeMix 2 without sample matrix to compare with the spiked salmon sample. Details regarding the spike mixes are outlined in *Appendix F*. However, during laboratory procedures, both SpikeMix 1 and SpikeMix 2 were mistakenly added to one of the blank samples, mimicking the spiked samples. Consequently, a blank spike sample was unintentionally generated, allowing for comparison with the standard spike sample containing fish tissue. It is worth noting that the specific exposure is unknown for the AQUAvit samples, and this is a general screening of environmentally relevant targets. Therefore, the spike mixes provide an estimation of the uptake of environmental toxins by farmed salmon.

In *Figure 41a*, the chromatogram of the salmon spike sample (ID: 2024-1582) prepared using DCM:Hex/GPC from AQUAvit is depicted, while *Figure 41b* shows the chromatogram of the spiked blank sample, prepared using QuEChERS. Comparing these two chromatograms allows for an assessment of the extent to which the components from the spikemix are present in the salmon spike sample.



(a) AQUAvit Spike 6 (ID: 2024-1587), prepared using DCM:Hex/GPC

(b) AQUAvit Blank 16 (ID: 2024-1584), prepared using QuEChERS

Figure 41: Chromatograms from AQUAvit Spike 6 and AQUAvit Blank 16

Several similar peaks are observed in both total ion chromatograms, indicating the presence of similar compounds. The salmon spike sample exhibits some overload and contamination from biotic compounds or fatty acids. Notably, peaks corresponding to the standard compounds are detected in both chromatograms, along with additional peaks originating from SpikeMix 1 and SpikeMix 2.

Additionally, comparison with AQUAvit Spikemix 4 (ID: 2024-1065), presented in *Figure 42*, which contains the same compounds as the spike samples but in different ratio, provides further insights into the composition of the spike samples.

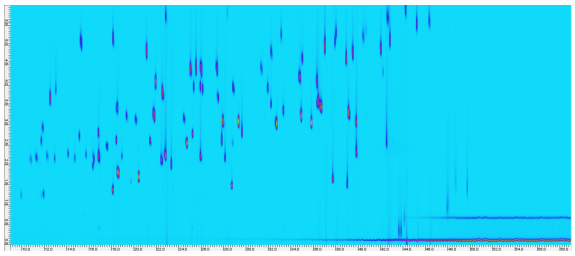


Figure 42: AQUAvit Spikemix 4 (ID: 2024-1065)

The comparison between the spike mix in the blank and the spike in the salmon sample represents the closest approximation, as the compounds added have the same ratio but different concentrations. This is particularly relevant considering the lack of precise information regarding the exact exposures of the AQUAvit samples. The blank sample, despite lacking a sample matrix, offers the best basis for comparison due to the addition of spike mixes. The spike mixes, SpikeMix 1 and SpikeMix 2, provide good estimations of the exposure, offering a consistent reference point for comparison.

4.10 Data Processing of GCxGC Chromatograms - Semi-Quantitative Analysis for AQUAvit

For screening of the samples from the AQUAvit project after GCxGC analysis, the template containing peak blobs is relevant. The selected blobs in the template corresponds to peaks from the OilMix and the standard ToxMix, and can be used to verify that similar components exists in the samples. The ToxMix contains components that are relevant for an environmental screening analysis, such as common PCBs, PAHs, PBDEs, PPCPs and plastic/rubber chemicals. *Figure 43* shows the blob-template overlaid over the ToxMix chromatogram.

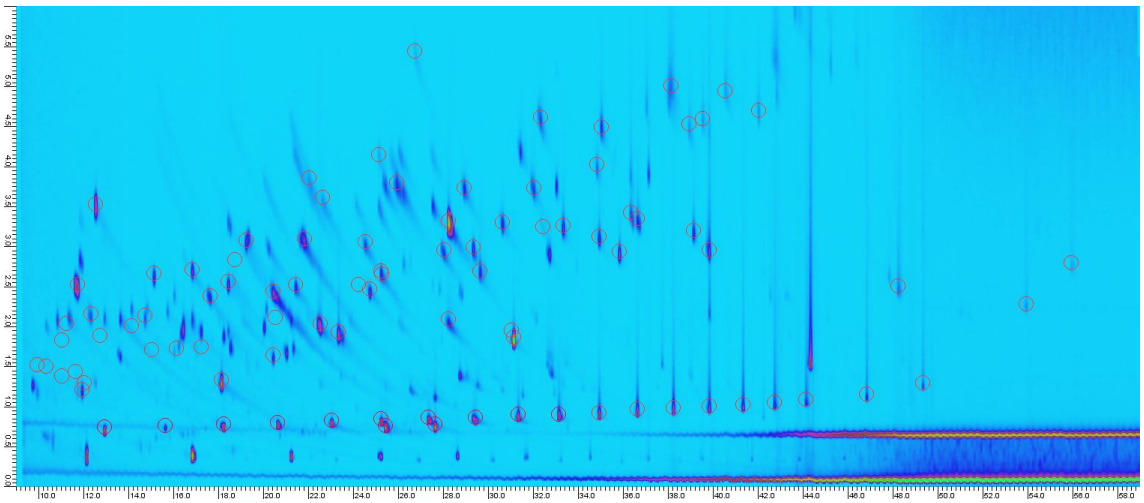


Figure 43: Standard ToxMix chromatogram with blob template

Table of identified compounds from standard ToxMix, with corresponding retention time is shown in *Appendix I*. Compounds identified from the OilMix is presented in *Appendix J*. The blobs corresponds to compounds detected by the *GC-Image*® tool "Interactive Blob (peak) Detection", with a relative volume sat to 40 cubic pixels. Out of the templates, 97 peaks, 7 of them were identified from the OilMix and the remaining from the ToxMix standard.

The blob template was employed on all samples to determine if the samples had corresponding peaks at the same retention time as the peaks detected and identified from the standards. There is no information regarding what AQUAvit samples have been exposed to, and therefore it is not certain that the detected peaks in the sample is the same compound identified from the standard.

4.10.1 AQUAvit Salmon Samples

The AQUAvit samples consist of 68 samples in total, which were prepared and run on the GCxGC. 34 of them was prepared using QuEChERS combined with dSPE clean-up and 34 with DCM:Hex/GPC clean-up. An overview of the AQUAvit sample replicates for both methods, QuEChERS and DCM:Hex/GPC is shown in *Table 14*.

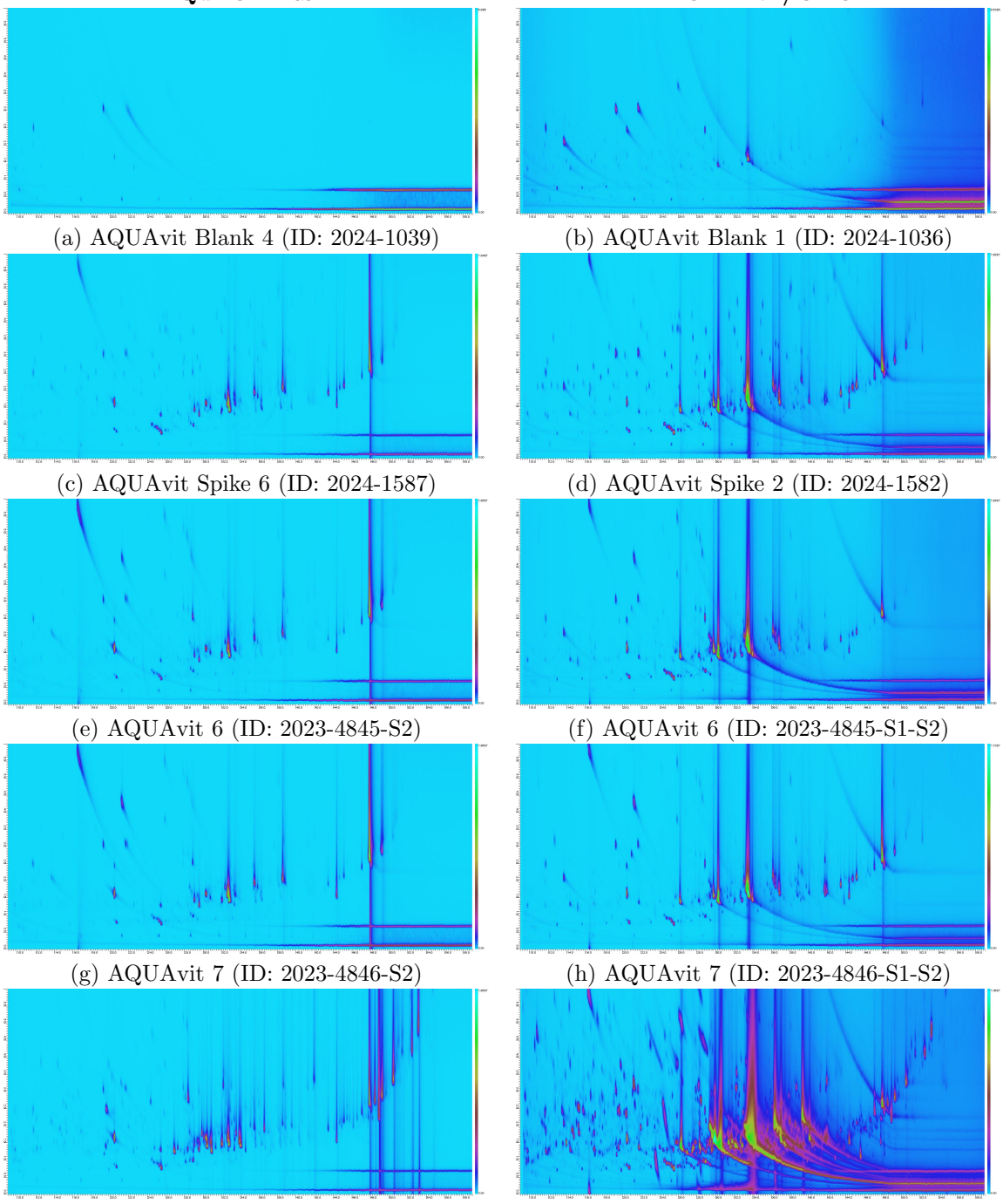
Table 14: Overview of AQUAvit sample replicates from QuEChERS and DCM:Hex/GPC

Sample type	**Replicates from each method**	
	QuEChERS	DCM:Hex/GPC
Salmon muscle tissue	20	20
Feed pellets	3	3
Spikes	3	3
Blanks	8	8

A full overview of all AQUAvit samples with their respective sample-ID and information regarding sample preparation is shown in *Appendix* B.

All chromatograms generated from GCxGC analysis for the AQUAvit samples are presented in *Appendix K.* In *Figure 44*, five total ion chromatograms from each preparation method; QuEChERS and DCM:Hex/GPC, are presented. One set of blanks (a, b), one set of spike samples (c, d) one set of lipid rich salmon tissue (e, f), one set of lean salmon tissue (g, h) and one set of feed samples (i, j).

QuEChERS **DCM:Hex/GPC**



(a) AQUAvit Blank 4 (ID: 2024-1039)

(b) AQUAvit Blank 1 (ID: 2024-1036)

(c) AQUAvit Spike 6 (ID: 2024-1587)

(d) AQUAvit Spike 2 (ID: 2024-1582)

(e) AQUAvit 6 (ID: 2023-4845-S2)

(f) AQUAvit 6 (ID: 2023-4845-S1-S2)

(g) AQUAvit 7 (ID: 2023-4846-S2)

(h) AQUAvit 7 (ID: 2023-4846-S1-S2)

(i) AQUAvit Feed 4 (ID: 2024-1577)

(j) AQUAvit Feed 1 (ID: 2024-1573)

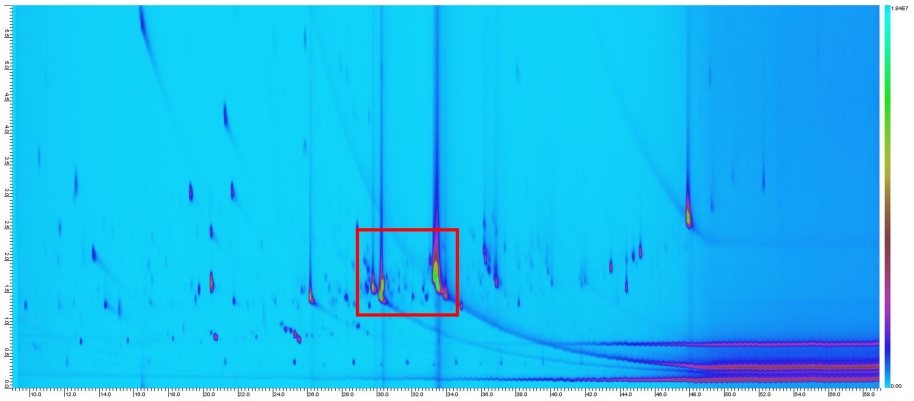
Figure 44: Chromatograms of selected AQUAvit samples

4.10.2 Comparison of QuEChERS and DCM:Hex/GPC

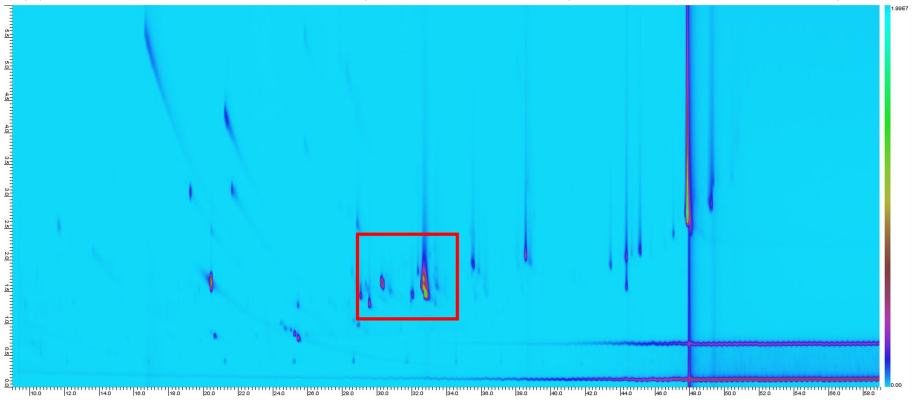
The AQUAvit samples were analysed on the GCxGC. The chromatograms showed a trend where samples prepared with DCM:Hex/GPC had more detected compounds, overload, contamination and column bleeding compared to the samples prepared using QuEChERS. Some parallels had more significant differences than others. In *Appendix K*, additional chromatograms from the AQUAvit project are presented, allowing for a more extensive qualitative comparison between the methods.

A comparison of samples with the least and the most differences between the methods are drawn as an example here.

Both of the chromatograms from AQUAvit 5 (ID: 2023-4844), which showed the least visual differences compared to each other are presented in *Figure 45a* and *Figure 45b*. The C16 and C18 fatty acids are marked in the chromatograms with a red square.



(a) Chromatogram of AQUAvit 5 (ID: 2023-4844-S1-S2) prepared using DCM:Hex/GPC



(b) Chromatogram of AQUAvit 5 (ID: 2023-4844-S2) prepared using QuEChERS

Figure 45: Chromatograms of AQUAvit 5 (ID: 2023-4844) with drawn red square around C16 and C18 fatty acids

Chromatogram of AQUAvit 5 (ID: 2023-4844-S1-S2), prepared using DCM:Hex/GPC have more detected components and a general higher peak intensity. Visually, the C16 and C18 fatty acid groups are more prominent in the chromatogram, indicating higher volume/concentration compared to the QuEChERS chromatograms (*Personal communication* [130]). There is some overload in chromatograms from DCM:Hex/GPC samples, especially in the second dimension (second column).

To assess how many analytes were extracted during each method, comparing the sample chromatograms with the chromatogram of ToxMix is relevant. The QuEChERS chromatogram looks cleaner and have less detected peaks compared to ToxMix, than samples prepared using DCM:Hex/GPC. *Figure 46* shows the total ion chromatogram of ToxMix standard, which consist of a large amount of environmental toxins, presented in *Appendix E*. The comparison with the ToxMix chromatogram is useful due to the lack of information about the AQUAvit sample exposure, resulting in suspect screening procedure. The relative overload in the chromatograms are also much lower in the QuEChERS samples.

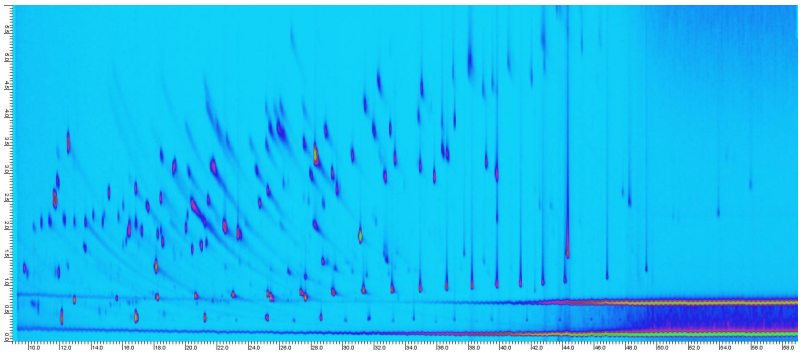
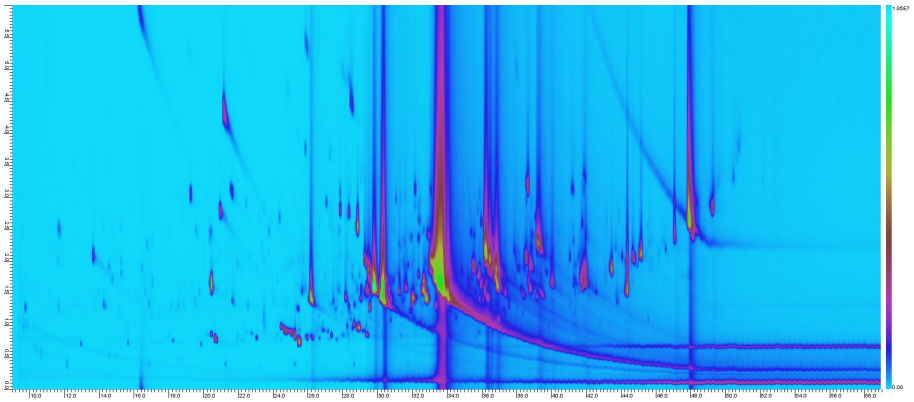
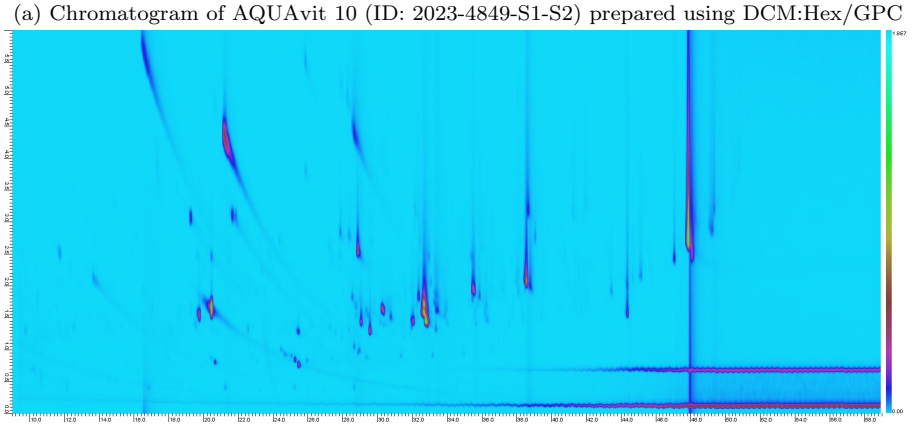


Figure 46: Total ion chromatogram of standard ToxMix

Both of the chromatograms from AQUAvit 10 (ID: 2023-4849), which showed the greatest visual differences compared to each other are presented in *Figure 47a* and *Figure 47b*.



(a) Chromatogram of AQUAvit 10 (ID: 2023-4849-S1-S2) prepared using DCM:Hex/GPC



(b) Chromatogram of AQUAvit 10 (ID: 2023-4849-S2) prepared using QuEChERS

Figure 47: Chromatograms of AQUAvit 10 (ID: 2023-4849)

There is a difference of peak intensity in the total ion chromatograms from AQUAvit 10 prepared using DCM:Hex/GPC and AQUAvit 10 prepared using QuEChERS. In the DCM:Hex/GPC sample a larger amount of the peaks have yellow and purple intensity, which means that the relative volume of these components are high compared to the other detected components in the chromatogram, and also causes overlap with neighbouring peaks.

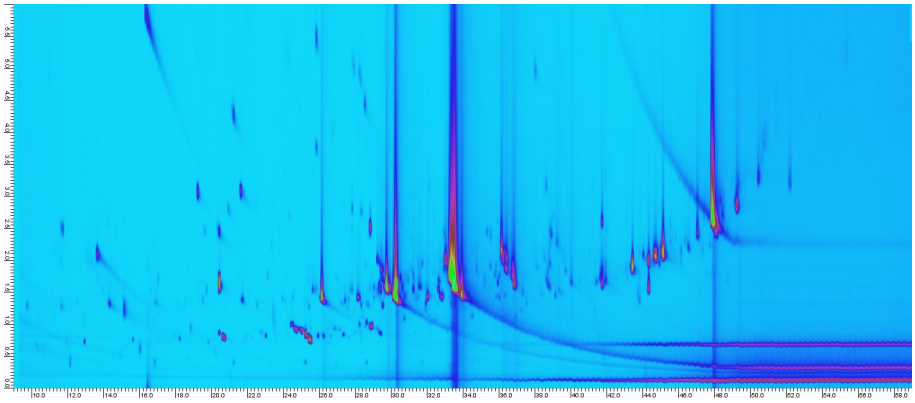
Since the goal is to recover as many of the standard components as possible, the DCM:Hex/GPC sample preparation is preferred, due to the number of detected peaks. Even if the DCM:Hex/GPC chromatograms have more overload and debris, the GCxGC processing software, *GC Image®*, still detects a lot of components. The downside of overload and large overlapping peaks from biological compounds are that smaller target compounds or compounds of interest with similar retention times may be difficult to detect. The issue with QuEChERS may be that not enough compounds are extracted in the extraction phase, this may lead to fewer components being discovered, especially in non-target screening.

The different solvents have different interactions with the analytes. This can contribute to the reason why there is observed a relatively large difference between the chromatograms from these two methods.

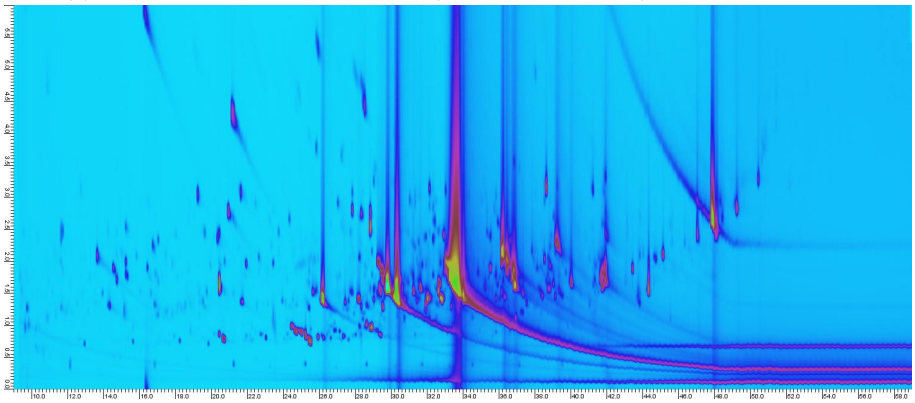
4.10.3 The Difference Between Lipid-Rich and Lean Tissue

The removal of lipids is one of the main challenges when preparing biological samples for trace compound analysis [67, 68]. From each salmon, two samples of the muscle was collected. One sample from the lipid rich part and one from the leanest part of the muscle. After GPC clean-up the lipid fraction was evaporated completely dry and weighed to determine the lipid content. Lipid weight was calculated as percentage lipid fraction of the salmon tissue wet weight. The average lipid content with standard deviation for the ten lean tissue samples is $6.1(\pm 2.3)\%$, and for the ten lipid rich tissue samples it is $13.4(\pm 1.5)\%$. The lipid content measured in both lean and lipid-rich tissue samples aligns with the assumed distinction made by the samplers between fatty and non-fatty tissue. An overview of lipid content collected in the lipid fraction after the GPC on all AQUAvit samples are presented in *Appendix M*. Even numbered samples are collected from the lipid rich part, and odd numbered samples are collected from the lean part of the sample.

The GCxGC chromatogram from lipid rich and lean tissue is compared to determine if the lipid percentage affected what was separated and detected. *Figure 48a* shows the chromatogram of AQUAvit 1 (ID: 2023-4840), a sample collected from the leaner part of the fish. *Figure 48b* shows the chromatogram of AQUAvit 2 (ID: 2023-4841) taken from the more lipid rich part of the fish. Both these samples are prepared using DCM:Hex/GPC.



(a) Chromatogram from AQUAvit 1 (ID: 2023-4840-S1-S2) - lean salmon tissue



(b) Chromatogram from AQUAvit 2 (ID: 2023-4841-S1-S2) - lipid rich salmon tissue

Figure 48: Chromatograms of AQUAvit 1 and AQUAvit 2

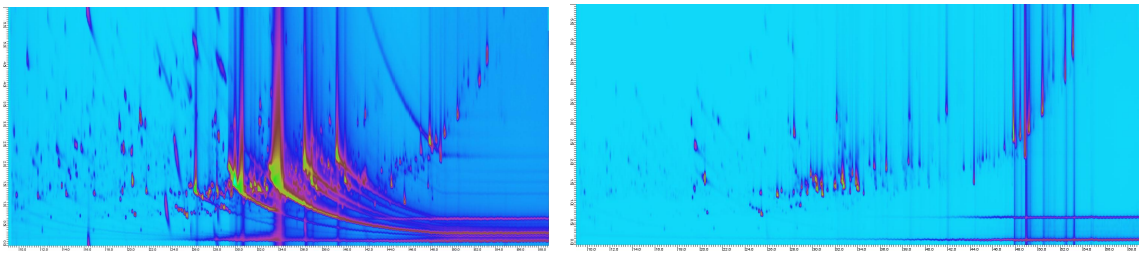
Both total ion chromatograms show overload in the first and second dimensions, with AQUAvit 2 exhibiting slightly more overload. The overload may originate from a concentration/volume overload in respect to these compounds. This could been improved by dilution of the samples or reduction of injection volume from 1 to 0.5 µL. Particularly the stronger peak intensity of the lipid-rich sample (AQUAvit 2). The intensity differences are greatest for the lipid peaks, while peaks originating from other compounds appear relatively similar in concentration between the samples. This is expected due to the difficulty of removing lipids from the samples. Since they are prepared in the same way, an equal amount of lipids can be expected to be removed in the process, resulting in a higher lipid content in the chromatograms from lipid-rich tissues.

This visual trend is consistent across all fish muscle samples prepared using both methods. To account for the different lipid content in samples, different amounts of solvent and chemicals in the sample preparation step could have been used. For instance, the amount of Z-Sep used in QuEChERS in the preparation of lipid rich tissue could have been increased, to observe if this would further remove biological compounds.

4.10.4 Comparison between Feed Pellets and Salmon Tissue

Regardless of sample preparation method, the feed sample chromatograms displayed a higher content and number of more fatty acids and biological components compared to the muscle tissue chromatograms. *Figure 49a* shows the chromatogram of AQUAvit feed 3 (ID: 2023-1575) that has been prepared using DCM:Hex/GPC and *Figure 49b* shows chromatogram of AQUAvit feed 5 prepared using QuEChERS.

Figure 49a have significant overload in both first and second dimensions. The detected peaks to the left in the chromatogram appear quite smeared out across a large portion of the chromatographic space.



(a) AQUAvit Feed 3 (ID: 2023-1575) prepared using DCM:Hex/GPC

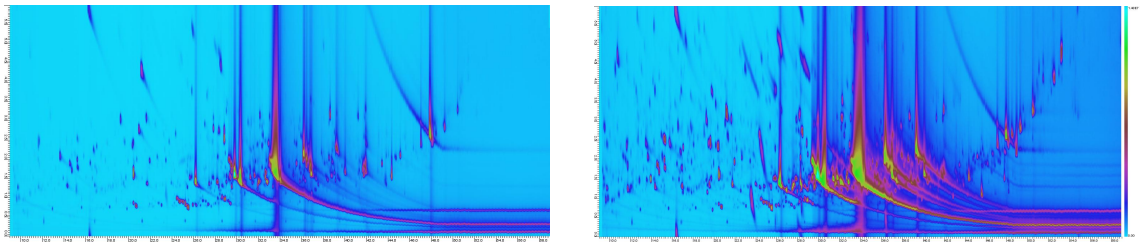
(b) AQUAvit Feed 5 (ID: 2024-1578) prepared using QuEChERS

Figure 49: Chromatograms from AQUAvit feed samples 3 and 5

The chromatogram from Feed 3 shows large groups of C16 and C18 fatty acids in the middle of the first dimension axis. The colour intensity of the peaks also refer to a large amount of these compounds being present. This can be related back to the color of the GPC fractions presented in *Figure 28* in *Chapter 4.4.1*. Indicating that lipids were not fully removed from the analyte fractions during GPC clean-up, and subsequently analysed on GCxGC resulting in these chromatograms with great overload and visible fatty acids.

The QuEChERS method have managed to remove a substanital part of the biological compounds from the feed compared to DCM:Hex/GPC, whilst still retaining some of the fatty acids which are detected in the lower middle part of the Feed 5 chromatogram. Feed pellets tend to have a high fat content, this may be the reason why the removal of all lipids can be difficult.

It is a useful observation to compare the feed pellet chromatograms against the chromatograms from the salmon muscle. *Figure 50a* and *Figure 50b* shows the chromatogram from AQUAvit 2 (ID: 2023-4841-S1-S2) and Feed 1 (ID: 2024-1573), samples prepared using DCM:Hex and GPC. The chromatogram from the feed pellets have a higher peak intensity and the overload from the chromatograms are more dominant in the feed sample, than in the salmon muscle. The column overload, overlap and peak size is also greater in the feed sample.

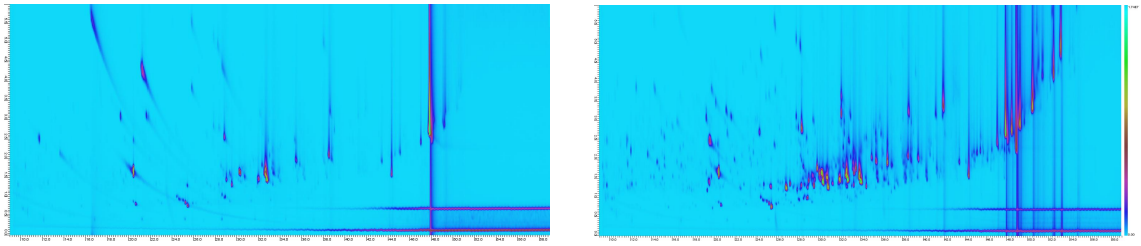


(a) AQUAvit 2 (ID: 2023-4841-S1-S2)

(b) Feed 1 (ID: 2024-1573)

Figure 50: Chromatograms from AQUAvit 2 and Feed 1 prepared using DCM:Hex/GPC

The same comparison was done to QuEChERS samples. *Figure 51a* and *Figure 51b* shows AQUAvit 2 (ID: 2023-4841-S2) and Feed 6 (ID: 2024-1579). The observations in the DCM:Hex/GPC samples are also present in the QuEChERS samples. There are more visual components in the feed sample and greater overload, especially in the second column.



(a) AQUAvit 2 (ID: 2023-4841-S2)

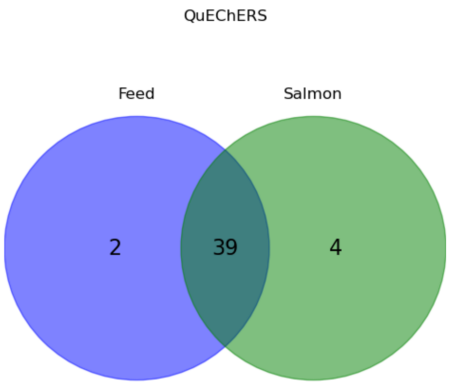
(b) Feed 6 (ID: 2024-1579)

Figure 51: Chromatograms from AQUAvit 2 and Feed 6 prepared using QuEChERS

Regardless of sample preparation method, the feed samples have more visual components than the salmon sample. The overload is generally higher in feed samples as well. There are some similarities between the feed samples and the salmon tissue samples, some of the components are present in all the chromatograms. These components can derive from the added standards, but also be transfer of components from the feed to the salmon when ingested. Between the different sample preparation methods, the observed difference is that the QuEChERS method removes more of the lipid contents in the sample compared to DCM:Hex/GPC.

The venn diagrams in *Figure 52* illustrates how many compounds were detected in the samples for both DCM:Hex/GPC and QuEChERS. It shows the number of compounds in common between feed samples and salmon samples, as well as how many unique compounds were detected in each sample. The diagram is based on the data presented in *Appendix H*, and is further described in *Chapter 4.12.1*. The biggest difference between the diagrams are the number of unique compounds inn each sample. DCM:Hex/GPC have more unique compounds in the feed samples, and QuEChERS have more in the salmon samples.

(a) Detected compounds in feed and salmon samples, prepared using DCM:Hex/GPC



(b) Detected compounds in feed and salmon samples, prepared using QuEChERS

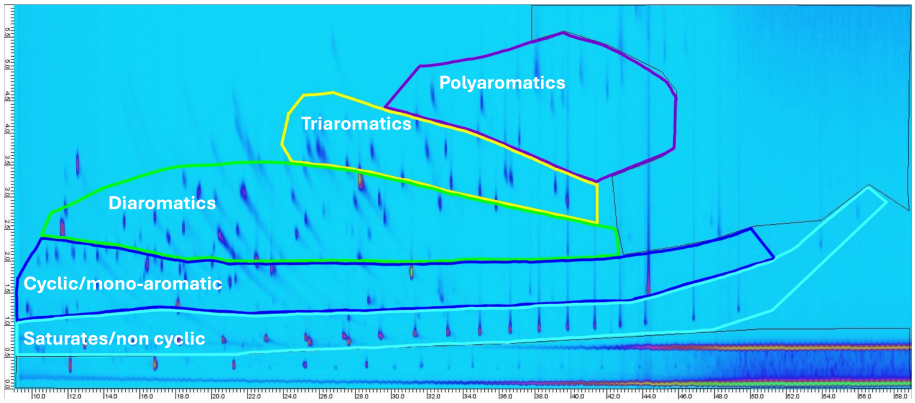
Figure 52: Venn diagrams representing the number of detected compounds in fish feed and salmon samples

A possible source of error here can be that the methods employed on the feed samples have not been optimized based on the feed samples characteristics. This could be the reason for overload in the samples. However, if the methods were optimized for feed samples, the comparisons would not be as straight forward.

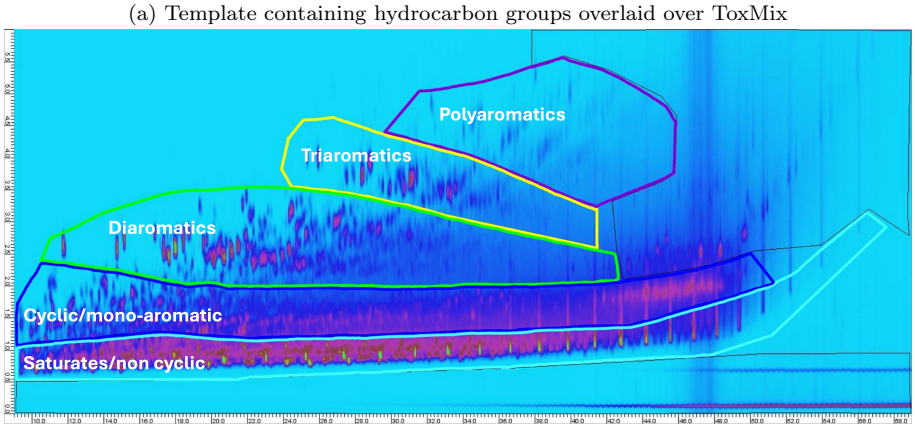
4.11 Identification and Data Processing of GCxGC Chromatograms - Qualitative and Semi-Quantitative Analysis for ToxiGen

For compound identification and semi-quantification in GCxGC a template in the processing software, *GC Image®*, was used. The template is often constructed from known samples and/or standard mixtures and are then applied to the sample chromatograms for semi-automated processing. The template was created as described in *Chapter 3.11.1* and a total of 97 peaks were marked based on the contents of the ToxMix standard and OilMix chromatograms, presented in *Figure 53a* and *Figure 53b*, respectively.

For the ToxiGen samples the aim is to semi-quantify the different classes of aromatic hydrocarbons. The template containing hydrocarbon groups were used for the purpose of estimating relative volume of different classes of hydrocarbons from oil. The template containing group names is presented in *Figure 53a*, where it is overlaid on the ToxMix chromatogram, and in *Figure 53b*, where it is overlaid on the OilMix chromatogram.



(a) Template containing hydrocarbon groups overlaid over ToxMix



(b) Template containing hydrocarbon groups overlaid over OilMix

Figure 53: Hydrocarbon group template overlaid over ToxMix and OilMix

4.11.1 ToxiGen Polar Cod Samples

Out of the 20 ToxiGen samples produced in relevance to this thesis, 17 of them were run on GCxGC after sample preparation using DCM:Hex followed by NH_2-fractionation. After analysis the template was added to each of the total ion chromatograms, to detect compounds and quantify the volume of hydrocarbon groups marked in the template, presented in *Figure 53a*.

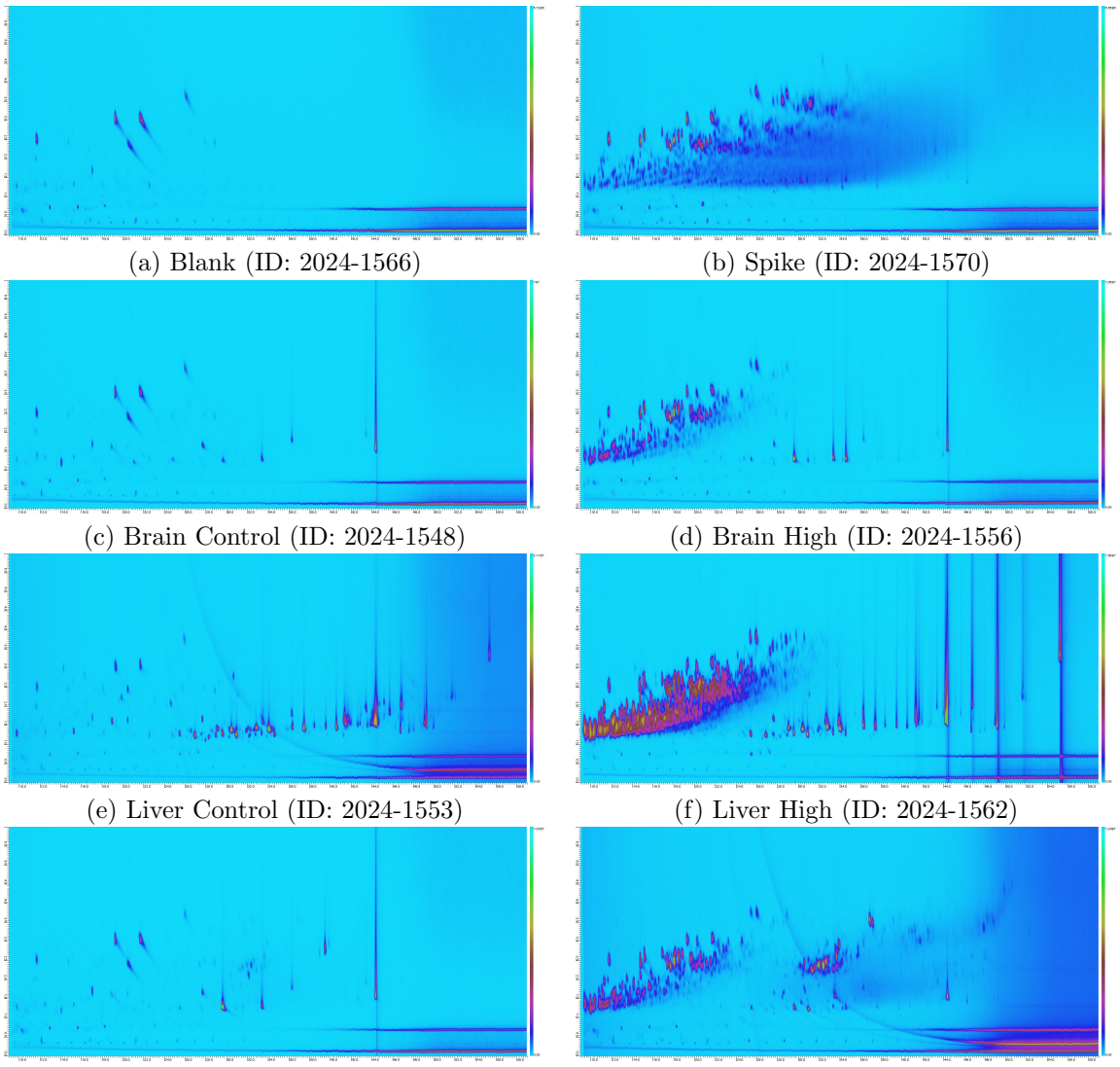
An overview of sample replicates associated with the ToxiGen project is presented in *Table 15*, for the high exposed and control samples.

There are two different sample types containing tissue. In the high exposed samples, the polar cod have undergone exposure of OilMix at relatively high concentrations. The total ion chromatogram of the oil is shown in *Figure 18*. For comparison to these exsposed samples, unexposed control tissue samples are used.

Table 15: Overview of ToxiGen sample replicates

Tissue type	**Sample type**	**Replicates**
Gonad	Control	2
	High	4
Liver	Control	3
	High	4
Brain	Control	2
	High	2
Spike	*N/A*	1
Blank	*N/A*	2

A full overview of all samples from ToxiGen prepared in relevance to this thesis are shown in *Appendix A*, and all the total ion chromatograms generated from GCxGC analysis are presented in *Appendix L*. 8 of the ToxiGen total ion chromatograms are presented in *Figure 54*.



(a) Blank (ID: 2024-1566)

(b) Spike (ID: 2024-1570)

(c) Brain Control (ID: 2024-1548)

(d) Brain High (ID: 2024-1556)

(e) Liver Control (ID: 2024-1553)

(f) Liver High (ID: 2024-1562)

(g) Gonad Control (ID: 2024-1550)

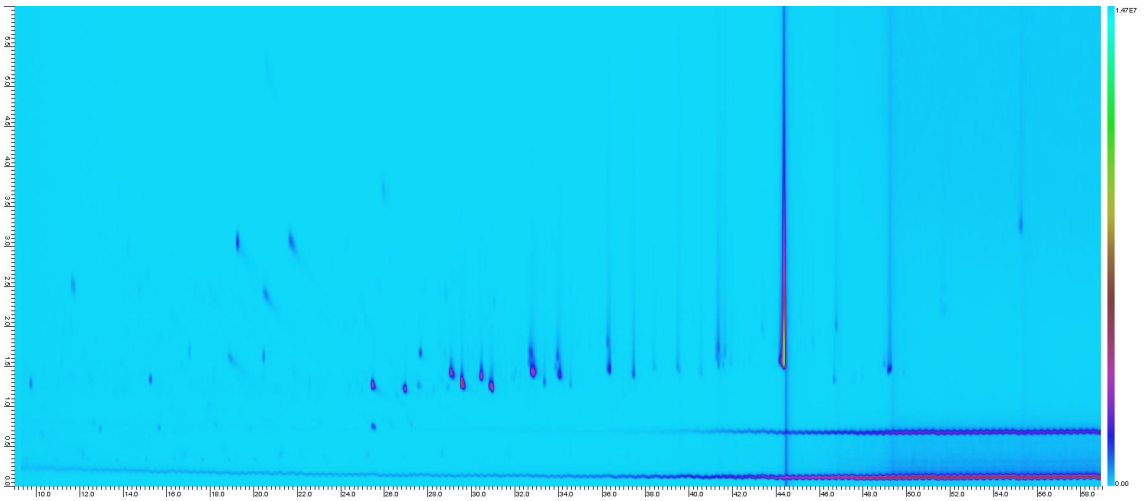
(h) Gonad High (ID: 2024-1559)

Figure 54: Chromatograms of selected ToxiGen samples

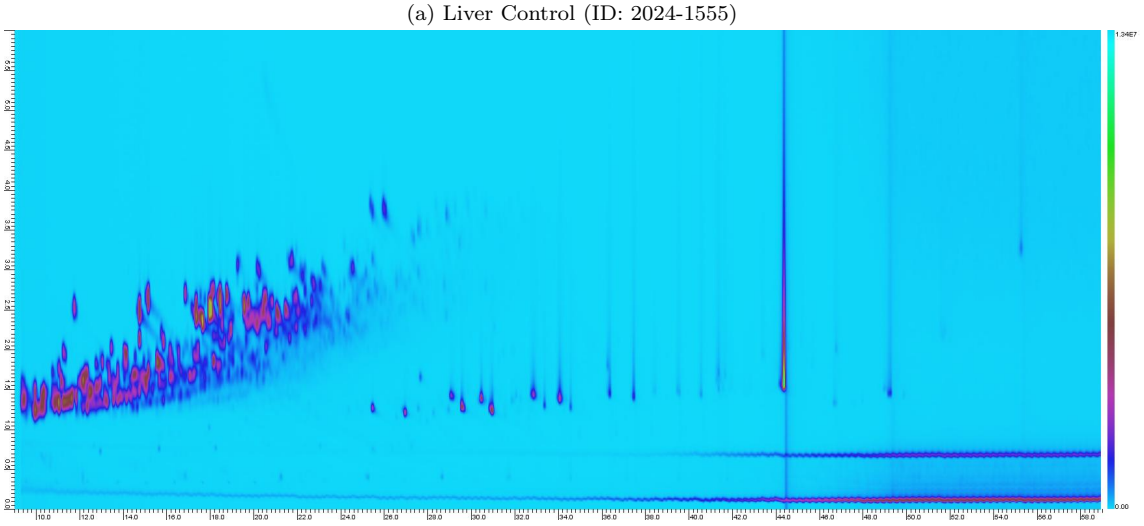
4.11.2 Comparison between Test Control and Test High Conditions

The aim of comparing chromatograms of control and high exposed polar cod liver samples, is to assess the uptake of pollutants following exposure to oil. The focus lies on clarifying the extent of uptake, if any, through a visual examination of the chromatograms.

Visually, the chromatograms for exposed cod samples have more peaks than control samples. This result is as expected because control samples are not exposed to oil, whereas high samples have been exposed. *Figure 55a* shows the chromatogram from a control sample of liver (ID: 2024-1555), and *Figure 55b* shows the chromatogram of an exposed liver sample (ID: 2024-1564).



(a) Liver Control (ID: 2024-1555)



(b) Liver High (ID: 2024-1564)

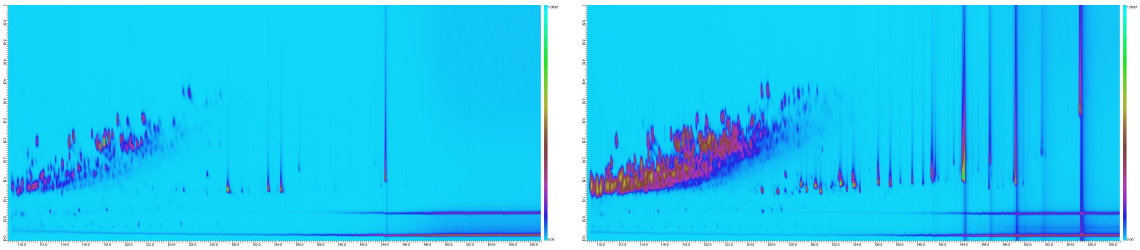
Figure 55: Chromatograms of Liver Control and Liver High

The distinct difference between these two samples are the group of hydrocarbons presumably from the exposure oil, to the left in the chromatogram. The horizontal line of detected compounds appear similar in both the samples, these compounds are probably biogenic components or compounds from the internal standards.

4.11.3 Comparison between Liver Tissue and Brain Tissue

To find out if there is a differences in uptake of hydrocarbons from oil exposure between the tissue types of polar cod, exposed samples from liver and brain is compared. The tissue composition vary between these samples, with the liver tissue containing more lipids than the brain. Lipids needs to be removed from sample matrices before analysis to ensure that biogenic material do not create a shadow over the target analytes in the chromatogram. However, removing lipids might also remove the analytes in question. *Figure 56* shows chromatograms of brain high (ID: 2024-1556) and liver high (ID: 2024-1562).

Visually, the hydrocarbon groups and overload from biogenic compounds are greater in the liver compounds. Indicating both higher lipid content and uptake from the oil exposure.



(a) Brain High (ID: 2024-1556)

(b) Liver High (ID: 2024-1562)

Figure 56: Chromatograms of Brain High and Liver High

The difference of compounds detected in the different tissues are an interesting observation. The reasons for this may be that the tissues have a different composition, especially when it comes to lipid content, so that the storage of toxins may be higher in lipid rich tissues. A study done by *Jackson et al.* in 2018 showed that toxins, such as PAHs, PBDEs, and PCBs, accumulated more rapidly in general adipose tissue [131]. This is an explanation to why the hydrocarbon groups are more prominent in the liver chromatogram.

4.12 Screening and Detection of Target Analytes

4.12.1 AQUAvit

Due to time consuming analysis, 28 AQUAvit samples were randomly picked out. There were 14 samples from each of the methods, where the sample distribution were 2 feed, 6 salmon samples, 2 spike samples and 4 blank samples. These were cross-checked with the detected peaks from ToxMix-standard chromatogram, presented in *Figure 19*, to see which identified compounds were detected in the samples. By overlooking components that are not present in the ToxMix standard, this approach can be considered more of a target or suspect screening, in contrast to non-targeted screening.

The average number of identified peaks across the different sample types (salmon tissue, feed, spike, and blank) and preparation methods are depicted in the bar chart in *Figure 57*. The data is based on the full overview of detected compounds presented in *Appendix H*. The green bars represent samples prepared using DCM:Hex as solvent and GPC clean-up, while the blue bars represent samples prepared with the QuEChERS method. The standard deviation of the randomly selected samples for each sample type is calculated and represented in the bar chart with the black error bars.

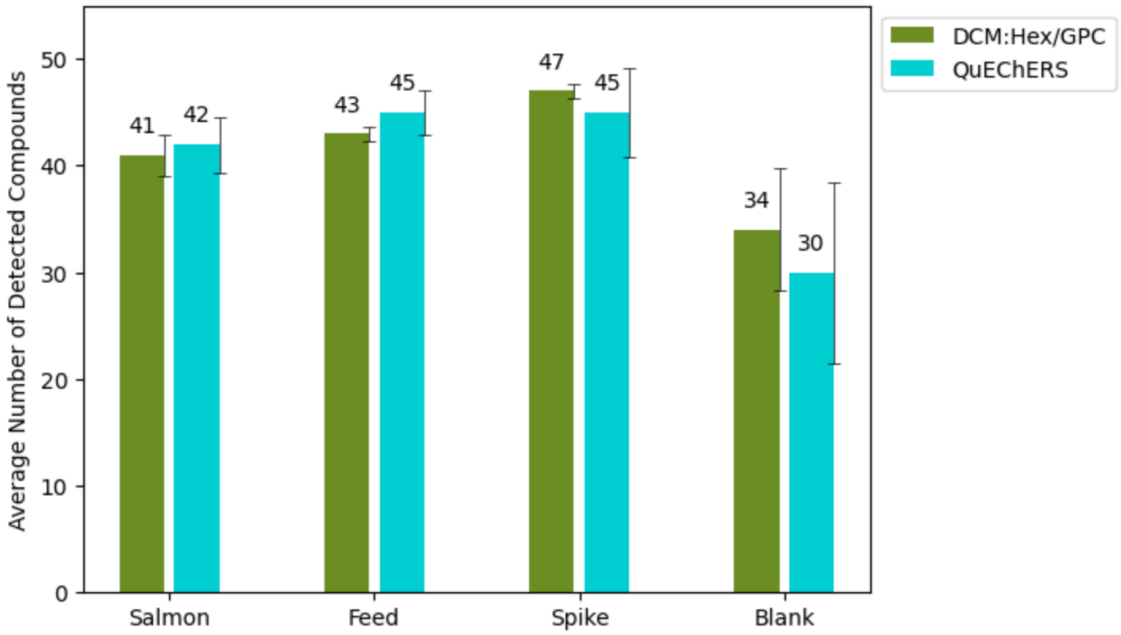


Figure 57: Average number of detected compounds from AQUAvit samples across the different solvent and clean-up method combinations. The green bars represent DCM:Hex and GPC, blue bars represent QuEChERS, and standard deviations are represented by the black error bars. Detailed detection list in *Appendix H*.

There are no notable differences in the number of identified peaks between the two methods. Average number for detected peaks with standard deviation for DCM:Hex/GPC salmon sample is 41.0($\pm$1.9), and for QuEChERS salmon sample is 42.0($\pm$2.6). These values are quite similar, with QuEChERS detecting a few more compounds on average. The spike samples have the highest share of compounds, as expected, with 47.0($\pm$0.7) and 45.0($\pm$4.2) for DCM:Hex/GPC and QuEChERS, respectively.

The components from the standards, presented earlier in *Table 9*, such as Naphthalene-$d8$ and Acenaphthene-$d10$ was found in all samples with a relatively large volume. The recovery of deuterated standard components show that the sample preparation procedure is adequate.

There were significantly fewer detected peaks in the blank samples, although they also exhibited the most variation, indicated by the standard deviation of 34.0($\pm$5.7) and 30.0($\pm$8.5). The blank samples prepared for feed and spike triplicates contained more peaks compared to those for the regular salmon samples, consequently increasing the overall average.

4.12.2 ToxiGen

As a screening approach for detecting and identifying hydrocarbon groups from oil exposure in ToxiGen samples, the template was employed on the samples. This is a suspect screening approach based on the screening of predefined groups, not specific compounds or using a compound library. The combined relative volume of identified peaks in each compound group was measured in both a set of control samples and a set of high-exposure samples for comparison. The compound groups include saturates/non-cyclic, cyclic/monoaromatics, diaromatics, triaromatics, and polyaromatics. The hydrocarbons included in these groups are relevant for the ToxiGen project aim of studying oil exposure.

A bar chart of the relative total volume of compound groups adjusted by sample weight for samples gonad control (ID: 2024-1550), gonad high (ID: 2024-1559), brain control (ID: 2024-1548), brain high (ID: 2024-1556), liver control (ID: 2024-1554), and liver high (ID: 2024-1563) is presented in *Figure 58*. The liver samples were diluted x10 due to high concentrations. Despite this, the vertical axis has a logarithmic scale due to the large differences in relative total volume between liver samples and the remaining samples. Statistics are not preformed due to the lack of replicates in this sample set. The data used for this bar chart is collected from *Table 30* as presented in *Appendix N*.

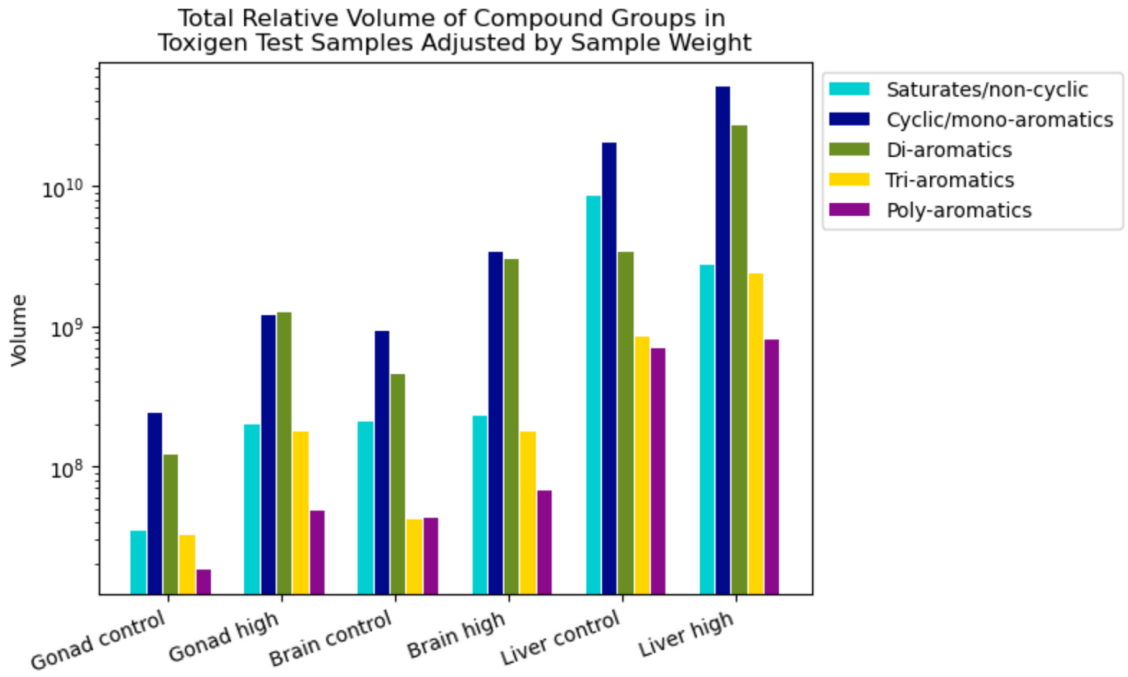


Figure 58: Total relative volume of compound groups in ToxiGen samples adjusted by sample weight bar chart with logarithmic scale. Compound group and colors: Saturates/non-cyclic (turquoise), cyclic/monoaromatics (dark blue), diaromatics (green), triaromatics (yellow), and polyaromatics (purple)

As anticipated, the control samples exhibited notably lower volumes across all groups compared to the high-exposure samples, reflecting the expected outcome due to sample exposure. This aligns with the ToxiGen project's aim of studying how oil exposure effect different tissues.

The tissue-specific differences observed in the bar chart is worth noting. Certain tissues exhibit higher total volumes of specific compound groups compared to others. The high-exposed liver tissue is significantly larger than the others, with the dark blue bar (cyclic/monoaromatics group) being almost 47 times higher than the next highest exposed sample; Gonad high. This could indicate differences in uptake, metabolism, or susceptibility to oil exposure among different tissues. For more detailed discussion the chromatograms needs to be quantified in order to eliminate systematic contamination and baseline levels.

The findings from this analysis provide valuable insights into the distribution and abundance of hydrocarbons within biological tissues following oil exposure. These insights can inform risk assessments, environmental monitoring efforts, and management strategies related to oil spills and contamination events.

4.13 Data Analysis Tools and Screening

The *Blob detection* tool was predominantly used for AQUAvit samples, as the defined hydrocarbon groups in the template were not relevant to the project's objectives. Compounds were detected from volume sat at 40, acknowledging the relative nature of volume measurement in this context. This approach detected peaks, some of which may not correspond to specific compounds, illustrating the challenge of accurately detecting and identifying peaks. Due to the bachelor projects time limit, the blob detection method was insufficient at identifying specific compounds. In order to be able to identify targets in the samples, the mass specter of each peak would have to be evaluated carefully.

For ToxiGen samples, the *GC Image®* tool "*Blob set table*" was used, which can calculate relative volume of compounds inside drawn groups. Different groups of hydrocarbons were drawn into the table, based on known chemical properties of hydrocarbons in oil. The advantage of this tool is that it allows for a semi-quantification of relative volume of the groups, without the need of accurately identify each of the components present. The weakness of this tool is that it does not exclude biological matrix components and debris that have not been fully removed from the sample, or non-organic components with similar retention times. This can lead to a calculated volume being higher than the actual volume of hydrocarbons in sample.

A suspect screening approach was employed for both project, but ToxiGen had a slight shit towards targeted screening due to the predefined hydrocarbon groups in the template. However, with more time available, non-targeted screening could have been explored in AQUAvit by analyzing individual mass spectra of peaks to identify compounds. Further analysis for ToxiGen can be done by optimizing the template by detecting specific peaks so that components that do not belong in the group are excluded. This will lead to a more targeted screening approach, and subsequently more accurate volumes of the hydrocarbon groups.

Nonetheless, the bachelor project's main focus remained on the sample preparation methods rather than advancing into more extensive analyses. While the potential for broader screening approaches existed, they were not pursued in alignment with the project's objectives, which prioritized refining sample preparation techniques.

5 Conclusion

The absence of standardized methods for preparing biotic tissue for extraction and analysis of environmental toxins, such as contaminants of emerging concern, legacy pollutants, pesticides etc., coupled with the increasing presence of chemicals in ecosystems, underscores the immediate need for innovative approaches in environmental research.

Our research highlight the necessity for tailored approaches based on tissue type. Even the initial step of homogenizing samples are not standardised. The work in this bachelors project has explored several combinations of solvent and salt addition and for the samples herein the best results were achieved with the addition of either DCM:*n*-Hexane or acetonitrile before, and Na_2SO_4 or NaCl or both after homogenization.

The clean-up methods, being the main objective for optimisation in this work, gave the most interesting and differential results, with the chosen methods aligning with the specific aim of each project. NH_2-fractionation is suitable for extracting analytes from liver, gonad and brain from polar cod samples from ToxiGen and GPC or QuEChERS is efficient for salmon tissue from AQUAvit. The choice between GPC and QuEChERS hinges on various factors, including time and cost, with QuEChERS offering advantages in efficiency and GPC provides the best analyte recovery for further analysis. There remains room for improvements in further development of the methods, particularly with the extraction of analytes in QuEChERS. The duration of the bachelor project did not allow for a more comprehensive method development and testing.

Due to restricted time limits for this project, lack of in depth quantitative and qualitative analysis limits the capability to detect, identify and quantify toxin levels accurately. Chromatograms across different sample types and methods showed both visible patterns and differences. This illustrates the need for tailored, innovative approaches for sample preparation in environmental toxin analysis.

6 Further Work

The short project limits the extent of method testing. To further develop and compare these methods, here are some suggestions:

- QuEChERS method: compare three extraction steps combined with the use of ultrasonication bath to one-step extraction.

- Further quantitative analysis to detect and identify specific quantities of environmental toxins.

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A Full Overview of ToxiGen Samples

Table 16: Full overview of ToxiGen samples

ToxiGen				
Name	**Type**	**LIMSID**	**Method**	**Weight [g]**
ET060-65 Gonad	High	2024-669	DCM:Hex-GPC	5,50544
ET060-65 Brain	High	2024-665	DCM:Hex-GPC	0,51629
ET060-65 Liver	High	2024-673	DCM:Hex-GPC	3,68211
ET021-30 Brain	Control	2024-1547	DCM/NH2	0,71003
ET057-68 Brain	Control	2024-1548	DCM/NH2	0,83295
ET021-30 Gonad	Control	2024-1550	DCM/NH2	4,14659
ET057-68 Gonad	Control	2024-1551	DCM/NH2	5,2226
ET021-30 Liver	Control	2024-1553	DCM/NH2	3,38288
ET057-68 Liver	Control	2024-1554	DCM/NH2	2,79206
ET093-101 Liver	Control	2024-1555	DCM/NH2	3,71819
ET024-32 Brain	High	2024-1556	DCM/NH2	0,77416
ET024-32 Gonad	High	2024-1559	DCM/NH2	4,48191
ET045-56 Gonad	High	2024-1560	DCM/NH2	4,79961
ET096-107 Gonad	High	2024-1561	DCM/NH2	4,39435
ET024-32 Liver	High	2024-1562	DCM/NH2	3,89065
ET045-56 Liver	High	2024-1563	DCM/NH2	4,83956
ET096-107 Liver	High	2024-1564	DCM/NH2	2,53918
Blank		2024-1565	DCM/NH2	-
Blank		2024-1566	DCM/NH2	-
Spike		2024-1570	DCM/NH2	-

B Full Overview of AQUAvit Samples

Table 17: AQUAvit samples prepared using DCM:Hex followed by GPC

Aquavit				
Type	**#**	**LIMSID**	**Sample-ID**	**Method**
Feed	1	2024-1573	302006501-6-1	DCM:Hex-GPC
Feed	2	2024-1574	302006501-6-1	DCM:Hex-GPC
Feed	3	2024-1575	302006501-6-1	DCM:Hex-GPC
Salmon	1	2023-4840-S1	302006501-6-1	DCM:Hex-GPC
Salmon	2	2023-4841-S1	302006501-6-1	DCM:Hex-GPC
Salmon	3	2023-4842-S1	302006501-6-1	DCM:Hex-GPC
Salmon	4	2023-4843-S1	302006501-6-1	DCM:Hex-GPC
Salmon	5	2023-4844-S1	302006501-6-1	DCM:Hex-GPC
Salmon	6	2023-4845-S1	302006501-6-1	DCM:Hex-GPC
Salmon	7	2023-4846-S1	302006501-6-1	DCM:Hex-GPC
Salmon	8	2023-4847-S1	302006501-6-1	DCM:Hex-GPC
Salmon	9	2023-4848-S1	302006501-6-1	DCM:Hex-GPC
Salmon	10	2023-4849-S1	302006501-6-1	DCM:Hex-GPC
Salmon	11	2023-4850-S1	302006501-6-1	DCM:Hex-GPC
Salmon	12	2023-4851-S1	302006501-6-1	DCM:Hex-GPC
Salmon	13	2023-4852-S1	302006501-6-1	DCM:Hex-GPC
Salmon	14	2023-4853-S1	302006501-6-1	DCM:Hex-GPC
Salmon	15	2023-4854-S1	302006501-6-1	DCM:Hex-GPC
Salmon	16	2023-4855-S1	302006501-6-1	DCM:Hex-GPC
Salmon	17	2023-4856-S1	302006501-6-1	DCM:Hex-GPC
Salmon	18	2023-4857-S1	302006501-6-1	DCM:Hex-GPC
Salmon	19	2023-4858-S1	302006501-6-1	DCM:Hex-GPC
Salmon	20	2023-4859-S1	302006501-6-1	DCM:Hex-GPC
Spike	1	2024-1581	302006501-6-1	DCM:Hex-GPC
Spike	2	2024-1582	302006501-6-1	DCM:Hex-GPC
Spike	3	2024-1583	302006501-6-1	DCM:Hex-GPC
Blank	1	2024-1036	302006501-6-1	DCM:Hex-GPC
Blank	2	2024-1037	302006501-6-1	DCM:Hex-GPC
Blank	3	2024-1038	302006501-6-1	DCM:Hex-GPC
Blank	7	2024-1051	302006501-6-1	DCM:Hex-GPC
Blank	8	2024-1052	302006501-6-1	DCM:Hex-GPC
Blank	9	2024-1053	302006501-6-1	DCM:Hex-GPC
Blank	13	2024-1572	302006501-6-1	DCM:Hex-GPC
Blank	15	2024-1580	302006501-6-1	DCM:Hex-GPC

Table 18: AQUAvit samples prepared using QuEChERS

Aquavit				
Type	**#**	**LIMSID**	**Sample-ID**	**Method**
Salmon	1	2023-4840-S2	302006501-6-1	QuEChERS
Salmon	2	2023-4841-S2	302006501-6-1	QuEChERS
Salmon	3	2023-4842-S2	302006501-6-1	QuEChERS
Salmon	4	2023-4843-S2	302006501-6-1	QuEChERS
Salmon	5	2023-4844-S2	302006501-6-1	QuEChERS
Salmon	6	2023-4845-S2	302006501-6-1	QuEChERS
Salmon	7	2023-4846-S2	302006501-6-1	QuEChERS
Salmon	8	2023-4847-S2	302006501-6-1	QuEChERS
Salmon	9	2023-4848-S2	302006501-6-1	QuEChERS
Salmon	10	2023-4849-S2	302006501-6-1	QuEChERS
Salmon	11	2023-4850-S2	302006501-6-1	QuEChERS
Salmon	12	2023-4851-S2	302006501-6-1	QuEChERS
Salmon	13	2023-4852-S2	302006501-6-1	QuEChERS
Salmon	14	2023-4853-S2	302006501-6-1	QuEChERS
Salmon	15	2023-4854-S2	302006501-6-1	QuEChERS
Salmon	16	2023-4855-S2	302006501-6-1	QuEChERS
Salmon	17	2023-4856-S2	302006501-6-1	QuEChERS
Salmon	18	2023-4857-S2	302006501-6-1	QuEChERS
Salmon	19	2023-4858-S2	302006501-6-1	QuEChERS
Salmon	20	2023-4859-S2	302006501-6-1	QuEChERS
Feed	4	2024-1577	302006501-6-1	QuEChERS
Feed	5	2024-1578	302006501-6-1	QuEChERS
Feed	6	2024-1579	302006501-6-1	QuEChERS
Blank	14	2024-1576	302006501-6-1	QuEChERS
Blank	16	2024-1584	302006501-6-1	QuEChERS
Blank	10	2024-1054	302006501-6-1	QuEChERS
Blank	11	2024-1055	302006501-6-1	QuEChERS
Blank	12	2024-1056	302006501-6-1	QuEChERS
Blank	4	2024-1039	302006501-6-1	QuEChERS
Blank	5	2024-1040	302006501-6-1	QuEChERS
Blank	6	2024-1041	302006501-6-1	QuEChERS
Spike	4	2024-1585	302006501-6-1	QuEChERS
Spike	5	2024-1586	302006501-6-1	QuEChERS
Spike	6	2024-1587	302006501-6-1	QuEChERS

C Risk Assessment

RISIKOANALYSE

Institutt:	Institutt for materialteknologi	**Dato opprettet:**	05/02/2024
Ansvarlig linjeleder (navn):	Ida Westermann	**Sist revidert:**	08/02/2024
Ansvarlig for aktiviteten som risikovurderes:	Lisbet Sørensen og Mari Creese (veiledere SINTEF), Lene Østby (veileder NTNU)		
Deltakere:			
Beskrivelse av den aktuelle aktiviteten, området mv.:	Marie Ibrekk og Rikke Torvanger		

Det skal gjøres forsøk på opparbeidling av fiskevev for analyse på GCxGC-HRMS i SINTEF Ocean sine laboratorier på SeaLab. Dette involverer løsemidler og annet utstyr som kan forårsake risiko.

Aktivitet/arbeidsoppgave	Mulig uønsket hendelse	Eksisterende risikoreduserende tiltak	Vurdering av sannsynlighet (S)	Vurdering av konsekvens(K) *Vurder en konsevenskategori om gangen. Menneske skal alltid vurderes.*				Risikoverdi (S x K)	Forslag til forebyggende og/eller korrigerende tiltak *Prioriter tiltak som kan forhindre at hendelsen inntreffer (sannsynlighetsreduserende tiltak) foran skjerpet beredskap (konsekvensreduserende tiltak)*	Restrisiko etter tiltak (S x K)
			(1-5)	Menneske (1-5)	Øk/materiell (1-5)	Ytre miljø (1-5)	Omdømme (1-5)			
Bruk av diklormetan	Kontakt med hud, øyne og innånding	Avtrekksskap, PPE, overflatepapir, ansvarlig avhending	2	3	1	3	2	6	Minimere eksponeringstid og forsiktig håndtering - derav lavere sannsynlighet	3
Bruk av acetonitrile	Kontakt med hud, øyne og innånding	Avtrekksskap, PPE, overflatepapir, ansvarlig avhending	2	2	1	1	1	4	Minimere eksponeringstid og forsiktig håndtering - derav lavere sannsynlighet	2
Bruk av metanol	Kontakt med hud, øyne og innånding	Avtrekksskap, PPE, overflatepapir, ansvarlig avhending	2	2	1	1	1	4	Minimere eksponeringstid og forsiktig håndtering - derav lavere sannsynlighet	2
Bruk av n-heksan	Kontakt med hud, øyne og innånding	Avtrekksskap, PPE, overflatepapir, ansvarlig avhending	1	3	1	1	1	3		
Bruk av glassutstyr	Knust glass kan føre til glasskår og personskade	Ansvarlig bruk	1	1	1	1	1	1		
Bruk av gass til GCxGC-HRMS	Gasslekasje	Gassensor	1	3	1	1	1	3		
Bruk av skalpell	Kuttskader	Ansvarlig bruk	1	2	1	1	1	2		
Oppholde seg lenge på lab	Høyt støynivå fra instrumenter	Bruk av hørselsvern	1	1	1	1	1	1		
Ultralydbehandling av prøver og utstyr	Høyfrekvent lyd	Bruk av hørselvern, plassere ultralydbadet i avtrekksskap for å minimere støy	1	1	1	1	1	1		
Bruk av kjeramisk ovn til baking av glassutstyr ved 450 grader	Brannskade fra varm ovn eller varmt utstyr	Ovnen åpnes ikke før temperaturen er lav nok, utstyrt med hengelås for å unngå at den åpnes for tidlig, varsel lapp henges på under kjøring, bruk av egnede hansker.	1	2	1	1	1	2		
Analysevekt for utveten av giftige kjemikalier	Eksponering	Benytt maske og punktavsug. Steng skyvedøren	1	1	1	1	1	1		

Kjemikalier	Hazard statements	Precautionary statements
Diklormetan	H315, H319, H336, H35	P202, P261, P264, P302 + P352, P305 + P351 + P338, P308 + P313
Acetonitrile	H225, H320 + H312 + H332, H319	P210, P280, P305 + P351 + P338, P403 + P235
n-heksan	H225, H315, H336, H361f, H373, H411	P202, P280, P303 + P361 + P353, P304 + P340, P308 + P313
Metanol	H225, H320 + H312 + H332, H370	P233, P280, P301 + P310, P303 + P361 + P353, P304 + P340 + P311

D SINTEF SOP GPC Clean-Up

SOP – GPC-clean-up

1. Check that there is enough DCM (lines A and B) and the waste container is empty
2. Check that the correct method is loaded
3. Turn the DAD on (software)
4. Open the flush port and flush with DCM at 5 mL/min for 5 minutes (check the line for absence of air bubbles)
5. Turn the flow to 1 mL/min and close the flush port, make sure to have a collection vial for the flow coming from the injector capillary
6. If the system was in isopropanol: Flush with DCM for 5 minutes in main path and another 5 minutes in bypass mode – check for leaks. If not, 30 sek in each setting is enough.
7. Turn the flow off (0 mL/min)
8. Connect pre-column and column to the system (quickly) – tighten without overtightening
9. Turn the flow on 1 mL/min – pressure ok (~6 bar)? No leaks?
10. Leave at 1 mL/min for 1 minute, turn to 2 mL/min and leave for 1 minute, continue until 5 mL/min – leave for 5 minutes to equilibrate. Pressure ok (~38-40 bar)? No leaks?
11. Reset fraction volumes (software)
12. Save sequence as – write sequence (remember SINTEF-ID in the sample name) – save sequence

    Remember 1 blank sample between each sample (injection of DCM, collection of 5 mL) – not used for further analysis, just to clean the system, all can be collected in the same empty collection vial
13. Place sample vials in the autosampler and labelled collection vials (2 mL hexane in each) in the fraction collector. Double-check locations.
14. Run sequence
15. Collect samples
16. Turn off pump and DAD/System in standby
17. Disconnect and plug the columns
18. Empty the waste
19. Reset fraction volumes

If you are *not* planning to use the instrument the next day – flush the system with isopropanol:

1. Open the flush port and flush with at 5 mL/min for 5 minutes – make sure to use the right line (B,C,D) (check the line for absence of air bubbles)
2. Turn the flow to 1 mL/min and close the flush port
3. Flush for 5 minutes in main path and another 5 minutes in bypass mode
4. Turn the flow off
5. Hang a warning on the pump that the system is set in isopropanol

E Compounds in ToxMix Standard

Table 19: Compounds with retention times in ToxMix standard

Compound class	Compound Name	Short name	CAS#	Rt (1D)	Rt (2D)
alk	Decalin, cis		91-17-8	9,9601	0,914
alk	Decalin, trans		91-17-8	10,0101	0,894
alk	Methyldecalin		2958-76-1	11,6101	0,894
alk	Pristane		1921-70-6	25,4837	0,534
alk	Phytane		638-36-8	27,6156	0,592
alk	5@Androstane		438-22-2	31,0887	1,511
alk	17.alpha.(H).21.beta.(H)-Hopane		13849-96-2	48,2092	2,085
arom	o-Terphenyl		84-15-1	28,2684	2,761
arom	1-Phenyldodecane		123-01-3	28,6813	1,145
degr	Benzamide. 3.4-fluoro-		85118-04-3	14,6387	4,237
Metabolites	1-Pyrenol		5315-79-7	38,2101	5,319
n-alkanes		nC12	112-40-3	12,9491	0,534
n-alkanes		nC13	629-50-5	15,6127	0,542
n-alkanes		nC14	629-59-4	18,2012	0,586
n-alkanes		nC15	629-62-9	20,6641	0,594
n-alkanes		nC16	544-76-3	22,9911	0,592
n-alkanes		nC17	629-78-7	25,2146	0,639
n-alkanes		nC18	593-45-3	27,3294	0,654
n-alkanes		nC19	629-92-5	29,3649	0,654
n-alkanes		nC20	112-95-8	31,2641	0,689
n-alkanes		nC21	629-94-7	33,1164	0,703
n-alkanes		nC22	629-97-0	34,8815	0,688
n-alkanes		nC23	638-67-5	36,5768	0,714
n-alkanes		nC24	646-31-1	38,2085	0,757
n-alkanes		nC25	629-99-2	39,7101	0,774
n-alkanes		nC26	630-01-3	41,2462	0,794
n-alkanes		nC27	593-49-7	42,7195	0,815
n-alkanes		nC28	630-02-4	44,1119	0,834
n-alkanes		nC30	638-68-6	46,7851	0,894
n-alkanes		nC32	544-85-4	49,3101	1,014
n-alkanes		nC34	14167-59-0	53,8212	1,881
n-alkanes		nC35	630-07-9	55,9101	2,334
NSO	Benzo(h)quinoline		230-27-3	25,9997	3,601
NSO	Xanthone		90-47-1	27,3726	3,618
OCPs	Tripropyl phosphate	TPP	513-08-6	16,8976	1,779
OCPs	Tributyl phosphate	TBP	126-73-8	23,3101	1,591
OCPs	Tris-(2-chloroethyl)phospate	TCEP	115-96-8	25,0539	3,571
OCPs	Triphenyl phosphate	TPhP	115-86-6	36,9815	4,057
OCPs	Tris(2-ethylhexyl)phosphate	TEHP	78-42-2	38,9351	1,134
PAH	Naphthalene		91-20-3	11,8006	2,092
PAH	1-Methylnaphthalene		90-12-0	15,1184	2,211
PAH	Biphenyl		92-52-4	16,8346	2,253
PAH	2,3-Dimethylnaphthalene		581-40-8	17,5866	1,985
PAH	2,6-Dimethylnaphthalene		581-42-0	18,3934	2,211
PAH	Acenaphthylene		208-96-8	18,4407	2,801
PAH	Acenaphthene		83-32-9	19,3873	2,596
PAH	Dibenzofuran		132-61-91	20,1611	2,554
PAH	2,3,5-Trimethylnaphthalene		2245-38-7	21,3298	2,044
PAH	Fluorene		86-73-7	21,7101	2,671
PAH	1-Methylfluorene		1730-37-6	24,5333	2,590
PAH	1,2,5,6-Tetramethylnaphthalene		2131-43-3	25,2117	2,268
PAH	Dibenzothiophene		132-65-0	25,3084	3,286
PAH	Phenanthrene		85-01-8	25,9703	3,262
PAH	4-Methyldibenzothiophene		7372-88-5	27,4777	3,043
PAH	1-Methylphenanthrene		832-69-9	28,8634	3,226
PAH	Phenanthrene. 3.6-dimethyl-		1576-67-6	30,6868	2,872
PAH	Fluoranthene		206-44-0	31,3434	3,661

PAH	1,2-Dimethylphenanthrene		20291-79-2	31,9101	3,241
PAH	Pyrene		129-00-0	32,2409	3,970
PAH	2,6,9-Trimethylphenanthrene		66271-32-7	33,4658	2,787
PAH	1-Methylpyrene		2381-21-7	34,8718	3,914
PAH	1,2,6,9-Tetramethylphenanthrene		204256-39-3	36,3101	2,900
PAH	Chrysene		218-01-9	37,9001	4,146
PAH	Benz[a]anthracene		56-55-3	37,9768	4,254
PAH	1-Methylchrysene		3351-28-8	40,4101	4,314
PAH	Benzo[b]fluoranthene		205-99-2	42,5530	4,768
PAH	Benzo[e]pyrene		192-97-2	43,5275	5,266
PAH	Benzo[a]pyrene		50-32-8	43,6351	5,274
PAH	Perylene		198-55-0	43,9901	5,454
PAH	Indeno[1,2,3-cd]pyrene		193-39-5	47,7768	5,754
PAH	Dibenz[ah]anthracene		224-41-9	47,9101	5,724
PAH	Benzo[ghi]perylene		191-24-2	48,6101	0,309
PBDEs	2,4,4'-Tribromodiphenyl ether	BDE-28	41318-75-6	34,7601	3,414
PBDEs	2,2',4,4'-Tetrabromodiphenyl ether	BDE-47	5436-43-1	38,9101	3,834
PBDEs	2,3',4',6-Tetrabromodiphenyl ether	BDE-66	189084-62-6	39,4351	3,984
PBDEs	2,2',4,4',6-Pentabromodiphenyl ether	BDE-100	189084-64-8	41,9351	4,089
PBDEs	2,2',3,4,4'-Pentabromodiphenyl ether	BDE-85	182346-21-0	42,8101	4,194
PBDEs	2,2',4,4',5-Pentabromodiphenyl ether	BDE-99	60348-60-9	44,2101	4,874
PBDEs	2,2',4,4',5,5'-Hexabromodiphenyl ether	BDE-153	68631-49-2	45,1601	4,644
PBDEs	2,2',4,4',5,6'-Hexabromodiphenyl ether	BDE-154	207122-15-4	46,3101	4,674
PCBs	2,4,4'-Trichlorobiphenyl	PCB-28	7012-37-5	27,9701	2,478
PCBs	2,2',5,5'-Tetrachlorobiphenyl	PCB-52	35693-99-3	29,3726	2,499
PCBs	2,2',4,5,5'-Pentachlorobiphenyl	PCB-101	37680-73-2	32,6539	2,454
PCBs	2,3',4,4',5-Pentachlorobiphenyl	PCB-118	31508-00-6	34,8351	2,619
PCBs	2,2',3,4,4',5-Hexachlorobiphenyl	PCB-138	35065-28-2	35,8030	2,471
PCBs	2,2',4,4',5,5'-Hexachlorobiphenyl	PCB-153	35065-27-1	36,6601	2,794
PCBs	2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB-180	35065-29-3	39,1101	2,714
Pesticide	Hexachlorobenzene	HCB	118-74-1	24,6795	2,034
Phenol	2,4-Dimethylphenol		105-67-9	10,7815	1,743
Phenol	4-Ethylphenol		123-07-9	11,2372	1,779
Phenol	3,5-Dimethylphenol		108-68-9	11,3801	1,728
Phenol	2,4,6-Trimethylphenol		527-60-6	12,2789	1,779
Phenol	2,3,5-Trimethylphenol		697-82-5	14,1101	1,854
Phenol	4-tert-Butylphenol		98-54-4	14,6914	1,742
Phenol	4-Isopropyl-3-methylphenol		3228-02-2	15,7101	1,854
Phenol	4-tert-Butyl-2-methylphenol		98-27-1	16,4768	1,674
Phenol	4-n-Pentylphenol		14938-35-3	18,9101	1,794
Phenol	2,6-Di-tert-butyl-4-methylphenol		128-37-0	20,4101	1,329
Phenol	2,6-Di-tert-butylphenol		128-39-2	21,3101	1,434
Phenol	4-Hexylphenol		2446-69-7	21,3226	1,794
Phenol	4-tert-Octylphenol		140-66-9	22,4601	1,674
Phenol	4-Heptylphenol		1987-50-4	23,7101	1,749
Phenol	4-n-Octylphenol		1806-26-4	25,9101	1,754
Phenol	4-n-Nonylphenol		104-40-5	28,0664	1,734
Phthalates	Diethyl phthalate	DEP	84-66-2	21,7993	2,662
Phthalates	Di-n-butylphthalate	DBB	84-74-2	29,6101	2,264
Phthalates	Benzyl butyl phthalate	BBP	85-68-7	36,2434	3,284
Phthalates	Bis(2-ethylhexyl)adipate	DEHA	103-23-1	37,7101	1,254
Phthalates	Di-n-octyl phthalate	DNOP	117-84-0	42,5101	2,004
Plastic/rubber chemical	Benzothiazole		95-16-9	12,5231	3,035
Plastic/rubber chemical	n-Cyclohexylformamide		766-93-8	13,0601	3,174
Plastic/rubber chemical	Phthalide		87-41-2	15,3070	4,209
Plastic/rubber chemical	2,4,7,9-Tetramethyl-5-decyne-4,7-diol		126-86-3	18,1042	1,070
Plastic/rubber chemical	Phthalimide		85-41-6	18,1780	4,254
Plastic/rubber chemical	Methylparaben		99-76-3	18,5101	2,826

Plastic/rubber chemical	3-Methyl-2(3H)-Benzothiazolone		2786-62-1	21,1101	3,999
Plastic/rubber chemical	2-(Methylthio)benzothiazole		615-22-5	21,9164	3,324
Plastic/rubber chemical	n-Butylbenzenesulfonamide		3622-84-2	25,5101	3,667
Plastic/rubber chemical	Bisphenol A	BPA	80-05-7	33,1101	4,297
Plastic/rubber chemical	Bumetrizole		3896-11-5	39,7684	2,461
PPCP	Isoeugenol		97-53-0	18,5601	2,214
PPCP	Benzophenone		119-61-9	22,5284	3,124
PPCP	Atrazine		1912-24-9	25,0789	2,994
PPCP	Caffeine		58-08-2	26,6201	4,842
PPCP	Allethrin		584-79-2	32,1101	1,794
PPCP	Triclosan		3380-34-5	32,2039	2,829
PPCP	Fluconazole		86386-73-4	32,4101	3,834
PPCP	17β-Estradiol	Estradiol	50-28-2	40,6501	4,434
PPCP	Praziquantel		55268-74-1	44,9601	5,274
PPCP	Nicotine		54-11-5	16,1101	2,034

F Compounds and Concentrations in Spike Mixes

Table 20: Compounds and concentrations in SpikeMix 1

	EmPolMix3						10 mL	
	Stock solution	Solvent Stock	Stock made/bought date	Conc. stam (mg/mL)	Stock withdrawal (mL)	Total volume	Solvent	Conc. µg/mL
ADD	S-319 Phthalimide	DCM	23/08/2019	1,001	0,1	10 mL	DCM	10,01
ADD	S-320 2-(methylthio)benzothiazole	DCM	23/08/2019	1,005	0,1			10,05
PPCP	S-321 Triclosan	DCM	23/08/2019	1,003	0,1			10,03
ADD	S-322 Benzophenone	DCM	23/08/2019	1,001	0,1			10,01
OCP	S-323 Tris(2-thylhexyl)phosphate	DCM	23/08/2019	1,003	0,1			10,03
OCP	S-325 Tripropyl phosphate	DCM	26/08/2019	0,994	0,1			9,94
DIV	S-326 1-Phenyldodecane	DCM	26/08/2019	1,007	0,1			10,07
OCP	S-327 N-butylbenzenesulfonamide	DCM	26/08/2019	1,006	0,1			10,06
OCP	S-328 Tris(2-chloroethyl)phosphate	DCM	26/08/2019	1,007	0,1			10,07
OCP	S-329 Triphenyl phosphate	DCM	26/08/2019	0,998	0,1			9,98
ADD	S-335 4-tert-octylphenol	DCM	04/09/2019	1,005	0,1			10,05
ADD	S-336 4-n-nonylphenol	DCM	04/09/2019	0,998	0,1			9,98
ADD	S-337 Benzothiazole	DCM	04/09/2019	1,007	0,1			10,07
ADD	S-338 Acetophenone	DCM	04/09/2019	1,008	0,1			10,08
ADD	S-339 Phthalide	DCM	04/09/2019	1,005	0,1			10,05
ADD	S-340 N-cyclohexylformamide	DCM	04/09/2019	1,005	0,1			10,05
ADD	S-341 Bisphenol A	DCM	04/09/2019	1,014	0,1			10,14
ADD	S-345 2,6-Di-tert-butyl-4-methylphenol	DCM	10/09/2019	1,023	0,1			10,23
ADD	S-346 4-n-octylphenol	DCM	10/09/2019	1,036	0,1			10,36
ADD	S-352 2,6-di-tert-butylphenol	DCM	20/09/2019	1,022	0,1			10,22
ADD	S-467 6-PPD-quinone	DCM	24/03/2021	0,11	1,0			11
ADD	S-497 3-methyl-2(3H)-Benzothiazolone	DCM	07/05/2021	0,998	0,1			9,98
DIV	S-498 Xanthone	DCM	07/05/2021	1,003	0,1			10,03
DIV	S-499 Benzo(h)quinone	MeOH	11/05/2021	1,004	0,1			10,04
ADD	S-501 6PPD	DCM	11/05/2021	0,114	1,0			11,4
ADD	S-505 Bumetrizole	DCM	18/05/2021	1,029	0,1			10,29
ADD	S-506 Tetramethyl-5-decyne-4,7-diol	DCM	18/05/2021	0,989	0,1			9,89
OCP	S-507 Tributyl phosphate	DCM	18/05/2021	1,085	0,1			10,85
ADD	S-508 Methylparaben	DCM	18/05/2021	1,031	0,1			10,31
PPCP	S-635 Ibuprofen	DCM	25/01/2024	1,005	1,0			100,5
PHT	S-636 Diisobutylphthalate	DCM	25/01/2024	1,008	0,1			10,08
PPCP	S-637 Hexachlorobenzene	DCM	25/01/2024	1,006	0,1			10,06
ADD	S-638 2-Hydroxybenzothiazole	DCM	25/01/2024	1,002	0,1			10,02
ADD	S-639 1,3-Diphenylguanidine	DCM	25/01/2024	0,998	0,1			9,98
PPCP	S-640 Estradiol	DCM	25/01/2024	0,996	0,1			9,96
PCB	STD-322/1 Dutch seven PCBs	DCM	05/08/2019	0,1	1,000			10
BDE	STD-323/1 PBDE mix of nine tri- to-heptabrominated compounds	Isooctane	27/05/2020	0,1	1,0			10
PHT	STD-324/1 Phthalate mix of seven compounds, surfactants Mix 1	Isooctane	05/08/2019	1,0	0,1			10

Table 21: Compounds and concentrations in SpikeMix 2

	Custom mix PAHs	Custom mix C0-C3 phenol	Custom mix C4-C5 Phenol	16-PAH	QA-PAH	QA-PAH
Standard-ID	**STD-270/1**	**STD-282/1**	**STD-283/1**	**STD-267/6**	**A707**	**A766**
Kons	**~500**	**~100**	**~100**	**~100**	**5**	**0,05**
Enhet	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**	**µg/mL**
	100	500	500	500		
					500µL STD-267/6 100µL STD-270/1 500µL STD-282/1 500µL STD-283/1 total 10mL	100µL A707 total 10mL
Naphthalene-d8						
Phenanthrene-d10						
Perylene-d12						
Chrysene-d12						
Phenol-d6						
p-cresol-d8						
4-n-propylphenol-d12						
Acenaphthene-d10						
Fluorene-d10						
Decalin (cis+trans)	1002				10,02	0,1002
C1-decalins (1-methyldekalin)					0,00	0,0000
C2-decalins					0,00	0,0000
C3-decalins					0,00	0,0000
C4-decalins					0,00	0,0000
Benzo(b)thiophene (Benzothiophene)	499				4,99	0,0499
Naphthalene				100	5,00	0,0500
C1-naphthalenes (1-methylnaphthalene)	500				5,00	0,0500
C2-naphthalenes (2,3-Dimethylnapthalene)	505				5,05	0,0505
C3-naphthalenes (2,3,5-Trimethylnapthalene)	498				4,98	0,0498
C4-naphthalenes (1,2,5,6-tetramethylnaphthalene)					0,00	0,0000
Biphenyl	498				4,98	0,0498
Acenaphthylene				100	5,00	0,0500
Acenaphthene				100	5,00	0,0500
Dibenzofuran	499				4,99	0,0499
Fluorene				100	5,00	0,0500
C1-fluorenes (1-methylfluorene)					0,00	0,0000
C2-fluorenes					0,00	0,0000
C3-fluorenes					0,00	0,0000
Phenanthrene				100	5,00	0,0500
Anthracene				100	5,00	0,0500
C1-phenanthrenes/anthracenes (1-methylphenanthrene)	499				4,99	0,0499
C2-phenanthrenes/anthracenes (1,2-dimethylphenanthrene)					0,00	0,0000
C3-phenanthrenes/anthracenes (2,6,9-trimethylphenanthrene)					0,00	0,0000
C4-phenanthrenes/anthracenes (1,2,6,9-tetramethylphenanthrene)					0,00	0,0000
Dibenzothiophene	498				4,98	0,0498
C1-dibenzothiophenes (4-methyldibenzothiophene)					0,00	0,0000
C2-dibenzothiophenes	500				5,00	0,0500
C3-dibenzothiophenes					0,00	0,0000
C4-dibenzothiophenes					0,00	0,0000
Fluoranthene				100	5,00	0,0500
Pyrene				100	5,00	0,0500
C1-fluoranthrenes/pyrenes (1-methylpyrene)	502				5,02	0,0502
C2-fluoranthenes/pyrenes	502				5,02	0,0502
C3-fluoranthenes/pyrenes					0,00	0,0000
Benz(a)anthracene				100	5,00	0,0500
Chrysene				100	5,00	0,0500
C1-chrysenes (1-methylcrysene)					0,00	0,0000
C2-chrysenes					0,00	0,0000
C3-chrysenes					0,00	0,0000
C4-chrysenes					0,00	0,0000
Benzo(b)fluoranthene				100	5,00	0,0500
Benzo(k)fluoranthene				100	5,00	0,0500
Benzo(e)pyrene	498				4,98	0,0498
Benzo(a)pyrene				100	5,00	0,0500
Perylene	499				4,99	0,0499
Indeno(1,2,3-c,d)pyrene				100	5,00	0,0500
Dibenz(a,h)anthracene				100	5,00	0,0500
Benzo(g,h,i)perylene				100	5,00	0,0500
Phenol		100			5,00	0,0500
C1-Phenols (o- og p-cresol)		204			10,20	0,1020
C2-Phenols (4-ethylphenol?)		100			5,00	0,0500
C3-Phenols (2,4-dimethylphenol?)		101			5,05	0,0505
C4-Phenols (4-n-butylphenol?)			99		4,95	0,0495
C5-Phenols (2-tert-butyl-4-methylphenol?)			100		5,00	0,0500

G Lipid Weight Extract

Table 22: Weight of lipid after extraction with ACN

ACN test sample #	Initial sample weight [g]	Kimax wo/extract [g]	Kimax w/extract [g]	Extract weight [g]	w% extract [%]
1	1,09	11,41968	11,43138	0,01170	1,1
2	1,07	11,44059	11,45139	0,01080	1,0
3	0,98	11,02600	11,03537	0,00937	1,0
4	1,03	11,47575	11,48450	0,00875	0,8
5	1,01	11,42809	11,43623	0,00814	0,8
6	1,13	11,04827	11,05579	0,00752	0,7

Table 23: Lipid weight after extraction with DCM:Hex

DCM:Hex test sample #	Initial sample weight [g]	Kimax wo/extract [g]	Kimax w/extract [g]	Extract weight [g]	w% extract [%]
1	1,02	11,12181	11,31351	0,1917	18,8
2	1,01	10,98499	11,17528	0,19029	18,8
3	1,01	11,13562	11,34098	0,20536	20,3
4	1,03	11,18063	11,31042	0,12979	12,6
5	1,01	11,46195	11,60584	0,14389	14,2
6	1,01	11,27392	11,42397	0,15005	14,9

Table 24: Average weight percentage of extract and standard deviation after extraction with ACN and DCM:Hex

Solvent	Average w% of extract	Standard deviation
ACN	0,9	0,15
DCM:Hex	16,6	3,11

H Detected Compounds in Selected AQUAvit Samples

Table 25: Detected compounds in DCM samples

			Feed 1	Feed 3	DCM 1	DCM 2	DCM 9	DCM 10	DCM 19	DCM 20	Spike 1	Spike 3	Blank 2	Blank 8	Blank 13	Blank 15
Identified compound name	**RT1**	**RT2**	**2024-1573**	**2024-1575**	**2023-4840-S1**	**2023-4841-S1**	**2023-4848-S1**	**2023-4849-S1**	**2023-4858-S1**	**2023-4859-S1**	**2024-1581**	**2024-1583**	**2024-1037**	**2024-1052**	**2024-1572**	**2024-1580**
	10,7992	2,1116														
	11,2992	2,1321														
Naphthalene-d8	11,6992	2,5216	1	1	1	1	1	1	1	1	1	1	1		1	1
	11,8992	1,2095	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	12,2992	2,1526	1	1		1	1	1	1	1	1	1			1	
	12,4992	3,5261	1	1	1	1		1		1	1	1		1	1	1
	14,6992	2,1321	1		1	1	1	1	1	1	1	1	1		1	1
	15,0992	2,6651	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	16,7992	2,7061	1	1	1	1	1	1	1	1	1	1			1	
	17,5992	2,3781														
	18,0992	1,3325	1	1	1		1	1	1	1	1	1	1	1	1	1
	18,3992	2,5626	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Acenaphthene-d10	19,1992	3,0751	1	1	1	1	1	1	1	1	1		1	1	1	1
	20,3992	1,6400	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	21,3992	2,5216	1	1	1	1	1	1	1	1	1	1				1
	21,7992	3,0956	1	1	1	1	1	1	1	1	1	1	1		1	1
	21,9992	3,8541	1		1	1			1		1	1	1	1	1	1
	22,4992	1,0296	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	22,5992	3,6081	1	1	1	1	1	1	1		1	1		1	1	1
	23,2992	1,9270	1	1	1	1	1	1	1	1	1	1	1	1		
Fluorene-d10	24,4992	3,0546	1	1	1	1	1	1		1	1	1		1	1	1
	24,6992	2,4601	1	1		1	1	1	1	1	1	1	1	1	1	1
	25,0992	4,1411	1	1	1	1	1	1		1	1	1			1	
	25,1992	2,6856	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	25,3990	0,7590	1	1	1	1	1	1	1	1	1	1	1			1
Phenanthrene-d10	25,8992	3,7926	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	26,6992	5,4326	1	1	1	1	1	1	1	1	1	1	1		1	1
	27,5990	0,7790	1	1		1	1	1	1	1	1	1				1
	27,9992	2,9521	1	1	1	1	1	1	1	1	1			1	1	1
	28,1992	3,3211	1	1			1	1	1	1	1	1	1	1		1
	28,1992	2,0911	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	28,8992	3,7311	1	1	1	1			1	1	1	1	1		1	1
	29,2992	2,9931	1	1	1	1	1	1		1	1	1	1		1	1
	29,5992	2,6856		1		1		1	1		1	1	1	1	1	1
	30,5992	3,3006		1	1		1	1	1	1	1	1	1		1	1
	31,0992	1,8655	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	31,9992	3,7311	1	1	1		1		1	1	1	1	1	1		
	32,2992	4,6126		1	1		1		1		1	1	1	1		1
	32,3992	3,2391	1	1	1	1	1	1	1	1	1	1	1		1	1
	32,6992	2,9316														
	34,7992	4,0181	1	1	1	1			1		1	1			1	1
	34,8992	3,1161			1	1	1	1	1	1	1	1			1	1
	34,9992	4,4896	1	1		1			1		1	1		1		
	35,7992	2,9316		1	1	1		1		1	1	1	1		1	1
	36,2992	3,4236	1		1	1	1			1		1				1
	36,5992	3,3416			1		1		1	1	1	1	1		1	1
	37,6992	1,5580														
Chrysene-d12	38,0992	5,0021	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	38,8992	4,5306				1			1	1		1				
	39,0992	3,1981	1	1	1	1	1	1	1	1	1	1	1		1	1
	39,4992	4,5921	1	1		1	1	1	1	1	1		1	1	1	1
	39,7992	2,9521	1	1	1	1	1		1	1	1	1	1	1	1	1
	40,4992	4,9406	1	1		1	1		1	1	1	1				
	41,9992	4,6946	1	1		1	1	1	1	1	1	1	1		1	
	48,1992	2,5011														
		Total	42	43	39	43	41	38	43	43	47	46	33	26	38	40

Table 26: Detected compounds in ACN samples

			Feed 4	Feed 6	ACN 1	ACN 2	ACN 9	ACN 10	ACN 19	ACN 20	Spike 4	Spike 6	Blank 5	Blank 11	Blank 14	Blank 16
Identified compound name	**RT1**	**RT2**	**2024-1577**	**2024-1579**	**2023-4840-S2**	**2023-4841-S2**	**2023-4848-S2**	**2023-4849-S2**	**2023-4858-S2**	**2023-4859-S2**	**2024-1585**	**2024-1587**	**2024-1040**	**2024-1055**	**2024-1576**	**2024-1584**
	10,7992	2,1116														
	11,2992	2,1321														
Naphthalene-d8	11,6992	2,5216	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	11,8992	1,2095	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	12,2992	2,1526	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	12,4992	3,5261	1	1	1	1	1	1	1	1	1	1	1		1	1
	14,6992	2,1321		1	1	1	1	1	1	1	1	1	1	1		1
	15,0992	2,6651	1	1	1	1	1	1	1	1	1	1	1		1	1
	16,7992	2,7061	1	1	1	1	1	1	1	1	1	1		1		1
	17,5992	2,3781				1										
	18,0992	1,3325	1	1	1			1	1	1		1	1	1	1	1
	18,3992	2,5626	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Acenaphthene-d10	19,1992	3,0751	1	1	1	1	1	1	1	1	1	1	1		1	1
	20,3992	1,6400	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	21,3992	2,5216	1		1	1	1	1	1	1	1	1	1	1	1	1
	21,7992	3,0956	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	21,9992	3,8541	1	1	1	1	1	1	1	1	1	1	1	1		1
	22,4992	1,0296	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	22,5992	3,6081	1	1	1	1	1	1	1	1	1	1	1	1		1
	23,2992	1,9270	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Fluorene-d10	24,4992	3,0546	1	1	1	1	1		1	1		1				1
	24,6992	2,4601		1	1	1	1	1	1	1		1	1	1	1	1
	25,0992	4,1411	1	1		1		1		1	1	1		1		
	25,1992	2,6856	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	25,3990	0,7590	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Phenanthrene-d10	25,8992	3,7926	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	26,6992	5,4326	1	1		1			1			1				
	27,5990	0,7790	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	27,9992	2,9521	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	28,1992	3,3211	1	1	1	1	1	1		1	1	1		1		1
	28,1992	2,0911	1	1	1	1	1	1	1	1	1	1	1		1	1
	28,8992	3,7311		1	1	1	1	1	1	1	1	1				1
	29,2992	2,9931	1	1		1	1	1			1	1		1		1
	29,5992	2,6856	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	30,5992	3,3006	1	1	1	1	1	1	1	1	1	1				1
	31,0992	1,8655	1	1	1	1	1	1	1	1	1	1			1	
	31,9992	3,7311	1	1	1	1	1	1	1	1	1	1				1
	32,2992	4,6126	1	1	1		1	1	1	1	1	1				1
	32,3992	3,2391	1	1	1	1	1	1	1			1				
	32,6992	2,9316									1					
	34,7992	4,0181	1	1			1	1			1	1				1
	34,8992	3,1161		1	1	1	1		1			1				1
	34,9992	4,4896	1					1	1	1	1	1				1
	35,7992	2,9316	1	1	1	1	1	1	1	1	1	1		1		1
	36,2992	3,4236	1	1	1	1		1		1	1	1			1	
	36,5992	3,3416		1		1		1	1	1	1	1				1
	37,6992	1,5580														
Chrysene-d12	38,0992	5,0021	1	1	1	1		1	1	1	1	1	1	1	1	1
	38,8992	4,5306					1			1						
	39,0992	3,1981	1	1	1	1		1			1	1				1
	39,4992	4,5921	1	1	1	1		1	1		1	1	1		1	1
	39,7992	2,9521	1	1	1	1	1	1		1	1	1			1	1
	40,4992	4,9406	1	1				1		1	1	1				1
	41,9992	4,6946	1	1	1	1	1			1		1				1
	48,1992	2,5011														
		Total	43	46	41	44	39	44	39	42	42	48	26	26	26	43

I Detected Peaks from ToxMix Standard

Group	Name	RT1 (min)	RT2 (min)
alk	5@-Androstane	31,0992	1,8655
alk	17α(H), 21β(H)-Hopane	48,1992	2,5011
alk	Pristane	25,399	0,759
alk	Phytane	27,599	0,779
alk	Methyldecalin	11,8992	1,2095
aromatic	o-Terphenyl	28,1992	3,3211
n-alkanes	n-C12	12,8992	0,738
n-alkanes	n-C13	15,5992	0,7585
n-alkanes	n-C14	18,1992	0,779
n-alkanes	n-C15	20,9992	0,7995
n-alkanes	n-C16	22,9992	0,82
n-alkanes	n-C17	25,199	0,841
n-alkanes	n-C18	27,299	0,861
n-alkanes	n-C19	29,399	0,861
n-alkanes	n-C20	31,2992	0,9020
n-alkanes	n-C21	33,0992	0,9020
n-alkanes	n-C22	34,8992	0,9225
n-alkanes	n-C23	36,5992	0,9635
n-alkanes	n-C24	38,1992	0,984
n-alkanes	n-C25	39,7992	1,0045
n-alkanes	n-C26	41,2992	1,025
n-alkanes	n-C27	42,6992	1,0455
n-alkanes	n-C28	44,0992	1,0865
n-alkanes	n-C30	46,7992	1,148
n-alkanes	n-C32	49,2992	1,2915
n-alkanes	n-C34	53,8992	2,2756
n-alkanes	n-C36	55,8992	2,7881
OCP	Tributyl phosphate	23,2992	1,927
OCP	Tris-(2-chloroethyl)phospate	25,0992	4,1411
PAH	Naphthalene-d8	11,6992	2,5216
PAH	Acenaphthene-d10	19,1992	3,0751
PAH	1-Methylnaphthalene	15,0992	2,6651
PAH	2,3-Dimethylnaphthalene	17,5992	2,3781
PAH	2,6-Dimethylnaphthalene	18,3992	2,5626
PAH	2,3,5-Trimethylnaphthalene	21,3992	2,5216
PAH	1,2,5,6-Tetramethylnaphthalene	25,1992	2,6856
PAH	1-Methylfluorene	24,4992	3,0546
PAH	Phenanthrene-d10	25,8992	3,7926
PAH	1-Methylphenantrene	28,8992	3,7311
PAH	*3,6-Dimethylphenanthrene**	30,5992	3,3006
PAH	1,2-Dimethylphenantrene	31,9992	3,7311
PAH	1,2,6,9-Tetramethylphenantrene	36,2992	3,4236
PAH	Chrysene-d12	38,0992	5,0021
PAH	1-Methylchrysene	40,4992	4,9406
PAH	Biphenyl	16,7992	2,7061
PAH	Pyrene	32,2992	4,6126
PAH	1-Methylpyrene	34,9992	4,4896
PCB	2,2',4,5,5'-Pentachlorobiphenyl	32,6992	2,9316
PCB	2,2',5,5'-Tetrachlorobiphenyl	29,2992	2,9931
PCB	2,4,4'-Trichlorobiphenyl	27,9992	2,9521
PCB	2,2',3,4,4',5,5'-Heptachlorobiphenyl	39,0992	3,1981

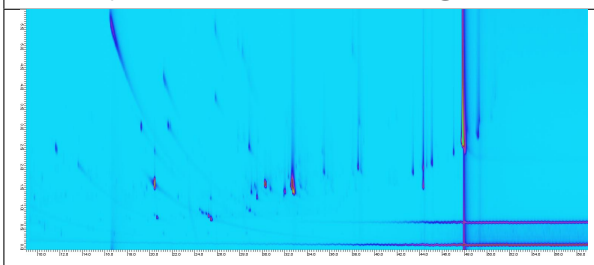
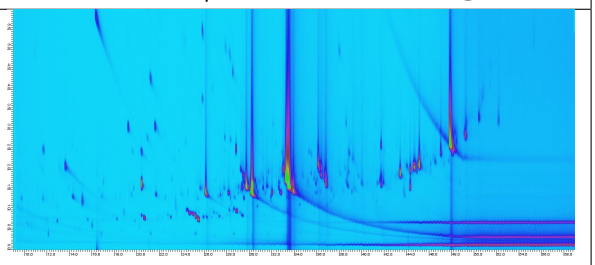
PCB	2,3',4,4',5-Pentachlorobiphenyl	34,8992	3,1161
PCB	2,2',3,4,4',5-Hexachlorobiphenyl	35,7992	2,9316
PCB	2,2',4,4',5,5'-Hexachlorobiphenyl	36,5992	3,3416
Phenol	2,4-Dimethylphenol	10,7992	2,1116
Phenol	3,5-Dimenthylphenol	11,2992	2,1321
Phenol	2,4,6-Trimethylphenol	12,2992	2,1526
Phenol	4-tert-Butylphenol	14,6992	2,1321
Phenol	4-tert-Octylphenol	22,4992	1,0296
Phenol	2,6-Di-tert-butyl-4-methylphenol	20,3992	1,6400
Phenol	4-n-Nonylphenol	28,1992	2,0911
Phtalates	Di-n-butylphthalate	29,5992	2,6856
Phtalates	Dietyl phtalate	21,7992	3,0956
Phtalates	Bis(2-ethylhexyl)adipate	37,6992	1,5580
Plastic/rubber chemical	2-(Methylthio)benzothiazole	21,9992	3,8541
Plastic/rubber chemical	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	18,0992	1,3325
Plastic/rubber chemical	Benzothiazole	12,4992	3,5261
Plastic/rubber chemical	Bumetrizole	39,7992	2,9521
PPCP	Caffeine	26,6992	5,4326
PPCP	Benzophenone	22,5992	3,6081
PPCP	Triclosan	32,3992	3,2391
PBDEs	2,2',4,4',6-Pentabromodiphenyl ether	41,9992	4,6946
PBDEs	2,3',4',6-Tetrabromodiphenyl ether	39,4992	4,5921
PBDEs	2,2',4,4'-Tetrabromodiphenyl ether	38,8992	4,5306
PBDEs	2,4,4'-Tribromodiphenyl ether	34,7992	4,0181

J Detected Peaks from OilMix

Table 27: Detected compounds from OilMix

Methylation:	Compound:	RT1:	RT2:	Mass:
C1	2,3-dihydro-5-methyl-1H-Indene	10,9992	1,8245	132
Tetraline	1,2,3,4-tetrahydronaphthalene	11,1992	2,0296	132
C2	2,3-dihydro-4,7-dimethyl-1H-Indene	14,0992	2,0091	146
C2	1,2,3,4-tetrahydro-5,7-dimethyl-naphthalene	14,9992	1,7015	160
C3	1,2,3,4-tetrahydro-1,1,6-trimethyl-naphthalene	16,0992	1,722	174
C5	2,3-dihydro-1,1,4,5,6-pentamethyl-1H-Indene	20,4992	2,1116	188

K Chromatograms from AQUAvit

QuEChERS chromatogram	DCM:Hex/GPC chromatogram
	
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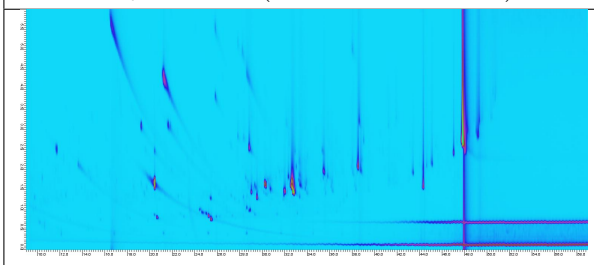	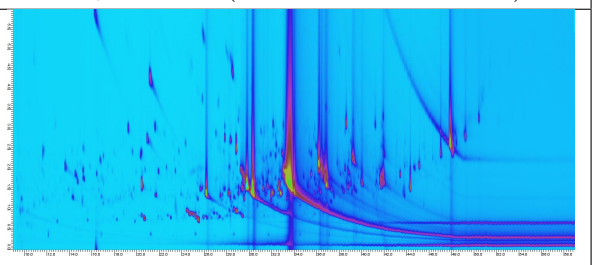
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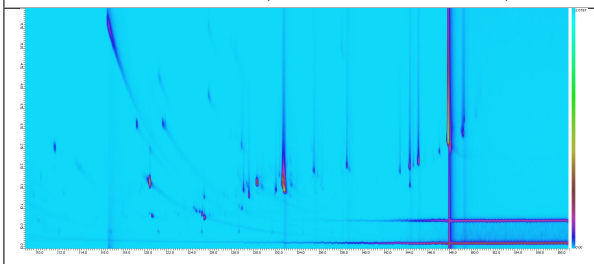	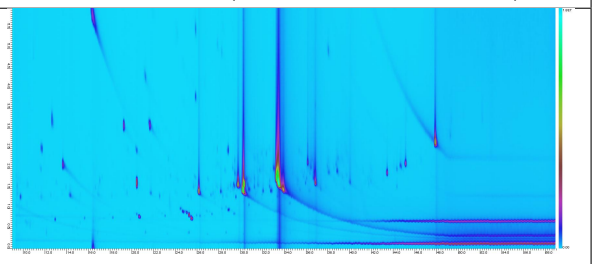
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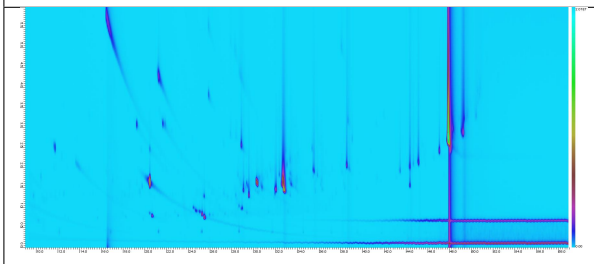	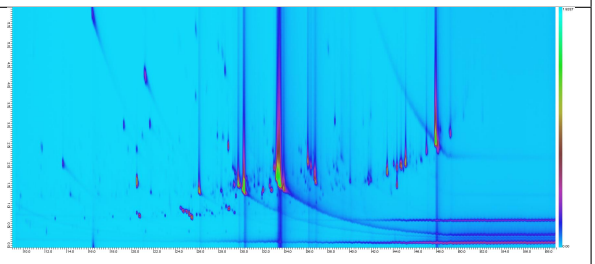
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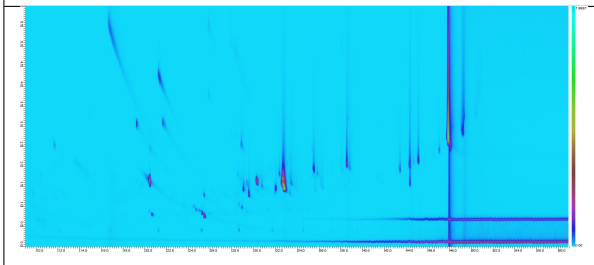	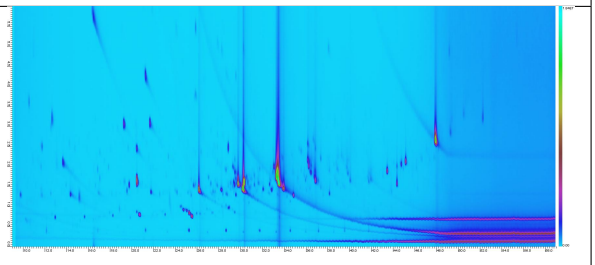
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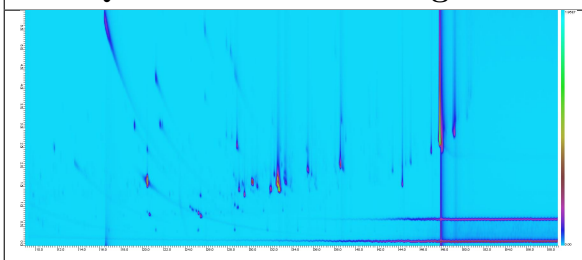
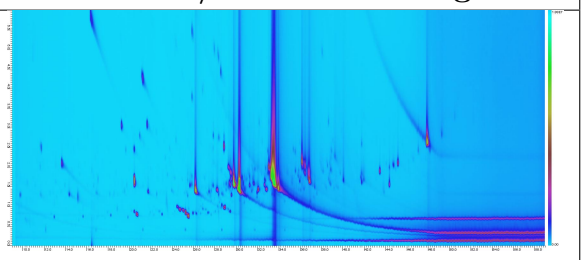
QuEChERS chromatogram	DCM:Hex/GPC chromatogram
	
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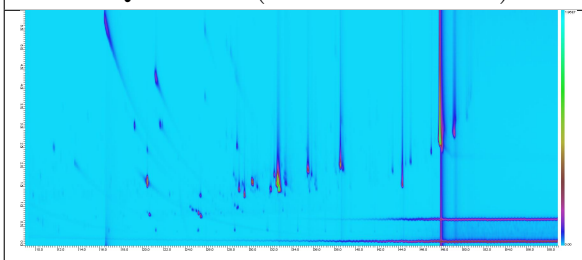	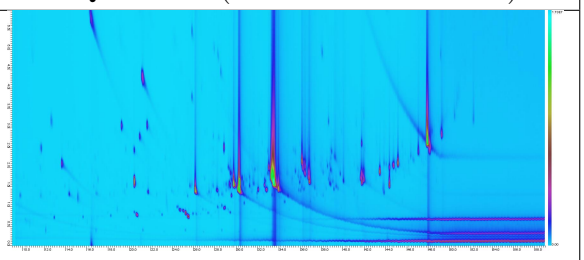
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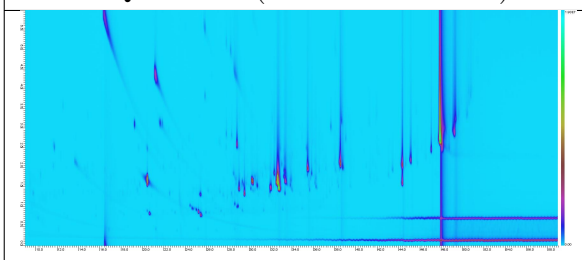	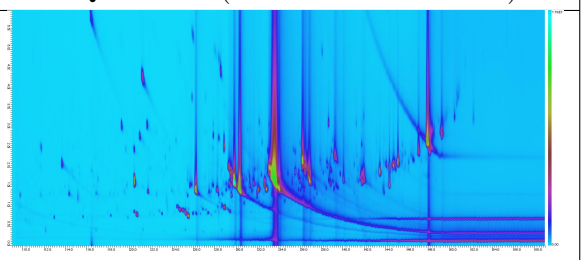
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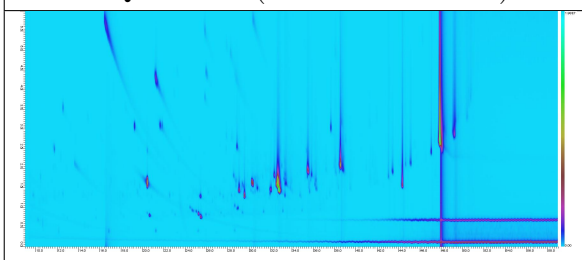	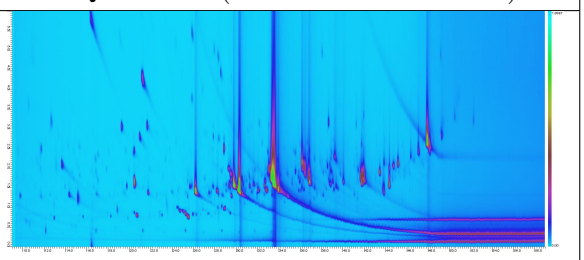
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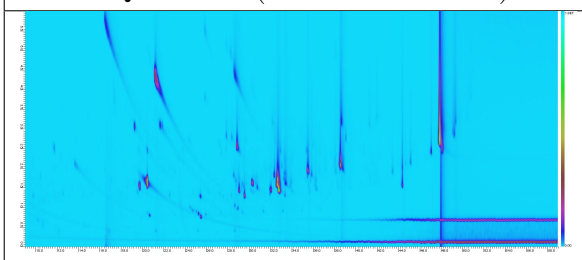	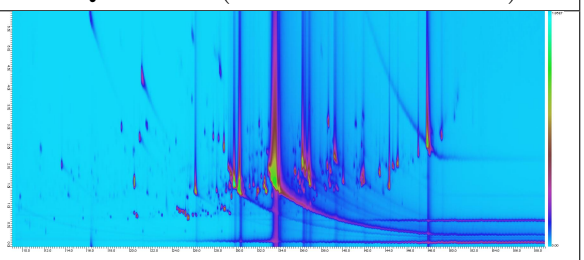
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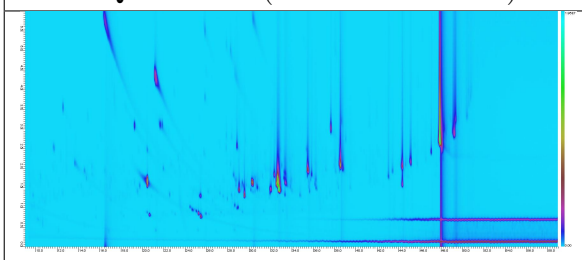	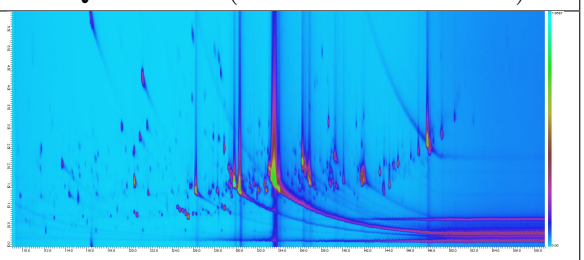
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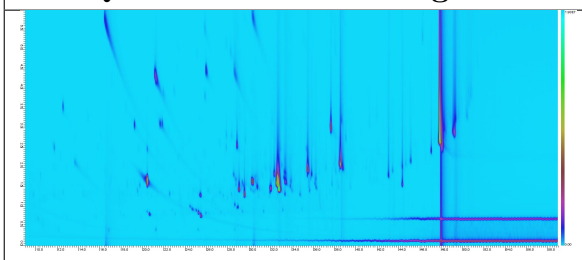
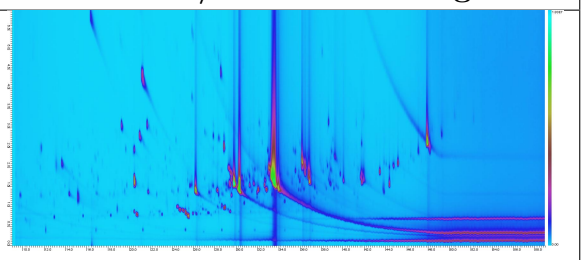
QuEChERS chromatogram	DCM:Hex/GPC chromatogram
	
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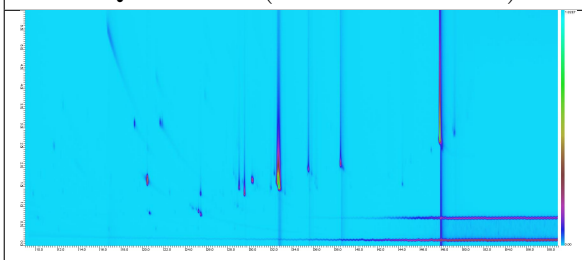	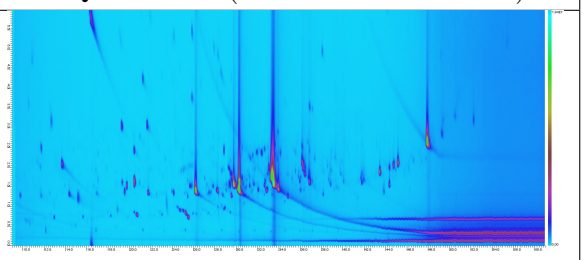
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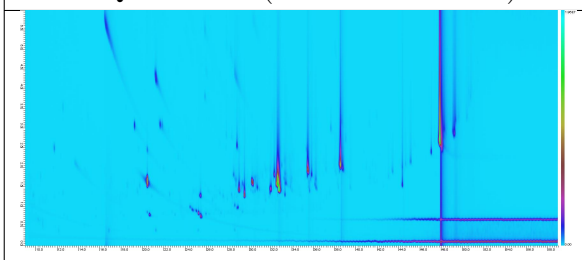	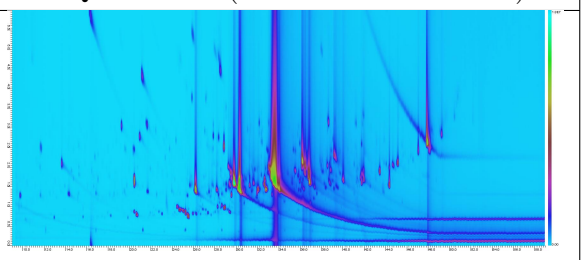
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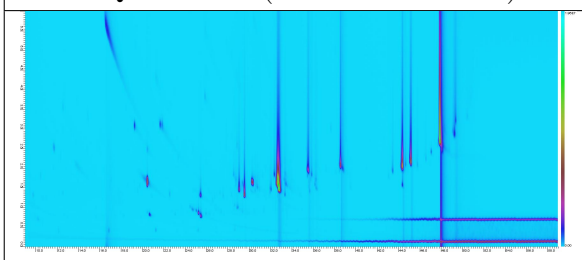	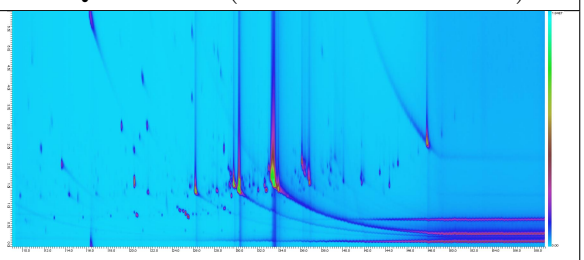
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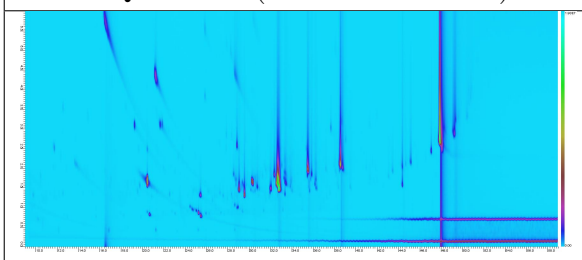	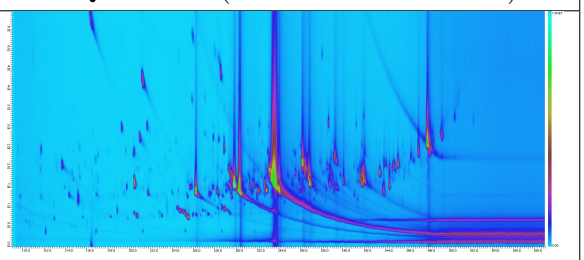
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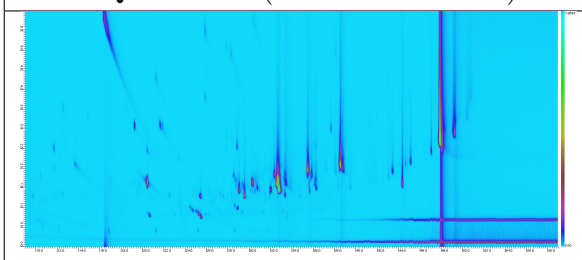	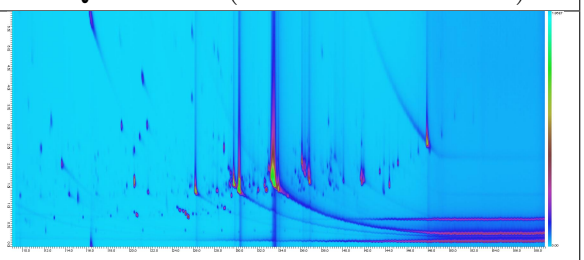
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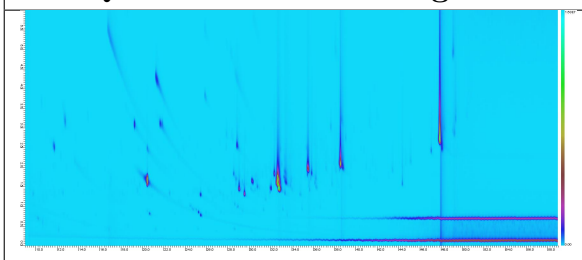
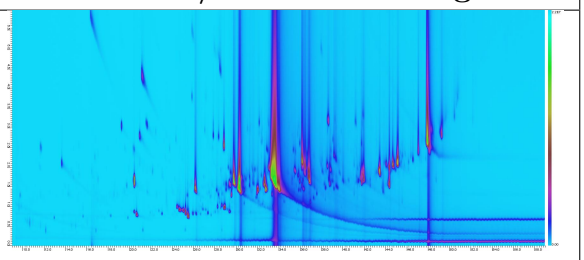
QuEChERS chromatogram	DCM:Hex/GPC chromatogram
	
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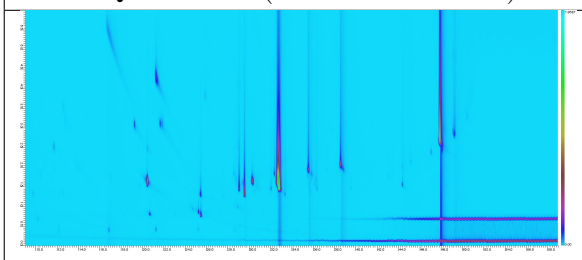	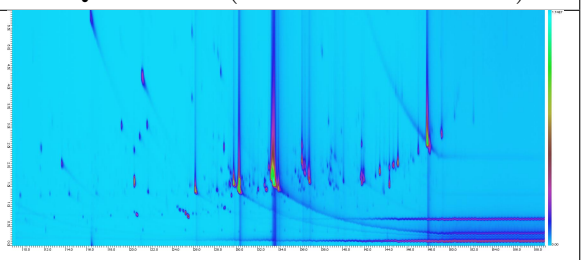
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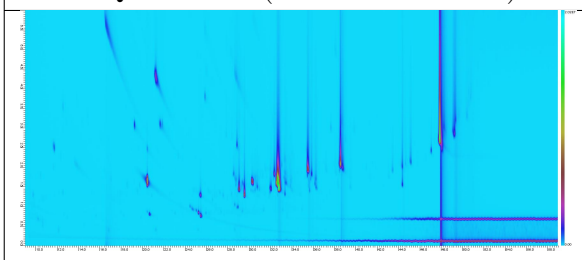	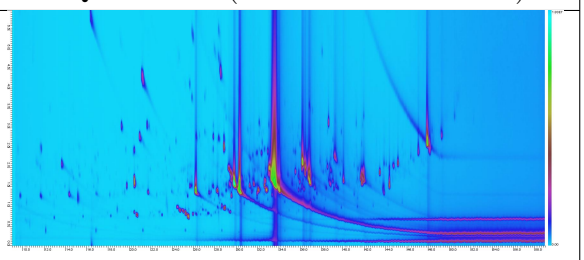
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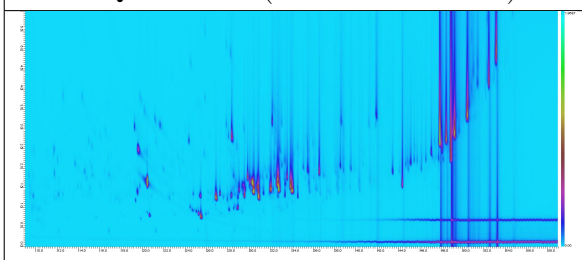	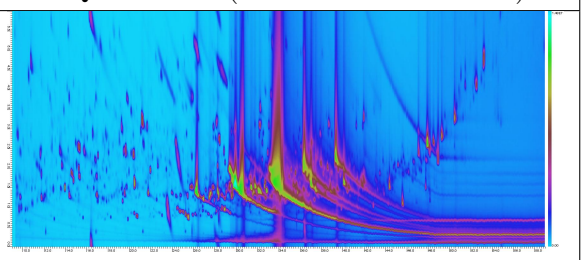
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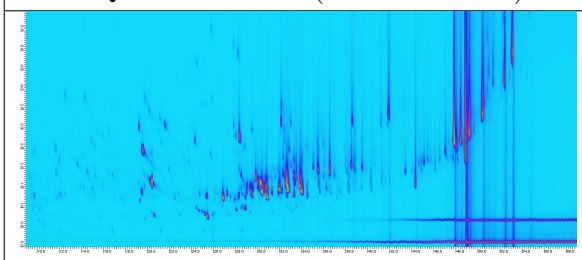	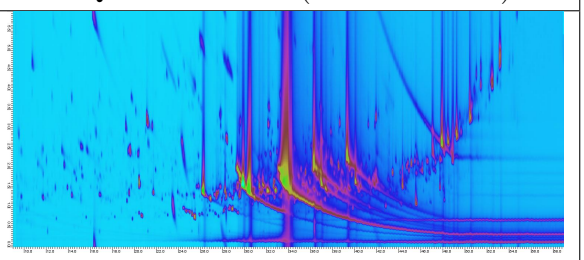
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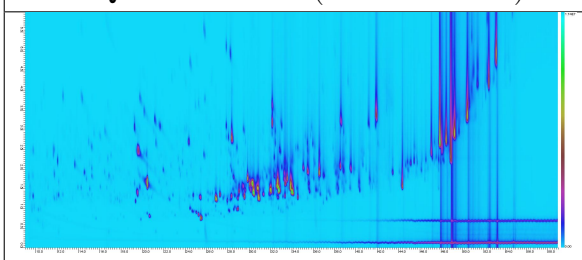	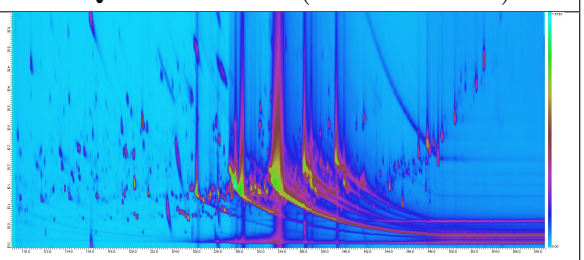
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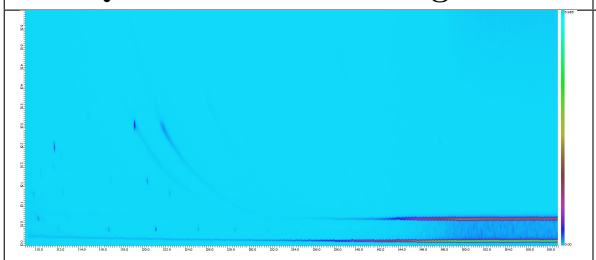
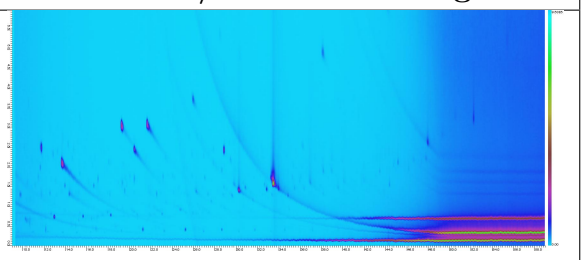
QuEChERS chromatogram	DCM:Hex/GPC chromatogram
	
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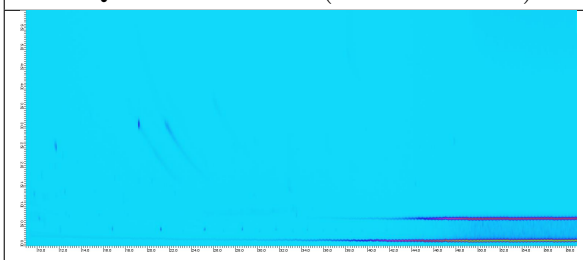
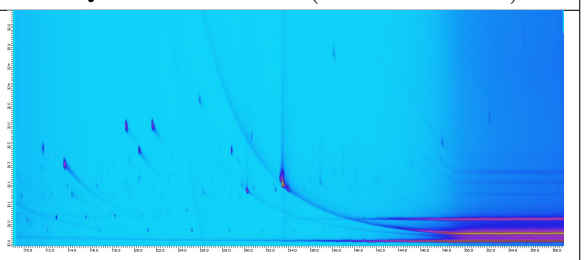
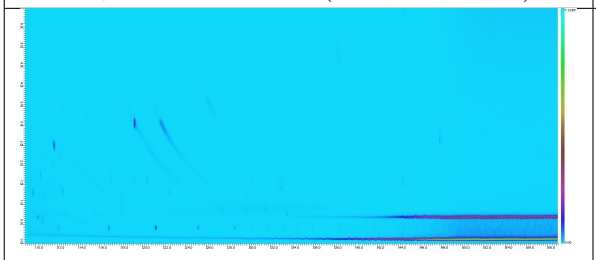
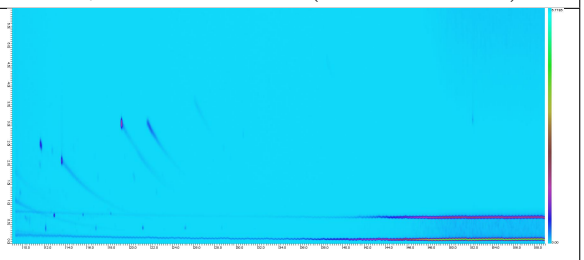
	
AQUAvit Blank 5 (ID: 2024-1040)	AQUAvit Blank 2 (ID: 2024-1037)
	
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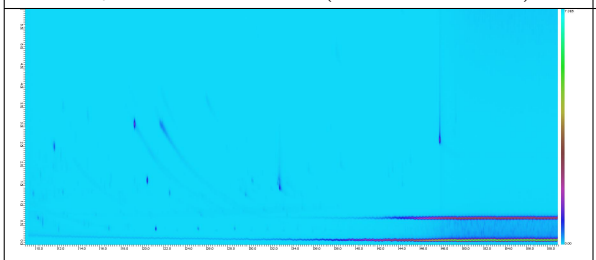
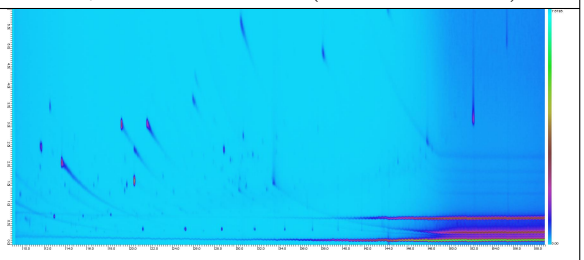
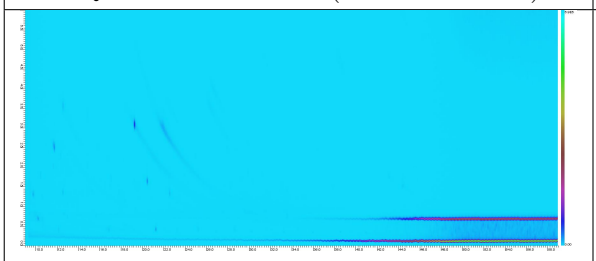
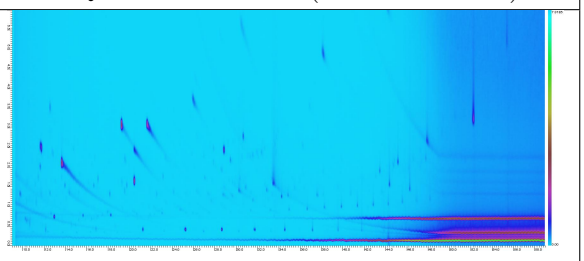
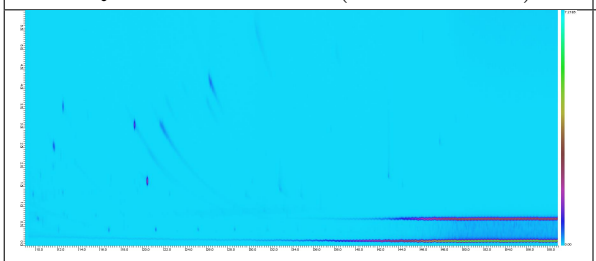
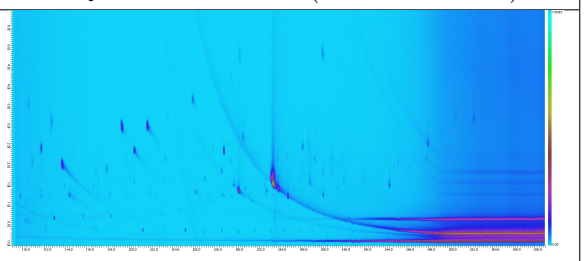
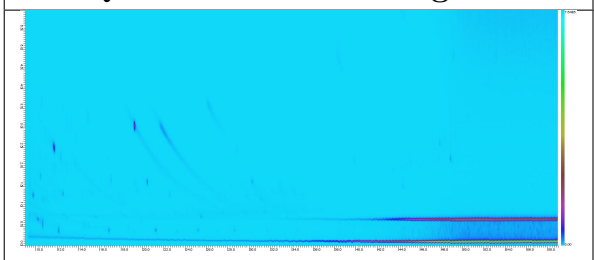
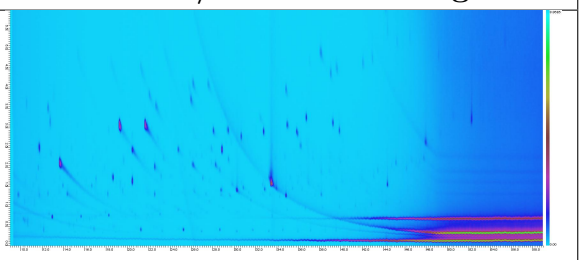
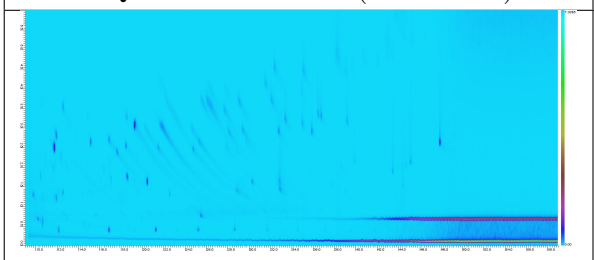
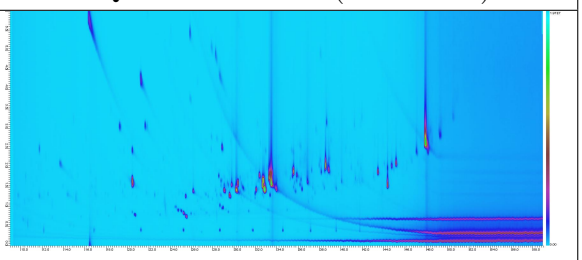
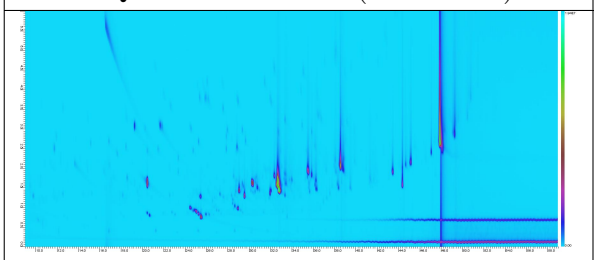
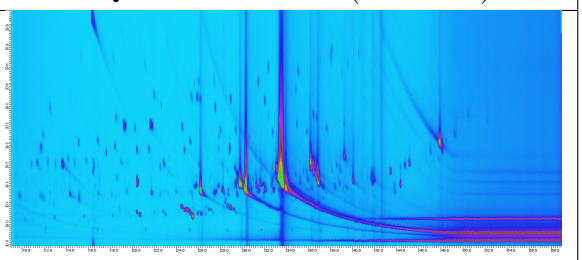
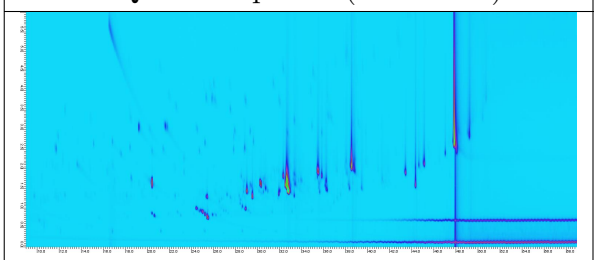
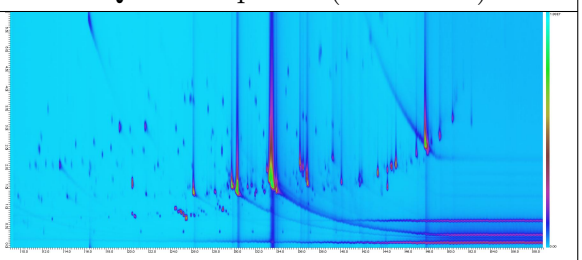
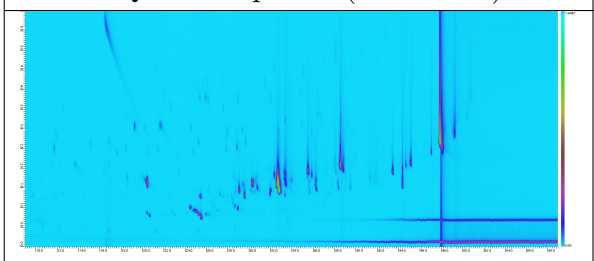
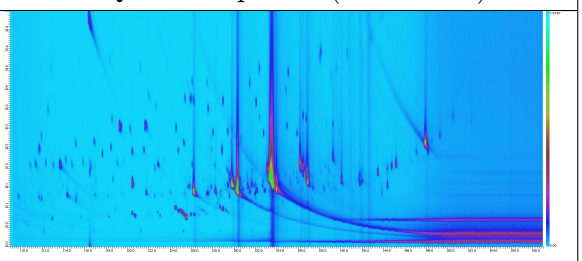
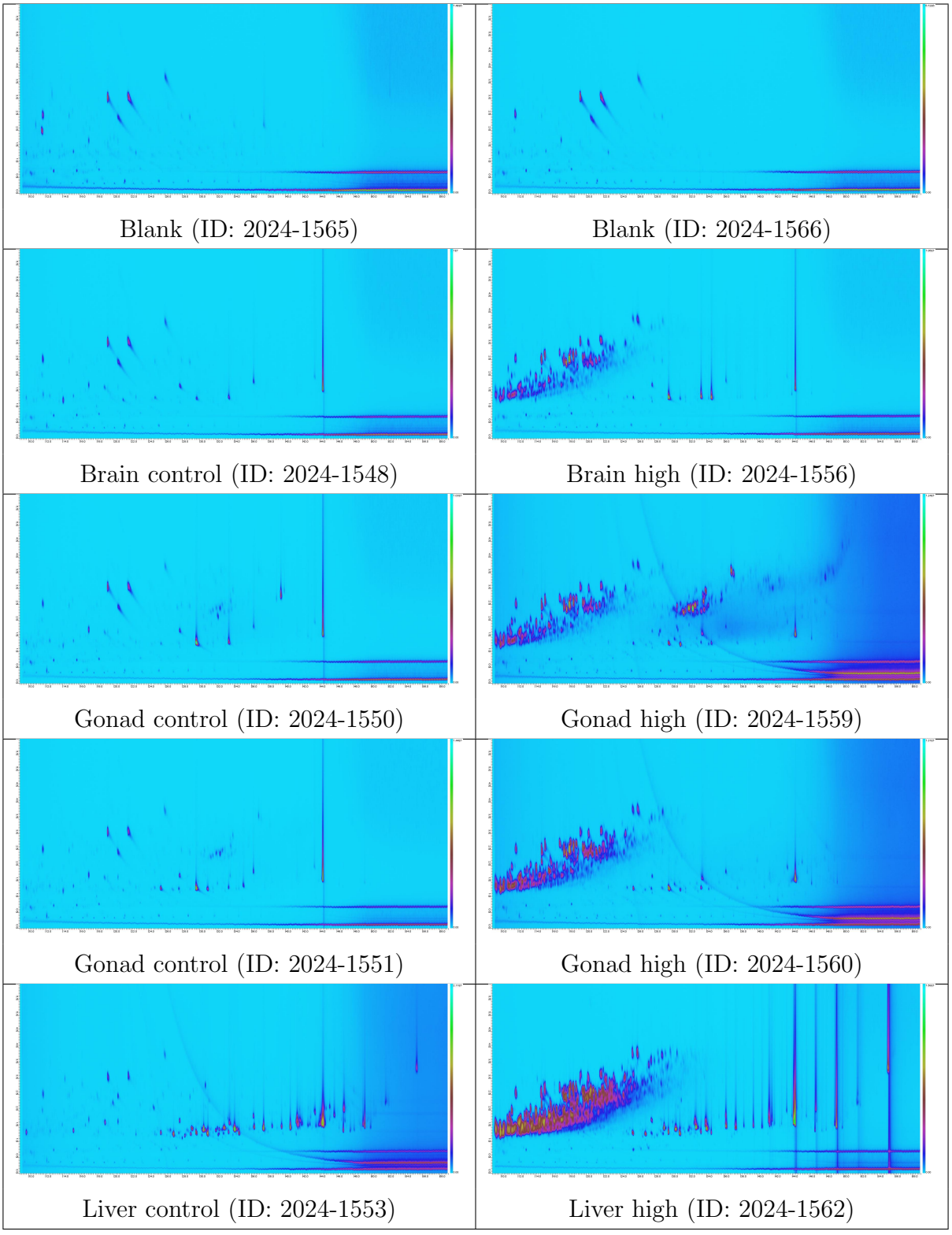
	
AQUAvit Blank 10 (ID: 2024-1054)	AQUAvit Blank 7 (ID: 2024-1051)
	
AQUAvit Blank 11 (ID: 2024-155)	AQUAvit Blank 8 (ID: 2024-1052)
	
AQUAvit Blank 12 (ID: 2024-1056)	AQUAvit Blank 9 (ID: 2024-1053)

QuEChERS chromatogram	DCM:Hex/GPC chromatogram
	
AQUAvit Blank 14 (2024-1576)	AQUAvit Blank 1 (2024-1572)
	
AQUAvit Blank 16 (2024-1584)	AQUAvit Blank 15 (2024-158)
	
AQUAvit Spike 4 (2024-1585)	AQUAvit Spike 1 (2024-1581)
	
AQUAvit Spike 5 (2024-1586)	AQUAvit Spike 2 (2024-1582)
	
AQUAvit Spike 6 (2024-1587)	AQUAvit Spike 3 (2024-1583)

L Chromatograms from ToxiGen



Blank (ID: 2024-1565)

Blank (ID: 2024-1566)

Brain control (ID: 2024-1548)

Brain high (ID: 2024-1556)

Gonad control (ID: 2024-1550)

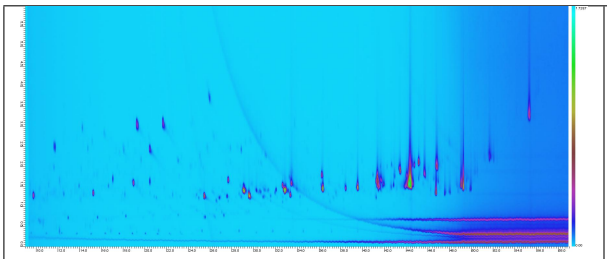
Gonad high (ID: 2024-1559)

Gonad control (ID: 2024-1551)

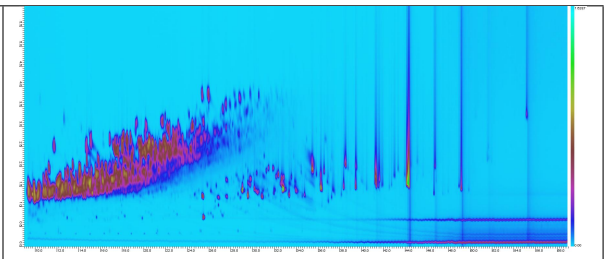
Gonad high (ID: 2024-1560)

Liver control (ID: 2024-1553)

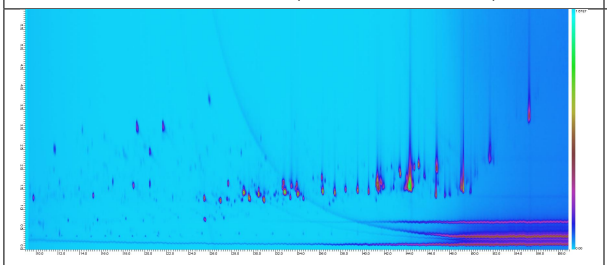
Liver high (ID: 2024-1562)



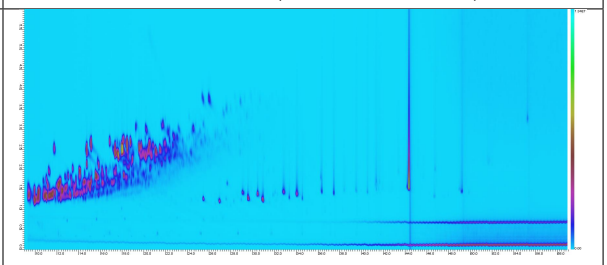
Liver control (ID: 2024-1554)



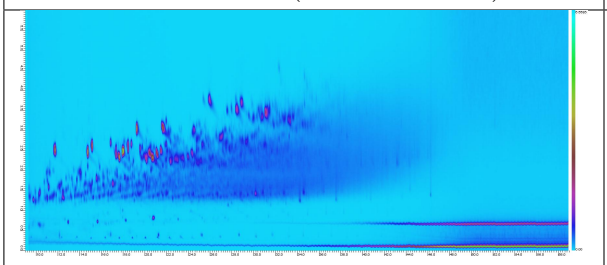
Liver high (ID: 2024-1563)



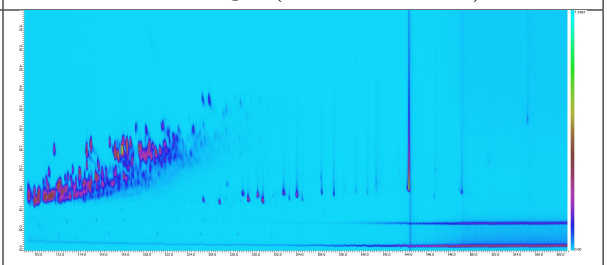
Liver control (ID: 2024-1555)



Liver high (ID: 2024-1564)



Spike (ID: 2024-1570)



Liver high (ID: 2024-1564)

M Post GPC Lipid Weights and Lipid Contents in Samples

Table 28: Lipid weight after GPC and evaporation

Post GPC Lipid 6 - 10				
Sample type	**SINTEF ID**	**Weight Kimax wo/ sample [g]**	**Weight Kimax w/ sample [g]**	**Lipid weight after GPC [g]**
Spike 1	2024-1581-S1	14,35163	14,41832	0,06669
Spike 2	2024-1582-S1	13,76363	13,82863	0,06500
Spike 3	2024-1583-S1	13,84682	13,89558	0,04876
Feed 1	2024-1573-S1	14,05538	14,16598	0,11060
Feed 2	2024-1574-S1	14,16437	14,36585	0,20148
Feed 3	2024-1575-S1	13,78703	13,99621	0,20918
Blank 1	2024-1036-S1	13,91644	13,91684	0,00040
Blank 2	2024-1037-S1	13,63075	13,63100	0,00025
Blank 3	2024-1038-S1	13,69461	13,69472	0,00011
Blank 7	2024-1051-S1	13,86239	13,86250	0,00011
Blank 8	2024-1052-S1	13,85014	13,85037	0,00023
Blank 9	2024-1053-S1	14,37544	14,37550	0,00006
Blank 13	2024-1572-S1	13,77500	13,77539	0,00039
Blank 15	2024-1580-S1	13,82048	13,82055	0,00007
Aquavit 1	2024-4840-S1	13,73449	13,83645	0,10196
Aquavit 2	2024-4841-S1	14,20018	14,34667	0,14649
Aquavit 3	2024-4842-S1	13,71479	13,75030	0,03551
Aquavit 4	2024-4843-S1	14,17114	14,29283	0,12169
Aquavit 5	2024-4844-S1	14,32890	14,36895	0,04005
Aquavit 6	2024-4845-S1	13,67808	13,79031	0,11223
Aquavit 7	2024-4846-S1	13,80390	13,87029	0,06639
Aquavit 8	2024-4847-S1	14,31218	14,44677	0,13459
Aquavit 9	2024-4848-S1	13,76663	13,82821	0,06158
Aquavit 10	2024-4849-S1	13,58736	13,75526	0,16790
Aquavit 11	2024-4850-S1	14,11838	14,22875	0,11037
Aquavit 12	2024-4851-S1	15,62053	15,75271	0,13218
Aquavit 13	2024-4852-S1	13,63585	13,68572	0,04987
Aquavit 14	2024-4853-S1	14,22106	14,34614	0,12508
Aquavit 15	2024-4854-S1	14,84787	14,91477	0,06690
Aquavit 16	2024-4855-S1	14,13586	14,30562	0,16976
Aquavit 17	2024-4856-S1	13,79942	13,86887	0,06945
Aquavit 18	2024-4857-S1	13,81357	13,97155	0,15798
Aquavit 19	2024-4858-S1	13,86377	13,93364	0,06987
Aquavit 20	2024-4859-S1	13,86644	14,00663	0,14019

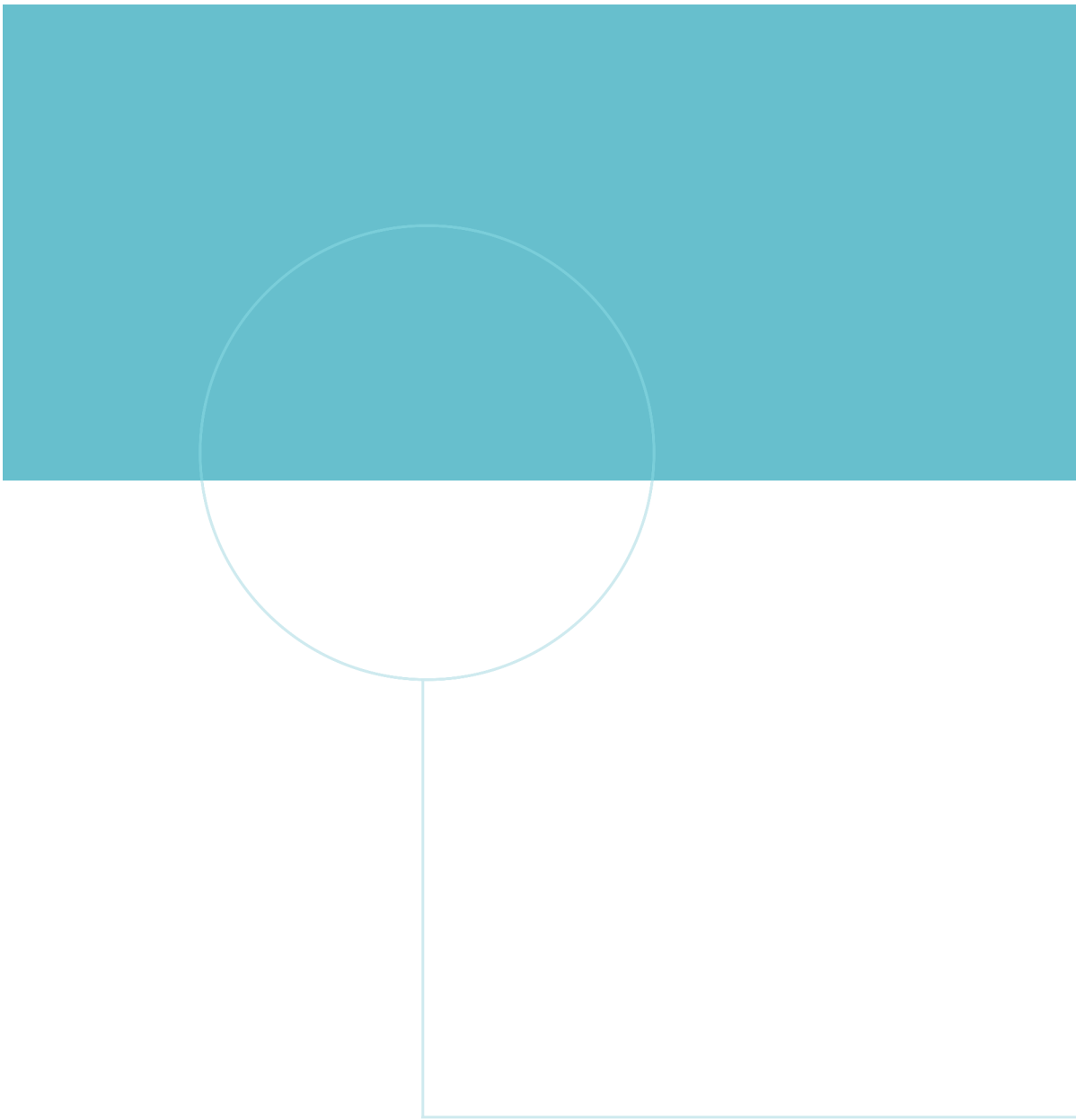
Table 29: Calculated lipid percentage in original sample

Sample	**Kimax [g]**	**Kimax + salmon [g]**	**Salmon [g]**	**Relative lipid content in sample [%]**
Aquavit 1	11,00659	12,06321	1,05662	9,6
Aquavit 2	11,43012	12,56475	1,13463	12,9
Aquavit 3	11,39639	12,50874	1,11235	3,2
Aquavit 4	11,17216	12,13141	0,95925	12,7
Aquavit 5	10,99876	12,1112	1,11244	3,6
Aquavit 6	10,98047	11,94357	0,9631	11,7
Aquavit 7	11,03774	12,08248	1,04474	6,4
Aquavit 8	11,4269	12,49523	1,06833	12,6
Aquavit 9	11,00748	12,12098	1,11350	5,5
Aquavit 10	11,01833	12,13206	1,11373	15,1
Aquavit 11	11,38365	12,47723	1,09358	10,1
Aquavit 12	10,49678	11,55174	1,05496	12,5
Aquavit 13	10,89809	11,94104	1,04295	4,8
Aquavit 14	11,32929	12,40631	1,07702	11,6
Aquavit 15	11,58862	12,74183	1,15321	5,8
Aquavit 16	11,06892	12,17497	1,10605	15,3
Aquavit 17	11,53013	12,60992	1,07979	6,4
Aquavit 18	11,03634	12,03686	1,00052	15,8
Aquavit 19	11,17424	12,52385	1,34961	5,2
Aquavit 20	11,58262	12,60372	1,0211	13,7

N Total Relative Volume of ToxiGen Test Samples

Table 30: Total relative volume of toxigen test samples raw data

Sample name	Sample ID	Group name	Relative volume (Total)
Gonad control	2024-1550	Saturates/non-cyclic	144649158,52
		Cyclic/mono-aromatics	990043657,63
		Tri-aromatics	133225188,40
		Poly-aromatics	76699863,27
		Di-aromatics	499146341,94
Gonad high	2024-1559	Saturates/non-cyclic	891749903,41
		Cyclic/mono-aromatics	5312224031,26
		Tri-aromatics	786711714,12
		Poly-aromatics	218190353,64
		Di-aromatics	5645962249,48
Brain control	2024-1548	Saturates/non-cyclic	173472941,07
		Cyclic/mono-aromatics	767908227,19
		Tri-aromatics	34679843,44
		Poly-aromatics	35851843,00
		Di-aromatics	377010471,45
Brain high	2024-1556	Saturates/non-cyclic	176190773,82
		Cyclic/mono-aromatics	2608514445,93
		Tri-aromatics	136509597,85
		Poly-aromatics	52329575,10
		Di-aromatics	2352932247,87
Liver control	2024-1554	Saturates/non-cyclic	2369427969,30
		Cyclic/mono-aromatics	5668258424,09
		Tri-aromatics	236183057,51
		Poly-aromatics	194100726,26
		Di-aromatics	946746915,33
Liver high	2024-1563	Saturates/non-cyclic	1312049120,59
		Cyclic/mono-aromatics	24826028266,45
		Tri-aromatics	1149638044,37
		Poly-aromatics	389249423,23
		Di-aromatics	13060703279,61



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