NON-TARGET ANALYSIS OF ANTIMICROBIAL RESIDUES AND THEIR TRANSFORMATION PRODUCTS IN FISH AND SHRIMP

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Abstract

Analytical techniques targeting specific analytes, i.e., targeted analysis, have long been established as the main methods in food safety and environmental analysis. On account of the large number of chemical contaminants with various physicochemical properties detected in the food and water, there is a need to adapt these methods to screen and identify a broader number of chemicals. Additionally, studies have shown a decrease in contaminant levels during cooking and with food, especially seafood, eaten following some sort of thermal processing, it is important to study the outcome of processing on food contaminants. The preferred technique to screen for, and study the fate of contaminants in food is non-targeted analysis (NTA) using high resolution mass spectrometry. The objective in this thesis was to develop a non-targeted method in food analysis, focusing on the determination of veterinary drugs and other pharmaceuticals (drugs used in human medicine) in fish and shrimp. Chapter 3 covers the first step in NTA, which is the selection of an extraction method. Malachite green (MG) exposed brook trout and pacific white shrimp were used as a case study to evaluate the impact of extraction methods on the determination of the veterinary drug. Based on the comparison parameters, e.g., matrix, ionization mode, a different extraction was considered more suitable. Although a compromise must be reached, based on specific research objectives, there is a need for a more harmonized approach on some aspects, like data filtering, e.g., blank subtraction, and data processing. Overall, QuEChERS extraction provided satisfactory results and was chosen to further study MG. In Chapter 4, a data analysis approach was validated for compound discovery from non-targeted data, focusing on the identification of antimicrobials and other pharmaceuticals in fish livers. The validated workflow was suitable, as it led to the identification of an antibiotic, azithromycin, and an anti-depressant metabolite, erythrohydrobupropion. Chapter 5 covered the

application of NTA for the identification of thermal transformation products (TPs) of MG and LMG in brook trout and shrimp, using the extraction and data analysis workflow validated in Chapters 3 &4. Boiling, canning and microwaving reduced MG levels in both brook trout and shrimp, with microwaving the only treatment successful in significantly reducing LMG levels. Different TPs were proposed between the two matrices and cooking treatments, with des-methylated forms and a benzophenone derivative tentatively identified. In chapter 6, NTA was used to study thermal transformation of another veterinary drug, oxytetracycline (OTC), in shrimp. OTC was identified as another commonly detected veterinary drug, particularly in shrimp, thus it was included in this research. The extraction method, based on solvent extraction/freezing, was selected following the same procedure as in Chapter 3. The relevance of using a "spiked" or "water" model to study the fate of OTC during cooking was deemed inadequate, as different TPs were identified between water and incurred/spiked tissues. This research has shown how NTA can be integrated alongside target analysis in both food safety and environmental applications. This type of analysis can describe the chemical "cocktail" to which consumers may be exposed to and it can lead to the discovery of new compounds, i.e., TPs. Through the identifications of these compounds, and evaluation of toxicity, regulatory agencies can better assess possible risks to human health. From an environmental perspective, this research showed how non-target analysis may be used to identify other contaminants present in the environment that may be toxicologically relevant to aquatic species.

Résumé

Les méthodes analytiques concentrées sur des analytes spécifiques, c.-à-d., analyse ciblée, sont les principaux outils dans l'analyse alimentaire et environnementale. En raison du grand nombre des nombre de contaminants avec des propriétés physico-chimiques différents, il y a un besoin d'adapter ces méthodes pour permettre leur criblage and identification. De plus, des études ont démontré une diminution des résidus des contaminants chimiques durant la cuisson, et étant donné que la plupart des aliments, particulièrement le poisson, sont consommés cuits, il est important d'étudier le devenir de ces composés durant la cuisson. La technique utilisée pour le criblage and l'étude du devenir des contaminants dans les aliments est l'analyse non-ciblée (abrév. ANC) basé sur la spectrométrie de masse-haute résolution. L'objectif de cette thèse était de développer une méthode non-ciblée pour la détermination des médicaments vétérinaires et autres composés pharmaceutiques (utilisés pour la médecine humaine) dans le poisson et la crevette. Chapitre 3 couvre la première étape dans l'ANC, soit la sélection d'une méthode d'extraction. La truite mouchetée et la crevette blanche du Pacifique, exposées au vert de malachite (VM), ont été utilisés pour étudier l'impact des méthodes d'extraction sur l'analyse du médicament vétérinaire. Basé sur des paramètres de comparaison, c.-à-d., la matrice alimentaire, le mode d'ionisation, une autre extraction était appropriée. Bien que la sélection de la méthode jugée optimale dépende des objectifs de recherche, il y a un besoin d'une approche standardisée sur certains facteurs, comme la filtration et traitement des données. La méthode basée sur QuEChERS a démontré des résultats satisfaisants et a été choisi pour étudier le VM. Dans le Chapitre 4, une approche d'analyse des données a été validée pour la découverte des composés des données non-ciblée, concentrée sur l'identification des antimicrobiens et autres pharmaceutiques dans les foies des poissons. L'approche validée a été jugée apte car elle a

permis d'identifier un antibiotique, l'azithromycine, et le métabolite d'un antidépresseur, l'erythrohydrobupropion. Dans le Chapitre 5, l'ANC a été appliquée pour identifier des produits de transformation (abrév. PT) thermale du VM et VLM dans la truite et la crevette cuites en utilisant l'extraction et analyse de données optimisées dans les chapitres 3&4. Le bouillonnement, mise en conserve et la cuisson au micro-ondes ont diminué les résidus du VM, tandis que seulement la cuisson au micro-ondes a diminué les résidus du VLM de façon significative. Des PT différents ont été proposés entre les deux matrices et les méthodes de cuisson. Enfin, l'ANC a été utilisé pour étudier la transformation thermale dans la crevette d'un autre médicament vétérinaire, l'oxytetracycline, fréquemment identifié dans la crevette. L'extraction à l'aide de solvants/étape de congélation, jugée optimale selon la même procédure qu'au chapitre 3 a été utilisé. La pertinence d'utiliser des matrices modèles, soit l'eau ou la crevette dopée, pour étudier le devenir de l'antibiotique, a été jugée inadéquate, car the PT différents ont été proposés pour les trois matrices. Ce travail de recherche a montré comment l'ANC peut être intégrée avec l'analyse ciblé dans l'analyse alimentaire et environnementale. Ce type d'analyse peut qualifier le « cocktail » chimique auquel les consommateurs peuvent être exposés et entraîner la découverte des nouveaux composés, comme les PT. En évaluant leur toxicité, les agences de réglementation peuvent mieux évaluer les risques à la santé humaine. Du côté environnemental, les résultats obtenus montrent comment l'ANC peut être utilisé pour identifier autres contaminants qui pourraient être pertinents du point de vue toxicologique pour des espèces aquatiques.

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Contributions of authors

This thesis is presented in manuscript form and consists of seven chapters. The first chapter offers a brief introduction to contaminants in aquatic biota and non-target analysis. A comprehensive review of the literature on five veterinary drugs, including their detection in seafood and current methods of analysis is covered in chapter 2. Chapters 3 to 6 are presented in manuscript form and are arranged sequentially through connecting text. Chapters 3 and 4 have been submitted for publication in the *Food Chemistry* and *Science of the Total Environment*, respectively. Chapter 5 will be submitted for publication to the journal *Food Research International*, while chapter 6 will be submitted to the journal *Food Control*. Chapter 7 offers a conclusion of the thesis and recommendations for future research.

The present author was responsible for the experimental design and work, data acquisition and treatment and manuscript preparation for all the chapters. Dr. Stéphane Bayen, the thesis supervisor oversaw the progression of the experimental work and was involved, along with all co-authors, in the editing of the manuscripts before submission. Dr. Céline Audet was responsible for the brook trout exposure to malachite green, and is co-author on both Chapter 3 "Evaluation of different extractions for the metabolite identification of malachite green in brook trout and shrimp" and 5 "Application of non-target analysis to study the thermal transformation of malachite and leucomalachite green in brook trout and shrimp". Gregory Ballash, Dixie Mollenkopf, Dr. Thomas Wittum and Dr. S. Mažeika Patricio Sulliván were involved with fish liver sample collection, methodology, manuscript editing and are listed as co-authors of Chapter 4, "Suspect screening of pharmaceuticals in fish livers based on QuEChERS extraction coupled with high resolution mass spectrometry".

Conference presentations

- Baesu A., Ballash G., Mollenkopf D., Wittum T., Bayen S. Identification of Erythrohydrobupropion and other PPCPs in Fish Livers using Nontargeted Screening. Society of Environmental Toxicology and Chemistry (SETAC) North America 41st Annual Meeting, virtual, 15-19 November 2020. (Oral)
- Baesu A., Ballash G., Mollenkopf D., Wittum T., Bayen S. Application of suspect screening for the identification of erythrohydrobupropion and other PPCPs in in fish livers. 16th Annual Workshop On Emerging High-Resolution Mass Spectrometry (HRMS) And LC-MS/MS Applications In Environmental Analysis And Food Safety. Virtual, 15-16 October 2020. (Oral)
- Baesu A., Ballash G., Mollenkopf D., Wittum T., Bayen S. Suspect screening of pharmaceuticals in fish livers. 36th Annual Trent Conference on Mass Spectrometry. 10-11 August 2020, Virtual, Canada. (Oral, best speaker award)
- Baesu A., Bayen S. Application of non-target analysis for identification of thermal degradation products of oxytetracycline in pacific white shrimp. 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019) 5-8 November 2019, Prague, Czech Republic. (Poster)
- Baesu A., Audet C., Bayen S. Influence of food matrix on the fate of malachite and leucomalachite green during cooking in brook trout and shrimp. 9th International Symposium on Recent Advances in Food Analysis (RAFA 2019) 5-8 November 2019, Prague, Czech Republic. (Poster)

- Baesu A., Bayen S. Application of non-targeted analysis to study the fate of malachite and leucomalachite green in shrimp following thermal treatment. Tri Conference of 1st Eastern Canada Mass Spectrometry (MS) Conference - 10th International Symposium on Enabling Technologies - 35th Trent Conference on MS, Sherbrooke, Canada, August 4 – 7, 2019. (Oral)
- Baesu A., Bayen S. Comparison of a non-targeted screening approach for the detection of malachite green and metabolites in brook trout and shrimp. 102nd Canadian Chemistry Conference and Exhibition. Quebec, Canada. 4-7 June 2019. (Poster)
- Baesu A., Bayen S. Analysis and fate of oxytetracycline in shrimp using a non-targeted screening approach. 63rd International Conference on Analytical Sciences and Spectroscopy. Montreal, Canada. 25-28 June 2019. (Oral)
- Baesu A., Audet C., Bayen S. Non-targeted screening malachite green and metabolites in brook trout using UHPLC-QTOF-MS. 62nd International Conference on Analytical Sciences and Spectroscopy. Toronto, Canada. 11-14 June 2018. (Oral)
- Baesu A., Audet C., Bayen S. Optimization of a non-targeted approach to screen malachite green and its metabolites in brook trout using UHPLC-QTOF-MS. 2e édition du Symposium des étudiants de l'INAF. Quebec, Canada. 23-24 April 2018. (Poster)

Abbreviations

CFIA: Canadian Food Inspection Agency

CTC: Chlortetracycline

CVMP: Committee for Medicinal Products for Veterinary Use

DAD: Diode array detector

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

EU: European Union

FAO: Food and Agriculture Organization

HPLC: High Performance Liquid Chromatography

JEFCA: Joint FAO/WHO Expert Committee on Food Additives

LC: Liquid Chromatography

LD_{50}: Lethal dose

LIN: Lincomycin

LMG: Leucomalachite green

LOQ: Limit of quantification

MDL: Method detection limit

MG: Malachite green

MME: Mass measurement error

MRL: Maximum residue limits

MS: Mass Spectrometry

NOAEL: No observed adverse effects level

OECD: Organization for Economic Co-operation and Development

OTC: Oxytetracycline

PAH: Polyaromatic hydrocarbons

POP: Persistent organic pollutants

PSA: Primary Secondary Amine

QA: Quality assurance

QC: Quality control

QuEChERS : quick, easy, cheap, effective, rugged and safe

RASFF: Rapid Alert System for Food and Feed

SCX: Strong-Cation Exchange

SFM: Sulfadimethoxine

SPE: Solid phase extraction

TP: Transformation product

USDA: United States Department of Agriculture

US EPA: United States Environmental Protection Agency

WHO: World Health Organization

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Chapter 1: Introduction

1.1 General introduction

Fish and crustaceans can be exposed in the environment to a variety of chemical contaminants, released in water through agricultural activities and wastewater (Kummerer 2009). In addition, chemical residues may be found in aquatic species owing to their use as therapeutants in animal production (Kummerer 2009), which include veterinary drugs, like antibiotics/antimicrobials, pesticides (anti-parasitic), disinfectants and anti-fungals (Government of Canada 2008, Sapkota et al. 2008). In order to simplify the terminology, therapeutants will be referred to as veterinary drugs in this thesis. Regulations have set out which veterinary drugs are authorized and the maximum residue limits (MRLs) that are tolerated in tissues (Health Canada 2010). However, some drugs that are banned continue to be detected in seafood (Love et al. 2011) posing health risks to consumers. Some water contaminants, usually associated with human medicine, e.g., antidepressants, can also be easily taken up by aquatic species through the gills and accumulated in tissues (Arnnok et al. 2017). Not only could this pose adverse health effects to humans but can also lead to adverse effects in fish species (Brodin et al. 2013). Recent studies have demonstrated that the levels of some seafood contaminants decrease following cooking (Bayen et al. 2005, Tian and Bayen 2018). Seafood is usually consumed cooked and although this may lower the levels to which consumers are exposed, there are little qualitative studies that describe what happens to these chemicals during heating. This information is vital to better outline the risks to humans from consumption of contaminated seafood. Since any transformation products (TPs) formed during heating are unknown, analytical techniques must be developed for their extraction and identification.

One technique reported to study the fate of contaminants from field to fork, both quantitively and qualitatively, is based on non-targeted analysis (NTA) (Stadler et al. 2019, Tian

et al. 2020, von Eyken and Bayen 2020). Although confirmation of transformation products (TPs) may be limited to the availability of analytical standards, formulas and tentative structures can be proposed using NTA methods (von Eyken and Bayen 2020). The terms non-targeted analysis and untargeted analysis are both commonly used in the literature (Schrimpe-Rutledge et al. 2016, Bletsou et al. 2015, Cajka and Fiehn 2016, Ballin and Laursen 2019). Non-targeted analysis (NTA) will be used throughout the thesis. The workflow upon which NTA is based on can allow not only for the identification of unknown compounds, but also for the screening of a large number of chemicals (Bletsou et al. 2015, Knolhoff and Croley 2016). This is achievable with the simple, unselective extraction methods which can extract and identify compounds from different chemicals families and with different properties, e.g., size and polarity (Vuckovic 2012, Knolhoff and Croley 2016). Therefore, as the first step in the development of NTA methods, sample preparation is very important as it can affect the interpretation of the data (Vuckovic 2012). However, there is no standard approach on the comparison of extractions in NTA, or generally on the development of NTA methods. Data processing has also been demonstrated to impact the identification of some target chemicals (Tian et al. 2019). Some approaches for the comparison of extractions in food NTA for example have been adapted from metabolomics (Creydt et al. 2018) but there is limited information on the impact of sample preparation or data processing in NTA applied to fish or crustaceans.

1.2 General objectives

The overall objective of this study was to develop and apply a NTA workflow to investigate the fate of food contaminants, focusing on antibiotic residues in fish and shrimp, from field to fork, notably during cooking. Specifically, the objectives were:

(i) Review the literature on the occurrence, fate and current methods of analysis of key antimicrobials in seafood (Chapter 2)

(ii) Evaluate the impact of the sample extraction and data processing on the determination of veterinary drugs in brook trout and pacific white shrimp (Chapter 3)

(iii) Validate a data processing workflow for the identification of chemical contaminants such as antibiotics using fish livers as a case study (Chapter 4)

(iv) Apply the optimal extraction method and data processing workflow to evaluate the fate (reduction percentage and identification of TPs) of one veterinary drug, malachite green, during cooking of shrimp and brook trout muscle and assess the impact of the food matrix on the transformation mechanism (Chapter 5)

(v) Apply the optimal extraction method and data processing workflow to evaluate the fate (reduction percentage and identification of TPs) of another veterinary drug, oxytetracycline, during cooking of shrimp muscle and shell and assess the impact of the food matrix on the transformation mechanism (Chapter 6)

Chapter 2: Literature review

2.1 Introduction

One of the fastest growing food producing sectors is aquaculture, with an increase from world production of 3 million tonnes in 1970 to 66.63 million tonnes in 2013 (FAO 2016). Fish represents a high-quality protein source and accounts for 20% of the average protein intake for almost 3.1 billion people (FAO 2016). Intensive farming methods are often practiced to meet the increased demand consisting of high density fish populations and increase use of antimicrobials (Sapkota, Sapkota et al. 2008). For instance, in Vietnam, 813 products were registered with government authorities as veterinary drugs (Thi Kim Chi et al. 2017). Nowadays, the terms antimicrobial/antibiotic are used interchangeably (Wang et al. 2012). Antibiotics can be broadly defined as chemotherapeutic agents that "inhibit or abolish the growth of microorganisms, such as bacteria, fungi or protozoa"(Kummerer 2009). Beside their intentional use in disease prevention and treatment, pharmaceuticals like antibiotics, including those used for veterinary purposes, may be detected in the environment following wastewater release (Archer et al. 2017). The occurrence of veterinary drugs and other pharmaceutical drugs in the aquatic environment and biota is problematic for various reasons. First, the widespread use of antimicrobials is responsible for a rise in antimicrobial resistance (AMR) in bacteria (Sapkota et al. 2008). Second, some compounds discharged in the environment may have toxic effects for aquatic species. For example, anti-depressants may alter behaviour such as lower aggression (McCallum et al. 2017). Third, many of these compounds can be taken up by fish through the gills and accumulate in muscle (Ramirez et al. 2009, Alvarez-Munoz et al. 2015). The presence of these residues may cause adverse health effects to consumers. Low levels of antimicrobials may impact the human intestinal flora leading to possible bacterial infections and decreased immune response (Cabello 2006). They may also trigger allergic symptoms in individuals that are already

allergic to antibiotics or cause sensitivity in workers that handle antibiotics on a daily basis (Cabello 2006). Finally, seafood is mostly consumed following some kind of processing, e.g., grilling and studies have shown the reduction of some contaminants in seafood following cooking (Bayen et al. 2005, Uno et al. 2010). Understanding the fate of these compounds in foods following processing is very important to obtain a better representation of exposure levels (WHO 2009) and to identify newly formed TPs which may be more or equally toxic as the parent compounds (Nguyen et al. 2015).

In the past years, analytical methods have shifted from a targeted to a non-targeted approach. Traditional multi residue methods (Villar-Pulido et al. 2011, Sapozhnikova and Lehotay 2013, Dasenaki and Thomaidis 2015, Carmona et al. 2017) are focussing on specific compounds disregarding other possible contaminants, metabolites or TPs. In this context, the emerging field of metabolomics and the associated analytical techniques can be applied. Metabolomics can be defined as the study of "the whole small metabolite composition of a particular system or organism" (Castro-Puyana et al. 2017). It is currently frequently based on NTA using high resolution mass spectrometry (HRMS), following simple, generic sample treatment steps, that looks to cover the maximum number of compounds (Castro-Puyana et al. 2017). These approaches can allow for the extraction and identification of compounds for which no information is available (Knolhoff and Croley 2016) such as thermal TPs of food contaminants (Tian et al. 2020, von Eyken and Bayen 2020). The high mass accuracy achieved using HRMS can help generate molecular formulae which can be used to screen chemical databases e.g. Chemspider, in order to identify the compounds of interest (Bletsou et al. 2015). This workflow in NTA is also sometimes referred to as suspect screening (Bletsou et al. 2015).

In this first chapter, a literature review was conducted on one anti-fungal drug (malachite green) and four antibiotics (oxytetracycline, chlortetracycline, lincomycin and sulfadimethoxine). These compounds were chosen based on their frequent detection in water sources and fish and crustaceans. For example, tetracyclines, sulfonamides and lincomycin have been detected in waste and coastal waters (Biel-Maeso et al. 2018) and fish tissues (Zhao et al. 2015) while malachite green is one of the main veterinary drugs responsible for non-compliances in fish and crustaceans (Love et al. 2011). Their toxicity, current methods of analysis, occurrence in water and biota and information on the fate of the drugs during processing are covered in this review. The goal was to identify gaps in the current knowledge that may be answered with the research through this project, in order to have a better representation of the human exposure and possible health risks associated with the consumption of seafood contaminated with these compounds.

2.2 Chemical properties, toxicity and aquaculture applications

Physicochemical properties such as hydrophobicity and pH can influence the distribution of veterinary drugs within the water column (Alygizakis et al. 2016) and their accumulation in different fish tissues (Liu et al. 2017). Analytical methods should be developed based on these properties to make sure residues are fully extracted from the food matrix. The chemical structure could also indicate possible toxicity, for example triphenylmethane dyes like gentian violet and pararosaniline are possible carcinogens due to their arylamine groups (Doerge et al. 1998). In this section physicochemical properties along with the usage of these five veterinary drugs in aquaculture, like forms of administration of the drugs, will be discussed. Some key chemical properties, like pK_a and log K_{ow} are listed in Table 2.1. Other metabolites of the antimicrobials

identified in fish tissues will also be covered. This information will ensure that the methodology used in this study, like sample preparation and controlled exposure experiments, is well suited for the extraction and quantification of the veterinary drugs.

Table 2.1: Chemical properties of the antimicrobials reviewed

	Malachite green	Leucomalachite green	Lincomycin	Chlortetracycline	Oxytetracycline	Sulfadimethoxine
Molecular formula	$C_{23}H_{25}N_2^+$	$C_{23}H_{26}N_2$	$C_{18}H_{34}N_2O_6S$	$C_{22}H_{23}N_2O_8Cl$	$C_{22}H_{24}N_2O_9$	$C_{12}H_{14}N_4O_4S$
Monoisotopic mass	329.2017	330.2096	406.2137	478.1143	460.1481	310.0735
log K_{ow}	0.60	5.72	0.20	-0.62	-0.90	1.63
pK_a	5.9	5.5	7.6	3.3, 7.4, 9.3	3.3, 7.3, 9.1	2.0, 6.7

Information obtained from Pubchem https://pubchem.ncbi.nlm.nih.gov/

2.2.1 Malachite green

Malachite green (MG) (Figure 2.1) is a positively charged dye part of the triphenylmethane family of dyes. It has been traditionally used in the textile and paper industry (FAO JEFCA 2009). Starting from the 1930s, it was used in aquaculture for its effectiveness against fungi like *Saprolegnia* spp. and protozoan ectoparasites, particularly *Ichthyophthirius multifiliis* (EFSA 2016). Treatment is frequently administered as water baths considering the relatively high solubility of the MG chloride or oxalate salts (FAO JEFCA 2009). When present in aqueous solutions, as is the case during water treatments, MG is in equilibrium with its colorless carbinol base which is less water soluble, but more lipid soluble, than the chromatic ionized form (FAO JEFCA 2009). It has been hypothesized that this carbinol form is taken up by the fish (Plakas et al. 1996) and metabolized to leucomalachite green (LMG), which is more lipophilic and persistent in the muscle of organisms such as trout and carp (Bajc et al. 2011).

Demethylated forms of LMG have been proposed as other metabolites in catfish (Doerge et al. 1998).

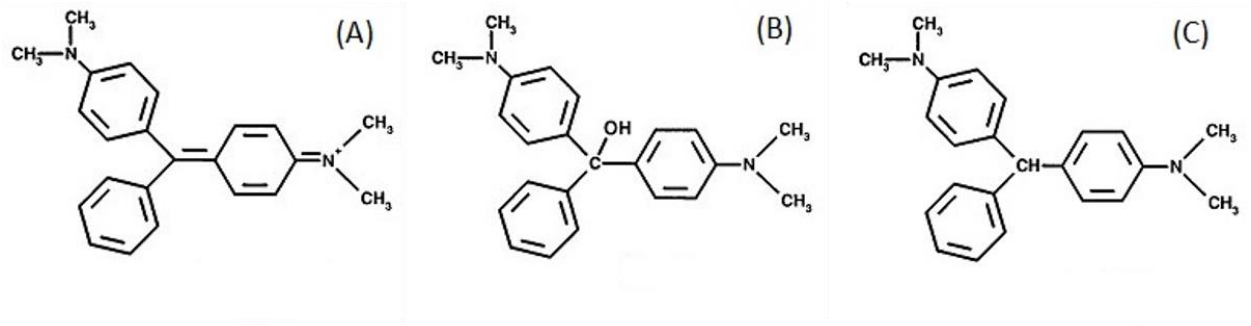

Figure 2.1: Chemical structures of MG (A), MG-Carbinol (B) and LMG (C) (Plakas et al. 1996)

Both compounds have been studied for their potential toxicity in laboratory animals (Table 2.2). But there is little information on the direct effects of MG in humans. One case has been reported where a three-year-old girl ingested about 45 mg of MG that was in the form of an aquarium product (EFSA 2016). She was admitted to hospital with signs of cyanosis, such as blue legs and arms. Oxygen saturation was 47.4 % with levels of methaemoglobin at 50.6 %, indicating some form of hematological toxicity in humans. Indeed, hematological toxicity characterized by a significant increase in lymphocytes and a decrease in neutrophils has been observed in female rats at dietary daily MG exposure doses of 1000 ppm (equivalent to 94.5 mg/kg bw/day) (Clemmensen et al. 1984, EFSA 2016). From this, a NOAEL of 9.4 mg/kg body weight can be found (EFSA 2016), as this corresponds to the next lowest exposure dose (100 ppm). In the same study, a LD_{50} of 275 mg/kg body weight was determined for rats (Clemmensen et al. 1984). Decrease in hemoglobin and hematocrit was also observed in female rats fed MG for 28 days at dose levels of 1200 ppm, corresponding to 190 mg/kg body weight,

and in male rats fed LMGat 1160 ppm, corresponding to 115 mg/kg body weight (Culp et al. 1999, NTP 2004, EFSA 2016). Other adverse health effects noticed in animal studies included increases in liver weights in rats and skeletal malformations in rabbits (EFSA 2016). One mode of action proposed for MG is as an inducer of reaction oxygen species (ROS) as it is considered an electron accepting/transferring compound (EFSA 2016). These species are associated with DNA damage and cytotoxicity which appears to be supported by the various adverse effects observed in animals (EFSA 2016).

Based on various side effects observed in experimental studies, including detection of DNA adducts in the liver of rats, induction of DNA strand breaks in hamster embryo cells, increase in hepatocellular adenomas in female rats and an increase in cellular adenomas of the testis in male rats, MG and LMG are considered possible carcinogens (EFSA 2016). Therefore, MG has not been approved as a veterinary drug for use in aquaculture by the European Union, US Food and Drug Administration or Health Canada (Cha et al. 2001, EFSA 2016, Health Canada 2017). But due its low cost and availability and high efficacy against parasites and fungi, MG is still being used in a lot of countries (EFSA 2016) . The *Codex Alimentarius* has not recommended a MRL for MG or LMG as there is no safe level that would present an acceptable risk to consumers (Codex Alimentarius 2018). The European Union has set a minimum required performance limit of 2 μg/kg as the sum of MG and LMG to be detected and confirmed, in other words the limit of quantification (EFSA 2016). Canada has set a more conservative limit of 0.5 μg/kg as an interim limit of quantification (Health Canada 2017). Based on their risk assessment, Health Canada concluded that there is low risk to humans based on consumption of fish with concentrations of MG and/or LMG below 1 μg/kg. Aquaculture products with MG or LMG concentrations above 1 μg/kg would not be permitted for sale in Canada (Health Canada 2017).

Table 2.2: Reported toxicities for MG and LMG

Toxicity	Exposure duration	Compound	Animals	NOAEL	Reference
Haematological (erythrocyte, hemoglobin count)	28 days	MG	Female rats	9.4 mg/kg bw/day	Clemmensen et al. (1984)
Developmental (skeletal abnormalities)	9 days	LMG	Pregnant female rats	10 mg/kg bw/day	Wan et al. (2011)
Hepatic (hepatocyte vacuolization)	28 days	MG	Female rats	75 mg/kg bw/day	Culp et al. (1999)
Developmental (body weight)	28 days	MG	Female mice	120 mg/kg bw/day	Culp et al. (1999)

2.2.2 Lincomycin

Lincomycin (LIN, Figure 2.2) is part of the lincosamide group of antibiotics which are characterized by a common structure containing an amino acid group and a sulfur-containing galactoside (Wang et al. 2012). It is a weak base; the pKa value has been determined at 7.5 (Wang et al. 2012) and it is considered a very stable compound in aqueous solutions under normal environmental conditions (FAO JEFCA 2000). As LIN is approved for use as an antibiotic in humans, there is some information in the literature on adverse health effects observed following treatment. Antibiotics often impact the intestinal microflora which may in turn lead to gastrointestinal problems. A frequent adverse health effect of oral treatment with LIN is pseudomembraneous colitis, a sometimes-fatal disease characterized by diarrhea and inflammation and hemorrhage of the colon (Scott et al. 1973, Pittman et al. 1974, Smart et al. 1976). Similar toxicity has been observed in laboratory animals. In rabbits, overgrowth of pathogenic bacteria like *Clostridium perfringens* following LIN treatment led to enteritis, another

disease characterized by inflammation of the colon (Morris 1995). In acute toxicity studies, LD_{50} for rats were determined to be more than 4000 mg/kg following oral administration of the antibiotic (Gray et al. 1964) suggesting a lower toxicity in animals.

In Canada, it has been approved as a drug for both human and veterinary use but it is not approved as a drug in aquaculture (Health Canada 2018). The *Codex Alimentarius* has set MRLs for chicken and pig liver, kidney, muscle and fat and for cow's milk (Codex Alimentarius 2018).

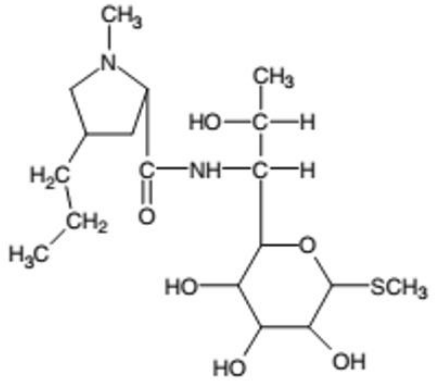

Figure 2.2 Chemical structure of LIN (Kuchta and Cessna 2009)

2.2.3 Sulfadimethoxine

Sulfadimethoxine (SFM) (Figure 2.3) is an antibiotic part of sulfonamide family of compounds. Sulfonamides have a general structure composed of a benzene ring, an amine group and an sulfonamide group (Batchu et al. 2014). The first pKa, 2.0, is associated with the deprotonation of the amine group with a pKa of 6.7 for the loss of a proton from the sulfonamide group.

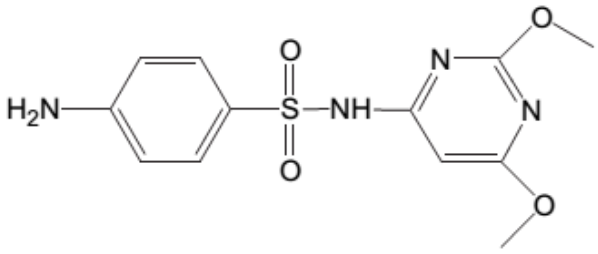

Figure 2.3 Chemical structure of SFM (Won et al. 2011)

Little information could be found on the toxicity of SFM specifically. LD_{50} values have been determined for other sulfonamide antibiotics (Baran et al. 2011). For sulfafurazole, reported LD_{50} were 10000 mg/kg in rats, 2000 mg/kg in rabbits and 5700 mg/kg in mice. For sulfacetamide, LD_{50} in rats was reported at 16500 mg/kg (Baran et al. 2011). For sulfanilamide and sulfathiazole, LD_{50} in mice were reported as 4200 and 4500 mg/kg, respectively. The high LD_{50} indicate low acute toxicity. Acetylated metabolites of sulfonamides are less water soluble and may accumulate in the kidneys, causing renal damage (Baran et al. 2011).

In Canada, SFM is approved for use in aquaculture in salmonids for the treatment against *Aeromonas salmonicida* with a MRL of 0.1 ppm (Health Canada 2010) . The drug should not be applied when the water temperature is below 10°C and treated fish should be kept for at least 42 days after the last treatment before being marketed (Health Canada 2010). In Europe, the MRL has been set 0.1 ppm as the sum of all sulfonamides (as parent drugs) in all food producing species, including fish (Won et.al 2011, EU Commission Regulation 37/2010). A similar MRL has been set in the United States (Done and Halden 2015).

In mammals, sulfonamides are generally metabolized through oxidation, acetylation or hydroxylation at the N^4 atom (nitrogen in the amine group of the molecule) or glucuronidation at

the N^1 (amide group) or N^4 atoms (Baran et al. 2011). This type of metabolism has been observed in aquatic organisms like trout (Uno et al. 1993) or lobsters (Barron and James 1988).

2.2.4 Tetracyclines

The focus of this review was on two tetracycline antibiotics, oxytetracycline (OTC) and chlortetracycline (CTC) (Figure 2.4). CTC is an amphoteric compound that can react as an acid or a base (Wang et al. 2012). Three pKa values have been determined for the compound: 3.3, 7.4 and 9.3 (Wang, MacNeil et al. 2012). There appears to be some differences in the literature between the assignment of the pKa values to specific groups within the structure. The first pKa has been associated with the loss of proton at the hydroxy group of carbon 3 (Qiang and Adams 2004, Anderson et al. 2005). The second and third pKa are associated with the loss of protons from the β-diketone and dimethylammonium groups but the specific assignment of each pKa to a group varies (Qiang and Adams 2004). The loss of hydroxyl group on carbon 6 and a hydrogen on carbon 5a forms anhydro-CTC. This loss of water can also occur on the 4-epi-CTC to form 4-epi-anhydro-CTC. Under alkaline conditions, the bond at carbon 6 can cleave to form iso-CTC. Tautomerism between keto-enol forms on carbon 12 and 11 can also occur in alkaline conditions (Halling-Sorensen, Sengelov et al. 2002). In alkaline conditions and if oxygen is present, desmethyl-iso-CTC can be formed (Halling-Sorensen et al. 2002).

In the case of OTC, as the structure is similar to CTC, three pK_a values have been determined: 3.3, 7.3 and 9.1 (Wang et al. 2012). OTC can undergo the same reactions like CTC, such as epimerization at carbon 4 under the pH range 2-6 (Anderson et al. 2005). In alkaline conditions and in the presence of metals, 4-epi-OTC will revert back to its parent structure (Anderson et al. 2005). Anhydro-OTC can be formed under highly acidic conditions (Anderson

et al. 2005). However, compared to other anhydrotetracyclines, anhydro-OTC is less stable because of its hydroxy group on carbon 6 which leads to the formation of α-apo-OC and β-apo-OTC (Anderson et al. 2005). Tetracyclines are also known to bond to divalent cations, e.g. Ca^{2+} (Anderson et al. 2005). This reactivity of tetracyclines influences greatly their analysis notably its stability during the sample preparation and extraction steps.

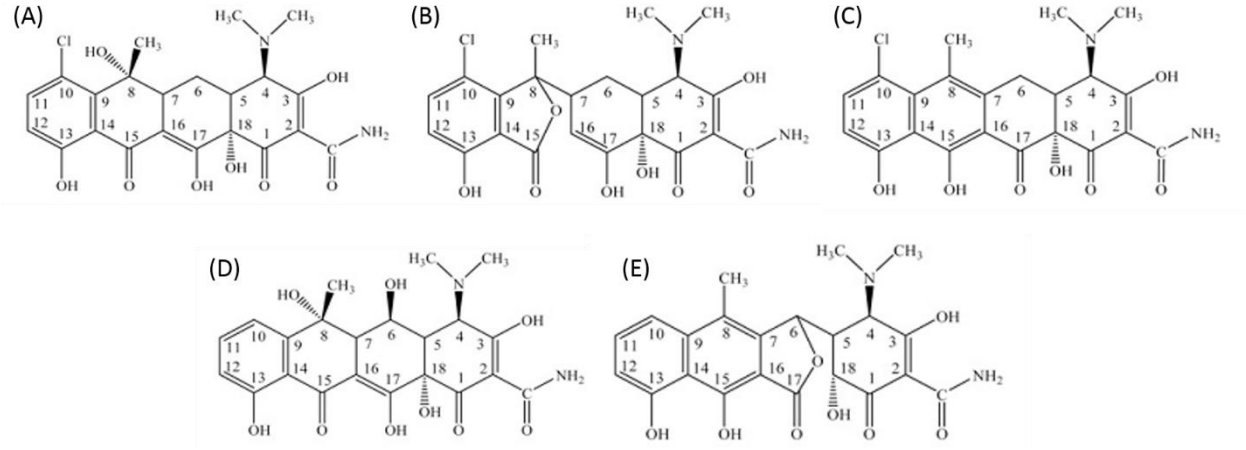

Figure 2.4: Chemical structures of CTC (A), iso-CTC (B), anhydro-CTC (C), OTC (D) and α-apo-OTC (E) (Loftin, Adams et al. 2008)

Similar to LIN, CTC may inhibit growth of bacteria in the intestinal flora and allow bacteria such as *Clostridium difficile*, leading to pseudomembranous colitis (FAO JEFCA 1999). Based on animal studies in mice rats, CTC was considered as low acute toxicity, based on LD_{50} values between 2150 and 5000 mg/kg body weight (CVMP 1995). Low acute toxicity has also been observed for OTC. LD_{50} values in mice vary between 3600 and 7200 mg/kg body weight (CVMP 1995). No observed effect level (NOEL), established based on the induction of resistant intestinal bacteria, has been established at 2 mg/day in a human study (FAO JEFCA 1999). Neither compound is considered carcinogenic (CVMP 1995).

CTC has not been approved for use in aquaculture in Canada. MRLs for kidney, muscle, liver of cattle, sheep, poultry and pig as well as eggs and the milk of cattle and sheep have been set by the *Codex Alimentarius* (Codex Alimentarius 2018). On the other hand, OTC is approved for use in aquaculture species, specifically lobsters and salmonids, with some restrictions (trout and salmon) (Health Canada 2010). In lobsters, OTC may be used for the treatment of gaffkemia (red tail) caused by the bacteria *Aerococcus viridans* but the treated lobsters should not be marketed for at least 30 days after the last treatment. In salmonids, OTC is applied as a treatment against *Haemophilus piscium, Aeromonas salmonicida, Chondrococcus (Flexibacter) columnaris, Cytophaga psychrophila* and *Yersinia ruckeri* (Health Canada 2010). MRLs have been established at 0.2 ppm in the muscle of lobsters and salmonids (Health Canada 2010). *Codex Alimentarius* standards also establish an MRL of 0.2 ppm for OTC in the muscle of fish and giant prawns (*Paeneus monodon*) (Codex Alimentarius 2018). In the United States, OTC has also been approved for use in catfish, beside salmonids and lobsters at a maximum tolerance level of 2 ppm with a withdrawal period of 21 days (Bernardy et al. 2003). In Vietnam, the MRL has seen set at 0.1 ppm (Li et al. 2017). In Europe, the MRL has been set at 0.1 ppm (reported as the sum of the parent drug and its 4-epimer) in all food producing species (Charitonos et al. 2017).

2.3 Methods of analysis

Analysis of food contaminants, including veterinary drugs, can follow two workflows, target or non targeted analysis (NTA) (Figure 2.5) (Díaz et al. 2012, Knolhoff and Croley 2016). NTA also includes suspect screening, where extracted compounds are screened against existing chemical databases using specific information like molecular formula and mass, to identify

compounds which may be further confirmed using standards (Krauss et al. 2010, Díaz et al. 2012). With the development of HRMS methods, the implementation of non-targeted analysis for environmental and food applications is increasing as it can identify high risk compounds from various chemical families (Fu et al. 2017, Asghar et al. 2018, Blanco-Zubiaguirre et al. 2020). The goal in this section was to identify the current methods of analysis, based on both target and non-target approaches, for the reviewed veterinary drugs, e.g., solvents used for extraction, matrix clean-up strategies and quantification techniques. The advantages and limitations of each approach will be discussed.

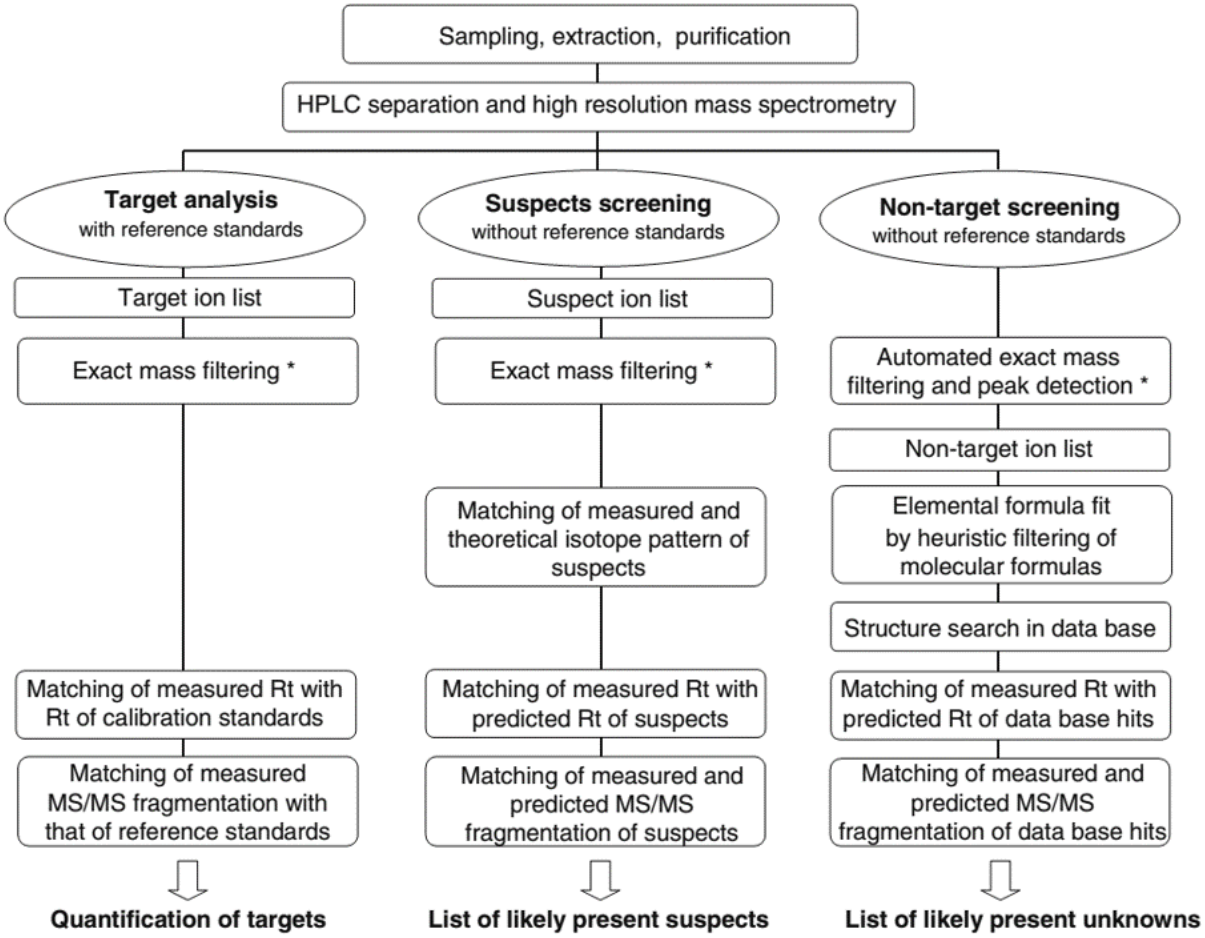

Figure 2.5: Workflow for target and non-target analysis (Krauss et al. 2010)

2.3.1 Targeted analysis

Extraction and quantification of veterinary drugs residues from seafood tissues is regularly based on liquid extraction with organic solvents followed by some sample clean-up to remove interferences like lipids, and analysis based on HPLC or LC-MS (Canada-Canada et al. 2009, Chen et al. 2019). Methods are validated for the target compounds using criteria like recovery, repeatability, MDLs and LOQs. The most obvious disadvantage of target analysis is its limitation to specific analytes. Too much clean-up of samples removing interferences may remove other compounds of interest, like other contaminants or metabolites.

2.3.1.1 Malachite green

Numerous studies have been published in the literature for the targeted analysis of MG and LMG (Table S2.1). Several steps must be taken to ensure correct detection and quantification of the compound, due to its chemical properties. Standard solutions of MG should be stored in amber flasks as they are prone to photodegradation (EFSA 2016). Particular attention must be paid to the pH of the extraction. If the pH is below the pK of 6.9 of MG, then the chromatic ionized form will more likely be extracted. If the pH is above 6.9, then the MG extracted is more likely to be in the un-charged, colourless, carbinol base.

Homogenized fish tissues are extracted usually with an organic solvent. The most frequently used solvent in the methods available in the literature is acetonitrile. Acetonitrile is usually mixed with a buffer, such acetate buffer (pH around 4.5) (Mitrowska et al. 2007). McIlvaine buffer, which is a mixture of citric acid and disodium hydrogen phosphate, at pH 3.0 has also sometimes been used (Scherpenisse and Bergwerff 2005, Valle et al. 2005, Chen and

Miao 2010). Acidic media is not only useful for keeping malachite green in the ionized form but also to denature proteins present in fish tissues so *p*-toluenesulfonic acid has been added along to acetonitrile extraction mixture for this purpose (Bergwerff and Scherpenisse 2003, Chen and Miao 2010). Hydroxylamine can also be added to acetonitrile as a reductant to prevent conversion into carbinol base (Mitrowska et al. 2007, Hurtaud-Pessel et al. 2011). Ascorbic acid and *N*,*N*,*N*,*N*-tetramethyl-1,4-phenylenediamine hydrochloride have also been added during sample extraction as they can prevent demethylation of the malachite green (Scherpenisse and Bergwerff 2005, Valle et al. 2005, Chen and Miao 2010).

Further isolation and purification to remove interferences can be achieved using SPE or with liquid extraction using dichloromethane (Halme et al. 2004, Mitrowska et al. 2007, Lee et al. 2010). The disadvantage when using organic solvents for extraction is that LMG extraction may be favorized over MG extraction, as LMG is more hydrophobic (Bergwerff and Scherpenisse 2003). Defatting of sample is also necessary and alumina or hexane have been employed for this purpose (Andersen et al. 2006, Tittlemier et al. 2007). Solid phase extraction can also be useful in concentrating the analyte, as MG is usually present in low ppb levels. As malachite green is charged, SPE columns used include SCX (strong-cation exchange) (Mitrowska et al. 2007), molecularly imprinted polymers (Guo et al. 2011), alumina and propylsulfonic acid (Halme et al. 2004), aromatic sulfonic acid (Scherpenisse and Bergwerff 2005, Valle et al. 2005). While most methods analyse simultaneously for MG and LMG, some methods have included the *in-situ* oxidation of LMG to MG, so that quantification is done only with the parent compound. This is achieved by adding 2,3-dichloro-5,6-cyano-1,4-benzoquinone (Andersen et al. 2006, Lee et al. 2010, Guo et al. 2011).

Quantification is achieved using mostly HPLC or LC-MS for confirmatory analysis. HPLC-DAD or with fluorescence detection is used, with wavelength settings at 620 nm for MG and 265 nm for LMG (FAO/WHO Joint Committee, 2009). Some methods using LC also use PbO_2 pre- or post-columns for the oxidation of LMG into MG so that a stronger signal is obtained (Valle et al. 2005, Ascari et al. 2012). ELISA kits have also been used for analysis of malachite green (Bilandžić et al. 2012, Xu et al. 2013, Oliveri Conti et al. 2015). Acceptable recoveries of around 80% have been reported with ELISA.

2.3.1.2 Lincomycin

Contrary to MG, LIN is a much more stable compound and it does not require the addition of other compounds to stabilise it during analysis. After homogenization of sample, addition of a mixture of acid and organic solvent is added for extraction of the antibiotic. Acids like formic acid are added to precipitate fish proteins (Dasenaki and Thomaidis 2015) followed by a centrifugation step to separate the proteins from the sample, as proteins are larger particles and will sediment at the bottom. Buffers may also be used to separate proteins. In one method, sodium phosphate buffer at pH 4.5 was used, as this pH was closed to the isoelectric point of fish proteins (Luo et al. 1996). Extraction of LIN is done by organic solvents such as acetonitrile and methanol (Moats 1991, Smith et al. 2009, Dasenaki and Thomaidis 2015). Defatting of samples can be achieved through addition of hexane (Sin et al. 2004, Smith et al. 2009, Dasenaki and Thomaidis 2015). Solid phase extraction can be done for purification of samples and concentration of the analyte. HLB or C18 cartridges eluted with polar compounds like water and methanol have been successfully applied to the analysis of lincomycin (Adams et al. 2009, Kowalski et al. 2014). The quantification of LIN is often using LC coupled with MS or UV

detectors (Tolgyesi et al. 2012). Luo et al. (1996) reported a method using gas chromatography for the analysis of lincomycin in salmon tissues. In that case, lincomycin was derivatized with *N*-*O*-bis(trimethylsilyl)trifluoroacetamide to form volatile trimethylsilyl derivatives that could be analysed by GC.

2.3.1.3 Sulfadimethoxine

Extraction of SFM from fish tissues is very similar to extractions of the compounds discussed above. Solid-liquid extraction with acetonitrile, or other organic solvents like ethyl acetate or acetone (Dasenaki and Thomaidis 2010), may be followed by sonication to promote breakdown of the matrix and release of the analyte into the solvent (Fais et al. 2017). In order to remove possible interferences like fats that are present in fish samples, LLE with hexane orSPE with hydrophilic lipophilic balance (HLB) or C_{18} phases can be employed (Won et al. 2011, Tong et al. 2014, Fais et al. 2017). Centrifugation can allow of removal of proteins, through sedimentation. There does not appear to be a significant difference in the recoveries of sulfonamides from acidic versus non acidic reactions (Dasenaki and Thomaidis 2010).

2.3.1.4 Tetracyclines

Both CTC and OTC have similar properties and are often included together during analysis of tetracyclines and other antibiotic residues. For this section on analytical methods, both compounds will be included. Because of the affinity of the compounds to metals that may be present in water or fish samples, care must be taken to inhibit the formation of these complexes. For this, EDTA is often included with the extraction solvent to improve recovery as

the EDTA will bind metals (Anderson et al. 2005). Acidic aqueous extractions using McIlvaine buffer or succinic acid at pH 4 are often employed (Anderson et al. 2005, Arikan 2008). Extraction pH can influence greatly the overall recovery of the analytes, not only because it may change the form under which they are present which can affect the solubility in solvents, but also because at neutral conditions, acidic analytes lose a proton and can interact with the protonated amino groups present in the matrix (Dasenaki and Thomaidis 2010). Acetonitrile has often been reported as an extraction solvent from meat and fish samples (Anderson et al. 2005, Santos et al. 2016). Tetracyclines have a higher solubility in alcohols like methanol, while organic solvents like ethyl acetate or methanolic trichloroacetic acid may also be used (Dasenaki and Thomaidis 2010). When defatting of samples is needed, hexane can be used as the tetracyclines are not soluble in hexane (Anderson et al. 2005). CTC can also form complexes with silanol groups in C18 columns employed during chromatography analysis and show tailing peaks affecting quantification (Wen et al. 2006). To inhibit this formation, acids like oxalic acid or EDTA are added to the mobile phases used and pre-treatment of the columns with EDTA is also applied (Oka et al. 2000, Anderson et al. 2005, Wen et al. 2006, Evaggelopoulou and Samanidou 2012). Solid phase extraction can be employed to improve cleanup of samples using HLB cartridges (Arikan 2008, Evaggelopoulou and Samanidou 2012). The Charm II test has been applied to tetracycline antibiotics (Al-Mazeedi et al. 2010). The method consists of a microorganism that has binding sites for antibodies is added to the sample along with a known concentration of radio labelled ^{13}C tetracycline. Tetracyclines present in the sample will compete with the ^{13}C labeled compound. The lower the concentration of tetracyclines, the higher the concentration of ^{13}C-tetracycline that will bind to the antibody sites on the microorganism (Al-Mazeedi et al. 2010).

2.3.2 Non-targeted analysis

The development of non-targeted methods, similar to targeted methods, starts with the sample extraction (Figure 2.5). However, since NTA is based on finding new compounds, sample extraction should be simple, unspecific with limited clean-up steps to extract compounds with different polarities, size and charge (Fu et al. 2017). In food safety, NTA has been used in food authenticity/adulteration studies (Kalogiouri et al. 2016, Dasenaki et al. 2019) and identification of TPs of veterinary drugs and plastic related chemicals (Tian et al. 2020, von Eyken and Bayen 2020). Whilst targeted methods are optimized to achieve the highest recoveries and best precision for the particular analytes (Mol et al. 2008), optimization for the extraction of "unknown" or "unexpected" compounds in NTA is challenging. Currently, there is no standard protocol for choosing the optimal extraction method in NTA. The choice of extraction will depend on the study objectives and the analytes of interest (Naz et al. 2014). Some criteria have been adapted from metabolomic studies, where extractions are compared based on extracted features, chemical coverage, repeatability and recovery of some target analytes of interest (Bekele et al. 2014, Sarafian et al. 2014, Knolhoff et al. 2019). However, limited information was found in the literature on the impact of sample preparation in NTA for the purpose of identifying TPs of general food contaminants. Martínez Bueno et al. (2013) compared QuEChERS with accelerated solvent extraction for the determination of carbamazepine and its TPs in mussels, based on recovery and precision. However, in this case, the TPs had already been identified in previous studies and analytical standards were available for method development.

Regarding the general non-targeted or suspect screening of the five antimicrobials studied in this review, there have been few studies published in fish and crustacean tissues. Turnipseed et al. (2017) developed a screening method for veterinary drugs in fish species, which included MG, LMG, LIN, OTC/CTC and SFM amongst the analytes with the extraction method optimized to achieve recoveries of a targeted list of analytes. This method was then applied to screen for other contaminants based on database searches which lead to the identification of other antibiotic metabolites. This appears to be the strategy often employed when developing an extraction method for suspect screening. Specific analytes, with different chemical properties and different compound families are spiked onto different matrices, and the extraction is optimized to cover all the analytes with good recoveries (Baduel et al. 2015). Jia et al. (2017) optimized a QuEChERS method for suspect screening of antibiotics in tilapia, which included OTC/CTC/LIN. Six other compounds, including pesticides and anti-parasitic drugs were identified. QuEChERS extractions are frequently used in targeted analysis of different classes of contaminants (e.g. antibiotics, PAHs) in a variety of matrices including fish (Gratz et al. 2011, Baduel et al. 2015, Kim et al. 2019), eggs, milk, infant formula (Bessaire et al. 2018) and honey (Bargańska et al. 2014). QuEChERS is based on solvent extraction, followed by partitioning (phase separation) using salts like $MgSO_4$ and dispersive solid phase extraction (dSPE) using clean-up sorbents (Figure 2.6) (Kim et al. 2019). PSA can be used to remove fatty acids and sugars, while for more fatty matrices, C_{18} or Z-Sep sorbents may offer better recoveries (Kim et al. 2019) Extraction parameters, e.g., solvent type, sorbent, pH, can easily be adapted to improve sensitivity, recovery and reduce matrix effects (Perestrelo et al. 2019). Other benefits include lower costs, sample amount and solvent volume (Perestrelo et al. 2019). Owing to these advantages, QuEChERS offers great potential in suspect screening and NTA. Accelerated

solvent extraction was another approach used for the development of a suspect screening method in fish livers (Du et al. 2017).

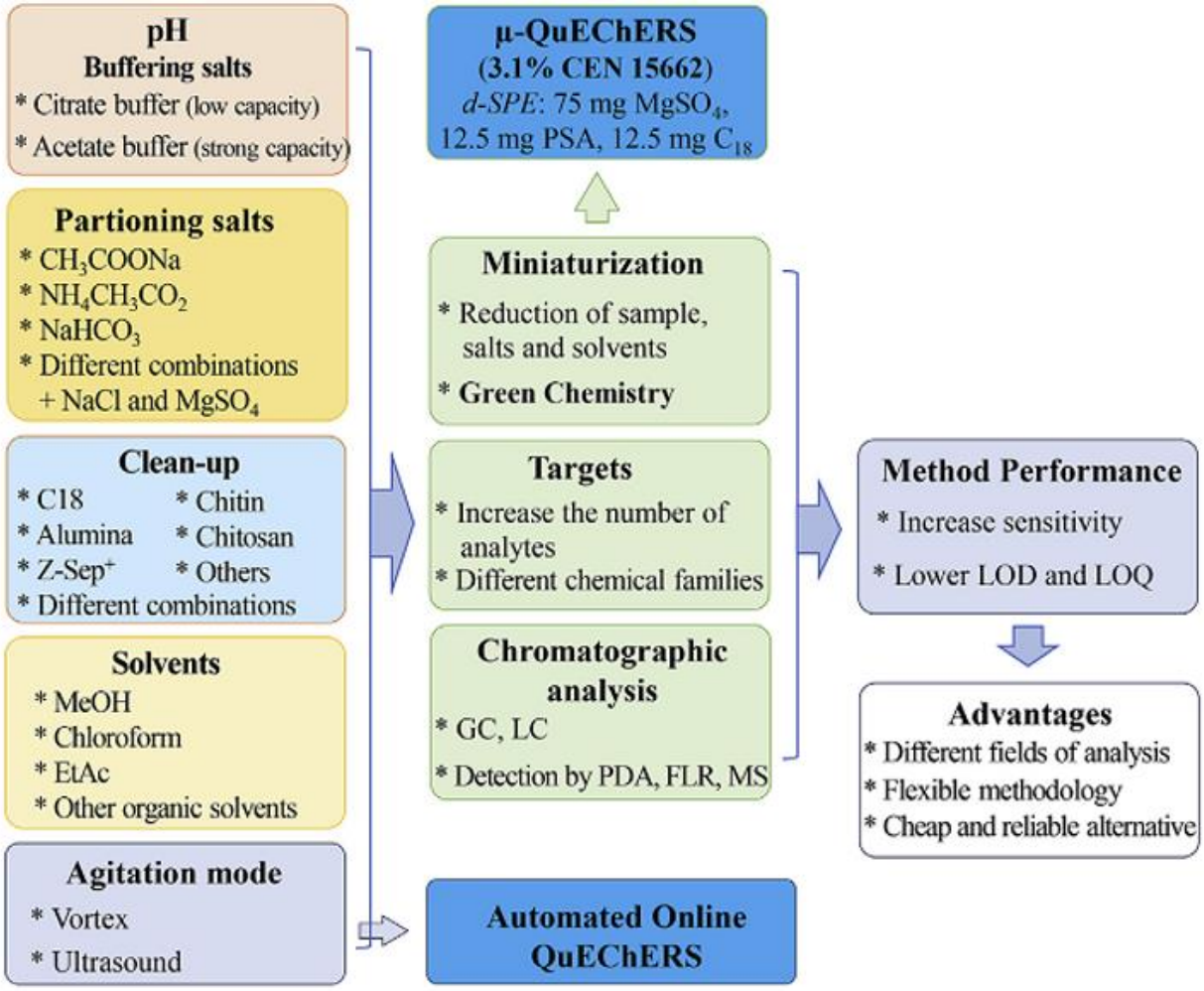

Figure 2.6: Overview of QuEChERS extraction

The next step following sample preparation is instrumental analysis. HRMS data in suspect or NTA can be acquired through full scan MS, Data Dependent Acquisition (DDA) or Data Independent Acquisition (DIA) modes (Ccanccapa-Cartagena et al. 2019, Guo and Huan 2020). Full scan MS allows to record *m/z* for extracted molecular features (Guo and Huan 2020). DIA (also known as All Ions MS/MS) and DDA offer an advantage over full scan MS mode, as

they allow to record both MS and MS^2 spectra, i.e., information on both precursor and fragment ions can be obtained (Turnipseed et al. 2017, Cccanccapa-Cartagena et al. 2019). Although better MS^2 spectra can be obtained with DDA, one limitation is it will register fragment information for ions only above a specific threshold (Guo and Huan 2020).

Another issue that must be taken into consideration with NTA is the data processing (Fu et al. 2017). Data obtained with non-target HRMS must undergo some treatment, like feature extraction and subtraction of compounds found in blanks, to identify compounds of interest (Fu et al. 2017). Similar as to the sample preparation step, there is no systematic approach to the data processing step in NTA. However, this step is very important in NTA because it may lead to false positive or false negatives, where compounds with potential risk may not be correctly identified (Díaz, et al. 2012).

2.4 Occurrence in water and biota

Drugs for human medicine or broader veterinary purposes beside aquaculture, can find their way in water sources (Calamari et al. 2003) and accumulate in edible aquatic species (Alvarez-Munoz et al. 2015). Depending on the compounds and the levels detected, there could be an important risk to human health from consuming contaminated seafood. Furthermore, safety issues or non-compliances in seafood may arise from differences between regulations governing the use of veterinary drugs in aquaculture between countries. For example, enrofloxacin is permitted for use in Korea, with an MRL of 0.1 μg/g (Kang et al. 2018) but is not allowed for use in Canada (Health Canada 2010). One of the objectives of this research is to study the impact of thermal processing on residues of veterinary drugs in seafood. Therefore, to identify which of the

veterinary drugs consumers are more likely to be exposed to through consumption of seafood, the literature was searched for studies that identified the compounds in fish muscle.

2.4.1 Malachite green

Despite the ban in the European Union, Canada and the United States, MG remains frequently detected in products such as trout and carp, as shown by various studies in Europe, Australia and Iran (Table 2.3). MG remains a cheap and effective treatment and it is readily available (Srivastava et al. 2004). Moreover, it can still be used as an industrial dye and may be found in the aquatic environment because of industrial activities (Schuetze et al. 2008, Belpaire et al. 2015).

The European Union has set up the RASFF Portal. It is a communication tool between European countries to allow them to share information, quickly, regarding the contamination of food products that may present a health risk to consumers (RASFF 2016). A search of the European Union RASFF Portal from 2006 until 2019 of notifications concerning residues of veterinary drugs in fish and fish products as well as crustaceans and products thereof yielded a total of 1139 results. Sixty-seven (5.9 %) notifications concerned MG and LMG with levels usually between 0 and 10 ppb, although one notification concerned the presence of LMG at 5680 ppb in eels (Figure 2.5). Other non-compliant products included shrimp, catfish, trout and pangasius.

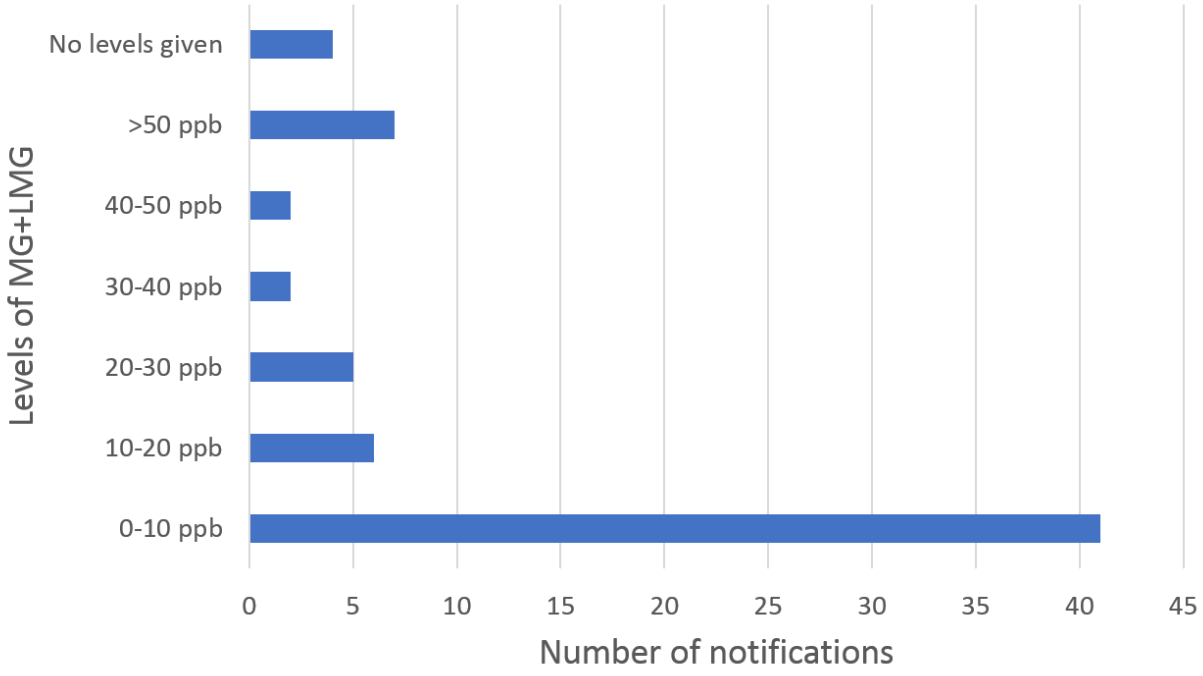

Figure 2.7: Range of MG and LMG levels in fish and crustaceans detected by the European Commission (RASFF Portal) between 2006 and 2019

A more extensive search of the literature, including government databases and direct requests to governmental agencies was conducted by Love et al. (2011) to study the violations of veterinary drugs in seafood products. Alongside nitrofurans and chloramphenicol, MG was one of veterinary drugs responsible for non-compliances in seafood products. In the period 2001-2006, an average of 105 violations of veterinary drug residues per year were found in the United States. Among these violations, 77% were because of MG residues in finfish. A similar trend in finfish veterinary drug violations concerning MG was observed in the European Union, Canada and Japan. In Canada, 68 % of violations concerned MG (for the period 2001-2009), in the European Union, the number of violations was 31% (for the 2001-2008 period) and 36 % in Japan (for the period 2000-2009). In shrimp and prawn products, MG violations were found in

Canada (3% of total violations) with less than 1 % for Japan. In shellfish, MG violations were found in Japan at 8%, no data was obtained for the European Union and Canada. Surprisingly, in Japan 66% of violations for crabs were regarding MG with no violations for the other regions. The data the authors obtained also numerical data regarding the concentrations of contaminants but only for the European Union and United States. Concentrations of MG ranged from 1 to 10 ppb.

Table 2.3: Occurrence of MG and LMG in seafood

Country	Matrix	Number of samples analysed	% Detection MG/LMG	Range (ng/g)	Reference
Canada	rainbow trout, shrimp	30	10	0.73-1.20	Tittlemier et al. 2007
Canada	salmon, tilapia, pangasius, white shrimp	56	13	0.049-0.90	Dinh et al. 2020
Australia	rainbow trout, perch, basa	60	16.7	5.0-138.0	FSANZ 2005
Croatia	carp, rainbow trout	72	18.1	0.3-1.07	Bilandžić et al. 2012
Iran	rainbow trout	144	48.6	0.3-146.1	Fallah and Barani 2014
Slovenia	trout	43	15.2	0.8-28	Bajc 2007
Netherlands	trout, eel, fresh, smoked and canned salmon	48	46.8	0.2-9.7	Bergwerff and Scherpenisse 2003
Malaysia	catfish, tilapia, barramundi, grouper	37	43.2	0.53-4.10	Kwan et al. 2018

2.4.2 Lincomycin

There is limited information on the presence of LIN in aquaculture products. In one of the few studies analysing LIN in aquaculture products, fish samples including tilapia, carp, catfish and bream were sampled in Chinese rivers (Zhao et al. 2015). The rivers chosen for sampling were part of the Pearl River Delta which receives large quantities of treated and untreated sewage waste. For fish samples, a total of 105 bile, 48 plasma, 91 liver and 128 muscle samples across the different species were analysed. LIN was detected in 92% of bile samples with a mean concentration of 8.80 μg/L, in 25% of plasma samples with a mean of 24.69 μg/L, in 9% of liver samples with a mean of 22.4 μg/kg and in 5% of muscle samples with a mean <0.88 μg/kg. In a study conducted in the United Kingdom, sampling of fish and shrimp imported from other countries were analysed for LIN (Fussell et al. 2014). Only one sample out of 10 samples of imported shrimp tested positive for LIN with a concentration of 1.1 ng/g.

On the contrary, as the antibiotic is used in livestock in human medicine, it has been detected in wastewater and agricultural run-off (Calamari et al. 2003, Kuchta and Cessna 2009, Forrest et al. 2011). For example, water samples collected near livestock facilities were contaminated with LIN at a frequency of 91% within a range of 0.2-355 ng/L (Lissemore et al. 2006). LIN has also been found in wastewater effluents with concentrations usually lower than 100 ng/L (Lin et al. 2008, Zuccato et al. 2010, Biel-Maeso et al. 2018).

2.4.3 Sulfadimethoxine

No notifications could be found on the RASFF Portal (RASFF 2016) concerning the detection of the SFM in fish and crustacean species. The withdrawal times specified in some

regulations (Health Canada 2018) are set so that any residues present in muscle will fall below the MRL. Some published studies, which will be discussed below, have analysed and detected SFM.

In a survey of seafood collected in the United States, SFM was found in only one farmed shrimp sample out of 27 total samples collected, at a concentration of 0.3 ng/g (Done and Halden 2015). Low detection frequency was also found in a study in Turkey, with only 1 out of 15 muscle samples of carp tested positive for SFM at a concentration of 0.0040 mg/kg (Varol and Sunbul 2017). Another matrix analyzed for SFM was mollusks, as part of a study in Bohoy Bay, China, as they are, like eels, good indicators of marine pollution (Li et al. 2012). Thirty-one percent of edible mollusks collected around coastal areas of Bohoy Bay, of a total sample of 191, showed the presence of SFM with a mean and a maximum concentration of 0.08 and 1.75 μg/kg dw.

SFM is not just approved for use in aquaculture animals, but also in other livestock animals. Similarly, to LIN, groundwater or surface water near livestock facilities may become contaminated with the antibiotic and may contaminate the aquatic environment through run-off. Indeed, ground water samples collected from wells nearby confined animal feeding operations in the United States contained SFM between 0.047 and 0.068 μg/L (Batt et al. 2006). Other studies have investigated the presence of SFM in water, however no levels were detected (Li et al. 2012), but other compounds from the sulfonamide family, such as sulfamethoxazole or sulfathiazole, have been detected (Zheng et al. 2012, Shimizu et al. 2013, Yan et al. 2013, Tong et al. 2014, Song et al. 2016).

2.4.4 Tetracyclines

As is the case with LIN, there is very little information in the literature on the presence of CTC in aquaculture products. A search of the RASFF portal (RASFF 2016) between 2006 and 2019 yielded two notifications on the presence of CTC in aquaculture products. Only one notification was found, alerting to the presence of the antibiotic in prawns at concentrations between 289 and 560 ppb in shrimps from China.

In their review on veterinary drug violations in seafood products, Love et al. (2011) found that between 2000 and 2009, 2% of veterinary drug violations in shrimp and prawns inspected in Japan concerned CTC. On the other hand, as mentioned in section 4.1, MG was responsible for 36% of drug violations in finfish.

Tilapia, carp, catfish and bream sampled in China showed less contamination of the fish by CTC as compared to LIN, erythromycin and norfloxacin (Zhao et al. 2015). CTC was not detected in muscle, bile and plasma but was detected in liver with a frequency of 2% (out of 91 samples) and at levels up to 326 μg/kg.

In other aquatic media, CTC is more often detected in sediment samples compared to water. For example, in their study of four rivers, Zhao et al. (2015) detected CTC in 80% of sediment samples compared to 33% of water samples. Concentrations up to 452 ng/L and147 μg/kg were recorded in water and sediments, respectively. Ionic interactions and chelation with metals may explain the binding of CTC to solid sediments (Monteiro et al. 2015). Other studies have identified CTC in river water and sediments (Jiang et al. 2014) and agricultural wastewater (Lin et al. 2008).

With OTC approved as a veterinary drug in aquaculture, it can be expected that the drug will be frequently detected in market products. However, as the regulations set a withdrawal

time, like SFM, in the majority of cases, levels will fall well below the limits set by regulatory bodies, as shown by the decreases in muscle concentrations in pharmacokinetic studies (Bernardy et al. 2003, Uno et al. 2006). Similar to the previous veterinary drugs discussed in the previous sections, the RASFF portal (RASFF 2016) was used to search notifications regarding OTC in the period 2006 to 2019. A total of 39 notifications concerning the presence of oxytetracycline above MRL, i.e., 0.1 ppm, were found. One notification concerning tiger prawns was discarded as the OTC concentration was given as $\mu g/dm^2$. Out of these notifications, 28 (72%) concerned shrimp, while the rest concerned fish feed, giant prawn, seafood mix, tilapia, perch and salmon. Concerning the levels detected (Figure 2.8), 49 % of notifications were between 100-200 ppb, 26 % between 200-300 ppb and 18 % above 500 ppb, including two notifications concerning shrimp from India and Indonesia at levels of 210 and 300 ppm.

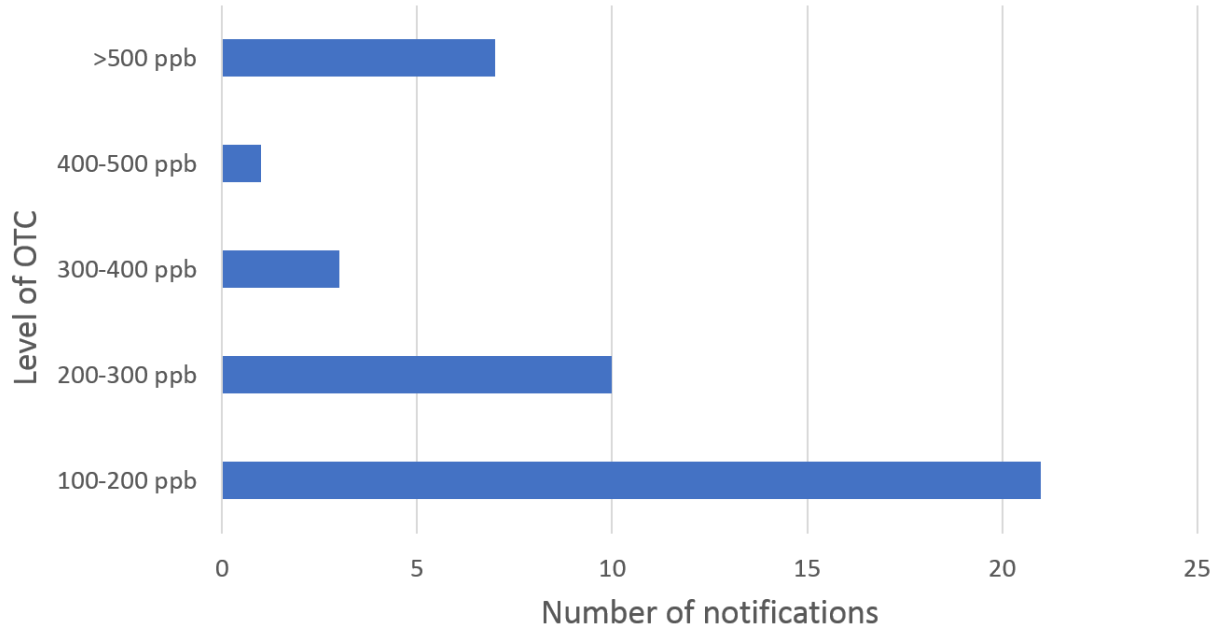

Figure 2.8: Range of OTC levels in fish and crustaceans detected by the European Commission (RASFF Portal) between 2006 and 2019.

The largest number of notifications referred products from Vietnam, where OTC has been found to be amongst the top three preferred antibiotics in a survey of shrimp farmers (Pham et al. 2015). Also in the European Union, 1% of veterinary drug violations in finfish between 2000 and 2008 were due to OTC (Love et al. 2011). Other monitoring studies in the Unites States and Iran found OTC in samples like shrimp, catfish and salmon as concentrations below 40 ng/g (Chafer-Pericas et al. 2010, Barani and Fallah 2015, Done and Halden 2015). In Korea, OTC was detected in 10.1% of seafood samples, including shrimp, but were judged compliant as the levels fell below the MRL of 0.2 μg/g (Kang et al. 2018).

Due to the low bioavailability of OTC in fish (Uno et al. 2006), most of administered drug will likely be excreted through feces in the surrounding water where it can bind to sediment particles due to the interactions between the compound and cations (Bebak-Williams et al. 2002). Medicated, uneaten feed may also be a factor in the accumulation of OTC in water and sediment (Rigos et al. 2004). Indeed, numerous studies have reported the presence of OTC in water and sediment samples collected from fish and shrimp farms (Chen et al. 2015). In Thailand, water and sediment were collected from the Tha Chin River where caged tilapia farming takes place (Rico et al. 2014). In total, 34 water samples and 31 sediment samples were collected. Twenty-five percent of water samples collected during the dry season contained OTC with a maximum concentration of 3.05 μg/L. On the other hand, 100% of samples collected during the wet season were positive for oxytetracycline with a maximum concentration of 1.76 μg/L. OTC was detected in all sediment samples, collected in both seasons, at higher concentration compared to water samples; 42.4 μg/kg and 12.4 μg/kg during the dry and wet season respectively. The differences between the concentrations during wet and dry season were attributed to several factors. Farmers reported a higher use of antibiotics during the wet season because of higher fish

stress associated with run-off episodes. Higher concentrations of organic matter during the wet season are correlated with a higher turbidity and can contribute to a better stability of the antibiotics in water. Lastly, changes in hydrodynamics can contribute to the desorption and re-suspension of antibiotics in sediment.

2.5 Stability during thermal treatment and transformation products

Both WHO and OECD recommend that processing should be taken into consideration in food contaminants risk assessments (OECD 2008, WHO 2009). Studies have shown that normal cooking conditions like baking, frying or microwaving can reduce levels of some contaminants, including antibiotics (Abou-Raya et al. 2013, Tian et al. 2017), phthalates (Fierens et al. 2012) and persistent organic pollutants (POPs) (Bayen et al. 2005). Generally, the levels of decrease differ among cooking treatments and food matrices (Perello et al. 2009). One limitation of these studies is that the focus has been on the parent compounds leading to large gaps in the characterization of the fate of these chemicals during cooking, i.e., formation of TPs. The identification of these compounds and evaluation of possible toxicity is essential for risk assessments to establish other possible health risks to human health. NTA has been shown as a suitable technique for the identification or these TPs in food (Stadler et al. 2019, Tian et al. 2020, von Eyken and Bayen 2020).

Regarding the antimicrobials under this review, some studies, relying on target analysis, investigated the concentration changes occurring during cooking and will be further described in this section. However, there were no studies found that applied NTA to further describe the outcome of the transformation occurring during cooking, i.e., no TP identification. As part of the review, photo and biotransformation studies were also included if they included identification of

TPs in their methodology. Transformation during these processes, particularly for phototransformation, is often due to oxidation, and with radicals formed also during cooking (Traore et al. 2012) there could be common mechanisms that could lead to the formation of the same TPs. The studies found were not reviewed in detail, but TPs described are included in Table S2.2.

2.5.1 Malachite green

Despite continuous detection of MG in aquaculture products, there is very little information in the literature on the fate of the compounds and its main metabolite in fish following cooking. In the studies found, MG was less stable to thermal processing compared to LMG.

Mitrowska et al. (2007) evaluated the stability of MG and LMG in incurred samples of carp muscle. The following cooking methods were applied to the samples: boiling to 100°C for 5, 10 and 15 minutes, baking at 180°C for 5, 10 and 15 minutes, microwaving at 2450 MHz/800W for 0.25, 0.5 and 1 minute. Temperature was monitored within the samples during cooking remaining below 100°C. For boiling, the percent reduction in MG levels was 15, 43 and 54% for the 5, 10- and 15-minute treatments respectively. For baking, a percent reduction of 12, 36 and 54% was achieved following treatments for 5, 10 and 15 minutes respectively. Overall, microwaving achieved the highest percent reduction of MG among the three treatments. For treatments of 0.25, 0.5 and 1 minute, percent reductions were 40, 52 and 61%. LMG did not exhibit the same degradation patterns as the parent compound. Boiling and baking only achieved a reduction between 3 and 5%, but microwaving for 1 minute achieved a 40 % reduction in the LMG residues. As the temperature within the fish samples never went above 100°C, the authors

hypothesized that the microwaving treatment was effective in reduction of LMG due to microwave energy. Also, the stability of the two compounds in cooking oil was also assessed. Solutions of MG and LMG were added to sunflower oil and heated at 150 and 210°C for 0, 10, 20, 30, 60, 90 and 120 minutes. When cooking in oil at 150°C, a 49% reduction in MG was observed after 10 minutes. At cooking temperature of 210°C, a 97% reduction in MG was observed after 10 minutes. LMG was stable over 120 minutes at 150°C cooking temperature while at 210°C it was reduced by 18% over 10 minutes and remained stable over the remaining 110 minutes. The products resulting from the degradation of the dye and its metabolite were not discussed.

A decrease of MG and LMG contents has also been observed in tilapia fish (Shalaby et al. 2016). Processing treatments were roasting at 200°C for 4, 8 and 12 minutes, frying at 160-180°C for 4, 6 and 8 minutes and microwaving at 0.5, 1 and 2 minutes. Degradation or MG was observed in all treatments and the percent degradation increased with increasing cooking time. Highest reduction percentages were obtained by roasting for 12 minutes (48% reduction), frying for 8 minutes (51.6% reduction) and microwaving for 2 minutes (80.8 % reduction). In this case, reduction of LMG was observed with all treatments, compared to only microwaving in the previous study by Mitrowska et al. (2007). Roasting for 12 minutes achieved 26.2 % reduction, frying for 8 minutes achieved 34.8 % reduction and microwaving for 2 minutes achieved 57.2 % reduction. The internal temperature of the fish did not go above 100°C. No qualitative analysis of possible TPs was done.

2.5.2 Lincomycin

The fate of LIN in seafood products following thermal treatment has yet to be investigated. It has been studied, however, in water and milk. The stability of LIN in water exposed to heat through a direct measurement of the concentration of the antibiotics and an assessment of the decrease in microbial activity was determined by Hsieh et al. (2011). LIN at concentrations of 50 and 200 μg/L in aqueous solution was exposed to heat treatment at 100 and 120°C for 15 minutes. A reduction of around 10% was achieved for LIN at both temperatures. The authors did not detect any new peaks (for the purpose of the study a new peak was defined to be at least 10 % of the peak of the parent compound). The method used was capillary electrophoresis and it is possible that any new TPs of lincomycin formed during the heat treatment are not detectable by that method. The microbial assay was applied to evaluate changes in the antimicrobial activity based on the minimum inhibitory concentration which is the concentration of the antibiotic that inhibits microbial activity. Based on the low increase in the minimum inhibitory concentration, LIN was considered to be heat stable. Beside temperature, the influence of pH has also assessed on the degradation of aqueous lincomycin, as it is a weak base. Different temperatures of 7, 22 and 35°C and pH values of 2, 5, 7, 9 and 11 were studied (Loftin et al. 2008). No decrease in the LIN content was observed at all temperatures and all pH values. The amide bond in LIN is stable to hydrolysis at the temperatures in the study (Loftin et al. 2008).

2.5.3 Sulfadimethoxine

Concerning the stability of SFM in processed fish, one study was identified that analysed the levels of the parent compound in catfish after cooking (Xu et al. 1996). Three cooking treatments were chosen: frying in canola oil at 190°C between 7 and 10 minutes, baking at 190°C for 45 minutes and smoking for 2 hours (1 hour at 160°C, 1 hour at 190°C) after filets were soaked in 25 % NaCl solution for one hour. SFM content decreased by 62, 42.3 and 52.6% for baking, frying and smoking respectively. No TPs were identified.

2.5.4 Tetracyclines

As OTC is allowed for use in aquaculture, its stability during cooking has been previously described (Kitts et al. 1992, Du et al. 1997, Uno et al. 2006, Uno et al. 2010). Black tiger shrimp (*Penaeus monodon*) including the muscle and shell was boiled, fried and baked (Uno et al. 2006). Boiling treatment consisted of immersion of shrimp into boiling water for 2, 4, 6, 8 and 12 minutes. Baking was done at 200°C for 4 minutes and frying was done in canola oil at 180°C for 1 minute. In muscle, frying and boiling for 4 minutes achieved the highest reduction in the levels of OTC between 50 and 60%. The highest percent reduction, 80%, was achieved by boiling at 12 minutes. Canola oil used in the frying and the water used for boiling were not analysed for possible migration of oxytetracycline from the muscle to the oil. The results of the cooking procedures show that compared to muscle, OTC is more resistant to thermal processing in shell compared to muscle; only 20 % reduction of oxytetracycline was achieved in shrimp shells. Likely, this is due to the formation of metal complexes between OTC and cations, e.g., calcium, in the shell. The study mentioned that no TPs of oxytetracycline, i.e., apo-OTC were

detected. There is the possibility that OTC can further degrade into other compounds following processing that were not detected by the HPLC analysis performed in this study. The same cooking treatment was applied to white shrimp (*Penaeus vannamei*) (Uno et al. 2010). Shell samples were also exposed to acid and alkaline treatment by exposure to 1 M NaOH and 1 M HCl at room temperature for one hour. Results obtained were similar with those obtained in black tiger shrimp. In muscle, boiling achieved a reduction of about 50%, with baking and frying achieving around 35% reduction. In shell, treatments achieved around 25-30% reduction, similar to alkaline treatment. OTC residues in acid treated shells were lower by 80%. In acid solutions, it is released from the calcium-OTC chelates.

In salmon, a reduction of approximately 35% was observed in fillets following heating in a water bath 100°C (Kitts et al. 1992). Salmon fillets were also fried for 15 minutes, with an internal temperature of 99.4 °C. A similar reduction of 32 % in OTC levels was achieved. No TPs were reported. Frying, baking and hot smoking treatments were also applied to catfish fillets (Du et al. 1997). Catfish fillets, breaded, were fried in canola oil at 190°C for 7-10 minutes, with an internal temperature measured at 71°C. The same temperature, 190°C was used for baking for 45 minutes. Fillets were also submitted to hot smoking for 2 hours, prior to being soaked in a 25% NaCl solution. Frying, baking and hot smoking achieved on average 6, 13, and 15 % reduction in OTC levels respectively. Higher reduction in baking and hot smoking were attributed to a longer cooking time compared to frying. The fried fillets achieved an internal temperature much lower than the set cooking temperature, which may not have been sufficient to degrade OTC. However, the internal temperature of smoked and baked fillets was not reported. Another important experimental parameter during the cooking of fillets that could have an

influence on the reported results is the thickness of fillets with thicker fillets possibly impeding heat penetration and distribution (Du et al. 1997).

In water, the degradation of OTC has been studied at temperatures of 62, 80 and 100°C (Rose et.al 1996). OTC was less stable with increasing temperature, with half-lives established as 120, 15 and 2 minutes. The comparison of the heating an OTC-sunflower oil solution at 110 and 180°C revealed that only a 25% decrease in the drug concentration was observed at 110°C. Similar to heating in water, faster reduction percentages were observed when heating in oil at a higher temperature. The stability of OTC in oil compared to water indicates that the main breakdown reaction is hydrolysis (Rose et.al 1996). Known TPs like epi-OTC and the apo forms of the compound formed less than 2 % of the breakdown products eluted during HPLC analysis.

2.6 Conclusion

In reviewing the five antimicrobials, overall, more information can be found on MG and OTC compared to LIN, SFM and CTC. Metabolism of LIN and CTC in fish has yet to be characterized. If the antibiotics are not metabolised or accumulated in fish tissues and excreted as parent compounds, studies on the fate of the compounds during cooking may not be applicable. OTC and MG are two veterinary drugs often responsible for non-compliances in seafood and were chosen to study in this project. Currently, as this review has shown, most information found in the literature deals with development and optimisation of analytical methods for extraction and quantification of the parent compounds. However, based on the very few studies that have looked at the stability of the compounds after cooking, residues present in muscle decrease, depending on the duration and type of treatment. Thermal TPs are important to study from a food safety perspective, as they can provide more information to assess the risk to consumers

associated with the presence of antimicrobial residues. Furthermore, these newly formed compounds may perhaps be used as markers of contamination, like in the case of thermally processed muscle, e.g., canned seafood. So far, no studies have identified any TPs in tissues. Based on the advantages of NTA using HRMS, e.g., high mass accuracy, unselective extractions, and previous studies that employed NTA to identify thermal TPs of food contaminants in fish (Tian et al 2020), it was hypothesized that this approach would be adequate in identifying thermal TPs of OTC and MG in fish and shrimp.

Therefore, the main objective of this research was to fill in the current knowledge gaps on the fate of MG and OTC during thermal processing, by identifying these compounds in white shrimp and brook trout, and investigating the effect of the matrix on the transformation pathway, i.e., if TPs formed are different between shrimp and trout for the same thermal treatment. The methodology employed will be based on NTA which has yet to be applied to study the fate of these two antimicrobials during cooking of seafood. Furthermore, although different approaches have proposed in the literature for method development in NTA, there is a lack of information on the impact of sample preparation and data processing for NTA applied for the identification of TPs. This research will be able to offer more information concerning the exposure of consumers to OTC and MG to regulatory bodies to better assess the risk to human health.

2.7 Supplementary material

Table S2.1: Methods of analysis for the reviewed compounds

Matrix	Analyte	Type of analysis	Extraction	Instrumental analysis	Reference
catfish, salmon, tilapia	MG/LMG	Targeted	-ammonium acetate buffer/acetonitrile/ dichloromethane -clean-up with alumina	LC-APCI-MS LC-VIS	Andersen et al. 2006
salmon, shrimp	MG/LMG	Targeted	-citrate acetate buffer/acetonitrile/ dichloromethane -clean-up with SPE SCX cartridges	LC-ESI-MS	Ascari et al. 2012
catfish	MG/LMG	Targeted	-McIlvaine buffer/ acetonitrile -clean-up with SPE Oasis MXC cartridges	LC-ESI-MS	Chen and Miao 2010
catfish, eel, turbot, trout, prawns	MG/LMG	Targeted	-McIlvaine buffer/acetonitrile/ dichloromethane -clean-up with SPE aromatic sulfonic acid	LC-ESI-MS	Bergwerff and Scherpenisse 2003
trout	MG/LMG	Targeted	-ammonium acetate buffer/acetonitrile/ dichloromethane -clean-up with SPE propylsulfonic acid	LC-ESI-MS	Halme et al. 2004
salmon	MG/LMG	Targeted	-McIlvaine buffer/acetonitrile/ dichloromethane -clean-up with SPE aromatic sulfonic acid	LC-APCI-MS	Valle et al. 2005
tilapia, eel, catfish, pangasius,	MG/LMG/SFM	Targeted	-McIlvaine buffer/acetonitrile/NaCl	LC-ESI-MS	Storey et al. 2014

shrimp, trout, salmon					
tilapia	MG/LMG	Targeted	-acetonitrile/acetic acid (QuEChERS) -clean-up with PSA sorbent	LC-ESI-MS	Hashimoto et al. 2012
trout	MG/LMG	Targeted	-acetonitrile/$MgSO_4$	LC-ESI-MS	Hurtaud-Pessel et al. 2011
bream	MG/LMG	Targeted	-acetate buffer/acetonitrile/ dichloromethane -clean-up with SPE alumina	LC-ESI-MS	Jiang et al. 2009
salmon, tilapia, trout	MG/LMG	Targeted	-dichloromethane/formic acid	LC-ESI-MS	Nebot et al. 2013
eel	MG/LMG	Targeted	-McIlvaine buffer/acetonitrile/ dichloromethane -clean-up with SPE aromatic sulfonic acid	LC-ESI-MS	Schuetze et al. 2008
carp, turbot, perch, mackerel	MG/LMG	Targeted	-McIlvaine buffer/acetonitrile -clean-up with SPE graphene	LC-ESI-MS	Chen et al. 2013
carp	MG/LMG	Targeted	-ammonium acetate/acetonitrile/ alumina/dichloromethane	LC-ESI-MS	Xie et al. 2013
shrimp	MG/LMG/OTC/ SFM	Targeted	-trichloroacetic acid/hydroxylamine/ sodium succinate -clean-up with SPE Oasis HLB cartridges	LC-APCI-MS	Li et al. 2006
tilapia, catfish, salmon, eel, shrimp	MG/LMG/OTC/ SFM/LIN	Targeted/ Non-target	-acetonitrile/acetic acid/ *p*-toluenesulfonic acid -clean-up with SPE HLB cartridges	LC-ESI-MS	Turnipseed et al. 2017
salmon, catfish, trout	MG/LMG/OTC/ SFM/LIN	Targeted	-acetonitrile/hexane	LC-ESI-MS	Smith et al. 2009
fish	LIN	Targeted	-metaphosphate:methanol/ hexane -clean-up SPE HLB cartridges	LC-ESI-MS	Tang et al. 2012
salmon, catfish	LIN	Targeted	-phosphate buffer -clean-up SPE C_{18} cartridges	GC-MS	Luo et al. 1996

sunfish	LIN	Targeted	-methanol/formic acid	LC-ESI-MS	Ramirez et al. 2007
salmon, shrimp, tilapia	LIN	Targeted	-phosphate buffer/acetonitrile -clean-up C_{18} cartridges	LC-ESI-MS	Dickson 2014
mosquito fish	LIN	Targeted	-ASE with dichloromethane -SPE Oasis MCX cartridges	LC-ESI-MS	Wang and Gardinali 2012
shrimp	OTC/CTC	Targeted	-succinic acid/NaCl/alumina -SPE HLB cartridges	LC-ESI-MS	Andersen et al. 2005
seabream	OTC/CTC	Targeted	-citrate buffer -SPE HPB cartridges	HPLC-DAD	Evaggelopoulou and Samanidou 2012
tilapia	OTC/CTC	Targeted	-acetonitrile/water/formic acid -SPE Captiva cartridges	LC-ESI-MS	Monteiro et al. 2015
shrimp	OTC/CTC	Targeted	-trichloroacetic acid -SPE C_{18} cartridges	HPLC-Fluorescence	Uno et al. 2006
salmon, trout, mackerel, eel, catfish	OTC/CTC/LIN/ SFM	Targeted	-acetonitrile/water -SPE StrataX cartridges	LC-ESI-MS	Peters et al. 2009
seabream, seabass	OTC/CTC/LIN/ SFM	Targeted	-acetonitrile/methanol/water/formic acid/hexane -clean up freeze step	LC-ESI-MS	Dasenaki and Thomaidis 2015
tilapia	OTC/CTC/LIN	Targeted/ Non target	-acetonitrile/water/acetic acid (QuEChERS) -clean-up with PSA, Z-Sep$^+$ sorbent	LC-ESI-MS	Jia et al. 2017
seabream, seabass	OTC/CTC/SFM	Targeted	-acetonitrile/methanol/formic acid	LC-ESI-MS	Dasenaki and Thomaidis 2010
catfish, shrimp, salmon	SFM	Targeted	-acetonitrile/methanol/acetic acid/ dichloromethane -SPE SCX cartridges	HPLC-Fluorescence	Gehring et al. 2006
carp	SFM	Targeted	-acetonitrile/methanol (QuEChERS) -clean-up graphene sorbent	LC-ESI-MS	Lu et al. 2016
salmon	SFM	Targeted/Non-targeted	-acetonitrile/water/acetic acid (QuEChERS)	LC-ESI-MS	Jia et al. 2018

			-clean-up with PSA, Z-Sep^{+} sorbent		
shrimp	SFM	Targeted	-acetonitrile/methanol -methanol/water/acetic acid	HPLC-DAD	Charitonos et.al 2017
tilapia	SFM	Targeted	-acetonitrile/formic acid -clean-up C_{18} sorbent	LC-ESI-MS	Kung et al. 2015
shrimp	SFM/MG/LMG	Targeted	-acetonitrile/acetic acid (QuEChERS) -clean-up PSA	LC-ESI-MS	Villar-Pulido et al. 2011

Table S2.2: TPs identified in photo and biotransformation studies

Compound	Neutral mass	Formula	Formation	Reference
MG	197.0841	$C_{13}H_{11}NO$	Phototransformation	Perez-Estrada et al. 2008
	210.0997	$C_{14}H_{13}NO$		
	213.0790	$C_{13}H_{11}NO_2$		
	225.1154	$C_{15}H_{15}NO$		
	227.0946	$C_{14}H_{13}NO_2$		
	239.0790	$C_{15}H_{14}NO_2$		
	241.1102	$C_{15}H_{15}NO_2$		
	259.1208	$C_{15}H_{17}NO_3$		
	273.1385	$C_{19}H_{17}N_2$		
	274.1470	$C_{19}H_{18}N_2$	Metabolite/biotransformation	Culp et al. 1999, Cha, et al. 2001
	287.1540	$C_{20}H_{19}N_2$	Phototransformation/metabolite/biotransformation	Perez-Estrada et.al 2008, Cha et.al 2001, Culp et.al 1999
	288.1626	$C_{20}H_{20}N_2$	Metabolite/biotransformation	Cha et.al 2001, Culp et.al 1999
	301.1700	$C_{21}H_{21}N_2$	Photodegradation/metabolite/biotransformation	Perez-Estrada et.al 2008, Cha et.al 2001, Culp et.al 1999
	302.1783	$C_{21}H_{22}N_2$	Metabolite/biotransformation	Cha et.al 2001, Culp et.al 1999
	306.1122	$C_{19}H_{16}NO_3$	Phototransformation	Perez-Estrada et.al 2008
	315.1861	$C_{22}H_{23}N_2$		
	316.1939	$C_{22}H_{24}N_2$		
	317.1646	$C_{21}H_{21}N_2O$	Phototransformation/metabolite/biotransformation	Perez-Estrada et.al 2008, Cha et.al 2001, Culp et.al 1999
	320.1280	$C_{20}H_{18}NO_3$	Phototransformation	

	321.1365	$C_{20}H_{19}NO_3$		Perez-Estrada et.al
	329.1648	$C_{22}H_{21}N_2O$		2008
	331.1804	$C_{22}H_{23}N_2O$		
	345.1961	$C_{23}H_{25}N_2O$	Phototransformation/metabolite	Perez-Estrada et.al 2008, Culp et.al 1999
	347.1753	$C_{22}H_{23}N_2O_2$	Phototransformation	Perez-Estrada et.al
	361.1908	$C_{23}H_{25}N_2O_2$		2008
	362.1994	$C_{23}H_{26}N_2O_2$		
	363.1705	$C_{22}H_{23}N_2O_3$		
	377.1858	$C_{23}H_{25}N_2O_3$		
LIN	323.1971	$C_{14}H_{27}N_2O_4$	Phototransformation	Calza et al. 2012
	343.1869	$C_{16}H_{27}N_2O_6$		
	373.1975	$C_{17}H_{29}N_2O_7$		
	375.2131	$C_{17}H_{31}N_2O_7$		
	391.2444	$C_{18}H_{35}N_2O_7$		
	405.2059	$C_{18}H_{33}N_2O_6S$		
	439.2114	$C_{18}H_{35}N_2O_8S$		
	455.2063	$C_{18}H_{35}N_2O_9S$		
SFM	235.0263	$C_6H_9N_3O_5S$	Phototransformation	Guerard et al. 2009
	246.1117	$C_{12}H_{14}N_4O_2$		
	295.0627	$C_{12}H_{13}N_3O_4S$		
	326.0685	$C_{12}H_{14}N_4O_5S$		
	327.0763	$C_{12}H_{15}N_4O_5S$		
	344.0791	$C_{12}H_{16}N_4O_6S$		
	352.0841	$C_{14}H_{16}N_4O_5S$	Metabolite	García-Galán et al.
	504.1162	$C_{18}H_{24}N_4O_{11}S$		2008
CTC	444.1523	$C_{22}H_{24}N_2O_8$	Phototransformation	Chen et al. 2012
	466.0779	$C_{20}H_{19}ClN_2O_9$		
	480.0936	$C_{21}H_{21}ClN_2O_9$		
	494.1092	$C_{22}H_{23}ClN_2O_9$		
	510.1041	$C_{22}H_{19}ClN_2O_{10}$		
OTC	272.1776	$C_{18}H_{24}O_2$	Phototransformation	Xue et al. 2020
	412.1271	$C_{21}H_{20}N_2O_7$		

414.1427	$C_{21}H_{22}N_2O_7$		Liu et al. 2016a, Liu et.al 2016b
428.1219	$C_{21}H_{20}N_2O_8$		
430.1376	$C_{21}H_{22}N_2O_8$		
432.1532	$C_{21}H_{24}N_2O_8$		
442.1376	$C_{22}H_{22}N_2O_8$	Phototransformation/Cooking	Liu et al 2016b, Nguyen et al. 2015
446.1325	$C_{21}H_{22}N_2O_9$	Phototransformation	Liu et al. 2016a, Liu et.al 2016b
448.1482	$C_{21}H_{24}N_2O_9$		
458.1325	$C_{22}H_{22}N_2O_9$		
459.1529	$C_{23}H_{25}NO_9$	Biotransformation	Migliore et al. 2012
462.1274	$C_{21}H_{22}N_2O_{10}$	Phototransformation	Liu et al. 2016b
474.1274	$C_{22}H_{22}N_2O_{10}$		Xue et.al 2020
475.1352	$C_{22}H_{23}N_2O_{10}$		
476.1430	$C_{22}H_{24}N_2O_{10}$		
478.1223	$C_{21}H_{22}N_2O_{11}$		
490.1223	$C_{22}H_{22}N_2O_{11}$		
492.1380	$C_{22}H_{24}N_2O_{11}$		
508.1329	$C_{22}H_{24}N_2O_{12}$		
524.1278	$C_{22}H_{24}N_2O_{13}$		
524.1278	$C_{22}H_{24}N_2O_{13}$		

Connecting paragraph

Chapter 2 covered a summary of the literature on the occurrence and current methods of analysis of five veterinary drugs in seafood, using both targeted and non-targeted approaches. Based on the review, malachite green was chosen as one seafood contaminant to be studied in this research. After determining that there is a lack of information on the development of non-target analysis methods for the chemical, specifically the effect of sample extraction and data processing, this third chapter covers the impact of sample preparation and data processing for the determination of malachite green and metabolites in brook trout and shrimp. This chapter has been submitted for publication to *Food Chemistry* as "Evaluation of different extractions for the metabolite identification of malachite green in brook trout and shrimp" (Baesu, A., Audet, C., Bayen, S., date of submission November 7th 2020).

Chapter 3: Evaluation of different extractions for the metabolite identification of malachite green in brook trout and shrimp

3.1 Abstract

Applications of mass spectrometry-based metabolomics in food science have developed fast in the last decade. Sample preparation and data processing are critical in non-target/metabolomic workflows but there is currently no standardized protocol for the development of these methods. The impact of data processing parameters or the inclusion of a different matrix is not often taken into account during the selection of an extraction. Thus, this study aimed to investigate the impact of different extractions, e.g., QuEChERS, and data processing on the determination of malachite green metabolites in two different organisms, brook trout and shrimp. The results obtained confirm the need for a harmonized approach for the validation of non-target workflows, as depending on the comparison criteria, the matrix, the mode of ionization or data processing, a different extraction could be chosen. This study also identified for the first time des-methylated leucomalachite green as another metabolite in the two organisms.

3.2 Introduction

Seafood has a lot of nutritional benefits as it is a good source of proteins, micronutrients such as calcium and iron, and unsaturated fats like omega-3 fatty acids, which can provide important health benefits in terms of prevention of cardiovascular diseases and aid in the development of the nervous system in children (FAO, 2016). In order to keep up with increased consumer demand, aquaculture production has greatly increased in the past years (FAO, 2016). Therapeutants are often used in aquaculture in response to stress conditions, such as high fish density and high ammonia/nitrite concentrations (EFSA, 2016). Unfortunately, regulations and enforcement differ between countries, and some banned compounds are still detected in seafood (Dinh et al., 2020). One such therapeutant is malachite green (MG), used as an anti-fungal treatment, which despite its ban in food producing animals continues to be detected in aquaculture products, on account of its high efficacy, low cost and widespread availability (EFSA, 2016). Furthermore, it continues to be used as an industrial dye, hence its presence in seafood could be due to uptake by the fish following release of wastewater from industrial activities (EFSA, 2016). Once absorbed, MG is rapidly metabolized in fish species such as catfish to the more lipophilic and persistent leucomalachite green (LMG) with des-methylated LMG identified as another metabolite in catfish (Doerge, Churchwell, Gehring, Pu, & Plakas, 1998) and rainbow trout (Dubreil et al., 2019). From a regulatory perspective, current action levels are set at 0.5 and 2 ng/g in Canada and Europe, respectively (Health Canada, 2017). With the compound still detected in seafood, a range of analytical methods have been reported in the literature for a variety of matrices, e.g., trout, shrimp and carp, that achieve the low detection limits required by regulatory levels to identify non-compliant products. These approaches involve an extraction step using a mixture of buffer and organic solvents (e.g., acetonitrile),

followed by liquid-liquid partitioning with dichloromethane to extract the less polar LMG and clean-up steps using solid-phase extraction (EFSA, 2016). Quantification is often achieved using liquid chromatography coupled to mass spectrometry (LC-MS) with electrospray or atmospheric pressure chemical ionization (Doerge et al., 1998). Extractions based on QuEChERS (quick, easy, cheap, effective, rugged and safe) or multi-residue screening methods have also been applied (Turnipseed et al., 2017; Villar-Pulido, Gilbert-Lopez, Garcia-Reyes, Martos, & Molina-Diaz, 2011). However, the focus of these methods is mostly on the parent compound MG and its main metabolite LMG, thereby disregarding other compounds of interest such as other contaminants, metabolites or possible transformation products formed during food processing or cooking. The extraction and identification of any of these compounds would be useful in better evaluating the risks to human health associated with consumption of contaminated seafood. Thorough sample treatment steps could remove some of these compounds of interest, thus simpler, more generic methods are preferred; methods which cover a wide range of compound classes and are applicable to different types of food matrices (Mol et al., 2008). When coupled to high-resolution mass spectrometry (HRMS), these extractions present more advantages as they can be used for suspect and non-target analysis in food analysis. Non target analysis allows for the identification of compounds not yet described and for which no previous information is available (Knolhoff & Croley, 2016). Suspect analysis or screening can be performed based on some existing information, such as mass and formula (e.g. known list of contaminants) and in both cases, HRMS can provide the information needed to identify compounds (accurate mass, isotope abundance) with structural information obtained from MS/MS fragmentation (Knolhoff & Croley, 2016). MS/MS information can also be obtained through All Ions MS/MS or data independent acquisition, in which both precursor and fragment ions are obtained. This has been

successfully applied for the screening of veterinary drug residues in seafood (e.g., fish and shrimp), including MG and LMG (Turnipseed et al., 2017). Non-targeted mass spectrometric methods have emerged as key methods in metabolomic studies for molecular fingerprinting (Arbulu, Sampedro, Gomez-Caballero, Goicolea, & Barrio, 2015; Perez-Miguez, Sanchez-Lopez, Plaza, Castro-Puyana, & Marina, 2018) and in food analysis for identification of contaminants (Kunzelmann, Winter, Aberg, Hellenas, & Rosen, 2018).

As non-targeted analysis is based on the identification of compounds for which there is limited information, designing and validating the extraction procedure can be challenging. Although no method will be able to offer full metabolome coverage, it should be robust, reproducible and efficient in extracting metabolites of interest (Antignac et al., 2011). Reproducibility is key for statistical analysis, like principal component or clustering analysis (Knolhoff, Kneapler, & Croley, 2019). There is currently no standardized procedure for the development and validation of extractions in non-target analysis/metabolomics (Rampler et al., 2021), including food metabolomics (Antignac et al., 2011). Different approaches were presented in the literature, depending on the application/goal of the study (Table 3.1). In metabolomics, the number of features and repeatability, often expressed as the number of features with a coefficient of variation (CV) below 20 or 30%, are two parameters used for comparison of extractions. The use of representative quality control (QC) samples has been proposed as a strategy in metabolomic studies to correct for changes in metabolite responses over time and ensure the data is robust and reproducible (Dunn et al., 2011). Pooled mixes of sample extracts or standard mixtures of compounds have been proposed as QCs (Dunn et al., 2011; Knolhoff et al., 2019; Perez-Miguez et al., 2018). For contaminant screening/suspect analysis, extraction protocols are often assessed in terms of recovery and precision for specific targeted

compounds. The number of detected features and their CV are less commonly used criteria in this case. Indeed, as most of the features extracted may be endogenous matrix components (e.g., amino acids, sugars, lipids), evaluating repeatability based on the percent features with a CV < 20% may not reflect the applicability of the method for trace contaminants. For screening approaches, optimization of extraction protocols will seek to improve recovery and precision of a target list of analytes from different compound classes (pesticides, antibiotics, etc.) which will then be applied to other samples to screen for the target analytes along with other contaminants present (Jia et al., 2017).

Another critical aspect in a metabolomics workflow is the data processing step (Fisher, Croley, & Knolhoff, 2021). Incorrect data processing parameters can introduce errors during data analysis and cause inaccurate interpretation of the data (Antignac et al., 2011; Fisher et al., 2021). Similar to validation of the sample preparation step, data processing parameters should also be optimized across a metabolomics workflow (Fisher et al., 2021). For example, data processing parameters may be optimized for compound identification, in terms of false positive and false negatives (von Eyken & Bayen, 2019). However, to the best of our knowledge, there are no studies that assessed the impact of data processing parameters on the selection of an extraction method. Specifically, if the modification of one processing parameter, like peak height, would change which extraction performed best in terms of the comparison criteria, e.g., number of extracted features.

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Table 3.1: Criteria used for extraction comparison in non target/screening analysis

Approach	Matrix	Application	Extractions tested	Criteria	Reference
Metabolomics	wine	identification of non-volatile/semi-volatile metabolites	centrifugation filtration direct injection	number of features repeatability (expressed as CV of features)	Arbulu et al., 2015
	rice	metabolome profiling and geographic discrimination	different solvents	extraction efficiency of various compound classes (lipids, sugars, lysophospholipids) ability to discriminate between different geographic rice samples	Lim et al., 2018
	green tea	metabolome profiling	accelerated solvent extraction benchtop extraction	extraction efficiency of catechins (expressed as concentration) repeatability (expressed as standard deviation of extracted catechins) metabolome profile (based on Principal Component Analysis clustering)	Kellogg, Wallace, Graf, Oberlies, & Cech, 2017
	apple	extraction polar metabolites	different solvents	extraction efficiency of target polar metabolites (expressed as ratio between metabolite response vs. maximum response across all methods) repeatability (expressed as relative standard deviation RSD of target metabolites) recovery of target metabolites	Bekele, Annaratone, Hertog, Nicolai, & Geeraerd, 2014
	grapes	metabolome profiling	different solvents	number of features repeatability (expressed as RSD of features)	Theodoridis et al., 2012
	coffee	metabolite identification related	different solvents	number of features	Perez-Miguez et al., 2018

		to the roasting process			
	plasma	lipid profiling	different solvents	protocol simplicity lipid recovery lipid coverage protein removal efficiency repeatability (expressed as CV of features)	Sarafian et al., 2014
	plasma	non lipid metabolome profiling	different solvents, SPE	recovery of target metabolites matrix effects number of metabolites detected repeatability of features (expressed as RSD)	Sitnikov, Monnin, & Vuckovic, 2016
	glioma cell lines	global metabolomics	different quenching solvents, cell disruption methods and solvent extraction	PCA analysis reproducibility and reliability (assessed as grouping of replicates in PCA and CV of metabolites) metabolite coverage extraction efficiency (expressed as intensity of 68 target metabolites)	Xu et al., 2019
Contaminant screening	shrimp, fish, eel	veterinary drug screening	acetonitrile/SPE extraction (with different levels of acids)	recovery of analytes	Turnipseed et al., 2017
	fish liver, water	organic contaminant screening	accelerated solvent extraction (different adsorbents and solvents) QuEChERS	number of features rate % false negatives	Du et al., 2017

	egg	antimicrobials and mycotoxins screening	QuEChERS (different solvents, pH, sample weight to solvent volume ratio)	recovery, matrix effect and RSD of target compounds	Capriotti, Cavaliere, Piovesana, Samperi, & Lagana, 2012
	tilapia	veterinary drug screening	QuEChERS (solvent volume, pH, amount sorbent)	recovery of target analytes	Jia et al., 2017
	fish, breast milk	PAHs, pharmaceuticals, PCBs, pesticides screening	QuEChERS (amount sorbent, pH) SPE	recovery of target analysis	Baduel, Mueller, Tsai, & Gomez Ramos, 2015
General	infant rice cereal, orange juice, yogurt	general chemical coverage	dilute and shoot acetonitrile extraction QuEChERS	number of features repeatability (expressed as CV of features) unique features chemical coverage (molecular weight, chromatographic retention)	Knolhoff et al., 2019

Overall, although there are studies in the literature that compared different extractions for metabolomic investigations (Table 3.1), the focus, especially for food metabolomics, has usually been on one sample type, and the food matrix is not often included as a comparison criterion during the sample preparation step. For MG-exposed organisms, metabolomics was found to be an appropriate strategy for the identification of other MG metabolites, e.g., des-methylated LMG in rainbow trout (Dubreil et al., 2019), but the impact of sample preparation for the purpose of metabolite identification was not studied. Furthermore, to the best of our knowledge, this metabolomic approach has yet to be used to determine MG metabolites in other species, such as shrimp, as residues have been detected in multiple species in markets in Montreal (Dinh et al., 2020). From a general non-target/suspect contaminant analysis approach, even though sample extraction is validated for different food matrices, is it often based on raw samples. Seafood is usually consumed following some kind of processing, and cooked samples should be included to account for the impact of thermal processing on the fate of contaminants and metabolites. Therefore, the objectives of the present study were to: (i) compare four extraction methods (from the literature) based on various criteria, including the impact of different matrices, for the determination of MG and metabolites in two exposed organisms: brook trout (*Salvelinus fontinalis*) and shrimp (*Litopenaeus vannamei*), (ii) evaluate the impact of data processing parameters on the choice of the extraction method and (iii) apply a metabolomics workflow to identify other MG metabolites in the two organisms. The novel aspects of this study are the identification of other MG metabolites in brook trout and white shrimp, a comparison of extractions for this purpose, along with the inclusion of different matrices as part of comparison criteria, and the assessment of the impact of data processing parameters on the choice of the extraction method.

3.3 Materials and methods

3.3.1 Chemicals

MG chloride (>96.0%) and LMG (>98.0%) analytical standards were obtained from Sigma Aldrich (St Louis, MO, USA). MG oxalate technical grade standards used for trout exposure was obtained from Fisher Scientific (Waltham, MA, USA) and for shrimp exposure, Acros Organics (Geel, Belgium). Labelled internal standards, d_3-diphenhydramine and d_3-6-acetylmorphine, were purchased from Cerilliant (Round Rock, TX, USA). HPLC grade acetonitrile, methanol, water, LC-MS grade formic acid, acetic acid and ammonium acetate were obtained from Fisher Chemical (Pittsburgh, PA, USA). Anhydrous magnesium sulfate and sodium acetate were purchased from Sigma Aldrich (St Louis, MO, USA). Primary secondary amine (PSA) sorbent was purchased from Agilent (Santa Clara, CA, USA). All glassware used was baked in an oven at 320°C for four hours and rinsed with methanol before use. Labelled internal standard solution of 0.4 µg/mL was prepared in methanol and stored at -20°C in amber vials. MG and LMG standards of 1 mg/mL and working standards of 20 µg/mL were prepared in methanol and stored at -20°C in amber vials. All standards were prepared fresh every 6 months. Five calibration standards, from 3 to 20 ng/mL, were prepared in water (0.1% formic acid).

3.3.2 Trout exposure

For MG exposure, two tanks of 250 L each (one control and one for exposure) were used with ten trout (1:1 male/female) in each tank. Trout (mean length 44.6±4.5 cm) weighed between 0.6 and 2.1 kg (mean weight 1.3±0.4 kg). Water temperature was between 4-5°C and pH 7.6. Trout were exposed to 2 mg/L MG for 90 minutes, after which they were sacrificed. Exposure

time, procedure, and euthanasia followed the normalized procedures accepted by the UQAR Animal protection committee. Briefly, trout were anesthetized using MS222 (tricaine methanesulfonate) and sacrificed by severing of the spine. Exposure time was established based on earlier studies. Mean MG and LMG levels in rainbow trout exposed to 1.5 mg/L MG for one hour were 528 and 2823 ng/g respectively one day after treatment (Bajc, Jenčič, & Šinigoj Gačnik, 2011). Comparable levels, 590 ng/g for MG and 1030 ng/g for LMG, were obtained for catfish exposed to 1 mg/L MG for one hour (Doerge et al., 1998). To account for discrepancies between fish weights amongst the different exposure studies and ensure detectable levels of MG and LMG, an experimental condition of 2 mg/L for 90 minutes was used in this study. Fish were filleted using stainless steel knives, wrapped individually in aluminum foil and polyethylene bags and stored at -80°C.

3.3.3 Shrimp exposure

Pacific white shrimp were obtained from Planet Shrimp facilities (Aylmer, ON, Canada) Two tanks of 60 L (one control and one for exposed) each filled with distilled water were used, with 60 shrimp per tank. Artificial seawater (16 g/L) was prepared with sea salt (Instant Ocean, Blacksburg, VA, USA) based on recommendations from Planet Shrimp facilities. Water pH was 8, temperature of 29°C and dissolved oxygen 5 mg/L. Shrimp were exposed to 0.4 mg/L MG for 2 hours. This level is in the range of those reported in the literature (0.2 mg/L for 2 hours) reported to lead to muscle concentrations of 20 and 79 ng/g for MG and LMG respectively (EFSA, 2016). At the end of exposure, shrimp were sacrificed by placing them on ice. Shrimp were individually wrapped in aluminum foil and polyethylene bags and stored at -80°C.

3.3.4 Sample extraction

Trout muscle was homogenized using a blender, while shrimp muscle was homogenized using a mortar and pestle.

Extraction 1 was adapted from Dasenaki & Thomaidis (2015). Briefly, 1.0 g of sample was weighed into a 50 ml centrifuge tube. Water, 2 mL (0.1% formic acid v/v) was added and the samples were vortexed for 1 minute. Acetonitrile (2 mL) followed by methanol (2 mL) were added, with samples vortexed for 1 minute between each solvent addition. Samples were centrifuged (Eppendorf, Hamburg, Germany) for 4400 rpm (3000 × *g*, 25°C) for 10 minutes. Supernatant was collected in new tubes and transferred to -20°C for 14.5 h for lipid precipitation. Samples were then centrifuged again for 10 minutes at 4400 rpm, filtered using a 0.22 μm PTFE filter (Canadian Life Science, Peterborough, ON, Canada) and stored in amber vials covered with aluminum foil at -20°C.

Extraction 2 (QuEChERS) was adapted from Jia et al., (2017). Briefly, 1.0 g of sample was weighed into centrifuge tubes, after which 5 mL (84:16 v/v) acetonitrile/water with 1% acetic acid was added and vortexed for 1 minute. To each sample, 1.0 g of $MgSO_4$ and 0.30 g sodium acetate were added, vortexed for 1 minute followed by centrifugation at 4400 rpm (3000 × *g*, 25°C) for 5 minutes. Supernatant (2 mL) was transferred to new tubes containing 0.24 g $MgSO_4$ and 25 mg PSA, vortexed for 1 minute and centrifuged for 5 minutes at 4400 rpm. Extracts were filtered using a 0.22μm PTFE filter and stored in amber vials at -20°C.

Extraction 3 was adapted from Nacher-Mestre, Ibanez, Serrano, Perez-Sanchez, & Hernandez (2013). In short, 5.0 g of trout muscle or 2.0 g of shrimp muscle was weighed into 50 mL centrifuge tubes. For trout extraction, 10 mL (80:20 v/v) acetonitrile/water with 0.1% formic acid was added, while for shrimp extraction 4 mL of the same solvent mixture was added.

Samples were vortexed and centrifuged at 4400 rpm (3000 × *g*, 25°C) for 10 minutes. Supernatant (2 mL) was transferred to new tubes, covered in aluminum foil and stored at -20°C for 2 hours. Extracts were centrifuged again for 10 minutes, 4400 rpm, filtered using 0.22µm PTFE filter and stored in amber vials at -20°C.

Extraction 4 followed the same protocol as extraction 3, except for no formic acid was added.

For each extraction, 10 replicates were prepared along with 5 procedural blanks. Blanks were prepared following the same protocols as described above, but with no sample added. Five QC injection samples were prepared by pooling 10 µL of all extracts and blanks from all four extractions. Extraction QCs (*n*=5) were prepared by pooling 20 µL of all five blanks and ten replicates for each extraction. For LC-MS analysis, 100 µL of each sample was diluted with water (1/10) and 50 µL of a 0.4 µg/mL solution of the labelled internal standards was added. Extraction QCs were diluted as such to obtain 0.01 g of matrix in the vials for direct comparison between extractions. Labelled standards were not added for quantification purposes, but rather to monitor the instrumental variability.

Recovery experiments (*n*=6) were completed for both raw and cooked tissues of trout and shrimp. Samples were spiked with MG/LMG to achieve a target concentration of 400 ng/g and 300 ng/g in trout and shrimp muscle respectively, and were allowed to equilibrate for 10 minutes before extraction. Extracts were prepared the same way as exposed samples for LC-MS analysis. Matrix effect and absolute recoveries were calculated according to the protocols set out by Matuszewski, Constanzer, & Chavez-Eng (2003).

3.3.5 Thermal treatment

To obtain cooked samples, homogenized shrimp and trout muscle were transferred to 40 mL amber vials, capped and placed in a water bath at 100°C. Trout muscle was boiled for 30 minutes, to ensure it was completely cooked. Shrimp was boiled for only 10 minutes, as a longer boiling time led to high water loss and too much disintegration of the muscle.

3.3.6 Instrumental analysis

Samples were analyzed using an Agilent UHPLC 1290 coupled with an Agilent 6545 QTOF-ESI-MS, in both positive and negative ionization modes. In positive mode, mobile phases were (A) H_2O with 0.1 % formic acid and (B) acetonitrile and in negative mode, mobile phases used were (A) 0.05 M ammonium acetate and (B) acetonitrile. For both positive and negative modes, the same gradient elution was used, starting from 1 min 5% B, from 1 to 15 min gradient to 100% B, from 15 to 20 min 100% B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. An InfinityLab Poroshell 120 (Pheny-Hexyl, 3.0 x 100 mm, 2.7 µm, Agilent Technologies) with a Poroshell (4.6 mm) Phenyl Hexyl pre-column was used. Flow rate was 0.2 mL/min, injection volume was 2 µL and column temperature was 20°C. The MS parameters were as follows: sheath gas temperature 275°C, drying gas temperature 325 °C, drying gas flow 5 L/min, sheath gas flow 12 L/min, nebulizer pressure 20 psi, capillary voltage 4000, nozzle voltage 2000 V, fragmentor voltage 175 V, skimmer voltage 65 V. All Ions MS/MS mode at collision energies of 0, 10, 20 and 40 V was used. Data was collected between 100 and 1700 *m/z* at a rate of 3 spectra/s.

Each sample type was considered an individual batch and was run at the same time, i.e., all four extractions for trout raw were run together (all replicates, blanks, extraction QCs and injection QCs). Samples were kept at 4°C in the multi sampler compartment.

3.3.7 Data treatment

SPSS Statistics software (v.26) (IBM, NY, USA) was used for statistical analysis to compare the four extractions. A three-way ANOVA was performed with the type of extraction, sample type (shrimp and trout) and process (raw and cooked) as the independent variables to evaluate differences between recovery and matrix effect values. To evaluate the impact of data processing parameters, a four-way ANOVA was performed with type of extraction, mass window, peak filter height and post-processing peak absolute height as the independent variables. Dependent variables considered were: (i) percentage of features with RSD<20%, (ii) percentage of features with RSD<30% and (iii) number of features present in all extraction QC samples at a ratio sample/blank>2 or not present at all in blanks.

Concentrations, based on external calibration, were computed using Agilent Mass Hunter Quantitative Analysis B.07.0. Method detection limit (MDL) and limit of quantification (LOQ) were calculated as 3σ and 10σ, respectively, of the procedural blanks integrated at the retention time of the target compounds. Data alignment and feature extraction were completed using Agilent Mass Hunter Profinder software B.08.0. A feature can be defined as an entity for which a neutral mass, retention time and abundance can be assigned. Ion species and isotopes are included. To evaluate each extraction individually, molecular feature extraction (MFE) was performed for each (10 replicates, 5 procedural blanks and 5 extraction QC samples), using the following default parameters: peak filter height 200 counts, retention time window ±0.30 min,

mass window ±10.00 ppm, post-processing peak absolute height 1000 counts, MFE score 80. Features were exported in Excel with only features present in all replicates at a ratio of sample/blank>2 used to calculate repeatability, expressed as CV or relative standard deviation.

To assess the impact of data filtering parameters, each parameter was changed one a time, while keeping the remaining parameters as default. The following values were assessed for each parameter: peak filter height 500 and 1000 counts, mass window ±5 and 50.00 ppm, post-processing peak absolute height 200 and 5000 counts. Peak filter height will set a threshold for chemical and background noise, which can be set at 100-300 counts (Du et al., 2017; von Eyken & Bayen, 2019). Post-processing peak absolute height is the minimum height at which a compound is considered to be found. For metabolite identification, control and exposed trout and shrimp samples were extracted using the Profinder default parameters and exported to .cef files. Files were imported into Mass Profiler Professional (v 14.8, Agilent Technologies) for statistical analysis (volcano plot, $p<0.05$, fold change>2) to identify statistically significant compounds that could be considered as other metabolites of MG.

3.4 Results and discussion

3.4.1 MG and LMG extraction

All four extraction methods extracted both MG and LMG from the raw tissues of exposed brook trout and shrimp with the average concentrations listed in Table S3.1. Figure 3.1 shows the chromatograms for MG and LMG in standard solutions and incurred shrimp extracts. For all four extractions, the extracted ion chromatograms showed clear peaks with little background signals. In general, somehow better LOQs (Table S3.2) were achieved for LMG compared to MG; for example, LOQ of 1.6 ng/g for LMG was determined for *Extraction 1*, compared to 3.0 ng/g for

MG. LMG MDLs for *Extraction 2* for trout and shrimp were below the set interim limit of quantification of 0.5 ppb set in Canada (Health Canada, 2017).

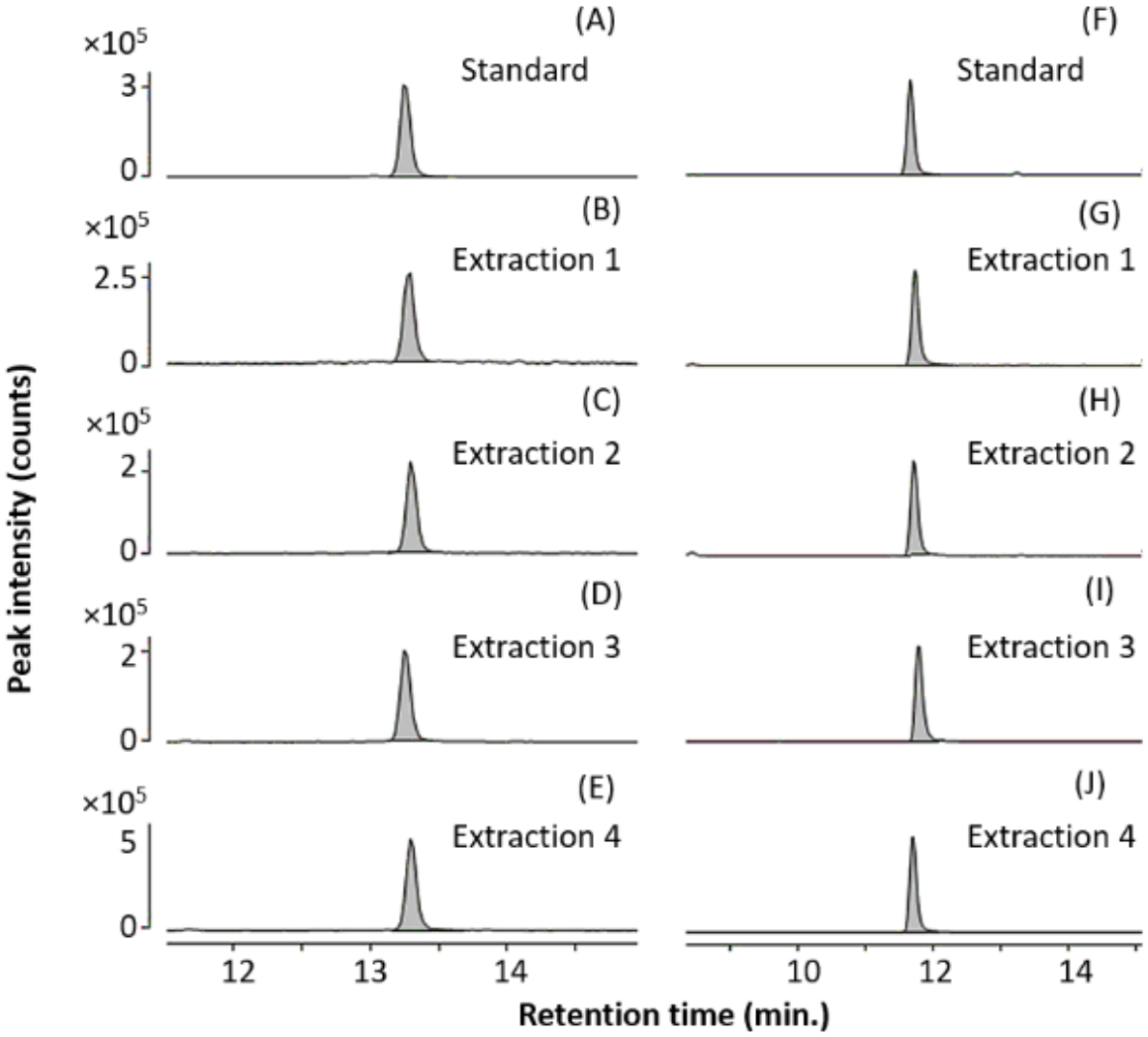

Figure 3.1: Extracted ion chromatogram for MG (*m/z* 329.2012; Fig. A-E) and LMG (*m/z* 331.2168; Fig. F-J) in extracted shrimp and pure solvent

Matrix effects for MG ranged between 82 and 106% in raw trout (Table S3.3). Values below 100% indicate signal suppression, while values above 100% indicate signal enhancement (Matuszewski et al., 2003). Slight ion suppression, with matrix effects for raw trout of 89 and 82%, was observed in *Extractions 3* and *4*, which can be expected as they are the simplest

extractions with very little clean-up. In cooked samples, further suppression was observed for *Extraction 4* e.g., matrix effects of 63%. *Extraction 2* (QuEChERS) showed little matrix effect, around 100%, for MG in both raw and cooked samples. Hurtaud-Pessel, Couedor, & Verdon (2011) reported a matrix effect for MG of 88% in raw rainbow trout based on a similar extraction with acetonitrile/magnesium sulfate, but no clean-up with sorbents. For shrimp, little matrix effect was observed for MG for any of the extractions, with values ranging between 91 and 110%. For *Extraction 2,* mean values observed of 109 and 110% for raw and cooked shrimp, respectively, are similar to those reported previously by Hurtaud-Pessel et al., (2011) for MG (i.e., 103.7 and 105% in raw and boiled shrimp, respectively).

In terms of MG recovery, the lowest values were found for *Extraction 1* (<50%), with *Extraction 2* providing the best recoveries for raw and cooked trout and shrimp (67-105%) and best precision (RSD<30%). Recoveries of MG between 48 and 78% (depending on the spiking level) have been reported in shrimp with a QuEChERS-like extraction based on acetonitrile/water and magnesium sulfate/sodium acetate, but with the sorbent clean-up step omitted (López-Gutiérrez, Romero-González, Plaza-Bolaños, Martínez-Vidal, & Garrido-Frenich, 2012). *Extractions 3* and *4* showed lower MG recoveries for shrimp (41-67%) and trout (37-69%). Statistical analysis (Table S3.4) showed no significant interaction, in terms of MG matrix effect and recovery between the three variables, type of extraction, sample type (trout or shrimp) and process (raw or cooked) ($p>0.05$). However, based on between-variables comparison, the type of extraction has a significant effect on MG matrix effect while a significant difference was found for MG recovery between raw and cooked samples.

For LMG, *Extraction 2* again appeared to provide the best results in terms of recovery and precision. For raw and cooked trout and shrimp, matrix effect ranging from 54 to 96% with

recoveries between 71 and 97 % were observed, similar to other studies where recoveries between 62-112% and 101-104.8% were found in shrimp and rainbow trout (Hurtaud-Pessel et al., 2011; López-Gutiérrez, Romero-González, Martínez Vidal, & Frenich, 2013).

For *Extractions 3* and *4*, the 2-hour freezing time appeared to be insufficient for removal of lipids and proteins, as precipitate formation was observed in the filtered extracts during storage at -20°C, even after a few days, which could have an effect on the matrix effect and impact quantification. Indeed, for raw trout, a matrix effect of 13% was measured for LMG (Table S3.3), indicating almost complete suppression. Another issue arising from the presence of precipitates and insufficient removal of proteins from samples is column clogging and poor performance associated with protein interactions (Sitnikov, Monnin, & Vuckovic, 2016). Large variability in the response for recovery samples was also observed for *Extractions 3* and *4*, leading to a poor precision in terms of both matrix effect and recovery. Statistical analysis (Table S4) found a significant interaction between the three variables for LMG recovery, with the type of extraction and process type (raw and cooked) having a statistically significant effect. Although the use of an internal standard could correct for the poor precision for LMG observed in *Extractions 3* and *4*, due to the precipitate formation throughout storage *Extraction 2* was considered to provide the best results for MG and LMG, in terms of recovery and reduced matrix effects.

3.4.2 Number of extracted features

The number of features is a common parameter used for comparison of extractions in metabolomic studies (Table 3.1), as it may reflect the metabolome coverage. The inspection of individual features revealed a large number present only in a single sample and many others

present in procedural blanks. Features retained for statistical analysis are often filtered based on their occurrence in all or in a minimum of replicates (e.g., two out of three) (Arbulu et al., 2015; Knolhoff et al., 2019; Sitnikov et al., 2016; Theodoridis et al., 2012). Completely removing features that are present in blanks may remove key molecular features, so features present in blanks or resulting from the chemical noise are often filtered based on a specific intensity ratio comparing samples and blanks (Knolhoff et al., 2019). In this study, for trout samples, features only present in all five replicates of each of the two fish samples and extraction QCs, and absent in blanks or present at a sample/blank ratio (based on peak height) above 2, were retained. For shrimp matrices, features only present in extraction QCs and absent in blanks or present at a sample/blank ratio (based on peak height) above 2 were retained. In general, the highest number of features for both raw and cooked trout and shrimp were observed in *Extraction 3* and *4* (Figure 3.2, Tables S3.7 to S3.12). As these extractions are the most generic extractions of the four, they may also extract other matrix components as showed by the higher number of features and confirmed by the more pronounced matrix effects observed for the two extractions, especially in the case of LMG in trout.

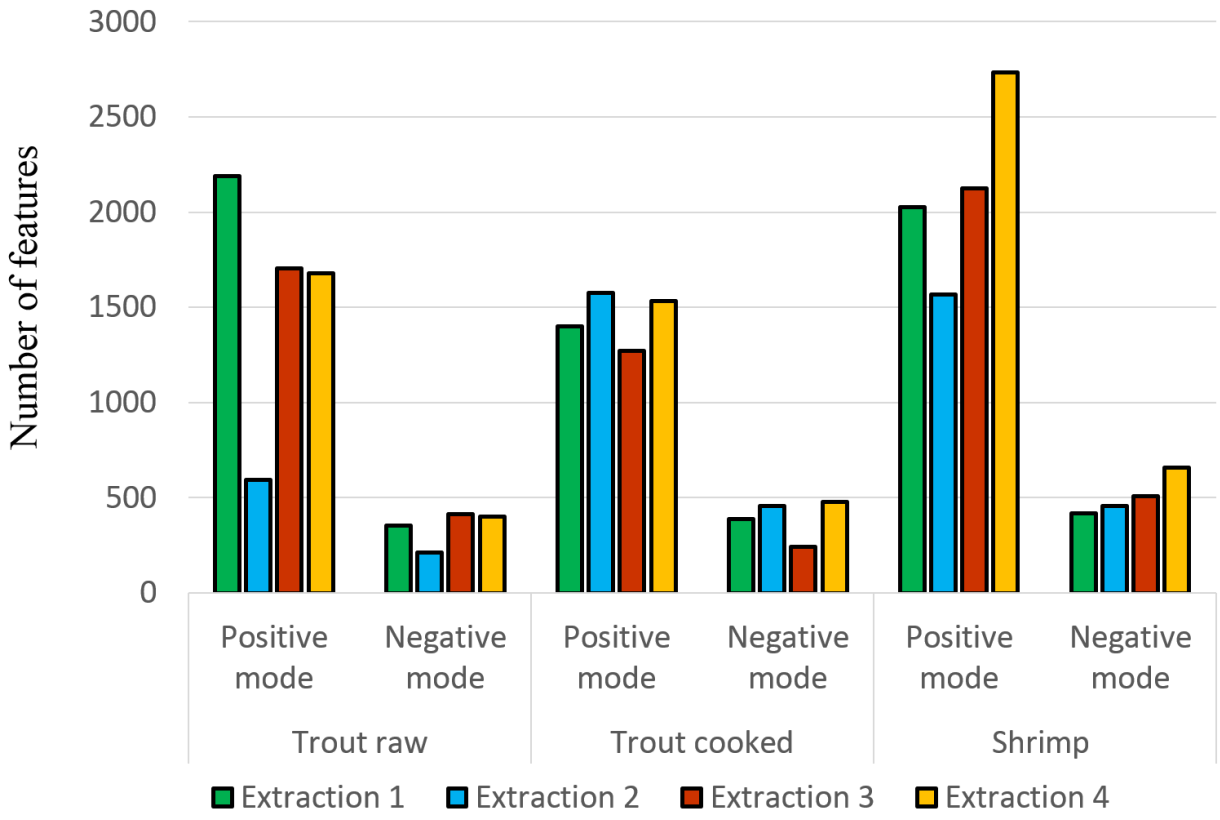

Figure 3.2: Number of molecular features extracted in trout and shrimp samples in different ionization modes

Modification of the MFE parameters, especially peak height and post-process peak absolute height significantly decreased the number of features extracted from the matrices (Tables S3.5, S3.7-S3.12). This can be expected as setting higher thresholds for peak height will eliminate smaller peaks (e.g., chemical noise). For example, for *Extraction 2* in cooked trout negative mode, increasing the peak height from 200 to 1000 counts decreased by more than half the number of detected molecular features. Still, with the above later threshold for peak height, *Extraction 2* performed the best amongst all four extractions. In some cases, such as shrimp positive mode (Table S3.11), a slightly higher number of extracted features were obtained for *Extraction 2* (868) compared to *Extraction 4* (804), when setting 5000 counts as the absolute

peak height, whereas *Extraction 4* had the highest extracted features when the default parameters are used. This indicates that features detected through *Extraction 4* had relatively lower intensities compared to *Extraction 2* and were not detectable with increasing absolute peak height. In the case of cooked trout positive mode, the highest number of features (1576) were obtained through *Extraction 2*, when using the default absolute peak height of 1000 counts. When the absolute peak height was increased to 5000 counts, it was *Extraction 4* that resulted in in a slightly higher number of features (875), compared to *Extraction 2*, through which only 760 features were extracted. Hence, for cooked trout samples, features detected through *Extraction 2* had lower intensities compared to *Extraction 4*, which is the opposite of what was observed in shrimp samples.

3.4.3 Repeatability

Repeatability, often expressed as the number of features present in all replicates with coefficient of variation (CV) or relative standard deviation (RSD) below 20 (Knolhoff et al., 2019) or 30% (Sitnikov et al., 2016) is another parameter used to compare extractions in non-target analysis. Particular attention should be paid to this parameter. If replicates are performed per more than one sample (i.e., one replicate per one individual fish), as opposed to all replicates performed per one sample (i.e., ten replicates per one individual fish), then high variability between features will not necessarily be due to poor method precision, but it could be due to variability among organisms (i.e., different metabolism, fat content). Sources of variability also include experimental preparation (i.e., extraction of replicates on different days). In this study, trout replicates were performed on two individuals (5 replicates/trout), while shrimp replicates were performed on individual shrimps, as their weights were too low to perform all replicates on

one individual. In trout, for the same extraction, different repeatability was observed between the two individuals. For example, in trout raw positive mode, for *Extraction 4*, 66.9% of features have an RSD<20% in fish 1. However, for fish 2, *Extraction 4* only has 38.8% of features with an RSD<20%. Similarly, for cooked trout samples analysed in negative mode, *Extraction 2* has 55.6% of features <20% for fish 1, but only 11.1% of features<20% for fish 2. Therefore, comparison of extractions based on the repeatability criterion was done based on CV of features detected in extraction QCs. Trout samples, both raw and cooked in positive mode, had around 50% of features with RSD<20% across all four extractions (Figure 3.3). On the other hand, in negative mode, *Extraction 2* had the lowest percent features with a CV<20% between the four extractions. This trend was different in shrimp samples; *Extraction 2* had the second highest % features (62.6%) in negative mode, but the lowest in positive mode.

The modification of peak heights parameters had a statistically significant impact on feature repeatability (Table S3.5). Increasing the noise threshold (peak height) should eliminate these smaller peaks and could theoretically improve the repeatability of features. Indeed, in some cases, for example in shrimp analyzed in positive mode, increasing the peak height from 200 counts to 500 and 1000 counts, increased the percent features with CV<20% in *Extraction 1* from 51.7% to 61.8 and 63.5% respectively. But, when comparing all CV values between the four extractions at the same parameter, e.g., 500 counts, *Extraction 1* still performed the best, an identical conclusion as with default parameters. However, it must be noted that extractions that have good precision for extracted features may not always have the best precision for some target compounds, which was the case in this study.

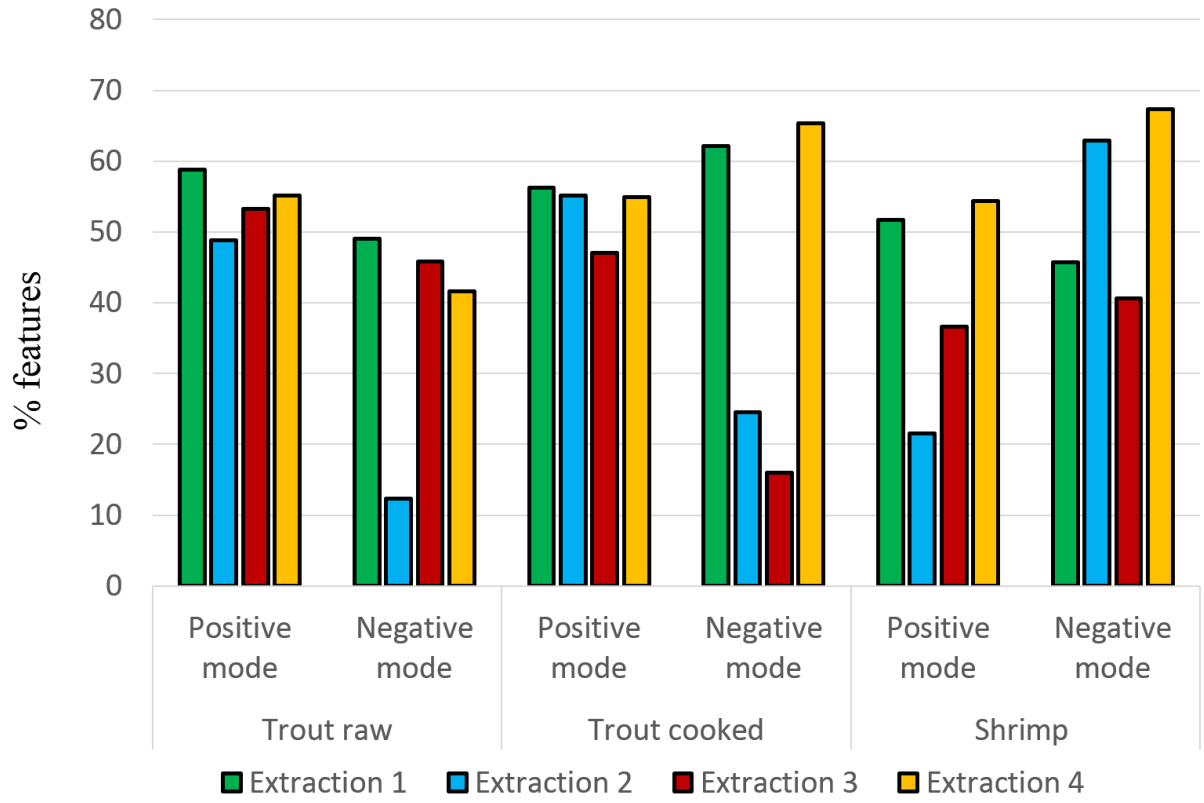

Figure 3.3: Percentage of molecular features with CV<20 % in extracted trout and shrimp in different ionization modes

3.4.4 Overall extraction comparison

The above results confirm that each comparison criteria varies with the matrix, the extraction method, the instrumental analysis conditions but also with the data processing approach. Overall, not one single extraction performed the best based on all comparison criteria investigated (Table 3.2, Figure 3.4) and depending on which criterion takes precedence a different extraction would be considered optimal for a metabolomics investigation of MG-exposed organisms. Moreover, there are different approaches in the literature on data treatment that may potentially impact the choice of sample preparation, e.g., treatment of features found in procedural blanks. Consequently, a harmonization of the approaches for metabolomics

workflows, that can offer some guidance on validation of the sample extraction method, is critically needed. Due to the high throughput of metabolomic studies, repeatability remains a key parameter in sample preparation (Bekele, Annaratone, Hertog, Nicolai, & Geeraerd, 2014). For example, Sarafian et al., (2014) used a point-based system for the optimization of extraction procedures in lipidomics, where the highest marked criteria (5 marks each) were given to repeatability and lipid recovery compared to 2 marks for lipid coverage (i.e. different lipid groups). In cases where the extraction that provided the highest number of features did not have the best repeatability, the latter was preferred over number of features when choosing the optimal extraction (Arbulu et al., 2015). However, despite the importance of this evaluation parameter, there are currently different ways of evaluating repeatability or reproducibility, with this being an aspect of sample preparation that would benefit from a systematic approach. For instance, Xu et al., (2019) used six biological replicates for their study on cell metabolomics with reproducibility evaluated based on the grouping of replicates in a PCA model and CV calculated for a targeted list of metabolites. In other cases, CV of features was also determined based on features extracted across three replicates of the same sample (Knolhoff et al., 2019). As this study has shown, different CVs for the extracted features were observed between two individual fish, therefore more representative samples are needed when assessing this parameter. For this purpose, there are several options proposed for determining feature repeatability. (i) based on pooled QC samples from each replicate after extraction or (ii) based on a pooled sample from each individual which is then extracted by all methods. For example, Theodoridis et al., (2012) prepared replicates for their solvent optimization study on grapes from a homogenous 1 kg sample. QC standard mixtures, composed of compounds with different chemical properties and present at high and low concentrations (Knolhoff et al., 2019) spiked before extraction could also

be used for assessment of repeatability, besides having other advantages. They have been used for further appraisal of data quality, e.g., mass accuracy and generation of formula for the spiked standards and could enable comparison of different data sets (Knolhoff et al., 2019). As this study has shown, data processing parameters e.g., peak height, had a statistically significant effect on the detectable molecular features and repeatability and should be taken into account as part of the sample preparation protocol for non-target analysis. The integration of the QC standard mixtures in routine non-target analysis can allow for optimization of the data processing parameters to improve compound identification and reduce false positives or false negatives. Another detail that must not be disregarded is the treatment of data obtained through negative ionization mode. The results in this study for negative ionization mode showed that while extractions were comparable in terms of molecular features, they were not in terms of feature repeatability. Although generally most compounds, including the two target compounds in this study, are ionized in positive mode, analysis in negative ionization mode could also be of benefit to identify other interesting compounds (Knolhoff et al., 2019). Therefore, the quality of data obtained through negative ionization mode and the effect of data treatment parameters must also be assessed.

Overall, despite a lower number of detected molecular features in raw positive mode observed for *Extraction 2* for trout, the generated data is still of good quality with good repeatability observed. *Extraction 2* also provided the best results amongst the four extractions in terms of precision and recovery for MG and LMG in both trout and shrimp. Good results were also obtained for cooked trout for *Extraction 2* which means that this extraction could also be applicable for other objectives, such as identification of possible thermal transformation products formed during cooking. Although *Extraction 2* extracted a lower number of features in shrimp

and had the lowest percentage of features with a CV<20% amongst all four extractions, since the same method should be applied to both matrices to identify common or unique compounds, it was chosen in this study as the suitable extraction for identification of metabolites of MG in exposed brook trout and shrimp.

Table 3.2*:* Optimal extraction of MG exposed trout and shrimp based on different criteria of comparison

Criteria	Best extraction
Recovery of target analytes	*Extraction 2* for both trout and shrimp
Precision of target analytes	*Extraction 2* for both trout and shrimp
Number of molecular features	Trout raw positive mode: *Extraction 1* Trout raw negative mode: *Extraction 3* Trout cooked positive mode: *Extraction 2* Trout cooked negative mode: *Extraction 4* Shrimp positive mode: *Extraction 4* Shrimp negative mode: *Extraction 4*
Repeatability of features	Trout raw positive mode: *Extraction 1* Trout raw negative mode: *Extraction 1* Trout cooked positive mode: *Extraction 1* Trout cooked negative mode: *Extraction 4* Shrimp positive mode: *Extraction 4* Shrimp negative mode: *Extraction 4*

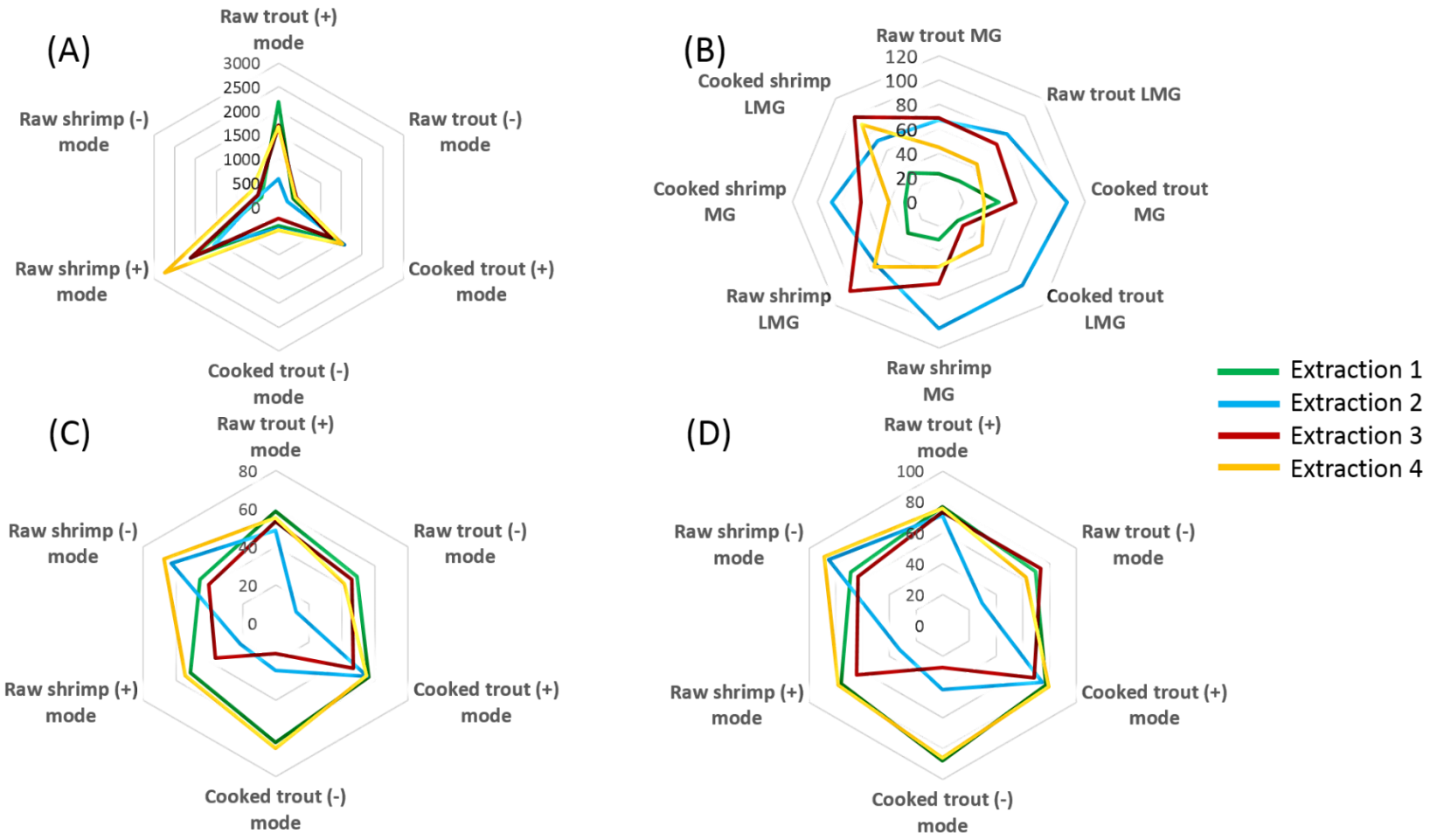

Figure 3.4: Extraction comparison based on: (A) number of features in QC ratio sample/blank>2 and absent in blanks, (B) recoveries MG and LMG, (C) features with CV<20% and (D) CV<30%

3.4.5 Metabolite identification

Ten exposed and ten control trout and shrimp samples were extracted using the selected QuEChERS method (*Extraction 2*). Following volcano plot analysis, 12 compounds with a matching score >70, beside MG and LMG were identified as present in statistically significant higher abundance in exposed compared to control organisms (Table S3.13). This match score is often used in non-target analysis for confident compound identification (Du et al., 2017). Although a search of the Agilent *PCDL Metlin* database yielded some possible matches, based on further MS/MS analysis, the identity of the compounds was not confirmed. Between those compounds, only four were common for both matrices. The mass and generated formula for *Compound 6* matches the mass and formula for des-methylated LMG, (ratio LMG/des-methyl LMG 6.6) which has been previously identified as a metabolite in rainbow trout (Dubreil et al., 2019) and catfish (Doerge et al., 1998). It was not found in exposure water samples but was retroactively detected in calibration standards with an average ratio LMG/des-methyl LMG of 12.7. *Compound 5* corresponds to des-methylated MG (ratio MG/des-methyl MG of 20.1) which was found in trout and shrimp exposure water samples with an average ratio MG/des-methyl MG of 1.3, compared to an average ratio of 2.5 across the calibration standards. The des-methylated forms of the parent compounds can also occur due to natural degradation of the compounds. Based on the higher abundance of des-methylated LMG in incurred tissues and the fact it was not detected in water samples, this indicates its presence in muscle is due to possible metabolism. Based on the generated formula, *Compound 1* is a possible product following cleavage of the conjugated structure to yield a benzophenone derivative. It has been described as a photodegradation product of MG and identified as 4-(dimethylamino)-benzophenone (DMBP) (Perez-Estrada, Aguera, Hernando, Malato, & Fernandez-Alba, 2008). MS/MS analysis and

database search through ChemSpider (Royal Society of Chemistry, 2020) provided a match but with a lower score of 80.6% for this benzophenone derivative. It was found in calibration standards with an average peak height of 4773 (ratio MG/DMBP 111) almost 15 times lower compared to the levels observed in exposed samples (ratio MG/DMBP 3.5). On the other hand, the peak height in exposure water samples was much higher (581425) with a ratio MG/DMBP of 0.58, which could be expected from a photodegradation product. The detection of this compound in muscle tissues could be due to oxidation of MG by hydroxy radicals. Its uptake by trout or shrimp directly from water needs to be further investigated.

4. Conclusion

Four extraction methods based on simple, solvent extraction were successfully applied for the screening for MG and LMG in brook trout and shrimp. Extractions were compared based on commonly used criteria in metabolomics and contaminant screening studies. Results show that based on different approaches, different extractions could be selected. A novel aspect of this study was the evaluation of data processing parameters on the number of features and repeatability in terms of extracted features. Peak height was found to significantly influence these two parameters, and even in some cases lead to a different extraction offering the best results. Consequently, some compromises might be required and although a “one approach fits all” is not always applicable, some standardization of the comparison criteria, for example on how to evaluate repeatability or blank subtraction (should features present in blanks not be considered at all or should there be a minimum ratio between replicate/blank) is needed. Further development on the inclusion and utilization of QC samples can allow for better assessment of the impact of data processing parameters and comparison of different data sets.

The optimal extraction method, based on QuEChERS, chosen in this study was used to extract pacific white shrimp and brook trout exposed to MG. Other metabolites beside LMG had yet to be described in these two matrices previous to this study. Based on statistical analysis, des-methylated LMG was tentatively identified and proposed as another metabolite of MG in muscle. This information is key for regulatory bodies involved in the surveillance of illegal dyes in seafood.

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3.7 Supplementary material

Table S3.1: Measured concentrations for MG and LMG in exposed raw trout and shrimp using various extraction approaches.

		MG ng/g	LMG ng/g
Extraction 1	Trout 1 (n=5)	701 (±41)	411 (±108)
	Trout 2 (n=5)	519 (±147)	898 (±349)
	Shrimp (n=10)	369 (±25)	952 (±612)
Extraction 2	Trout 1 (n=5)	1333 (±199)	1094 (±165)
	Trout 2 (n=5)	1232 (±100)	1895 (±195)
	Shrimp (n=10)	381 (±27)	751 (±180)
Extraction 3	Trout 1 (n=5)	346 (±76)	139 (±64)
	Trout 2 (n=5)	426 (±66)	951 (±417)
	Shrimp (n=10)	166 (±13)	753 (±283)
Extraction 4	Trout 1 (n=5)	313 (±22)	329 (±34)
	Trout 2 (n=5)	193 (±95)	298 (±198)
	Shrimp (n=10)	173 (±27)	1060 (±373)

Table S3.2: MDL and LOQ for MG and LMG in shrimp and trout

		MG		LMG	
		MDL ng/g	LOQ ng/g	MDL ng/g	LOQ ng/g
Extraction 1	Trout	0.9	3.0	0.5	1.6
	Shrimp	1.6	5.5	1.1	3.7
Extraction 2	Trout	0.9	3.1	0.5	1.6
	Shrimp	0.7	2.2	0.3	1.1
Extraction 3	Trout	1.1	3.6	0.2	0.8
	Shrimp	1.1	3.5	1.5	5.2
Extraction 4	Trout	0.9	2.9	0.2	0.5
	Shrimp	0.6	2.1	1.1	3.3

Table S3.3: Average matrix effect and recoveries for MG and LMG in brook trout and shrimp

				MG	RSD %	LMG	RSD%
Extraction 1	Trout	Raw	Matrix effect	98±5	5.6	39±19	49.5
			Recovery	23±4	19.7	24±9	42.6
		Cooked	Matrix effect	107±10	6.1	50±13	25.2
			Recovery	49±4	9.9	22±8	39.6
	Shrimp	Raw	Matrix effect	91±16	18.1	77±36	46.8
			Recovery	31±7	23.5	36±9	27.0
		Cooked	Matrix effect	94±8	8.4	77±25	32.9
			Recovery	28±4	19.4	34±8	24.6
Extraction 2	Trout	Raw	Matrix effect	106±7	6.6	91±6	7.1
			Recovery	67±10	15.6	79±10	13.5
		Cooked	Matrix effect	108±4	4.0	96±4	4.5
			Recovery	105±3	2.8	97±12	12.9
	Shrimp	Raw	Matrix effect	109±11	10.5	54±11	19.9
			Recovery	69±21	30.0	73±9	13.6
		Cooked	Matrix effect	110±12	10.1	64±22	35.1
			Recovery	88±7	8.1	71±14	20.7
Extraction 3	Trout	Raw	Matrix effect	89±19	21.9	13±2	15.5
			Recovery	69±6	8.1	67±8	11.3
		Cooked	Matrix effect	110±4	4.0	38±7	18.1
			Recovery	63±13	21.5	33±17	52.5
	Shrimp	Raw	Matrix effect	94±9	9.8	81±14	18.1
			Recovery	67±20	31.1	103±16	16.1
		Cooked	Matrix effect	95±15	16.1	89±49	55.8
			Recovery	64±22	34.9	98±42	42.8
Extraction 4	Trout	Raw	Matrix effect	82±20	25.0	59±40	67.5
			Recovery	45±9	21.3	44±13	29.6
		Cooked	Matrix effect	63±10	16.1	31±19	61.7
			Recovery	37±3	10.7	50±5	10.5
	Shrimp	Raw	Matrix effect	99±14	14.3	84±23	28.3
			Recovery	53±14	25.9	75±18	23.9
		Cooked	Matrix effect	91±24	26.6	85±27	32.3
			Recovery	41±25	62.3	90±37	41.5

Table S3.4: Three-way ANOVA p-values comparison extractions based on recovery and matrix effect

	Matrix effect MG	Recovery MG	Matrix effect LMG	Recovery LMG
Extraction*Sample type	p<0.0005	p=0.393	p<0.0005	p<0.0005
Extraction*Process	p=0.013	p<0.0005	p=0.240	p=0.051
Sample type*Process	p=0.477	p=0.031	p=0.852	p=0.261
Extraction*Sample type*Process	p=0.247	p=0.145	p=0.519	p=0.017

Table S3.5: Four-way ANOVA p-values comparison extractions based on data processing parameters

	Extraction	Mass window	Peak height	Absolute height
Trout raw positive mode				
Features	p<0.0005	p=0.698	p<0.0005	p<0.0005
% features with RSD<20%	p<0.0005	p=0.002	p<0.0005	p<0.0005
% features with RSD<30%	p<0.0005	p=0.077	p<0.0005	p<0.0005
Trout raw negative mode				
Features	p<0.0005	p=0.841	p<0.0005	p<0.0005
% features with RSD<20%	p<0.0005	p=0.700	p<0.0005	p<0.0005
% features with RSD<30%	p<0.0005	p=0.584	p=0.005	p=0.011
Trout cooked positive mode				
Features	p<0.0005	p=0.105	p<0.0005	p<0.0005
% features with RSD<20%	p=0.071	p=0.715	p=0.001	p=0.017
% features with RSD<30%	p<0.0005	p=0.281	p<0.0005	p<0.0005
Trout cooked negative mode				
Features	p<0.0005	p=0.466	p<0.0005	p<0.0005
% features with RSD<20%	p<0.0005	p=0.590	p<0.0005	p<0.0005
% features with RSD<30%	p<0.0005	p=0.136	p=0.029	p=0.002
Shrimp positive mode				
Features	p=0.002	p=0.442	p<0.0005	p<0.0005
% features with RSD<20%	p<0.0005	p=0.579	p=0.006	p=0.003
% features with RSD<30%	p<0.0005	p=0.524	p=0.045	p=0.015
Shrimp negative mode				
Features	p<0.0005	p=0.436	p<0.0005	p<0.0005
% features with RSD<20%	p<0.0005	p=0.795	p=0.003	p=0.001
% features with RSD<30%	p<0.0005	p=0.293	p=0.001	p<0.0005

Table S3.6: Description of table values in features analysis presented in tables S3.7-12

Raw number of features	Total features extracted through Profinder with no data filtering
Features present in samples at a ratio sample/blank >2 and no blanks	Features present in all five replicates of each sample (QC, Fish 1, Fish 2) with a ratio sample/blank>2 based on peak height and not present in blanks
% features present in samples with a ratio>2 and no blanks	Calculated as (number of features present in all five replicates of each sample (QC, Fish 1, Fish 2) with a ratio sample/blank>2 based on peak height and not present in blanks)/ (number of features found in all replicates of either QC, Fish 1, Fish 2)
% features with CV<20 or 30%	Calculated as (number of features with CV<20 or 30%)/ (Features present in all five replicates of each sample (QC, Fish 1, Fish 2) with a ratio sample/blank>2 based on peak height and not present in blanks

Table S3.7: Feature analysis in trout raw positive mode

		Mass window (±)			Peak height			Peak absolute height		
		5.0 ppm	10.0 ppm	50.0 ppm	200 counts	500 counts	1000 counts	200 counts	1000 counts	5000 counts
Raw number of features	Extraction 1	3531	3469	3408	3469	2092	1216	4680	3469	1216
	Extraction 2	3963	3782	3620	3782	2487	1368	5222	3782	1299
	Extraction 3	3350	3287	3245	3287	2070	1164	4499	3287	1152
	Extraction 4	3028	2969	2940	2969	1775	995	3948	2969	995
Features present in all replicates										
QC	Extraction 1	2187	2301	2393	2301	1396	788	2573	2301	938
	Extraction 2	1750	1890	2027	1890	1158	612	2032	1890	816
	Extraction 3	1722	1826	1924	1826	1119	623	2058	1826	777
	Extraction 4	1719	1816	1888	1816	1056	566	1999	1816	768
Fish 1	Extraction 1	1054	1079	993	1079	598	354	1007	1079	510
	Extraction 2	1289	1436	1583	1436	879	442	1505	1436	683
	Extraction 3	1040	1075	1097	1075	561	323	1032	1075	451
	Extraction 4	1092	1125	1133	1125	636	363	1130	1125	443
Fish 2	Extraction 1	1036	1078	1101	1078	332	358	1089	1078	561
	Extraction 2	1770	1926	2057	1926	1191	634	2102	1926	827
	Extraction 3	1204	1236	1257	1236	683	398	1401	1236	509
	Extraction 4	1036	1066	1074	1066	574	320	1186	1066	320
Features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	2090	2190	2268	2190	1334	761	2452	2190	884
	Extraction 2	553	592	607	592	359	189	622	592	275
	Extraction 3	1620	1706	1798	1706	1057	590	1930	1706	729
	Extraction 4	1600	1679	1729	1679	984	531	1859	1679	713
Fish 1	Extraction 1	315	326	277	326	186	109	306	326	171
	Extraction 2	266	289	320	289	179	85	425	289	182
	Extraction 3	323	329	340	329	154	91	367	329	165
	Extraction 4	957	531	534	531	324	199	562	531	243
Fish 2	Extraction 1	378	327	336	327	149	113	354	327	185
	Extraction 2	322	350	362	350	213	113	370	350	166
	Extraction 3	617	642	656	642	377	242	712	642	277
	Extraction 4	378	389	388	389	206	112	427	389	112
% features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	95.6	95.2	94.5	95.2	95.6	96.6	95.3	95.2	94.2
	Extraction 2	31.6	31.3	29.9	31.3	31.1	30.8	28.2	31.3	33.7
	Extraction 3	94.1	93.4	93.4	93.4	94.5	94.7	93.8	93.4	93.8
	Extraction 4	93.1	92.5	91.6	92.5	93.2	93.8	93.0	92.5	92.8
Fish 1	Extraction 1	29.9	30.2	27.8	30.2	31.4	30.8	30.4	30.2	33.5
	Extraction 2	20.6	20.1	20.2	20.1	20.3	19.2	29.9	20.1	26.6
	Extraction 3	31.1	30.6	31.0	30.6	27.5	28.1	35.6	30.6	36.6
	Extraction 4	87.6	47.2	47.1	47.2	50.5	54.8	49.7	47.2	54.8
Fish 2	Extraction 1	36.5	30.3	30.5	30.3	44.8	31.6	29.8	30.3	32.9
	Extraction 2	18.2	18.1	17.9	18.1	17.8	17.8	17.6	18.1	20.1
	Extraction 3	51.3	51.9	52.1	51.9	55.2	60.8	50.8	51.9	54.4
	Extraction 4	36.5	36.5	36.1	36.5	35.9	35.0	36.0	36.5	35.0

% features with RD<20%										
QC	Extraction 1	59.9	58.8	57.5	58.8	68.4	73.2	58.2	58.8	66.6
	Extraction 2	50.5	48.8	47.3	48.8	60.4	66.1	48.1	48.8	56.7
	Extraction 3	54.1	53.3	51.3	53.3	62.3	66.4	51.7	53.3	61.5
	Extraction 4	55.9	55.2	54.5	55.2	65.0	70.8	53.9	55.2	65.1
Fish 1	Extraction 1	69.8	69.0	76.5	69.0	73.1	77.9	50.0	69.0	55.6
	Extraction 2	7.1	6.3	5.3	6.3	6.7	5.9	27.3	6.3	3.9
	Extraction 3	44.3	44.4	43.5	44.4	51.3	53.8	31.6	44.4	35.2
	Extraction 4	62.6	66.9	66.9	66.9	70.7	72.4	46.6	66.9	50.6
Fish 2	Extraction 1	38.6	68.8	67.6	68.8	84.6	80.5	67.8	68.8	71.4
	Extraction 2	32.6	32.6	32.1	32.6	35.7	38.9	31.1	32.6	34.3
	Extraction 3	51.1	50.3	49.2	50.3	50.4	53.7	49.1	50.3	55.6
	Extraction 4	38.6	38.8	38.1	38.8	35.9	41.1	39.6	38.8	41.1
% features with RSD<30%										
QC	Extraction 1	76.9	76.6	75.1	76.6	80.9	82.5	77.2	76.6	75.9
	Extraction 2	72.8	71.1	70.1	71.1	80.2	80.4	70.4	71.1	70.6
	Extraction 3	74.4	73.6	71.6	73.6	78.8	80.8	73.9	73.6	74.6
	Extraction 4	76.5	75.8	75.1	75.8	79.8	79.8	76.4	75.8	76.3
Fish 1	Extraction 1	83.5	83.7	90.6	83.7	80.6	81.6	64.7	83.7	66.1
	Extraction 2	19.9	18.3	17.2	18.3	6.7	38.9	59.3	18.3	12.6
	Extraction 3	68.4	68.7	68.1	68.7	73.4	76.9	51.2	68.7	49.7
	Extraction 4	84.6	90.4	90.6	90.4	93.2	93.5	68.1	90.4	72.1
Fish 2	Extraction 1	70.9	84.7	83.6	84.7	90.6	87.6	83.9	84.7	79.5
	Extraction 2	63.6	61.7	59.1	61.7	68.6	67.3	60.8	61.7	63.3
	Extraction 3	78.9	78.2	77.1	78.2	80.1	82.3	78.5	78.2	80.5
	Extraction 4	70.9	70.6	70.1	70.6	68.9	72.3	72.1	70.6	72.3

Table S3.8: Feature analysis in raw trout negative mode

		Mass window (±)			Peak height			Peak absolute height		
		5.0 ppm	10.0 ppm	50.0 ppm	200 counts	500 counts	1000 counts	200 counts	1000 counts	5000 counts
Raw number of features	Extraction 1	3742	3487	3173	3487	3263	1818	4223	3487	1648
	Extraction 2	1827	1769	1666	1769	1617	1215	2005	1769	991
	Extraction 3	4102	3830	3422	3830	3491	1913	4223	3830	1707
	Extraction 4	4417	4151	3805	4151	3478	2211	4584	4151	1976
Features present in all replicates										
QC	Extraction 1	485	507	552	507	438	225	625	507	278
	Extraction 2	458	485	534	485	485	240	503	485	285
	Extraction 3	553	584	653	584	478	298	625	584	280
	Extraction 4	498	542	595	542	430	266	603	542	252
Fish 1	Extraction 1	1042	1199	1435	1199	1262	769	1107	1199	769
	Extraction 2	600	637	675	637	500	353	675	637	357
	Extraction 3	937	1090	1323	1090	1154	722	1107	1090	659
	Extraction 4	1116	1355	1578	1355	1399	873	1370	1355	875
Fish 2	Extraction 1	1030	1163	1367	1163	1198	654	1167	1163	736
	Extraction 2	494	521	564	521	398	288	535	521	486
	Extraction 3	1009	1143	1404	1143	1215	764	1167	1143	680
	Extraction 4	1085	1298	1541	1298	1281	803	1304	1298	829
Features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	354	354	365	354	329	173	454	354	193
	Extraction 2	200	212	235	212	212	112	220	212	122
	Extraction 3	416	415	426	415	355	230	454	415	196
	Extraction 4	392	401	403	401	347	221	438	401	183
Fish 1	Extraction 1	106	115	116	115	112	65	125	115	83
	Extraction 2	70	74	75	74	53	39	77	74	46
	Extraction 3	102	122	133	122	98	57	125	122	67
	Extraction 4	206	266	284	266	254	167	266	266	203
Fish 2	Extraction 1	156	175	195	175	158	67	184	175	104
	Extraction 2	116	120	123	120	88	67	124	120	75
	Extraction 3	166	179	182	179	168	101	184	179	90
	Extraction 4	225	280	322	280	230	137	281	280	205
% features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	73.0	69.8	66.1	69.8	75.1	76.9	73.0	69.8	69.4
	Extraction 2	43.7	43.7	44.0	43.7	43.7	46.7	43.7	43.7	42.8
	Extraction 3	75.2	71.1	65.3	71.1	74.3	77.2	72.6	71.1	70.0
	Extraction 4	78.7	74.0	67.7	74.0	80.7	83.1	72.6	74.0	72.6
Fish 1	Extraction 1	10.2	9.6	8.1	9.6	8.9	8.2	11.3	9.6	10.8
	Extraction 2	25.7	11.6	11.1	11.6	10.6	11.0	11.4	11.6	12.9
	Extraction 3	10.9	11.2	10.1	11.2	8.5	7.8	11.3	11.2	10.2
	Extraction 4	18.5	19.6	18.0	19.6	18.2	19.1	19.4	19.6	23.2

Fish 2	Extraction 1	15.1	15.0	14.3	15.0	13.2	10.2	15.8	15.0	14.1
	Extraction 2	14.7	23.0	12.2	23.0	22.1	23.2	23.2	23.0	15.4
	Extraction 3	16.5	15.7	26.4	15.7	32.1	25.8	15.8	15.7	13.2
	Extraction 4	20.7	21.6	20.9	21.6	18.0	17.0	21.5	21.6	24.7
% features with RD<20%										
QC	Extraction 1	48.0	49.1	45.7	49.1	52.9	54.9	44.5	49.1	52.9
	Extraction 2	12.0	12.3	11.5	12.3	12.3	16.1	11.2	12.3	13.9
	Extraction 3	44.3	45.8	43.7	45.8	51.3	59.1	44.5	45.8	56.6
	Extraction 4	42.3	41.6	42.2	41.6	47.3	55.7	42.3	41.6	47.5
Fish 1	Extraction 1	55.7	50.4	50.0	50.4	47.3	49.3	32.8	50.4	55.4
	Extraction 2	25.7	24.3	24.0	24.3	32.1	35.9	26.0	24.3	32.6
	Extraction 3	31.4	32.0	31.6	32	34.7	31.6	32.8	32.0	31.3
	Extraction 4	44.2	40.2	37.3	40.2	42.1	43.1	40.6	40.2	39.4
Fish 2	Extraction 1	19.9	17.1	16.9	17.1	17.7	25.4	27.7	17.1	22.1
	Extraction 2	14.7	14.2	12.2	14.2	12.5	26.9	23.2	14.2	6.7
	Extraction 3	30.1	27.4	26.4	27.4	32.1	25.8	27.7	27.4	28.9
	Extraction 4	12.9	11.8	10.2	11.8	10.4	8.8	11.7	11.8	7.8
% features with RSD<30%										
QC	Extraction 1	68.4	69.5	69.6	69.5	74.7	69.9	73.6	69.5	69.4
	Extraction 2	30.5	29.3	27.2	29.3	29.2	35.7	28.6	29.3	27.1
	Extraction 3	73.1	73.3	71.8	73.3	79.7	83.9	73.6	73.3	79.6
	Extraction 4	64.8	62.3	60.5	62.3	67.1	72.9	62.6	62.3	66.7
Fish 1	Extraction 1	77.4	76.5	76.7	76.5	78.6	75.4	62.4	76.5	78.1
	Extraction 2	72.9	71.6	69.3	71.6	83.0	82.1	71.4	71.6	78.3
	Extraction 3	62.8	62.3	58.6	62.3	61.2	59.6	62.4	62.3	55.2
	Extraction 4	83.5	79.0	77.1	79.0	83.5	85.6	40.6	79.0	80.8
Fish 2	Extraction 1	35.9	32.0	29.7	32.0	30.8	35.8	69.0	32.0	33.7
	Extraction 2	26.7	25.8	23.6	25.8	26.1	26.9	25.0	25.8	32.0
	Extraction 3	71.7	68.2	66.5	68.2	73.8	71.3	69.0	68.2	76.7
	Extraction 4	23.1	21.8	19.0	21.8	18.3	16.1	11.8	21.8	13.7

Table S3.9: Feature analysis in trout cooked positive mode

		Mass window (±)			Peak height			Peak absolute height		
		5.0 ppm	10.0 ppm	50.0 ppm	200 counts	500 counts	1000 counts	200 counts	1000 counts	5000 counts
Raw number of features	Extraction 1	6746	6307	5937	6307	4368	2268	8860	6307	2207
	Extraction 2	6956	6612	6206	6612	4891	2743	8456	6612	2390
	Extraction 3	7991	7560	7113	7560	5953	2659	9365	7560	2660
	Extraction 4	8565	8049	7586	8049	6532	3122	8049	8049	2851
Features present in all replicates										
QC	Extraction 1	1973	2205	2384	2205	1504	879	2359	2205	859
	Extraction 2	2018	2181	2354	2181	1628	988	2389	2181	1025
	Extraction 3	1526	1732	1921	1732	1108	618	1805	1732	1081
	Extraction 4	1882	2072	2203	2072	1353	767	2072	2072	1195
Fish 1	Extraction 1	1841	2112	2433	2112	1667	888	2216	2112	1052
	Extraction 2	1851	2175	2488	2175	1784	1018	2294	2175	1068
	Extraction 3	2494	2943	3434	2943	2458	1240	3020	2943	1485
	Extraction 4	2666	3147	3675	3147	2832	1453	3163	3147	1591
Fish 2	Extraction 1	1941	2262	2612	2262	1764	909	2383	2262	1092
	Extraction 2	1862	2150	2479	2150	1799	1017	2256	2150	1076
	Extraction 3	2535	2995	3534	2995	2633	1295	3081	2995	1470
	Extraction 4	2622	3163	3675	3163	2479	1457	3147	3163	1591
Features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	1300	1399	1412	1399	996	595	1492	1399	614
	Extraction 2	1504	1576	1604	1576	1254	891	1722	1576	760
	Extraction 3	1154	1271	1300	1271	843	492	1330	1271	800
	Extraction 4	1445	1534	1551	1534	1066	621	1534	1534	875
Fish 1	Extraction 1	695	797	878	797	566	333	841	797	390
	Extraction 2	565	619	666	619	514	311	649	619	322
	Extraction 3	1377	1642	1972	1642	1369	693	1686	1642	822
	Extraction 4	1573	1911	2226	1911	666	891	1923	1911	928
Fish 2	Extraction 1	772	886	982	886	657	358	944	886	414
	Extraction 2	542	613	685	613	511	301	640	613	317
	Extraction 3	1473	1765	2106	1765	1587	781	1814	1765	829
	Extraction 4	1577	1923	2216	1923	685	905	1911	1923	919
% features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	81.6	63.4	59.2	63.4	66.2	67.7	63.2	63.4	71.5
	Extraction 2	74.5	72.3	68.1	72.3	77.1	90.2	72.1	72.3	74.1
	Extraction 3	75.6	73.4	67.7	73.4	76.1	79.6	73.4	73.4	74.0
	Extraction 4	76.7	74.0	70.4	74.0	78.8	81.0	74.0	74.0	73.2
Fish 1	Extraction 1	37.5	37.7	36.1	37.7	33.9	37.5	37.5	37.7	37.1
	Extraction 2	30.5	28.5	26.7	28.5	28.8	30.5	28.3	28.5	30.1
	Extraction 3	55.2	55.8	57.4	55.8	55.7	55.9	55.8	55.8	55.3
	Extraction 4	59	60.7	60.6	60.7	23.2	61.3	60.8	60.7	72.7
Fish 2	Extraction 1	39.8	39.2	37.6	39.2	37.2	39.4	39.6	39.2	37.9

	Extraction 2	29.1	28.5	27.6	28.5	28.4	29.6	28.4	28.5	29.5
	Extraction 3	58.1	58.9	59.6	58.9	60.3	60.3	58.8	58.9	55.9
	Extraction 4	60.1	60.8	60.6	60.8	27.6	62.1	60.7	60.8	57.8
% features with RD<20%										
QC	Extraction 1	58.8	56.3	55.6	56.3	66.9	71.9	55.1	56.3	56.4
	Extraction 2	56.5	55.1	53.3	55.1	62.6	68.2	54.1	55.1	64.1
	Extraction 3	47.9	47.0	45.9	47.0	54.7	60.0	68.4	47.0	53.3
	Extraction 4	57.1	54.9	54.8	54.9	64.1	70.5	54.9	54.9	62.2
Fish 1	Extraction 1	37.4	35.6	32.1	35.6	39.6	47.0	33.3	35.6	43.9
	Extraction 2	37.1	35.1	33.9	35.1	42.8	47.6	34.5	35.1	41.9
	Extraction 3	37.2	34.0	31.0	34.0	39.3	51.5	33.8	34.0	44.9
	Extraction 4	48.9	35.1	40.9	35.1	33.9	59.4	44.3	35.1	56.7
Fish 2	Extraction 1	39.8	39.2	38.1	39.2	37.2	39.4	38.8	39.2	49.3
	Extraction 2	28.2	26.4	25.6	26.4	32.1	35.9	25.6	26.4	36.6
	Extraction 3	44.2	40.2	37.6	40.2	48.0	58.6	39.9	40.2	56
	Extraction 4	39.8	44.3	32.0	44.3	25.5	53.1	35.1	44.3	47.7
% features with RSD<30%										
QC	Extraction 1	78.9	77.5	77.3	77.5	83.4	84.7	77.5	77.5	75.4
	Extraction 2	74.8	74.4	73.1	74.4	78.9	82.7	74.6	74.4	75.9
	Extraction 3	69.3	68.7	67.9	68.7	72.1	72.4	68.6	68.7	71.3
	Extraction 4	80.3	79.3	78.7	79.3	83.7	86.6	79.3	79.3	83.1
Fish 1	Extraction 1	64.1	61.4	59.3	61.4	65.1	70.0	60.9	61.4	63.3
	Extraction 2	67.1	65.8	63.4	65.0	73.5	78.1	65.3	65.8	70.5
	Extraction 3	68.5	64.4	59.8	64.4	71.5	80.7	64.1	64.4	73.7
	Extraction 4	69.8	58.5	64.6	58.5	63.4	77.7	66.8	58.5	72.7
Fish 2	Extraction 1	66.1	64.9	63.4	64.9	68.1	67.6	64.5	64.9	68.6
	Extraction 2	54.8	51.7	49.5	51.7	57.7	55.8	50.6	51.7	58.0
	Extraction 3	71.8	68.3	64.3	68.3	76.0	83.5	68.1	68.3	77.0
	Extraction 4	63.8	66.8	54.1	66.8	49.5	74.8	58.5	66.8	68.9

Table S3.10: Feature analysis in cooked trout negative mode

		Mass window (±)			Peak height			Peak absolute height		
		5.0 ppm	10.0 ppm	50.0 ppm	200 counts	500 counts	1000 counts	200 counts	1000 counts	5000 counts
Raw number of features	Extraction 1	4680	4350	3903	4350	3311	1710	5196	4350	1720
	Extraction 2	5734	5365	4820	5365	3988	2126	6698	5365	2036
	Extraction 3	4465	4189	3854	4189	3251	1796	5008	4189	1669
	Extraction 4	4921	4591	4133	4591	3537	1767	5522	4591	1718
Features present in all replicates										
QC	Extraction 1	532	538	632	538	494	249	635	538	283
	Extraction 2	612	643	713	643	480	271	706	643	325
	Extraction 3	326	354	392	354	286	169	373	354	193
	Extraction 4	650	676	751	676	584	355	753	676	333
Fish 1	Extraction 1	1174	1409	1658	1409	1280	732	1460	1409	763
	Extraction 2	1576	1751	2028	1751	1705	1005	1843	1751	937
	Extraction 3	1211	1408	1651	1408	1273	777	1460	1408	791
	Extraction 4	1345	1548	1844	1548	1364	804	1605	1548	829
Fish 2	Extraction 1	1171	1358	1573	1358	1229	669	1411	1358	748
	Extraction 2	1342	1536	1830	1536	1446	842	1588	1536	879
	Extraction 3	1210	1396	1622	1396	1277	774	1450	1396	760
	Extraction 4	1370	1554	1836	1554	1378	779	1618	1554	828
Features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	378	388	407	388	351	187	420	388	181
	Extraction 2	441	456	484	456	366	208	499	456	219
	Extraction 3	230	244	244	244	213	126	254	244	122
	Extraction 4	473	478	495	478	438	280	536	478	222
Fish 1	Extraction 1	276	311	332	311	252	142	323	311	148
	Extraction 2	633	684	727	684	656	386	725	684	307
	Extraction 3	279	323	350	323	224	121	342	323	141
	Extraction 4	394	470	494	470	322	168	491	470	193
Fish 2	Extraction 1	252	282	299	282	220	115	295	282	140
	Extraction 2	420	486	527	486	391	236	495	486	264
	Extraction 3	269	299	321	299	216	109	317	299	126
	Extraction 4	432	495	532	495	367	194	519	495	204
% features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	71.1	66.6	64.4	66.6	71.1	75.1	66.1	66.6	63.5
	Extraction 2	72.1	70.9	67.9	70.9	76.3	76.6	70.7	70.9	67.4
	Extraction 3	70.6	68.9	62.3	68.9	74.5	74.6	68.1	68.9	63.2
	Extraction 4	72.8	70.7	65.8	70.7	75.0	73.6	71.2	70.7	66.7
Fish 1	Extraction 1	23.5	22.1	20.0	22.1	19.7	19.4	22.1	22.1	19.4
	Extraction 2	39.9	39.1	35.8	39.1	38.5	79.1	39.3	39.1	32.8
	Extraction 3	23.1	22.9	21.2	22.9	17.6	15.6	23.4	22.9	17.8
	Extraction 4	29.3	30.4	26.8	30.4	23.6	29.2	30.6	30.4	23.3

Fish 2	Extraction 1	21.5	20.8	19.0	20.8	17.9	17.2	20.9	20.8	18.7
	Extraction 2	31.3	31.6	28.8	31.6	27.0	28.2	31.2	31.6	30.0
	Extraction 3	22.3	21.4	19.8	21.4	16.9	14.1	21.9	21.4	16.2
	Extraction 4	31.5	31.9	29.0	31.9	26.6	46.9	32.1	31.9	24.6
% features with RD<20%										
QC	Extraction 1	62.4	62.1	59.7	62.1	66.4	75.4	61.4	62.1	69.1
	Extraction 2	24.9	24.6	24.2	24.6	31.4	49.5	24.4	24.6	29.2
	Extraction 3	16.5	16.0	15.6	16.0	19.2	20.6	16.1	16.0	14.8
	Extraction 4	66.2	65.3	60.4	65.3	67.8	73.6	62.7	65.3	72.5
Fish 1	Extraction 1	35.5	35.6	33.1	35.6	39.3	42.3	36.8	35.6	35.8
	Extraction 2	57.7	55.6	54.3	55.6	62	79.1	54.5	55.6	72.6
	Extraction 3	41.9	38.4	36.9	38.4	43.3	49.6	38	38.4	49.6
	Extraction 4	25.6	23	23.3	23.0	29.5	29.2	22.8	23.0	28.0
Fish 2	Extraction 1	21.8	20.2	20.7	20.2	23.6	19.1	20.9	20.2	22.9
	Extraction 2	11.2	11.1	11.2	11.1	13.8	11.9	11.3	11.1	9.0
	Extraction 3	47.6	44.5	42.3	44.5	54.6	63.6	43.2	44.5	56.3
	Extraction 4	36.1	35.0	32.3	35.0	41.7	46.9	34.5	35.0	43.6
% features with RSD<30%										
QC	Extraction 1	83.9	87.9	81.1	87.9	88	90.4	82.9	87.9	86.7
	Extraction 2	44.7	41.9	40.9	41.9	48.6	49.5	43.3	41.9	44.8
	Extraction 3	27.0	27.5	27.9	27.5	28.2	30.2	28.0	27.5	22.1
	Extraction 4	84.8	86.0	81.4	86.0	85.6	88.6	84.1	86.0	84.7
Fish 1	Extraction 1	72.8	72.7	69.0	72.7	78.6	80.3	73.4	72.7	75.0
	Extraction 2	83.6	81.8	80.6	81.8	87.2	94.5	81.2	81.8	89.6
	Extraction 3	68.1	62.8	60.6	62.8	73.2	68.6	63.2	62.8	69.5
	Extraction 4	48.7	46.2	45.8	46.2	50.9	44.1	45.8	46.2	42.0
Fish 2	Extraction 1	32.9	32.6	31.8	32.6	32.7	25.2	34.2	32.6	29.3
	Extraction 2	27.6	27.2	25.4	27.2	29.9	28.8	27.8	27.2	22.3
	Extraction 3	74.7	71.2	69.9	71.2	79.6	78.0	70.3	71.2	70.6
	Extraction 4	70.8	70.1	66.2	70.1	76.3	80.4	69.6	70.1	76.5

Table S3.11: Feature analysis in shrimp positive mode

		Mass window (±)			Peak height			Peak absolute height		
		5.0 ppm	10.0 ppm	50.0 ppm	200 counts	500 counts	1000 counts	200 counts	1000 counts	5000 counts
Raw number of features	Extraction 1	4105	3985	3856	3985	2113	985	8474	3985	1142
	Extraction 2	3423	3352	3287	3352	2260	1365	4650	3352	1312
	Extraction 3	4999	4819	4657	4819	2615	1172	8946	4819	1268
	Extraction 4	5645	5433	5298	5433	3114	1365	5246	5433	1412
Features present in all replicates										
QC	Extraction 1	2124	2323	2506	2323	1247	544	2808	2323	824
	Extraction 2	1756	1858	1966	1858	1216	714	1993	1858	1000
	Extraction 3	2151	2445	2711	2445	1337	575	2866	2445	874
	Extraction 4	2713	3038	3278	3038	1805	748	3047	3038	957
Features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	1845	2027	1850	2027	1076	468	2783	2027	692
	Extraction 2	1489	1567	1647	1567	1056	631	1673	1567	868
	Extraction 3	1870	2123	2352	2123	1149	483	2784	2123	760
	Extraction 4	2531	2732	2928	2732	1628	656	2735	2732	805
% features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	86.8	87.3	73.8	87.3	86.3	86.0	99.0	87.3	84.0
	Extraction 2	84.8	84.3	83.8	84.3	86.8	88.4	84.0	84.3	86.8
	Extraction 3	86.9	86.8	86.8	86.8	85.9	84.0	97.1	86.8	87.0
	Extraction 4	93.3	89.9	89.3	89.9	90.2	87.7	89.7	89.9	84.1
% features with RD<20%										
QC	Extraction 1	53.9	51.7	49.1	51.7	61.8	63.5	49.1	51.7	57.6
	Extraction 2	20.6	21.5	21.3	21.5	21.1	19.0	21.1	21.5	15.2
	Extraction 3	38.8	36.6	34.8	36.6	45.9	54.8	34.4	36.6	43.3
	Extraction 4	56.8	54.4	51.6	54.4	66.8	72.7	54.4	54.4	66.7
% features with RSD<30%										
QC	Extraction 1	76.5	75.9	73.1	75.9	79.4	79.9	74.9	75.9	73.4
	Extraction 2	33.2	32.1	34.1	32.1	32.6	30.3	34.7	32.1	24.3
	Extraction 3	66.1	64.4	62.4	64.4	71.5	74.7	63.1	64.4	64.1
	Extraction 4	79.7	78.2	75.9	78.2	86.4	87.0	78.2	78.2	81.9

Table S3.12: Feature analysis shrimp negative mode

		Mass window (±)			Peak height			Peak absolute height		
		5.0 ppm	10.0 ppm	50.0 ppm	200 counts	500 counts	1000 counts	200 counts	1000 counts	5000 counts
Raw number of features	Extraction 1	1504	1485	1449	1485	1056	604	2085	1485	540
	Extraction 2	1248	1228	1192	1228	977	548	1558	1228	473
	Extraction 3	1809	1795	1721	1795	1795	685	2643	1795	616
	Extraction 4	1915	1872	1810	1872	1218	720	2760	1872	627
Features present in all replicate samples										
QC	Extraction 1	543	576	610	576	384	201	661	576	264
	Extraction 2	536	553	585	553	418	236	612	553	249
	Extraction 3	600	630	663	630	630	224	724	630	290
	Extraction 4	754	785	815	785	533	305	921	785	328
Features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	393	418	440	418	354	153	480	418	195
	Extraction 2	443	457	470	457	347	201	506	457	204
	Extraction 3	485	509	529	509	508	196	576	509	222
	Extraction 4	634	660	684	660	466	270	771	660	273
% features present in samples with a ratio>2 and no blanks										
QC	Extraction 1	72.4	72.6	72.1	72.6	92.9	76.1	72.6	72.6	73.9
	Extraction 2	82.6	82.6	80.3	82.6	83.1	86.2	82.7	82.6	81.9
	Extraction 3	80.8	80.6	79.8	80.6	80.6	87.5	79.6	80.6	76.6
	Extraction 4	84.1	84.1	83.9	84.1	87.4	88.5	83.7	84.1	83.2
% features with RD<20%										
QC	Extraction 1	47.6	45.7	44.5	45.7	54.3	52.3	44.6	45.7	48.7
	Extraction 2	63.6	62.6	62.6	62.6	71.5	79.1	61.7	62.6	81.1
	Extraction 3	41.4	40.6	39.1	40.6	40.6	43.9	40.3	40.6	41.4
	Extraction 4	68.5	67.4	67.4	67.4	78.4	85.6	64.7	67.4	77.7
% features with RSD<30%										
QC	Extraction 1	71.0	68.9	67.7	68.9	74.0	73.9	69.4	68.9	66.7
	Extraction 2	85.6	85.3	84.9	85.3	87.9	91.5	84.6	85.3	87.7
	Extraction 3	63.7	63.4	61.6	63.4	63.4	64.3	64.2	63.4	60.8
	Extraction 4	90.1	88.9	89.2	88.9	93.6	96.7	89.1	88.9	90.5

Table S3.13: Compounds identified with statistically significant higher abundance in exposed compared to control trout and shrimp

Compound	Mass	*m/z*	R_t (min)	Formula (Score)	MME (ppm)	Match in Metlin database (Score)
1[ac]	225.1154	226.1227	13.5	$C_{15}H_{15}NO$ (99)	0.33	Navenone A (99)
2[b]	289.0807	290.0880	3.6	$C_{12}H_{11}N_5O_4$ (99)	-1.40	N/A
3[b]	302.2248	303.2316	15.7	$C_{20}H_{30}O_2$ (89)	0.63	8,15 Isopimaredien-18-oic acid (89)
4[ac]	314.1788	315.1860	12.3	$C_{22}H_{22}N_2$ (72)	1.57	N/A
5[ac]	315.1880	158.6011	11.2	$C_{22}H_{23}N_2$ (90)	6.07	N/A
6[ac]	316.1933	317.2012	10.3	$C_{22}H_{24}N_2$ (88)	-2.14	N/A
7[b]	346.1884	347.1954	15.6	$C_{17}H_{24}N_5O_3$ (95)	1.43	N/A
8[b]	402.2775	403.2849	15.4	$C_{25}H_{38}O_4$ (97)	-0.10	MG(0:00/22:6 (4Z,7Z,10Z,13Z,16Z,19Z/0:0) (97)
9[b]	682.4573	683.4647	18.3	$C_{41}H_{58}N_6O_3$ (98)	0.22	N/A
10[b]	686.4897	687.4971	18.9	$C_{43}H_{64}N_5O_2$ (97)	0.04	N/A
11[b]	729.5321	730.5396	18.6	$C_{40}H_{76}NO_8P$ (99)	0.29	PE(15:0/20:2 (11Z,14Z)) (95)
12[b]	776.4585	777.4658	18.4	$C_{55}H_{58}N_3O$ (92)	0.71	N/A

[a] present in higher abundance in exposed compared to control trout
[b] present in higher abundance in exposed compared to control shrimp
[c] common between the two organisms

Connecting paragraph

Chapter 3 showed that often a compromise will need to be reached when choosing the optimal extraction method in non-target analysis and some consensus is needed on the development of sample preparation methods. Based on the results obtained, QuEChERS showed satisfactory results in terms of extracted features and reproducibility. This extraction was used to identify, for the first time, des-methylated LMG as another metabolite of MG in brook trout and shrimp. In Chapter 4, a data analysis methodology was validated for the identification of chemical contaminants, focusing on general pharmaceutical drugs, including antibiotics and other drugs used in human/animal treatments. Fish livers were used as a case study, using the QuEChERS extraction and analysis approach described in Chapter 3. This chapter has been submitted to *Science of the Total Environment* as "Suspect screening of pharmaceuticals in fish livers based on QuEChERS extraction coupled with high resolution mass spectrometry" (date of submission: December 18, 2020).

Chapter 4: Suspect screening of pharmaceuticals in fish livers based on QuEChERS extraction coupled with high resolution mass spectrometry

4.1 Abstract

The presence of pharmaceuticals and personal care products (PPCPs) in aquatic environments is of increasing concern due to the presence of residues in fish and aquatic organisms, and emerging antibiotic resistance. Wastewater release is an important contributor to the presence of these compounds in aquatic ecosystems, where they may accumulate in food webs. The traditional environmental surveillance approach relies on the targeted analysis of specific compounds, but more suspect screening methods have been developed recently to allow for the identification of a variety of contaminants. In this study, a method based on QuEChERS extraction – using acetonitrile/water mixture as solvent and PSA/C_{18} for sample clean-up – was applied to identify pharmaceuticals and their metabolites in fish livers. Both target and suspect screening workflows were used and fish were sampled upstream and downstream of wastewater treatment plants from the Scioto River, Ohio (USA). The method performed well in terms of extraction of some target PPCPs, with recoveries generally above 90%, good repeatability (<20%), and linearity. Based on target analysis, lincomycin and sulfamethoxazole were two antibiotics identified in fish livers at average concentrations of 25.5 and 25.6 ng g^{-1} fresh weight, respectively. Using suspect screening, another antibiotic, azithromycin and an antidepressant metabolite, erythrohydrobupropion were identified (average concentrations: 27.8 and 13.8 ng g^{-1}, respectively). The latter, reported, to the best of our knowledge, for the first time in fish livers, was also found at higher concentrations in fish livers sampled downstream vs. upstream. The higher frequency of detection for azithromycin in benthic feeding fish species (63%) as well as clusters identified between different foraging groups suggest that foraging behavior may be an important mechanism in the bioaccumulation of PPCPs. This study shows how suspect screening

is effective in identifying new contaminants in fish liver, notably using differential analysis among different spatially distributed samples.

4.2 Introduction

The continuous detection of residues of pharmaceuticals and personal care products (PPCPs) in the aquatic environment and biota represents a growing problem in recent years (Bayen et al. 2014, Huerta et al. 2018, Xie et al. 2019). Their presence is concerning for various reasons. Fish can take up pharmaceuticals through the gills where they can accumulate and undergo metabolism in tissues (Valdes et al. 2016). Some pharmaceuticals, such as benzodiazepines, have been shown to cause behavioural changes such as less sociality in European Perch (*Perca fluviatilis*) (Brodin 2013) or reduced aggression in Round Goby (*Neogobius melanostomus*) exposed to wastewater effluent (McCallum et al. 2017). Pharmaceutical exposure can also affect dispersal and migration with population-level consequences (Brodin et al. 2014). Bioaccumulative effects of pharmaceuticals are also problematic owing to the fact that some effects do not manifest until later life stages, are multigenerational, or are only observed in fishes feeding at higher trophic levels (van der Oost et al. 2003).

While PPCPs concentrations are generally in the ppb range, there are concerns, especially for antibiotics, that bacteria exposed to sub-lethal (i.e., subinhibitory) concentrations can accumulate antibiotic resistant genes that can undermine the successful treatment of bacterial infections (Andersson and Hughes 2014). One important source of pharmaceuticals in the environment is through wastewater release as wastewater treatment measures are not always effective in reducing concentrations for some compounds (Botero-Coy et al. 2018). For example,

carbamazepine and erythromycin were found to be more stable to wastewater removal treatments with less than 20% removal rates in some cases (Botero-Coy et.al 2018). Even with different technologies, some compounds such as lorazepam have very poor removal efficiency (Greenham et al. 2019).

Investigations of PPCPs in aquatic biota commonly rely on the application of multi-residue analysis for a targeted list of compounds (Peña-Herrera et al. 2019). In this case, the identification and quantification are limited to a list of known target compounds based on use of pure analytical standards. However, with the increasing number of pharmaceuticals being detected, more generic methods that can cover a wide range of contaminant classes that can be applied to different matrices (Knolhoff and Croley 2016) can be advantageous. Especially when coupled with high-resolution mass spectrometry (HRMS), the resulting data can be used for non-target analysis or suspect screening. Both approaches are similar as there is no need for reference standards. However, suspect screening can be performed based on existing information, such as mass and formula, available through databases (Liu et al. 2019, Tian et al. 2019). Non-target analysis workflows identify compounds without *a priori* information for the discovery of transformation products, formed for example during UV exposure (Krauss et al. 2010). Structural information obtained from MS/MS fragmentation, along with accurate mass and isotope abundance information are used for compound identification (Knolhoff and Croley 2016).

Muscle is the matrix most often studied in fish contaminant studies due to the implications for human health (Rojo et al. 2019). However, for a better assessment of pollution levels, liver is key as it is a site of metabolism (Santos et al. 2020), with contaminants generally bioconcentrating at higher levels compared to muscle (Zhou et al. 2020). Some pharmaceuticals,

for example citalopram, have been detected mostly in livers compared to muscle tissues (Grabicova et al. 2017). There are limited studies in fish livers using non-target or suspect screening approaches. Du et al. (2017) used suspect screening to identify organic contaminants in fish livers following accelerated solvent extraction (ASE) while Liu et al. (2018a) used non-target analysis to identify perfluoroalkyl substances in fish livers. Likewise, in a target analysis study, ASE was preferred over QuEChERS for extraction of pharmaceuticals in fish livers (Huerta et al. 2013). QuEChERS type extractions are highly adaptable for different contaminants, e.g., pharmaceuticals, pesticides and mycotoxins for a variety of matrices in food and environmental analysis (Perestrelo et al. 2019). These methods use lower sample amounts and solvent volumes, are less costly, with clean-up steps easily adaptable for specific analytes to increase recoveries and lower matrix effects, through application of different dispersive sorbents like C_{18} or PSA (Perestrelo et al. 2019). For example, average recoveries and matrix effect values of 76 and 99% (indicating little matrix effect) were obtained for 99 compounds (antibiotics and other veterinary drugs) across different matrices such as salmon, shrimp, beef and chicken (Desmarchelier et al. 2018). Owing to these advantages, QuEChERS extractions offer great potential for suspect screening (Knolhoff and Croley 2016) and have been used for this purpose in fish muscle (Baduel et al. 2015, Jia et al. 2017).

The objective of this study was to demonstrate the potential of suspect screening workflows for unexpected contaminants, such as PPCPs, in fish livers. A QuEChERS-based extraction was first validated for the target analysis of 16 model PPCPs in fish livers. The validated workflow was then applied for suspect screening of PPCPs in fish sampled upstream and downstream of wastewater treatment plants (WWTP) in the Scioto River in Columbus, Ohio (USA). Based on previous studies that found pharmaceuticals to be more prevalent downstream

of WWTPs and in benthic-feeding organisms (Grabicova et al. 2015, Huerta et al. 2018), a third objective was to use statistical analysis, e.g., clustering analysis, to identify trends between downstream and upstream (of WWTP) fish and different foraging groups, using data acquired through suspect screening.

4.3 Materials and methods

4.3.1 Chemicals

Analytical standards used for method validation of tylosin A (97%) (TYL), lincomycin (>95%) (LIN), furazolidone (>99%) (FZL), sulfadimethoxine (>98.5%) (SDM), sulfamethazine (>99%) (SFM), sulfamethoxazole (>98%) (SFX), sulfanilamide (>99%) (SLM), cotinine (>99.5%) (COT), carbamazepine (>98%) (CBZ), acetaminophen (>99%) (ACT), thiamphenicol (>97%) (THP), florfenicol (>99%) (FLO), chloramphenicol (>99.8%) (CHP), caffeine (>99%) (CAF), trimethoprim (>98%) (TRI) and triclosan (>97%) (TCS) were obtained from Sigma Aldrich (St Louis, MO, USA). Labeled surrogate standards d_3-cotinine (>99%), d_3-triclosan (>97%), d_{10}-carbamazepine (>98%), d_3-trimethoprim (>99%), d_3-acetaminophen, d_3-caffeine (>99%) were purchased from CDN Isotopes (Pointe Claire, Canada). Azithromycin (>98%) (AZ) were purchased from Sigma Aldrich. Erythrohydrobupropion (>95%) (EHB) was purchased from Toronto Research Chemicals (Toronto, Canada). Labeled injection internal standards, d_3-diphenhydramine (>98%), d_3-6-acetylmorphine (>99%), ^{13}C-propylparaben (>99%) were purchased from Cerilliant (Round Rock, TX, USA) and Sigma Aldrich. HPLC grade acetonitrile, water, LC-MS grade formic acid, acetic acid, and ammonium acetate were obtained from Fisher Chemical (Pittsburgh, PA, USA). Anhydrous magnesium sulfate and sodium acetate were purchased from Sigma Aldrich. Primary secondary amine (PSA) and C_{18} sorbents were

purchased from Agilent (Santa Clara, CA, USA). All glassware used was baked in an oven at 320°C for 4 h and rinsed with methanol before use. Working standard solutions containing the 16 analytes (STD1) used in method validation and surrogate standard solutions (STD2) of 10 μg mL^{-1} were prepared in methanol and stored in amber vials at -20°C. Six calibration standards, from 10 to 80 ng/mL, were prepared in water. Solutions of 0.2 μg mL^{-1} ^{13}C-propylparaben (negative injection internal standard) and 0.4 μg mL^{-1} mixture of d_3-diphenhydramine and d_3-6-acetylmorphine (positive injection internal standard) were prepared in methanol and stored at -20°C.

4.3.2 Sample collection

Fish were sampled across 25 sampling sites (Figure S4.1), downstream and upstream of two WWTPs serving the city of Columbus, Ohio. Fish were sampled using both boat-mounted and backpack electrofishers depending on sampling location depth. After electroshocking, stunned fish were collected and identified to species. Three to four adult fish were sacrificed per sampling site for a total of 80 dispatched individuals. A total of 22 species (Table S4.1) were collected for analysis, selected to represent both benthic and water-column foraging strategies/groups. Sacrificed fish were transported to The Ohio State University on ice, where hepatic tissue was harvested nightly using sterile instruments. The collection of fish and their tissues was reviewed and approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC#: 2016A00000095 and 2009A0215-R2 CRE and ESBL Transmission in Fish held by TW). Liver samples were stored in amber vials at -80°C until extraction.

4.3.3 Sample extraction

Frozen fish livers were homogenized using a mortar and pestle. Extraction was adapted from Du et al. (2017) and Jia et al. (2017). Briefly, 0.50 g of fish liver was weighed into 50-mL centrifuge tubes to which 50 µL of the 10 µg mL^{-1} surrogate internal standard solution (STD2) was added and samples were allowed to equilibrate for 10 min Solvent – 2.5 mL (84:16 v/v) acetonitrile/water with 1% acetic acid was added and vortexed for 1 min – followed by the addition of 0.50 g of $MgSO_4$ and 0.15 g sodium acetate, vortexing for 1 min and centrifuging at 4400 rpm (3000 × *g*, 25°C) (Eppendorf, Germany). The supernatant (2 mL) was transferred to new tubes containing 150 mg $MgSO_4$, 50 mg PSA, 50 mg C_{18}, vortexed for 1 min and centrifuged for 5 minutes at 4400 rpm. Extracts were filtered using a 0.22 µm PTFE filter and stored in amber vials at -20°C. Five procedural blanks were prepared following the same protocol but with no sample added.

In order to test the efficiency of the extraction method, recovery and matrix effects were determined. For recovery experiments, three large livers from three different species [Smallmouth Buffalo (*Ictiobus bubalus*), Common Carp (*Cyprinus carpio*), and Black Redhorse (*Moxostoma duquesni*)] were selected. Three replicates were extracted for each liver. Samples were spiked with STD1 to achieve a target concentration of 0.100 µg g^{-1} in liver tissues, and allowed to equilibrate for 10 min before extraction.

Once the method was validated, the remaining 77 fish livers, along with 5 procedural blanks, were extracted using the same procedure described above. Due to the small liver sizes, one single replicate per fish liver was extracted; in cases where liver samples were smaller than 0.50 g, the whole liver was used for extraction. Twelve samples had weights lower than 0.50 g

(mean 0.38 ± 0.11 g). Five injection QC samples were prepared by pooling 20 µL of each fish liver extract (n = 77) and procedural blanks (n = 5). For LC-MS analysis, 100 µL of extract or pooled QC injection was diluted to 1 mL in water and 50 µL each of positive and negative injection internal standard solution was added. The injection internal standards were added for further monitoring of instrumental variability, not for quantification purposes. QC samples were analyzed throughout the LC-MS run. Currently, there is no standardized protocol for QA/QC in suspect or non-target analysis. QC samples composed of standard mixtures (Du et al. 2017, Knolhoff et al. 2020, Ng et al. 2020) or pooled extracts (Perez-Miguez et al. 2018, von Eyken et al. 2020) have been proposed to monitor variability and assess the quality of the data and were used in this study.

4.3.4 Instrumental analysis

Samples were analyzed using an Agilent UHPLC 1290 coupled with an Agilent 6545 QTOF-ESI-MS, in both positive and negative ionization modes. In positive mode, mobile phases were (A) H_2O with 0.1 % formic acid and (B) acetonitrile. In negative mode, mobile phases used were (A) 0.05 M ammonium acetate and (B) acetonitrile. For both positive and negative modes, the same gradient elution was used: 5% B (0-1 min), increase to 100% B (1-15 min), 100% B (15-20 min), decrease to 5% B (20.0-20.1 min), and finally 5% B (20.1-25 min). An InfinityLab Poroshell 120 (Phenyl-Hexyl, 3.0 x 100 mm, 2.7 µm, Agilent Technologies) with a Poroshell (4.6 mm) Phenyl Hexyl pre-column was used. Flow rate was 0.2 mL min^{-1}, injection volume was 5 µL and column temperature was 20°C. The MS parameters were as follows: sheath gas temperature 275°C, drying gas temperature 325°C, drying gas flow 5 L min^{-1}, sheath gas flow

12 L min^{-1}, nebulizer pressure 20 psi, capillary voltage 4000 V, nozzle voltage 2000 V, fragmentor voltage 175 V, skimmer voltage 65 V. All Ions MS/MS mode, where information on both precursor and fragment ions may be obtained, at collision energies of 0, 10, 20 and 40 V was used. Data were collected in the range of 100-1700 *m/z* at a rate of 3 spectra s^{-1}. The average resolution was 19,147 across positive and negative ionization modes. Samples were kept at 4°C in the multisampler compartment.

Although traditionally C_{18} columns are used in suspect screening analysis (Jia et.al 2017), some studies have successfully used phenyl-hexyl columns in suspect screening analysis of plastic related chemicals in pike (Tian et al. 2019), honey (von Eyken et al. 2020), and multi-residue analysis of antibiotics in wastewater (Karthikeyan and Meyer 2006) and fish (Dufresne et al. 2007). Good peak signals and chromatographic separation were observed for this column and were used in this study.

All Ions MS/MS data were manually screened to compare fragment ions between liver samples and standards. Additional MS/MS data were then acquired for suspected compounds through Targeted MS/MS mode to confirm the identity of specific compounds.

4.3.5 Data treatment

4.3.5.1 Recovery and matrix effect

For recovery and matrix effect, quantification for the targeted 16 PPCP compounds was performed using Agilent Mass Hunter Quantitative Analysis B.010.0. This software was used for quantification across the whole study. For compounds where a labeled surrogate standard was available (acetaminophen, cotinine, carbamazepine, caffeine, trimethoprim, triclosan), internal

standard calibration was used and recovery concentrations were calculated using the Relative Response Factor (RRF) (USEPA 2007) as listed in equation 1.

$$RRF = \frac{(area\ native\ compound\ x\ concentration\ labeled\ compound)}{(area\ labeled\ compound\ x\ concentration\ native\ compound)} \quad (Equation\ 1)$$

For the 10 remaining compounds, external calibration was initially used only for quantification of recovery and matrix effect samples. Matrix effect and recovery were calculated according to the protocols set out by Matuszewski et al. (2003). Matrix effect (ME) was determined by comparing the response of the analyte spiked post-extraction (B) with the response in a pure water solution standard (A) where $ME=B/A \times 100$. Absolute recovery (AR) was determined by comparing the response of the analyte spiked pre-extraction (C) with the response in a pure water solution standard (A) where $AR=C/A \times 100$.

As recovery tests were performed on three different fish species, sample *t*-tests (SPSS Software v.26, IBM, NY, USA) were run to evaluate if the differences between recovery and matrix effect values were significant between the three recovery species. Method detection limits (MDLs) and limits of quantification (LOQs) were calculated as 3σ and 10σ, respectively, where σ is the standard deviation, of the procedural blanks integrated at the retention time of the target compounds.

4.3.5.2 Targeted analysis

As described below, four compounds (LIN, SFX, AZ, EHB) were detected in actual fish liver samples. Procedural blanks were free of contamination. Since labeled surrogates were not

available for these compounds, matrix matched calibration (blank fish liver extracts; 3 to 30 ng mL^{-1}) was used for their quantification based on response factors (RF).

$$RF = \frac{area\ compound}{concentration\ compound} \qquad (Equation\ 2)$$

4.3.5.3 Suspect screening

Data alignment and feature extraction were completed on all 77 fish liver extracts, 5 procedural blanks, and 5 QC samples using Agilent Mass Hunter Profinder software B.010.0 using the following parameters: isotope model: common organic molecules; accepted adducts $[M+H]^+$ and $[M-H]^-$; peak filter height 200 counts; retention time window ±0.30 min; mass extraction window ±10 ppm, post-processing peak absolute height 1000 counts; MFE (Molecular Feature) score > 80%. Features were exported as Profinder archive (.pfa) files and imported into Mass Profiler Professional (MPP, v 15.0 Agilent Technologies) for statistical analysis and database searches. External scalar normalization was applied to reduce variability introduced by the liver sample weights used for extraction (Agilent Technologies 2019).

4.3.5.3.1 Compound identification

Imported features into MPP software were screened, using the ID Browser option, against the Agilent *Veterinary Drug PCDL* (2153 compounds) and *Water Screening PCDL* (1451 compounds) libraries, with 777 compounds in common between the two. Compounds with a matching score >70% in either of the databases and which were absent in blanks or present with a fold change >2 were selected and re-analyzed in Targeted MS/MS mode in order to confirm their identity. Next, MS/MS data files were extracted using the targeted MS/MS feature in Agilent Qualitative Analysis software B.010.0, exported as .cef files, and imported into Agilent

Molecular Structure Correlator (MSC). Compounds were screened using the MSC software against the two databases again to compare fragment ions information required for identification. Quantification of compounds identified through suspect screening and confirmed with analytical standards was done based on matrix-matched calibration, as per section *4.3.5.2*, using liver samples in which the suspect compounds were not detected.

4.3.5.3.2 Statistical analysis

Samples were grouped into two different parameters (Table S4.1): location (downstream vs. upstream) and foraging group. Foraging group was categorized as either benthic feeders or water-column feeders using Rice and Zimmerman (2019) as a guide, classified by their general feeding strategy vs. diet *per se*. All molecular features imported from Profinder were filtered based on 40, 70 and 100% of samples. Different filtering frequencies were used because pharmaceuticals and other contaminants may not be present in fish liver samples at 100% frequency. Thus, some compounds could be missed if data filtering is too stringent. Principal component analysis (PCA) was performed on filtered features. Volcano plot, using thresholds of 2 for fold change and 0.05 for statistical significance (Mann-Whitney unpaired) was performed in order to identify statistically significant features between downstream vs. upstream samples, and between benthic vs. water column feeder samples. Similar to the previous section on compound identification, these features were screened using MPP ID Browser and the two databases.

Correlation analysis was performed for all entities, not filtered by any frequency, using the default MPP parameters (normalized intensity values, hierarchical clustering algorithm, and Euclidean distance metric Ward's linkage rule).

4.4 Results and discussion

4.4.1 Method performance/QC

All spiked compounds were extracted from three fish liver (Smallmouth Buffalo, Common Carp and Black Redhorse samples (Figure 4.1). Recoveries were above 90% for all target PPCP compounds (Table S4.2), except for lincomycin, which had an average recovery of 73% across the three different liver species. MDLs were generally below 2 ng g^{-1} fresh weight (Table S4.3). The method achieved good precision, with RSD<20%, except for furazolidone which had an RSD of 21.1% in spiked Black Redhorse. Matrix effect results indicated some signal enhancement (i.e., >100%) for most compounds, especially for sulfamethoxazole and carbamazepine. Ion suppression has been observed in the literature for carbamazepine, especially with an increase in lipid content (Huerta et al. 2013). On the other hand, simple extraction using acidified acetonitrile/isopropanol coupled with freezing showed no matrix effects for carbamazepine in Common Carp livers (Grabicova et al. 2018). Regarding sulfamethoxazole, slight ion suppression was observed in fish livers following pressurized liquid extraction (Huerta et al. 2013). Ion suppression was observed in this study in Smallmouth Buffalo for sulfanilamide and triclosan with matrix effects of 90 and 76%, as well as sulfanilamide in Common Carp with a matrix effect of 75%. No statistical difference ($p > 0.05$) was noted for the matrix effect and recovery values among the three fish species.

In suspect screening, compound identification is possible due to the high mass accuracy achieved by high-resolution mass spectrometry with mass tolerances usually set at 5 ppm (Moschet et al. 2013). Mass measurement errors (MME) for the 16 compounds were calculated as described in Brenton and Godfrey (2010) and ranged between -0.51 to -3.94 ppm in solvent (water) and -0.71 to 3.44 ppm in spiked liver (Table S4.4). This threshold, of 5 ppm, has been

reported in other suspect screening methods in wastewater (Deeb et al. 2017), mussels (Liu et al. 2019), and pike (*Esox lucius)* (Tian et al. 2019). The overall method performance was satisfactory, with good recoveries and confident compound identification, and was applied to the remaining 77 fish livers.

Further QC measures to determine the quality of the data obtained from the LC-MS run of the 77 liver samples included the calculation of MME and reproducibility of internal standards, as well as PCA analysis. Retention time for all internal standards in QC samples was reproducible with RSD<1%. MME for all internal standards across the 5 QC replicates were also below 5 ppm. PCA clustering of injection QCs has been used as an indicator of consistency and lack of drift e.g., retention time shift, change in peak intensity, in non-target and suspect screening (Masson et al. 2010, Perez-Miguez et al. 2018, von Eyken et al. 2020). All the QC samples clustered together (Figure S4.5), which reflects a low variability introduced by the instrumental analysis.

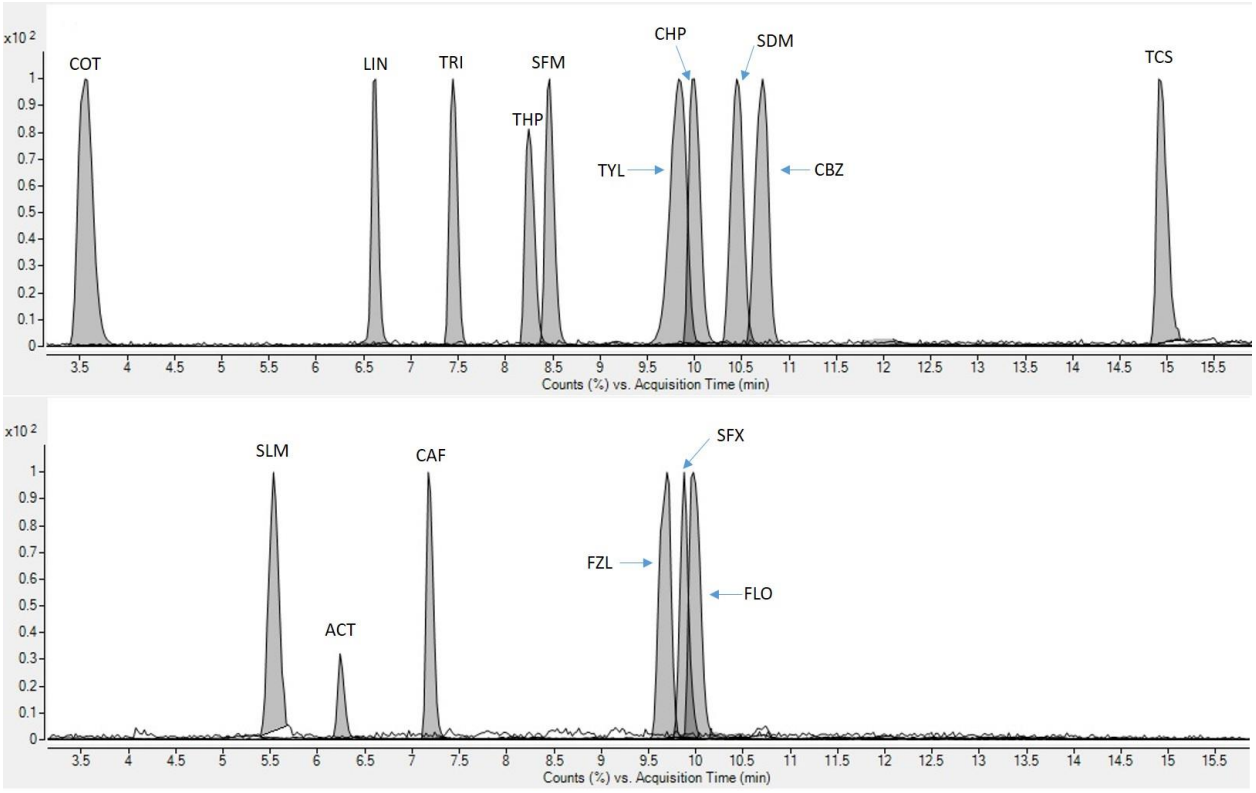

Figure 4.1: Extracted ion chromatograms (± 20 ppm extraction window) for the targeted compounds in spiked liver extract **(**0.100 µg g^{-1}**)**

4.4.2 Targeted analysis

Two compounds out of the 16 targets were detected across the 77 fish livers (Table S4.4). Lincomycin was detected in 15 out of 77 livers at an average concentration of 40.4 ng g^{-1} in both downstream and upstream samples, across different fish species. The highest concentration, 114.1 ng g^{-1}, was found in a Common Carp. Lincomycin has been previously reported in wastewater at 100% frequency with concentrations between 39-75 ng L^{-1} (Biel-Maeso et al. 2018) and in fish livers with a mean concentration of 22.4 ng g^{-1} (Zhao et al. 2015). Sulfamethoxazole was detected in three fish livers, Gizzard Shad (*Dorosoma cepedianum),* Golden Redhorse (*Moxostoma erythrurum),* and Northern Hogsucker (*Hypentelium nigricans*) at concentrations of 15.7, 19.4 and 41.7 ng g^{-1}, all collected upstream. Sulfamethoxazole has been reported previously in Argentina in the livers of catfish (*Hypostomus commersoni)* and Streaked Prochilods (*Prochilodus lineatus)*) collected from rivers receiving untreated wastewater within a range of 0.94-2.40 ng g^{-1} (Ondarza et al. 2019). Sulfonamide antibiotics, including sulfamethoxazole and their N^4-acetyl metabolites have been detected in wastewater effluents (Botero-Coy et al. 2018, Cui et al. 2020). A manual screening of all samples was run on N^4-acetylsulfamethoxazole (*m/z* 296.0699), N^4-acetylsulfadimethoxine (m/z 353.0919), N^4-acetylsulfamethazine (*m/z* 321.1021), N^4-acetylsulfathiazole (*m/z* 298.0314), N^4-acetylsulfadiazine (*m/z* 293.0702), some of which have been previously detected in water samples (Yuan et al. 2019, Cui et al. 2020). No discernable peaks were observed in the extracted ion chromatograms at the above specific *m/z* (mass window ±20 ppm) of any of these compounds. Carbamazepine is another compound often detected in wastewater samples (Biel-Maeso et al. 2018) and can undergo metabolism by fish to carbamazepine-10,11-epoxide (CBZ-EP) and 2-hydroxycarbamazepine, with the latter accumulating in brain and liver tissues and

CBZ-EP accumulating in muscle (Valdes et al. 2016). Carbamazepine-10,11-epoxide was part of the *Water Screening PCDL* but it did not match any features in MPP.

4.4.3 General screening of PPCPs in fish livers

The molecular feature extraction using Profinder resulted in 118,246 features in positive mode and 23,406 features in negative mode. Compounds for which matches were found through a search of the two Agilent database are listed in Table S4.5. Following further MS/MS analysis, as per Section *2.5.2.1,* the list of suspect compounds was narrowed to ten (Table S4.5). The identity of two of these compounds, azithromycin (Figure 4.2) and erythrohydrobupropion (which if further discussed in section *4.4.4.2*) was confirmed with analytical standards. Azithromycin was detected in 24 fish livers with a range of 5.0-169.8 ng g^{-1}, in both downstream and upstream liver samples. This antibiotic has been frequently detected in wastewater samples (Rodriguez-Mozaz et al. 2020). In biota, azithromycin has been reported in bivalves (*Mytilus spp.)* (Alvarez-Munoz et al. 2015) and in livers of marine species such as So-iuy Mullet (*Liza haematocheilus*), Sea Perch (*Lateolabrax maculatus*), and Dotted Gizzard Shad (*Konosirus punctatus*) with a maximum concentration of 56 ng g^{-1} (Liu et al. 2018b). In the present study, 15 of the 24 samples where azithromycin was detected were from benthic feeders: e.g., Black Redhorse, Common Carp. The highest concentration, 169.8 ng g^{-1} was found in a Golden Redhorse. It has been shown that some pharmaceuticals accumulate at higher levels in benthic compared to pelagic fish (Du et al. 2016). Due to their chemical properties, e.g., hydrophobicity, pharmaceuticals like antibiotics, including azithromycin can bind to sediments (Fernandes et al. 2020), where they can become available to benthic organisms (Grabicova et al. 2015) and this

could also be another possibility as to the higher detection of the antibiotic in benthic fish in this study.

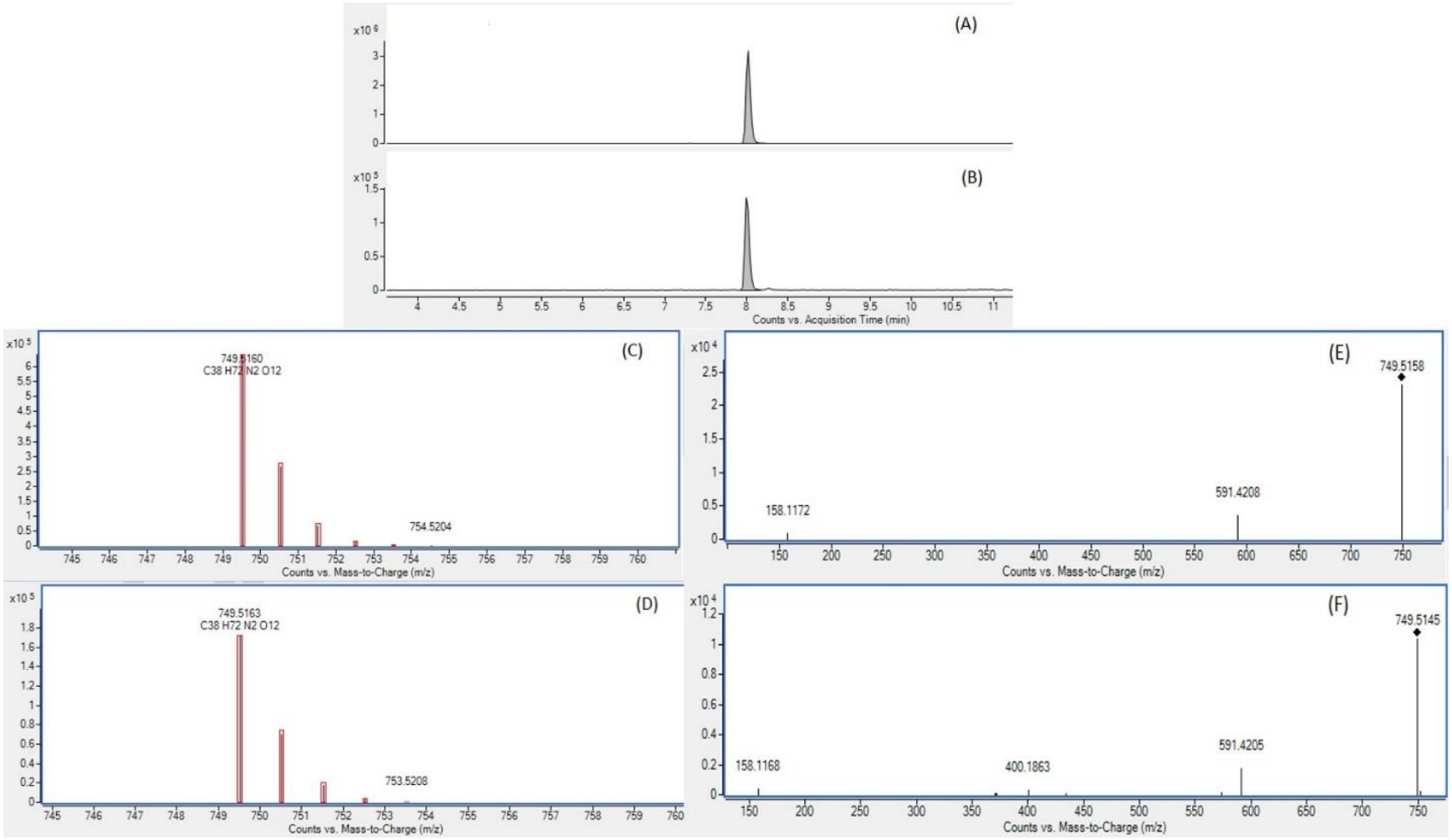

Figure 4.2: Extracted ion chromatograms for azithromycin *m/z* 749.5160 (±20 ppm) in (A) standard and (B) liver, isotope distribution pattern in (C) standard and (D) liver, MS/MS spectra in (E) standard and (F) liver

4.4.4 Statistical analysis

4.4.4.1 Clustering and principal component analysis

To identify patterns or groupings among samples and other variables (e.g., pharmaceutical concentrations), chemometric tools such as clustering, correlation, and principal component analysis can be applied (Santos et al. 2009, Al-Odaini et al. 2012). For example, Al-Odaini et al. (2012) applied clustering analysis to find similarities between different river sampling stations with formed clusters indicative of different pollution loads. Clustering analysis on all features, grouped based on the sampling location, i.e., downstream and upstream of WWTPs (Figures 4.3, S4.2) did not indicate two clear clusters between downstream and upstream samples, either in positive or negative ionization mode. However, some smaller clusters were formed, especially for positive ionization mode (Figure 4.3). Although fish were collected upstream and downstream of the wastewater plant, there is no physical barrier that would constrict the movement of fish between sampling locations. Thus, there is the possibility that fish caught downstream also traveled upstream and vice-versa. For example, in a tracking study in Australia, Common Carp were generally recaptured within a 5 km distance from their release site but some individuals traveled up to 203 km over a period over 400 days (Stuart and Jones 2006). Moreover, exposure to chemical contaminants, even at sub-lethal levels, may trigger spatial avoidance responses in some aquatic species (Araújo et al. 2016) that may explain, along with the absence of a physical barrier, the lack of distinctive grouping between downstream and upstream fish samples. A similar pattern was observed when samples were grouped based on foraging group (benthic vs. water column feeders), with small clusters observed (Figures S4.3 and S4.4). In this case, this pattern indicates that different fish species may be exposed to different levels of pollutants.

PCAs were first built based on all features to assess variability and possible grouping between samples based on the parameters in the study, i.e., sampling location (downstream or upstream) and the foraging group. Based on the PCA plot for the first 3 principal components, there was no clear clustering of the samples based on the sampling location (Figures S4.5 and S4.6). PCA was also performed without QC and blank samples, with no clear grouping between upstream and downstream samples (Figure S4.7 and S4.8). Features extracted from the livers included not only possible chemical contaminants but also endogenous metabolites, which may explain why the variability among samples did not appear to be predominantly driven by spatial differences, i.e., downstream vs. upstream. Although metabolite identification was not within the scope of the current study, some compounds matched (score > 70, mass tolerance < 5 ppm) through the Agilent *Metlin PCDL* (30322 compounds) are listed in Table S4.6. Other factors such as sex, differences in metabolic profiles, and habitat variability can potentially influence the chemical profiles (Ekman et al. 2015, Tsentalovich et al. 2019). For example, distinct groupings were found between male and female Fathead Minnows (*Pimephales promelas*) based on metabolites identified in skin mucus (Ekman et al. 2015).

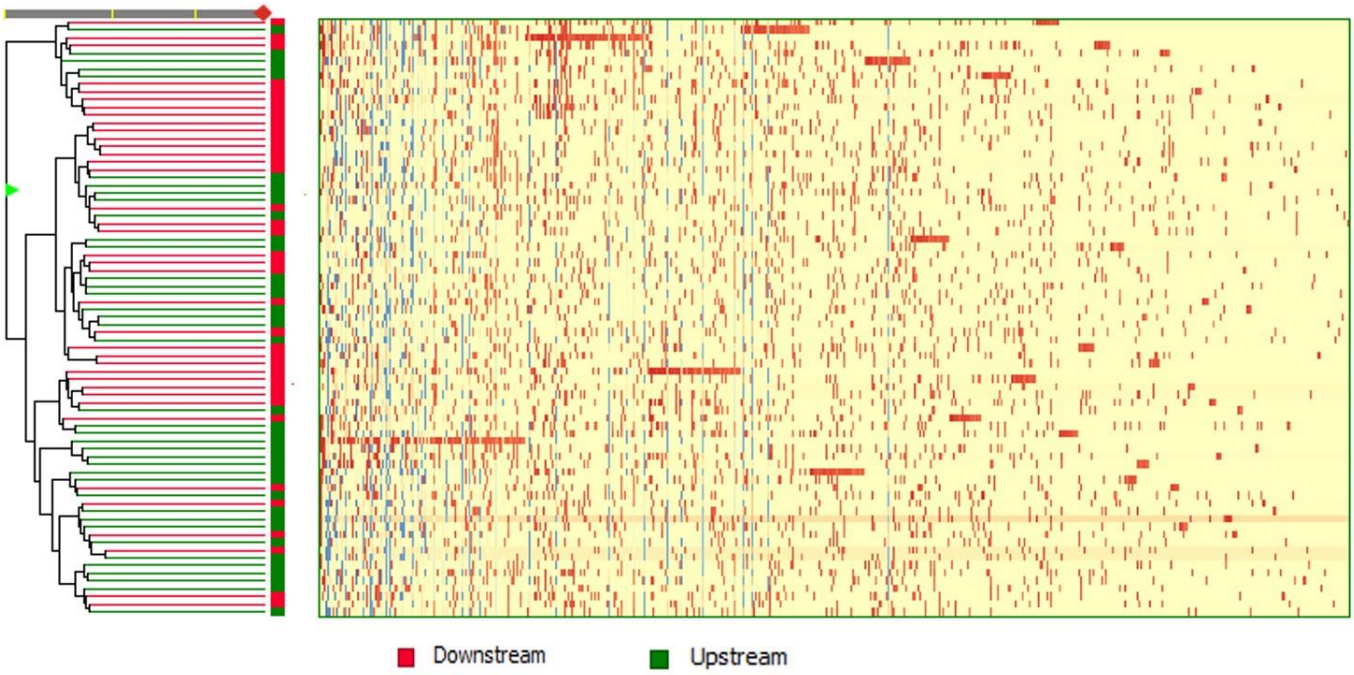

Figure 4.3: Clustering analysis of all features grouped based on downstream and upstream samples (positive ionization mode).

A PCA performed on all features filtered by foraging group also showed grouping between bottom-feeding and water-column feeding fish (Figures 4.4, S4.8-S4.10). Some pharmaceuticals may also be detected in sediments (Fernandes et al. 2020) through which benthic species may be exposed. Therefore, exposure to a similar habitat could impact the bioaccumulation of pharmaceuticals. To investigate this, PCA analysis was next performed based on the signals for molecular features putatively identified through the *Water Screening PCDL* only. All samples were screened with Profinder software using the *Batch Targeted Feature Extraction* (see Table S4.7 for data processing parameters). In total, 641 and 1,146 features were putatively identified as water contaminants in positive and negative modes, respectively. Features were then exported in MPP for PCA (Figures S4.11 and S4.12). The different foraging groups still clustered based on the features identified through the library

search. Although the identity of compounds obtained through the Profinder database search should be further confirmed, the grouping between the different foraging types indicated different contamination patterns among fishes, with the possibility of some compounds being used as markers of contamination between species.

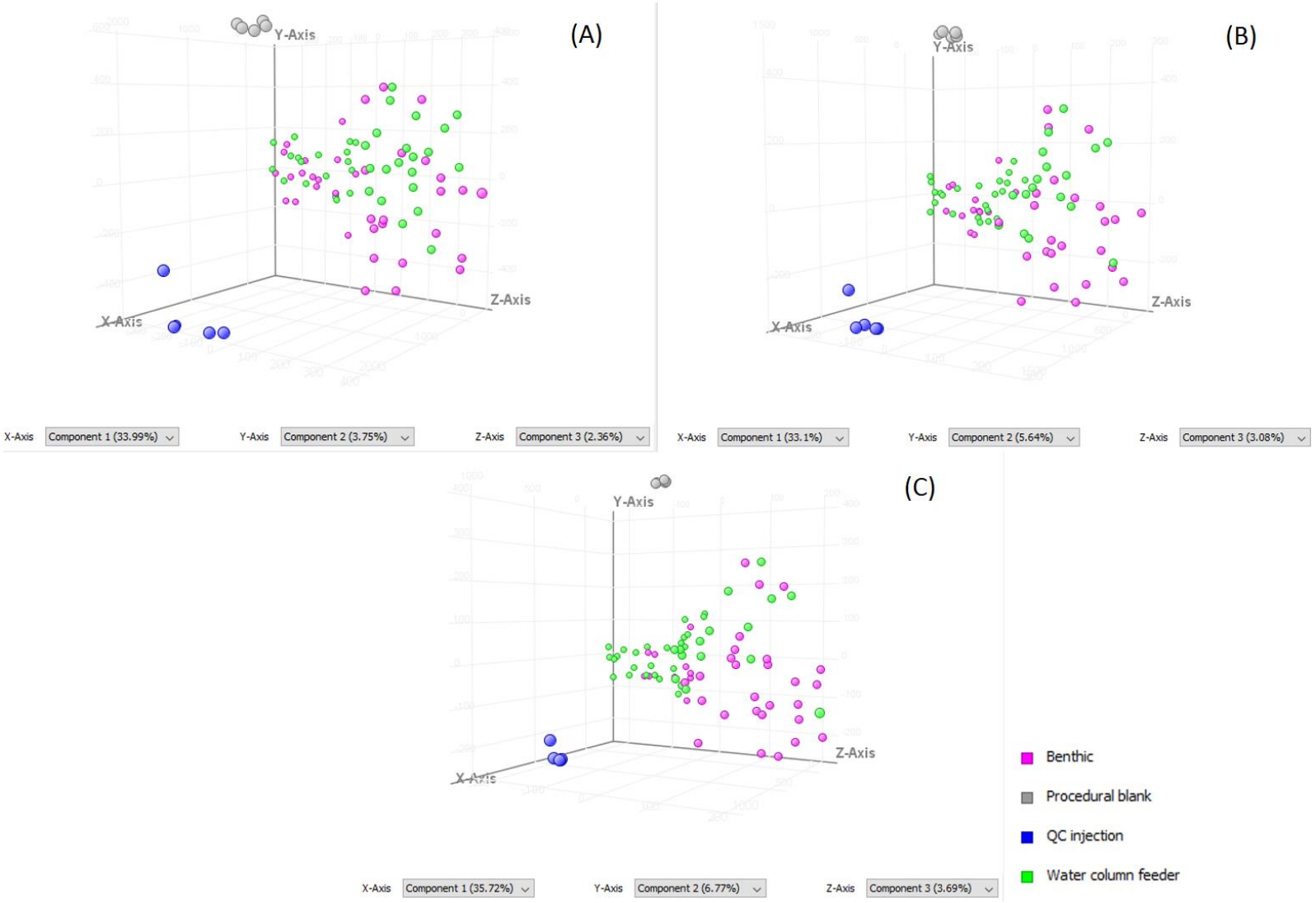

Figure 4.4: PCA on all entities (positive ionization mode) based on foraging group with molecular features present in (A) 40%, (B) 70%, or (C) 100% of samples.

4.4.4.2 Comparison between groups

The results of various volcano plot analyses are presented in Table S4.8. Fifteen compounds, including azithromycin, lovastatin, and lidocaine – potentially identified in the *Water Screening* and *Veterinary Drug PCDL* – were significantly higher in the liver of fish sampled downstream. Erythrohydrobupropion, for instance, exhibited a significant upstream-downstream difference (p = *6.19E-5*, log FC = 7.43). This compound was subsequently confirmed with a pure standard (Figure S4.13). It was detected in 43 out of 77 livers (12 upstream and 31 downstream samples) and across the two different foraging groups, with a mean concentration of 13.8±12.9 ng g^{-1}. Based on a two-way ANOVA, there was no difference between the two foraging groups and sampling locations (p = 0.858).

Erythrohydrobupropion, a metabolite of bupropion and an anti-depressant, has been detected at concentrations above 1000 ng L^{-1} in wastewater effluent (Writer et al. 2013). The parent compound, bupropion has been detected between 0.3 and 1.0 ng g^{-1} (dry weight) in Small (*Micropterus dolomieu*) and Largemouth (*Micropterus salmoides*) Bass livers sampled from the Niagara River (Arnnok et al. 2017) as well as White Sucker (*Catostomus commersonii*) brains collected from wastewater impacted streams in the United States (Schultz et al. 2010), thus reflecting assimilation in both benthic and water-column species.

It should be noted that the parent compound, bupropion, is also present in the *Water Screening PCDL* library, but no feature was identified as the parent compound. In an exposure study, bupropion both on its own and in a mixture of anti-depressants was shown to affect the predator behaviour of Fathead Minnows (*Pimephales promelas*) (Painter et al. 2009); further studies are needed to assess adverse effects on fish associated with the presence of its metabolite.

4.5 Conclusion

QuEChERS extraction was successfully applied to fish livers for the simultaneous target and suspect screening of pharmaceuticals and other PPCPs. Although the current method performed well in terms of recovery and precision of 16 target PPCPs it may be further tested, and consequently adapted, for the extraction of other PPCPs, e.g., antidepressants, β-blockers and antibiotics at different concentrations. Overall, lincomycin, sulfamethoxazole, azithromycin and erythrohydrobupropion were identified in fish livers based on target and suspect screening analysis.

In addition to allowing for the identification of a various contaminants, suspect screening may be useful in identifying possible patterns amongst different groups, as it has been shown in this study through cluster and principal component analysis where grouping by fish foraging group was observed based on all compounds as well as contaminants putatively identified through database searches. These findings imply that fish foraging behavior is likely related to bioaccumulation of pharmaceuticals in fish. Water and sediment sample analysis could be included in future research in order to obtain an improved representation of the chemical exposure of different foraging species. Owing to its advantages, suspect screening can be an important tool in ecological risk assessments.

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4.7 Supplementary information

Table S4.1: Sampling station, common and scientific names for sampled fishes, location of sampling stations relative to nearest wastewater treatment plant (WWTP), fish foraging group, and pharmaceutical concentrations in fish livers. *LIN-lincomycin, AZ-azithromycin, EHB-erythrohydrobupropion, SFX-sulfamethoxazole. Each row represents an individual fish. Empty columns represent Not Detected.

Sampling station	Location WWTP	Common Name	Scientific Name	Foraging Group	Concentrations (ng g^{-1} fresh weight)			
					LIN	AZ	EHB	SFX
26	upstream	Black Crappie	*Pomoxis nigromaculatus*	water-column feeder	10.1	ND	ND	ND
21	downstream	Black Crappie	*Pomoxis nigromaculatus*	water-column feeder	ND	ND	ND	ND
3	upstream	Black Crappie	*Pomoxis nigromaculatus*	water-column feeder	ND	ND	ND	ND
10	upstream	Black Redhorse	*Moxostoma duquesni*	benthic feeder	ND	38.7	3.6	ND
15	upstream	Black Redhorse	*Moxostoma duquesni*	benthic feeder	22.0	ND	ND	ND
10	upstream	Black Redhorse	*Moxostoma duquesni*	benthic feeder	ND	47.9	41.4	ND
5	upstream	Black Redhorse	*Moxostoma duquesni*	benthic feeder	ND	21.5	20.5	ND
9	upstream	Bluegill	*Lepomis macrochirus*	water-column feeder	ND	5.7	ND	ND
9	upstream	Bluegill	*Lepomis macrochirus*	water-column feeder	20.6	ND	ND	ND
14	upstream	Common Carp	*Cyprinus carpio*	benthic feeder	114.1	5.0	7.2	ND
8	upstream	Common Carp	*Cyprinus carpio*	benthic feeder	ND	ND	ND	ND

8	downstream	Common Carp	*Cyprinus carpio*	benthic feeder	ND	ND	5.7	ND
15	downstream	Flathead Catfish	*Pylodictis olivaris*	water-column feeder	ND	12.2	22.9	ND
12	downstream	Freshwater Drum	*Aplodinotus grunniens*	benthic feeder	ND	32.8	4.0	ND
22	downstream	Freshwater Drum	*Aplodinotus grunniens*	benthic feeder	35.0	21.6	ND	ND
18	downstream	Freshwater Drum	*Aplodinotus grunniens*	benthic feeder	ND	7.6	ND	ND
9	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	15.7
9	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	ND
9	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	ND
9	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	ND
10	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	79.5	14.9	ND
15	downstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	12.3	15.9	ND
16	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	15.7	ND
16	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	12.1	ND
9	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	ND
6	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	4.8	ND
6	upstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	9.9	3.2	ND

3	downstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	ND
26	downstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	ND	ND
12	downstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	11.5	14.6	ND
19	downstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	10.6	3.6	ND
19	downstream	Gizzard Shad	*Dorosoma cepedianum*	water-column feeder	ND	ND	5.4	ND
25	upstream	Golden Redhorse	*Moxostoma erythrurum*	benthic feeder	ND	ND	ND	19.4
23	downstream	Golden Redhorse	*Moxostoma erythrurum*	benthic feeder	ND	ND	5.2	ND
9	upstream	Golden Redhorse	*Moxostoma erythrurum*	benthic feeder	ND	ND	ND	ND
26	upstream	Golden Redhorse	*Moxostoma erythrurum*	benthic feeder	ND	ND	ND	ND
11	downstream	Golden Redhorse	*Moxostoma erythrurum*	benthic feeder	ND	169.8	28.7	ND
24	upstream	Hybrid White × Striped Bass	*Morone chrysops* × *Morone saxatilis*	water-column feeder	ND	ND	ND	ND
242	upstream	Hybrid White × Striped Bass	*Morone chrysops* × *Morone saxatilis*	water-column feeder	ND	ND	ND	ND
2	upstream	Largemouth Bass	*Micropterus salmoides*	water-column feeder	ND	ND	ND	ND
14	downstream	Mooneye	*Hiodontidae*	water-column feeder	ND	7.9	8.0	ND
23	upstream	Northern Hogsucker	*Hypentelium nigricans*	benthic feeder	ND	ND	2.5	41.7

4	upstream	Northern Hogsucker	*Hypentelium nigricans*	benthic feeder	ND	ND	14.9	ND
5	upstream	Northern Hogsucker	*Hypentelium nigricans*	benthic feeder	22.7	18.6	24.5	ND
13	downstream	Quillback Carpsucker	*Carpiodes cyprinus*	benthic feeder	ND	17.5	19.6	ND
11	downstream	Quillback Carpsucker	*Carpiodes cyprinus*	benthic feeder	9.1	63.6	15.3	ND
11	downstream	River Carpsucker	*Carpiodes carpio*	benthic feeder	ND	ND	6.3	ND
12	downstream	River Carpsucker	*Carpiodes carpio*	benthic feeder	ND	ND	73.7	ND
14	downstream	River Carpsucker	*Carpiodes carpio*	benthic feeder	ND	ND	9.2	ND
18	downstream	River Carpsucker	*Carpiodes carpio*	benthic feeder	ND	ND	30.6	ND
19	downstream	River Carpsucker	*Carpiodes carpio*	benthic feeder	ND	ND	30.4	ND
18	downstream	River Carpsucker	*Carpiodes carpio*	benthic feeder	ND	ND	3.7	ND
17	downstream	Sauger	*Sander canadensis*	water-column feeder	18.7	ND	9.2	ND
11	downstream	Saugeye	*Sander canadensis* × *Sander vitreus*	water-column feeder	79.6	ND	ND	ND
20	downstream	Saugeye	*Sander canadensis* × *Sander vitreus*	water-column feeder	ND	16.4	6.2	ND
20	downstream	Silver Redhorse	*Moxostoma anisurum*	benthic feeder	ND	ND	7.8	ND
9	upstream	Smallmouth Bass	*Micropterus dolomieu*	water-column feeder	22.6	ND	ND	ND

9	upstream	Smallmouth Bass	*Micropterus dolomieu*	water-column feeder	ND	ND	ND	ND
9	upstream	Smallmouth Bass	*Micropterus dolomieu*	water-column feeder	ND	ND	ND	ND
26	upstream	Smallmouth Bass	*Micropterus dolomieu*	water-column feeder	8.2	ND	ND	ND
10	upstream	Smallmouth Bass	*Micropterus dolomieu*	water-column feeder	ND	ND	28.5	ND
24	upstream	Smallmouth Bass	*Micropterus dolomieu*	water-column feeder	ND	ND		ND
21	downstream	Smallmouth Buffalo	*Ictiobus bubalus*	benthic feeder	ND	ND	3.2	ND
13	downstream	Smallmouth Redhorse	*Moxostoma breviceps*	benthic feeder	ND	20.7	3.8	ND
15	downstream	Smallmouth Redhorse	*Moxostoma breviceps*	benthic feeder	7.0	31.1	6.4	ND
20	downstream	Smallmouth Redhorse	*Moxostoma breviceps*	benthic feeder	ND	7.6	9.3	ND
12	downstream	Smallmouth Redhorse	*Moxostoma breviceps*	benthic feeder	ND	64.5	12.3	ND
20	downstream	Smallmouth Redhorse	*Moxostoma breviceps*	benthic feeder	ND	ND	8.2	ND
1	upstream	Spotted Sucker	*Minytrema melanops*	benthic feeder	53.2	ND	ND	ND
1	upstream	Spotted Sucker	*Minytrema melanops*	benthic feeder	ND	ND	ND	ND
1	upstream	Spotted Sucker	*Minytrema melanops*	benthic feeder	ND	ND	ND	ND
18	downstream	White Bass	*Morone chrysops*	water-column feeder	8.1	ND	12.6	ND
19	downstream	White Bass	*Morone chrysops*	water-column feeder	22.9	ND	10.8	ND

17	downstream	White Bass	*Morone chrysops*	water-column feeder	ND	ND	ND	ND
22	downstream	White Bass	*Morone chrysops*	water-column feeder	ND	ND	19.4	ND
21	downstream	White Crappie	*Pomoxis annularis*	benthic	ND	ND	2.8	ND
25	upstream	White Crappie	*Pomoxis annularis*	benthic	ND	ND	ND	ND

Table S4.2: Average recoveries, matrix effect, and interday precision (n = 3) for targeted PPCP compounds in fish livers

Compound	log P	Ionization mode	Theoretical m/z	Linearity		Average matrix effect			Average recovery		
						Smallmouth Buffalo	Common Carp	Black Redhorse	Smallmouth Buffalo	Common Carp	Black Redhorse
Acetaminophen	0.46	Positive	152.0711	0.99749		129±43	131±36	114±24	107±6	105±5	102±2
					RSD (%)	33.3	27.7	21.1	5.8	4.1	2.4
Caffeine	-0.07	Positive	195.0882	0.99801		122±40	110±20	111±19	103±4	108±5	102±4
					RSD (%)	33.2	18.2	17.5	4.2	4.5	4.2
Carbamazepine	2.45	Positive	237.1028	0.99823		176±35	159±48	156±12	110±4	108±6	100±3
					RSD (%)	20.1	29.6	8.3	4.0	5.6	3.2
Cotinine	0.07	Positive	177.1028	0.99657		119±46	100±17	101±34	106±5	106±4	102±2
					RSD (%)	39.2	17.2	33.9	4.7	4.1	2.1
Trimethoprim	0.91	Positive	291.1457	0.99878		131±27	142±32	121±29	107±6	106±6	104±3
					RSD (%)	35.8	21.9	24.5	5.0	5.1	2.9
Furazolidone	0.04	Positive	226.0464	0.99481		137±34	123±26	165±46	115±11	122±8	103±21
					RSD (%)	25.0	21.1	27.8	9.8	7.2	21.1
Lincomycin	0.2	Positive	407.2216	0.99036		114±11	116±34	116±23	77±6	76±7	67±5
					RSD (%)	9.8	29.8	20.3	7.7	10.1	6.7
Sulfadimethoxine	1.63	Positive	311.0814	0.99484		140±31	144±36	116±11	119±11	129±9	120±8
					RSD (%)	22.1	25.2	10.1	9.6	6.6	6.7
Sulfamethazine	0.14	Positive	279.0916	0.99414		128±25	126±19	111±9	124±10	113±11	117±6
					RSD (%)	20.1	15.6	8.2	8.1	8.1	4.9
Sulfamethoxazole	0.89	Positive	254.0599	0.99502		270±88	194±50	190±44	92±10	114±18	92±4
					RSD (%)	32.5	25.9	23.1	11.4	16.5	3.8
Sulfanilamide	0.62	Positive	173.0385	0.99544		90±27	75±5	106±25	108±11	96±4	103±3
					RSD (%)	30.4	9.1	24.0	10.1	9.7	2.9
Tylosin A	1.63	Positive	916.5269	0.99577		139±27	126±13	116±9	90±16	111±10	96±8
					RSD (%)	19.7	10.9	7.9	20.5	8.9	9.1
Triclosan	4.76	Negative	286.9433	0.99805		76±16	105±11	123±2	104±13	103±3	100±5
					RSD (%)	22.2	11.1	1.9	13.2	2.8	4.7

Chloramphenicol	1.14	Negative	321.0045	0.99280		142±32	128±23	148±34	124±15	101±12	106±9
					RSD (%)	22.5	17.5	23.1	11.5	11.8	8.3
Florfenicol	0.36	Negative	355.9926	0.99660		138±24	120±21	127±15	114±5	90±10	106±18
					RSD (%)	24.6	17.7	12.0	4.4	11.4	17.1
Thiamphenicol	0.27	Negative	353.9969	0.99387		136±27	129±22	122±7	115±17	97±10	107±12
					RSD (%)	20.0	17.2	6.1	14.5	10.9	10.6

Table S4.3: MDLs and LOQs for targeted compounds in fish livers.

Compound	MDL (ng g^{-1})	LOQ (ng g^{-1})
Acetaminophen	1.9	6.6
Caffeine	1.1	3.9
Carbamazepine	0.6	1.9
Cotinine	1.9	6.4
Trimethoprim	1.1	3.4
Lincomycin	0.4	1.2
Furazolidone	0.2	0.6
Sulfadimethoxine	2.1	7.0
Sulfamethazine	0.5	1.7
Sulfamethoxazole	1.1	3.9
Sulfanilamide	1.5	4.9
Tylosin A	0.1	0.5
Thiamphenicol	1.7	5.7
Chloramphenicol	1.1	3.9
Florfenicol	0.7	2.4
Triclosan	2.6	8.8

Table S4.4: Mass measurement errors for targeted compounds in solvent and spiked livers.

Compound	Mass measurement error in solvent (ppm)	Mass measurement error in liver extract (ppm)
Acetaminophen	-3.94±1.38	-3.43±0.64
Caffeine	-2.73±0.41	-3.76±0.36
Carbamazepine	-2.18±0.56	-2.91±0.74
Cotinine	-2.82±0.87	-3.19±0.28
Trimethoprim	-0.51±0.29	-0.57±0.34
Furazolidone	2.65±0.68	3.44±1.59
Lincomycin	-0.49±0.22	-0.74±0.30
Sulfadimethoxine	-2.04±0.78	-2.36±0.64
Sulfamethazine	-1.91±0.67	-2.11±0.42
Sulfamethoxazole	-0.72±0.63	-0.83±0.41
Sulfanilamide	-2.50±1.14	-0.71±1.82
Tylosin A	0.56±0.66	0.39±0.29
Triclosan	1.45±0.91	0.97±0.45
Chloramphenicol	2.39±0.87	1.45±0.66
Florfenicol	2.10±1.13	0.84±0.65
Thiamphenicol	2.26±0.78	0.99±0.67

Table S4.5: General screening of PPCPs in fish livers

Compound match in MPP	Ionization mode	*m/z*	mass	Retention time (min)	Compound match in MSC	Matching score in MSC	Mass difference (ppm)	Statistically significant	
								Up-regulated* Downstream vs. upstream	Up-regulated* Benthic vs. water column
Lidocaine	Positive	235.1804	234.1728	7.8	no			√	
Lovastatin	Positive	405.2611	404.2536	14.4	no			√	√
Viloxazine	Positive	260.1258	237.1365	7.8	no				
Rimiterol	Positive	224.1278	223.1206	7.3	no				
Fosinopril	Positive	564.3063	563.2990	12.2	no				
JWH-133	Positive	330.2791	312.2453	11.8	no			√	
Benzocaine	Positive	166.0862	165.0790	5.6	no				
Erythrohydrobupropion	Positive	242.1309	241.1233	9.1	yes	77.2	-3.7	√	
Azithromycin	Positive	749.5152	748.5076	7.9	yes	79.9	-0.7	√	√
Tolycaine	Positive	279.1703	278.1630	7.8	yes	77.5	-1.0		
Tulathromycin	Positive	806.5742	805.5625	17.0	no				
Fluoxetine	Positive	310.1420	309.1343	5.0	no				
Norcitalopram	Positive	311.1554	310.1483	10.0	no			√	√
Warfarin	Positive	309.1107	308.1049	11.8	no				√
Penicillin V	Positive	351.0994	350.0922	15.2	no				
Difloxacin	Positive	400.1478	399.1401	7.0	no				√
Simvastatin	Positive	419.2767	418.2701	15.1	no				
Scopolamine	Positive	321.1802	303.1466	15.3	no			√	
Allopurinol	Positive	137.0459	136.0387	4.0	no				
Atenolol	Positive	267.1676	266.1630	4.0	no				
Benomyl	Positive	291.1457	290.1378	7.5	yes	78.6	-3.5		
Metoprolol	Positive	267.1898	267.1834	8.1	no			√	
Oxycodone	Positive	333.1797	315.1471	15.4	no			√	
17β-estradiol	Positive	273.1555	272.1776	15.3	yes	64.5	-2.2	√	

Methyltestosterone	Positive	303.2322	302.2246	15.8	yes	60.2	-1.1		√
Butralin	Positive	296.1583	295.1532	5.5	yes	69.4	7.4	√	√
Camphor	Negative	151.1127	152.1199	11.8	yes	69.7	3.6	√	√
Z,E, 9,12-Tetradecadienyl acetate	Negative	251.2016	252.2089	13.3	yes	72.5	-8.1		
Homosalate	Negative	261.1497	262.1571	12.0	no				
Indole	Negative	116.0506	117.0577	6.2	yes	77.4	0.6	√	
Mephenesin	Negative	281.1421	236.1439	9.0	no			√	
16-α-methylprogesterone	Negative	327.2330	328.2402	14.2	no				
5-α-adrost-16-en3-one	Negative	391.2128	346.2145	8.3	no				

Table S4.6: General screening of metabolites in fish livers.

Compound match in MPP	Ionization mode	*m/z*	mass	Retention time (min)	Matching score
24R,25-dihydroxyvitamin D3	positive	417.3365	416.3292	9.8	99.8
N-palmitoylsphingosine	positive	538.5196	537.5132	18.8	97.8
10-Eicosene	positive	298.3468	280.3131	15.7	99.6
Glutaminyl-Isoleucine	positive	284.1427	259.1535	6.3	99.6
Alanyl-Isoleucine	positive	203.1389	202.1317	5.5	99.7
Stearamide	positive	284.2948	283.2875	16.6	99.4
N-Feruloylglycyl-L-phenylalanine	positive	416.1810	398.1475	11.6	88.9
Sterol-3-beta-D-glucoside	positive	428.3001	410.2665	10.8	94.9
Isoleucyl-phenylalanine	positive	279.1706	278.1630	8.1	99.8
Lyso-PE (0:0/20:5(5Z,8Z,11Z,14Z,17Z))	positive	500.2770	499.4695	12.1	98.7
20-COOH-Leukotriene B4	positive	382.2377	366.2043	10.6	98.4
Tetracosahexaenoic acid	positive	357.2786	356.2713	12.4	99.8
3,5-didecanoylpyridine	positive	387.3138	388.3211	14.1	99.9
Methionyl-Tryptophan	positive	336.1391	335.1317	7.2	88.4
Isoleucyl-Valine	positive	231.1704	230.1631	5.8	97.9
Cer(d18:1/18:11 (11Z))	positive	562.5351	569.5279	18.9	97.1
MG(0:0/18:2(9Z, 12Z)/0:0	positive	355.2842	354.2776	15.4	97.8
Threoninyl-Threonine	positive	221.1150	220.1078	10.8	83.0
Dihydrozeatin riboside	positive	354.1791	353.1719	9.4	89.1
5β Cyprinol sulfate	positive	555.2967	532.3074	9.8	99.0
Coprocholic acid	negative	450.3341	449.3270	10.1	98.8
Taurallocholic acid	negative	514.2844	595.1915	9.1	99.4
Tauroursodeoxycholic acid	negative	997.5861	499.2968	9.7	99.6
Chenodeoxycholic acid	negative	391.2853	392.2926	10.0	99.7

Table S4.7: Profinder data alignment parameters for *Targeted feature extraction* of liver samples based on *Water Screening PCDL.*

Isotope model	Common organic molecules
Accepted adducts	[H+] in positive, [H-] in negative
Match mass tolerance	±10 ppm
Expansion of values for chromatogram extraction	±35.0 ppm
Isotope abundance score	60.0
Absolute height	200 counts
Spectra to include	average scans>10% of peak height
Compound score absolute height	1000 counts
Compound score	70.0 and do not match if score<70.0

Table S4.8: Number of statistically significant features between the sampling locations and foraging groups and features matched in the databases

Grouping	Ionization mode	Sample frequency	Number of down-regulated compounds	Number of up- regulated compounds	Number of database matches with a score >70%	
					PCDL *Water screening*	*PCDL* Veterinary drugs
Location	Positive					
Downstream vs upstream		40%	468	1502	40	55
		70%	197	710	39	45
		100%	92	410	28	28
Foraging group	Positive					
Benthic vs. water column feeder		40%	2281	2053	99	100
		70%	920	1204	76	90
		100%	511	776	57	88
Location	Negative					
Downstream vs upstream		40%	216	233	21	20
		70%	118	145	13	11
		100%	68	88	10	6
Foraging group	Negative					
Benthic vs. water column feeder		40%	742	1253	59	64
		70%	455	810	48	50
		100%	293	522	36	36

Note: down-regulated compounds are present at statistically significant higher levels in upstream compared downstream samples or water-column feeders compared to benthic sample

Figure S4.1: Fish sampling locations (i.e., stations) in the Scioto River basin of Ohio, U.S.A. Sample sites were distributed upstream and downstream of two wastewater treatment plants near Columbus, Ohio

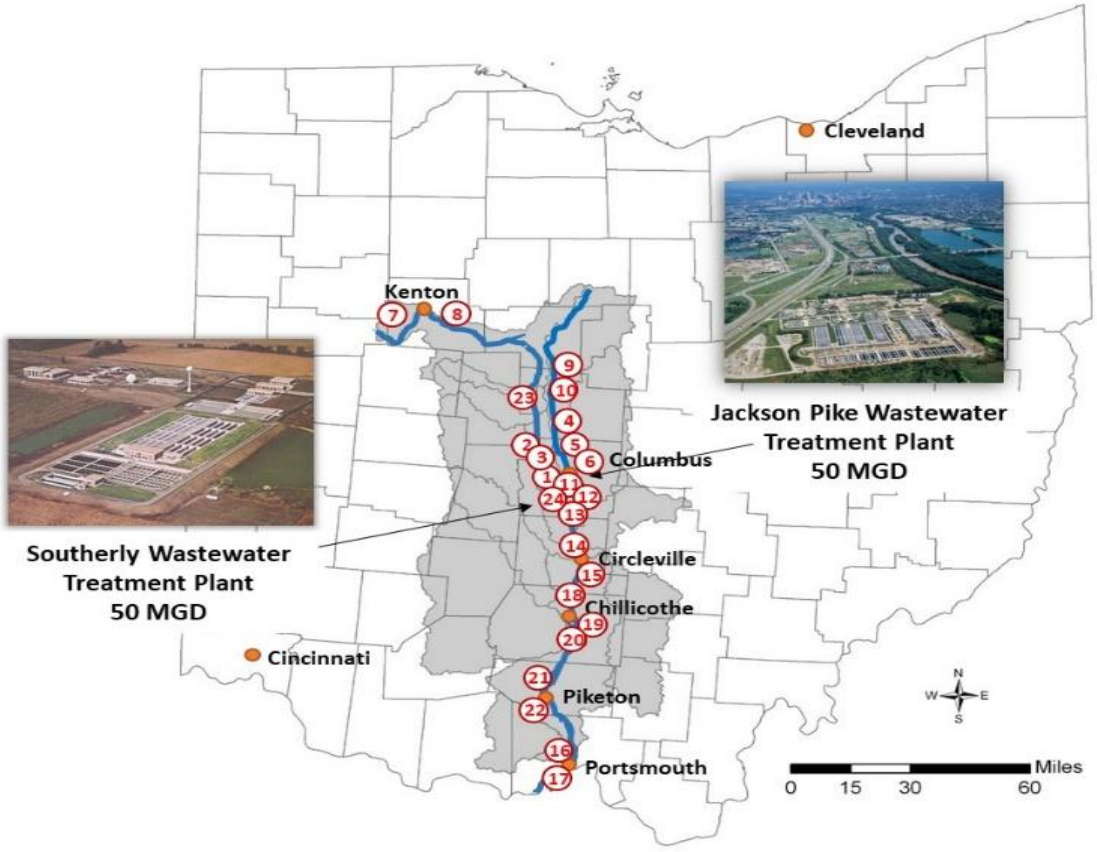


Figure S4.2: Clustering analysis of all features grouped based on downstream and upstream samples (negative ionization mode)

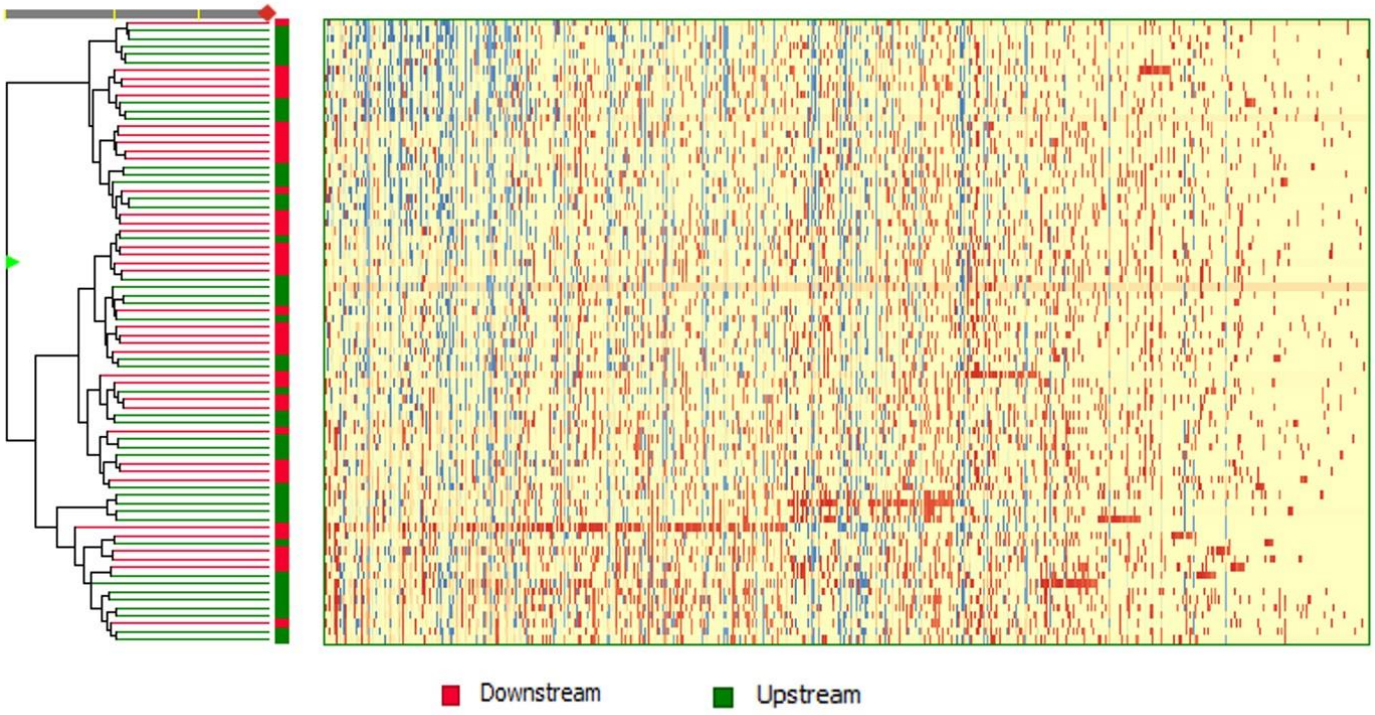


Figure S4.3: Clustering analysis of all features grouped based on benthic and water column feeders (positive ionization mode)

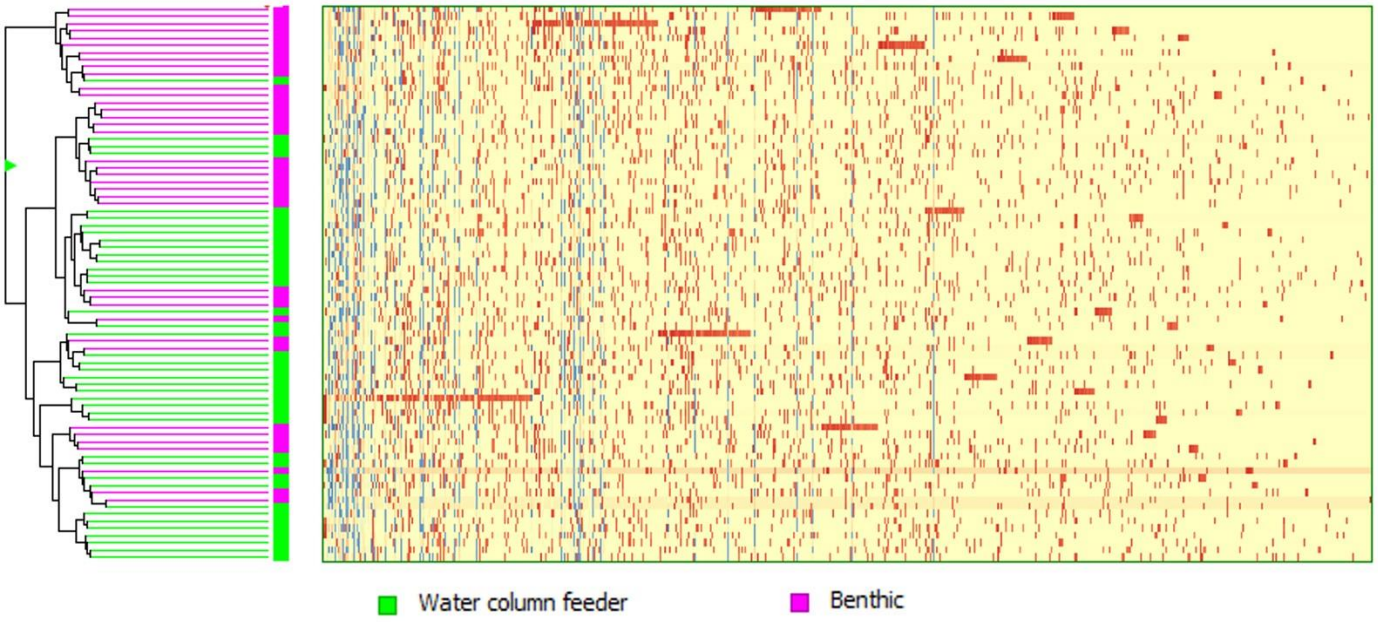


Figure S4.4: Clustering analysis of all features grouped based on benthic and water column feeders (negative ionization mode)

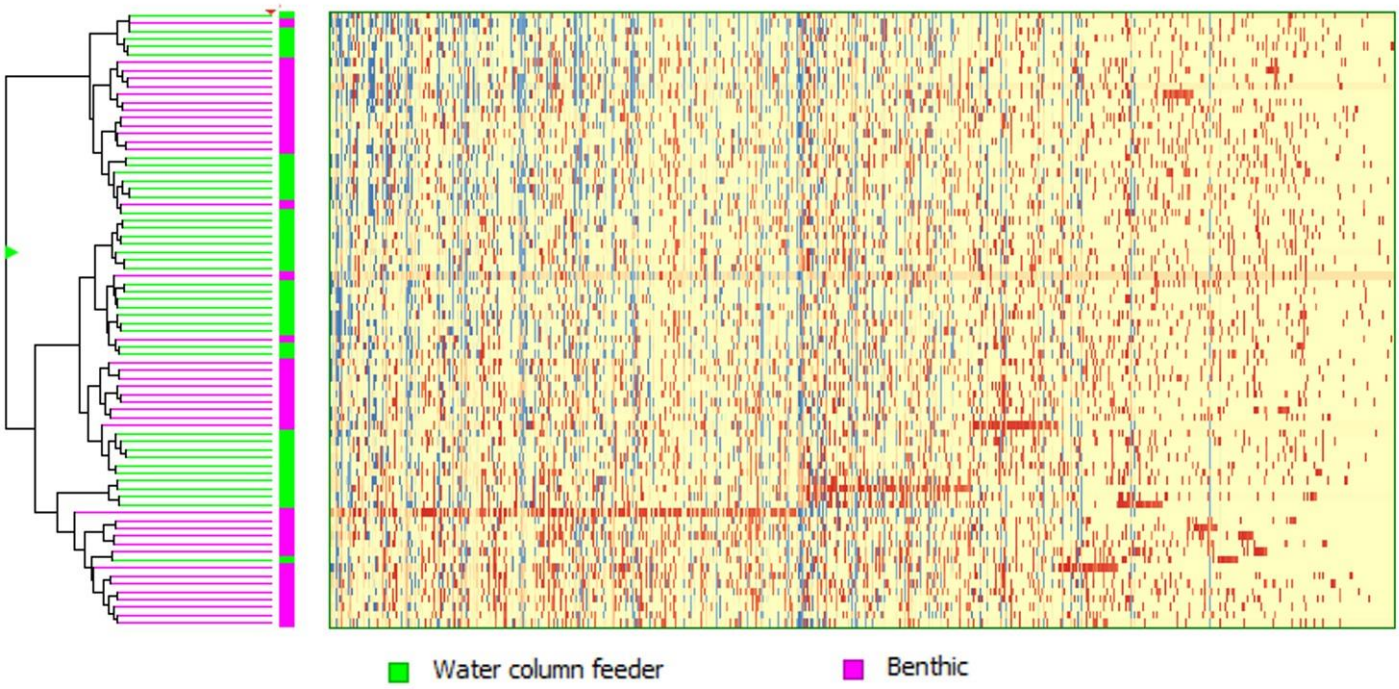


Figure S4.5: PCA on all entities (positive ionization mode) based on location in (A) 40%, (B) 70% or (C) 100% of samples.

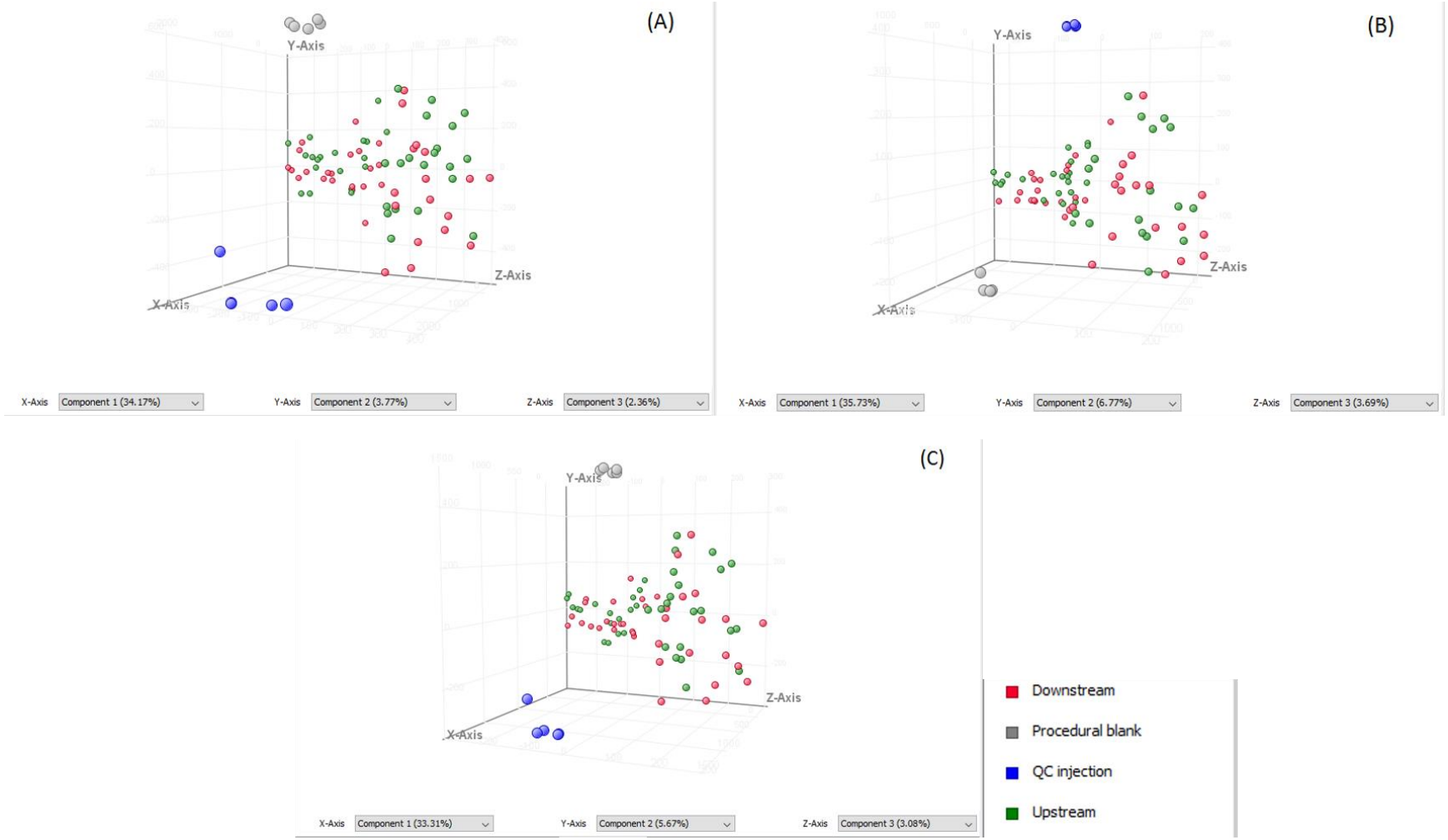


Figure S4.6: PCA on all features (negative ionization mode) based on location in (A) 40%, (B) 70% and (C) 100% of samples.

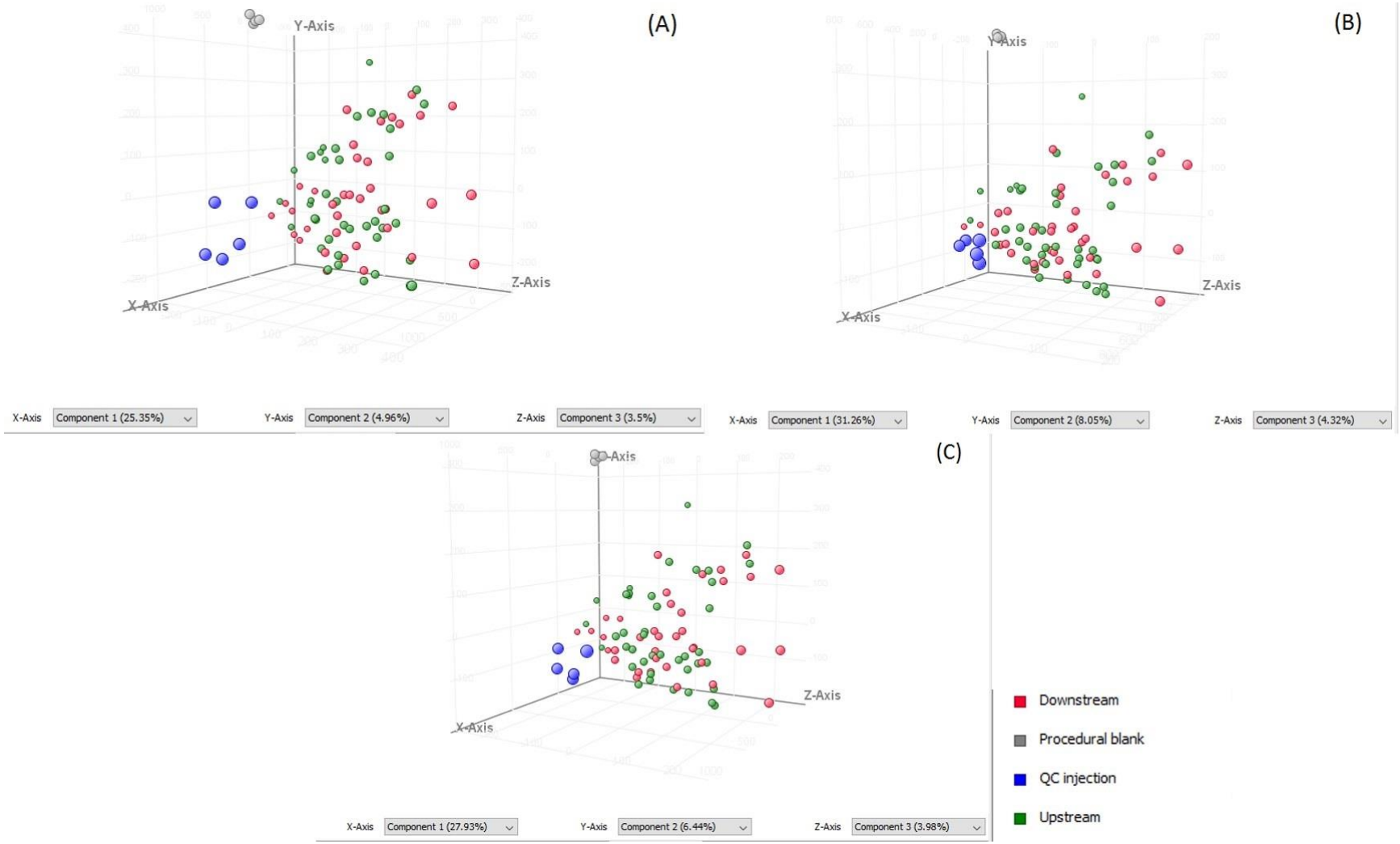


Figure S4.7: PCA on all features (positive ionization mode) based on location in (A) 40%, (B) 70%, (C) 100% of samples without QC or procedural blank samples

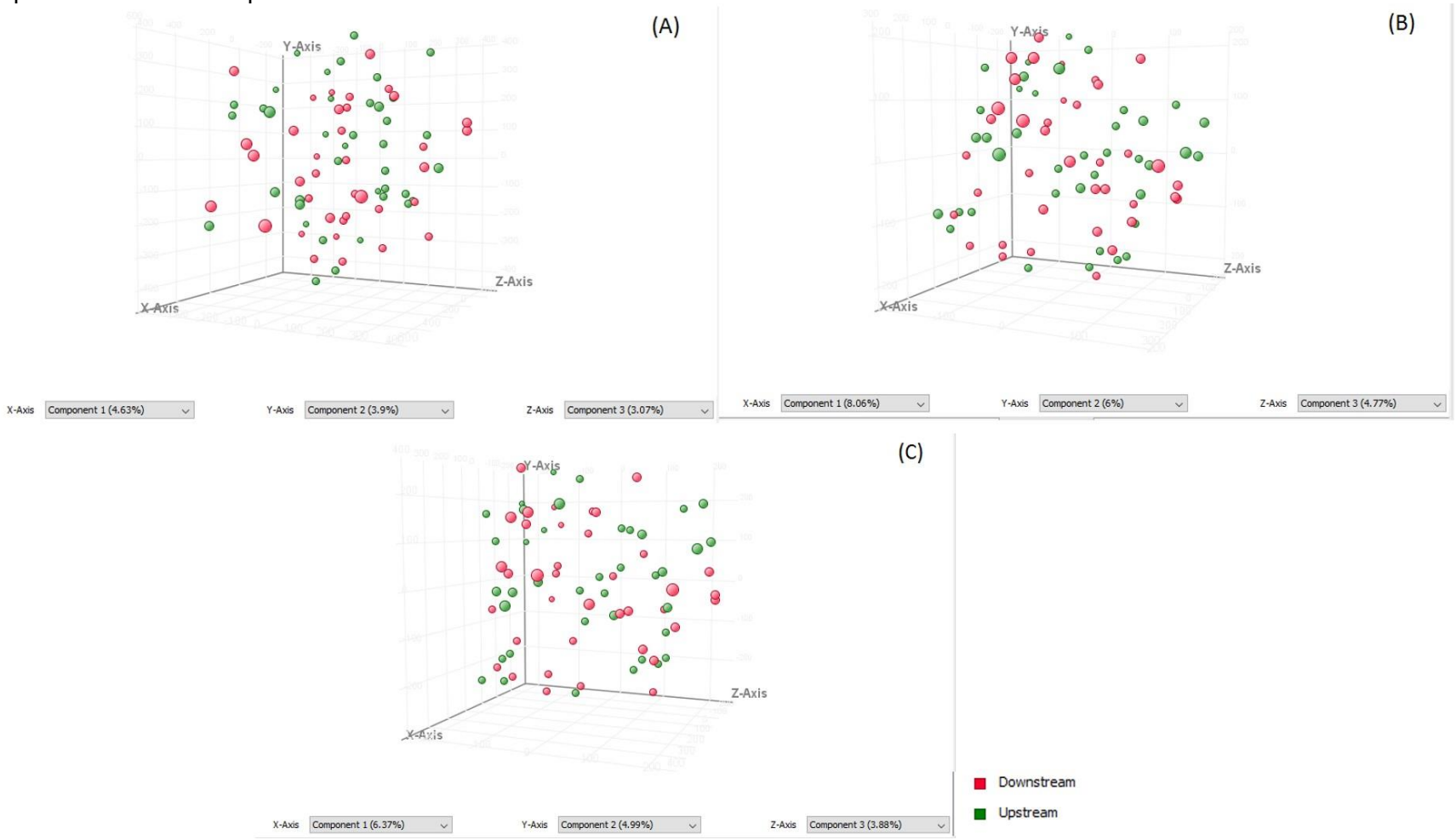


Figure S4.8: PCA on all features (negative ionization mode) based on location in (A) 40%, (B) 70%, (C) 100% of samples without QC or procedural blank samples

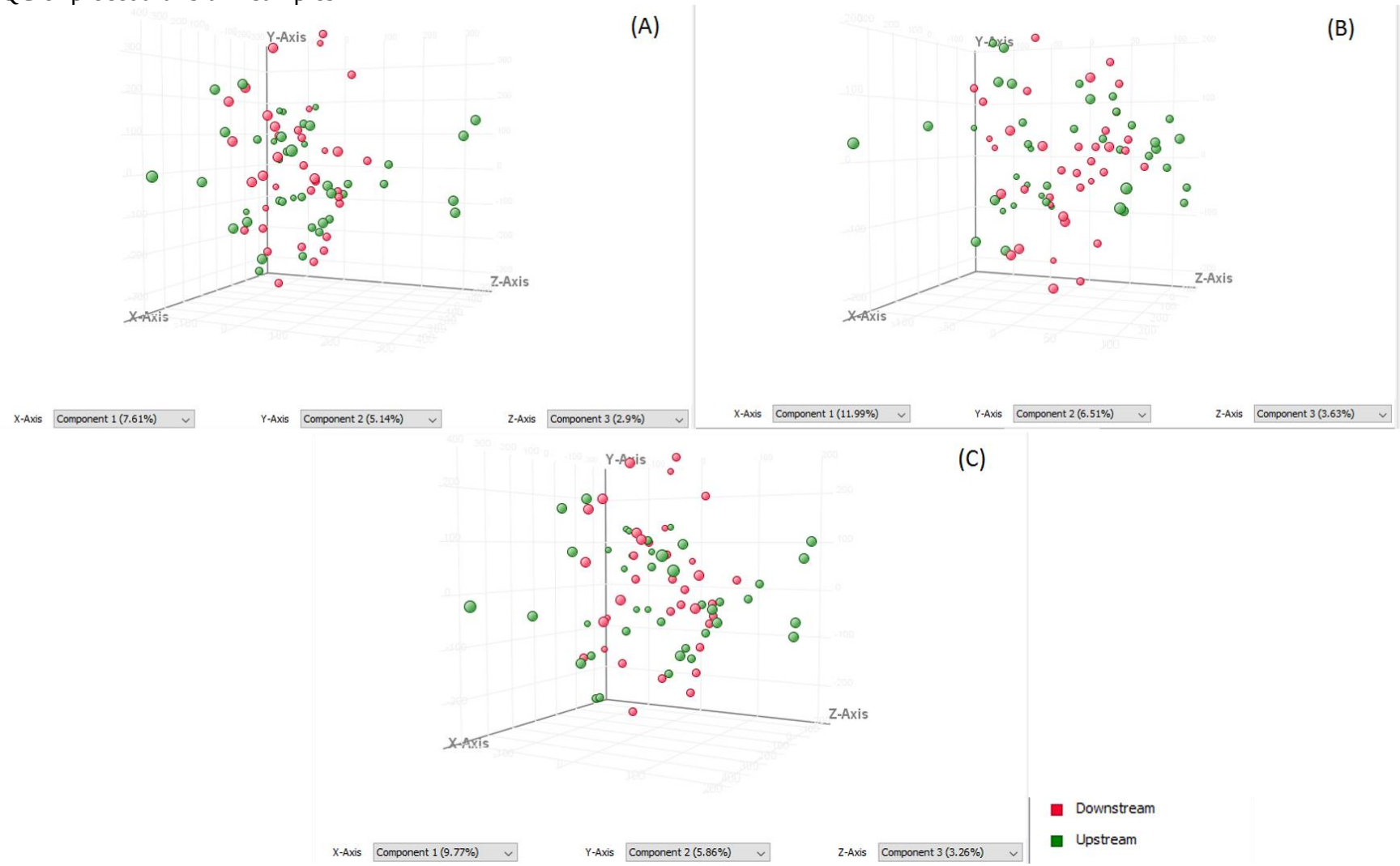


Figure S4.9: PCA on all features (negative ionization mode) based on foraging group in (A) 40%, (B) 70%, (C) 100% of samples

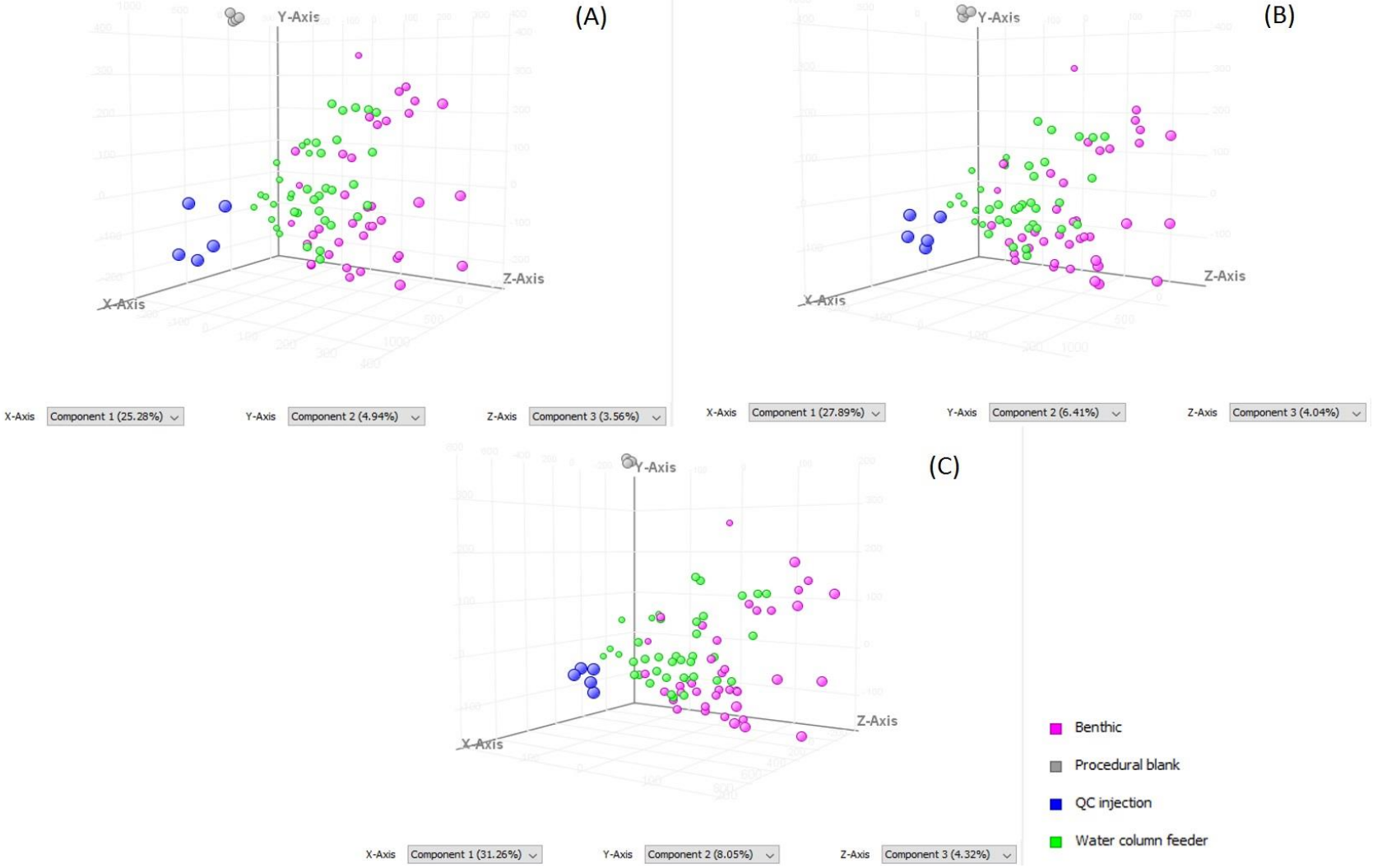


Figure S4.10: PCA on all features (positive ionization mode) based on foraging group in (A) 40%, (B) 70%, (C) 100% of samples without QC or procedural blank samples

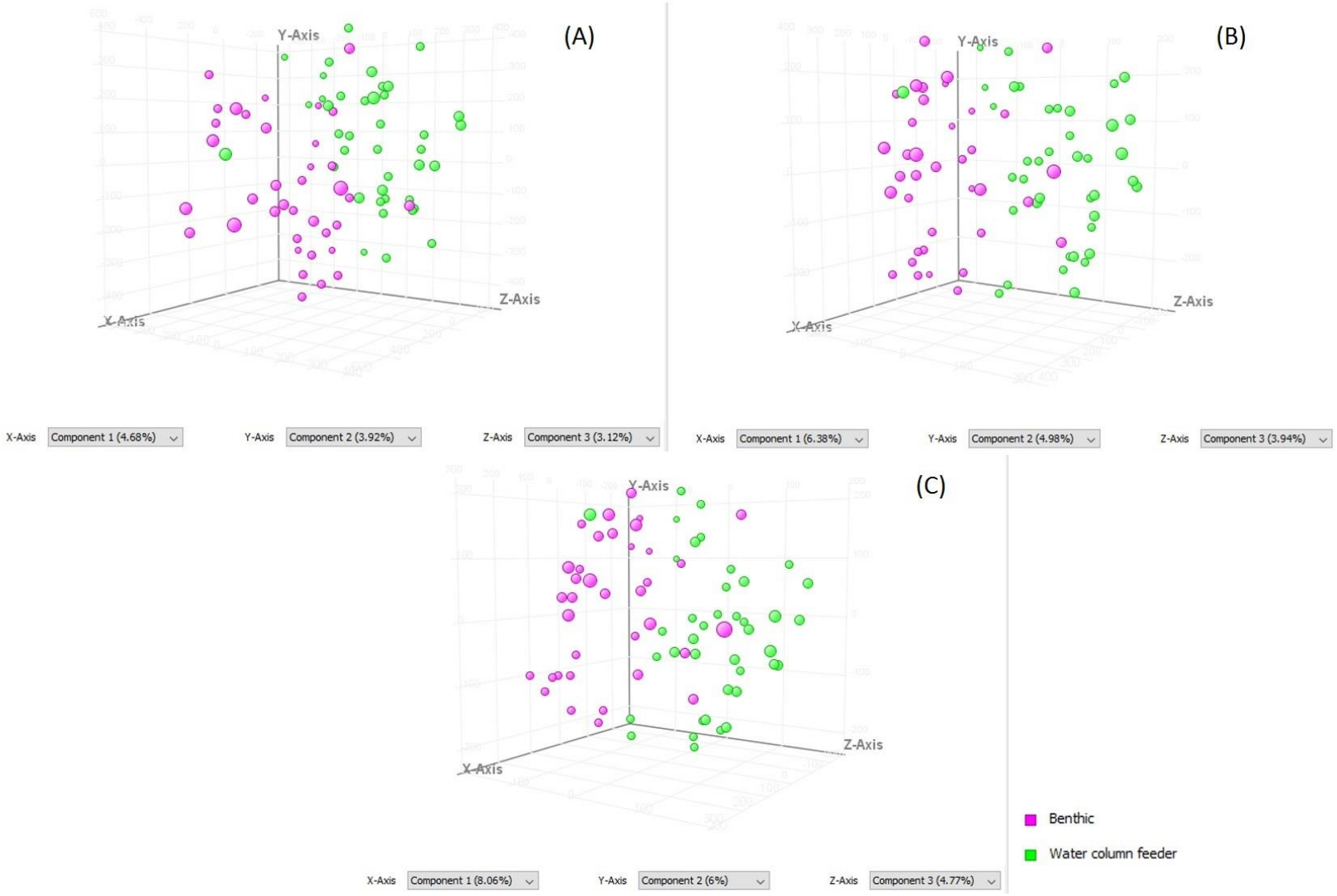


Figure S4.11: PCA on all features (negative ionization mode) based on foraging group in (A) 40%, (B) 70%, (C) 100% of samples without QC or blank samples

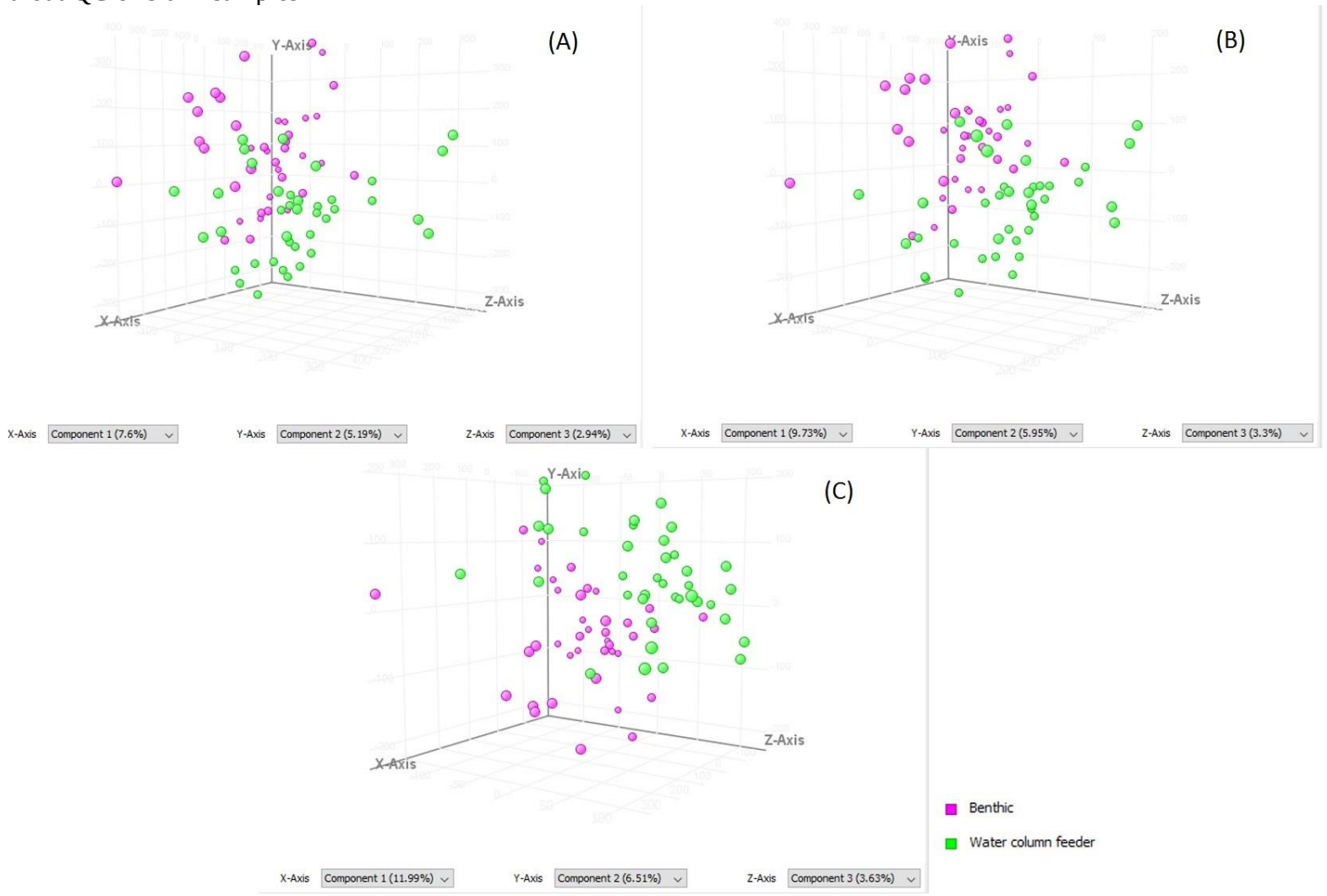


Figure S4.12: PCA on Profinder *Targeted feature extraction* (positive ionization mode) based on foraging group in (A) 40%, (B) 70%, (C) 100% of samples.

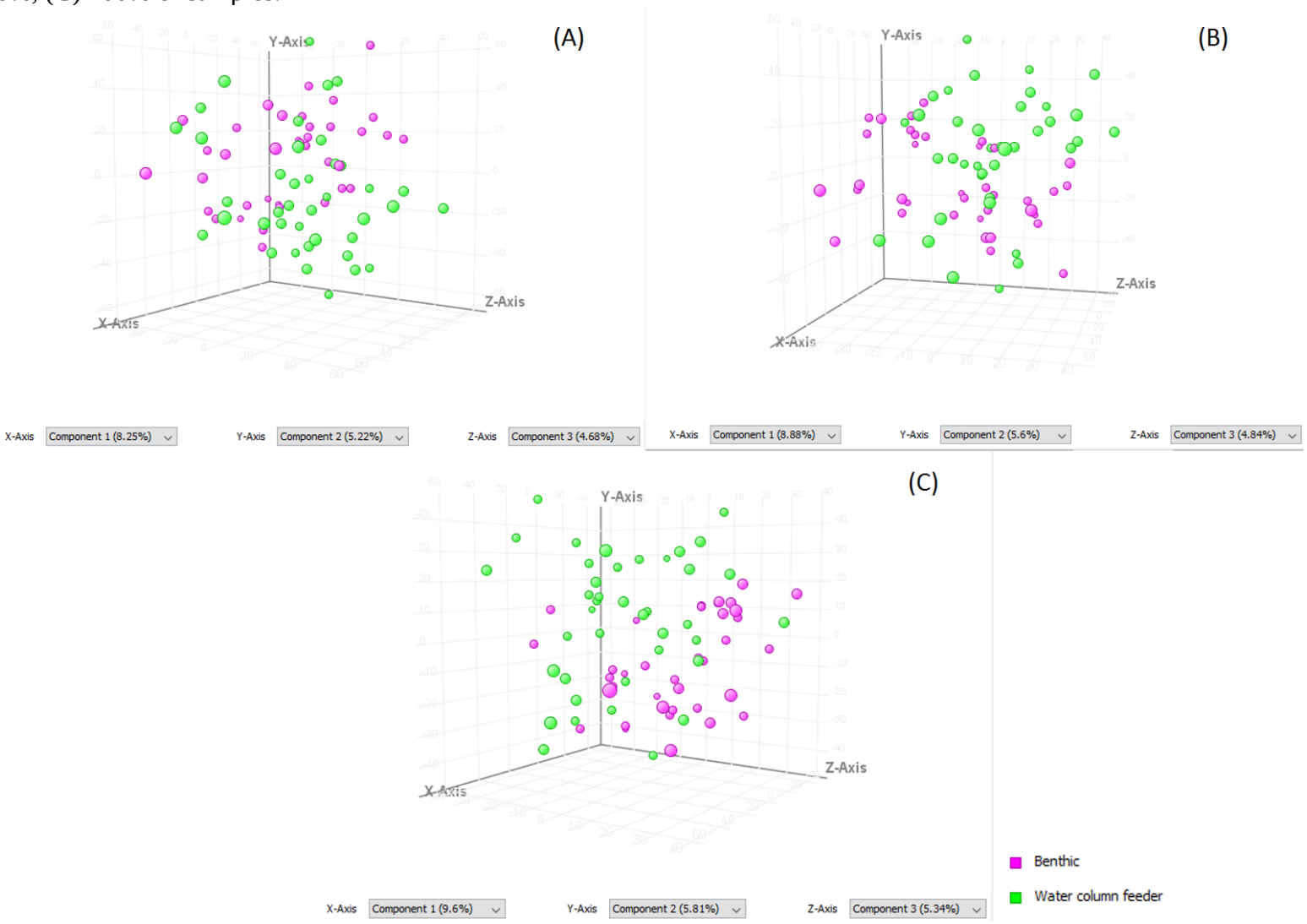


Figure S4.13: PCA on Profinder *Targeted feature extraction* (negative mode) based on foraging group in (A) 40%, (B) 70%, (C) 100% of samples

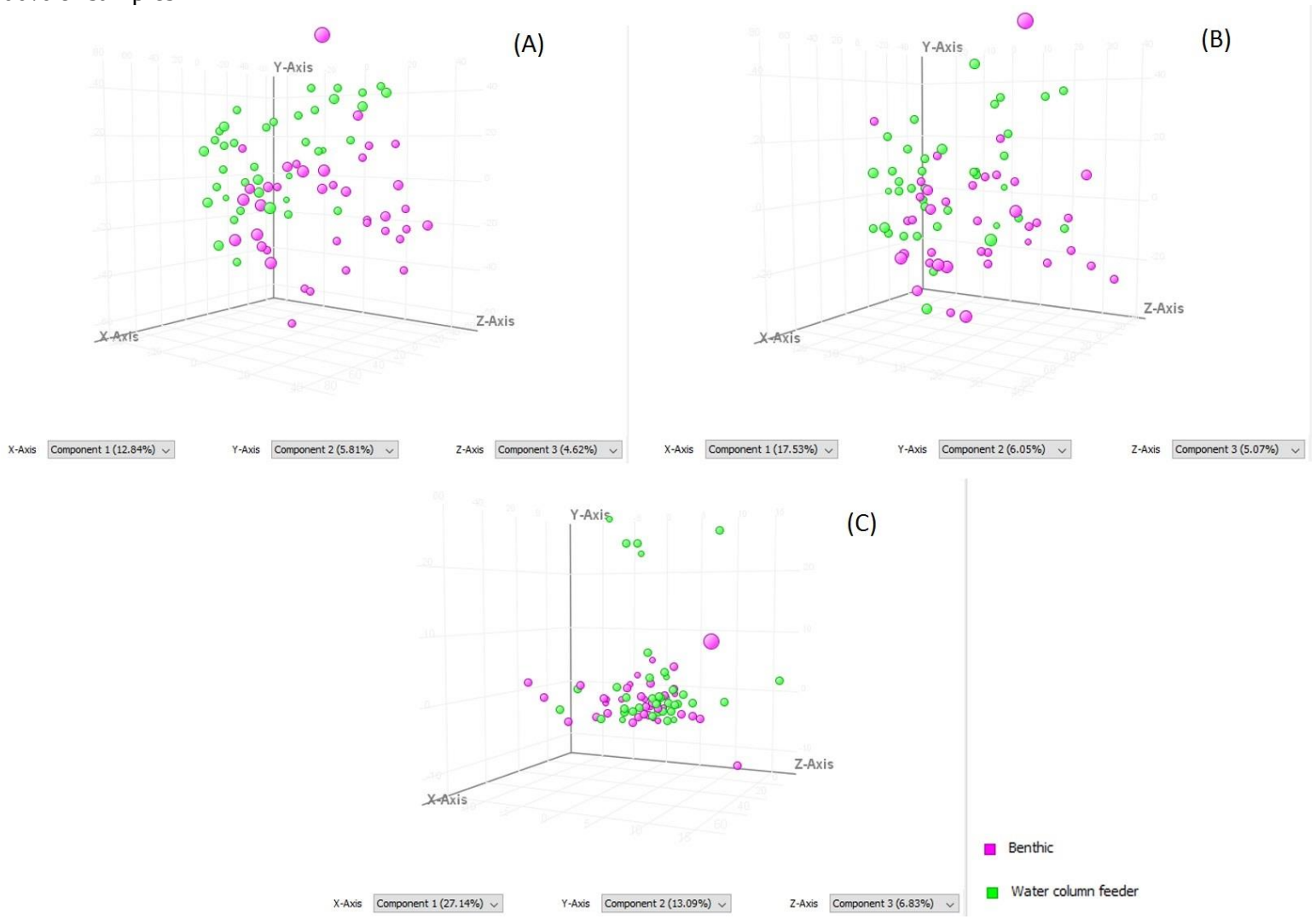
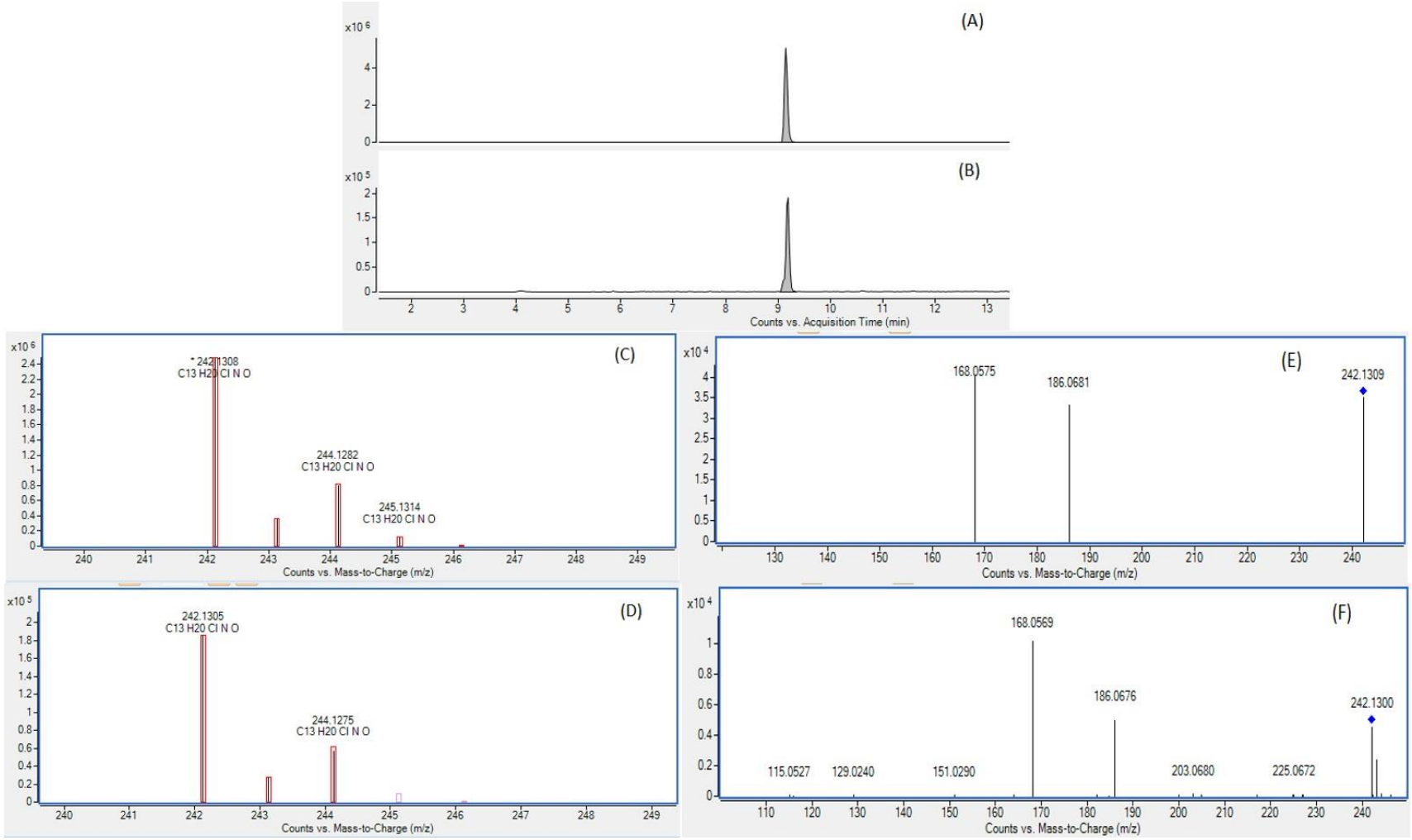


Figure S4.14: Extracted ion chromatograms for erythrohydrobupropion *m/z* 242.1302 (±20 ppm) in (A) standard and (B) liver, isotope distribution pattern in (C) standard and (D) liver, MS/MS spectra in (E) standard and (F) liver.



Connecting paragraph

In chapters 3 and 4, a non-target analysis method was developed for the determination of antimicrobials in fish and shrimp tissues with sample preparation and data analysis steps covered. This workflow allowed for the identification of antimicrobial metabolites and other pharmaceuticals, such as erythrohydrobupropion, which was identified for the first time in fish livers in this study. The optimized extraction and analysis workflow were applied in chapter 5 to study the fate of the antimicrobial during cooking of shrimp and brook trout muscle. This chapter will be submitted for publication to *Food Research International* as "Application of non-target analysis to study the thermal transformation of malachite and leucomalachite green in brook trout and shrimp".

Chapter 5: Application of non-target analysis to study the thermal transformation of malachite and leucomalachite green in brook trout and shrimp

5.1 Abstract

The fate of malachite green and its main metabolite leucomalachite green during thermal treatment was examined in seafood (brook trout and white shrimp) using non-target analysis. Samples were extracted using QuEChERS and analyzed using liquid chromatography coupled with quadruple time of flight mass spectrometry. Malachite green levels were reduced in meat during boiling (~40%), microwaving (64%), and canning (96%). Only microwaving was successful in significantly decreasing leucomalachite green levels in brook trout. The reduction percentages of the two target analytes were not significantly different ($p<0.05$) in shrimp (mean fat content = 0.8±0.3%) and in brook trout (mean fat content = 3.5±1.7%), suggesting that a higher fat content may not affect the reduction of the more lipophilic leucomalachite green in these two matrices. Three transformation products were tentatively identified in the cooked tissues, resulting from the cleavage of the conjugated structure or through demethylation. Further research is needed to determine possible adverse health effects. The findings of this study show how non-target analysis can complement targeted methodologies in identifying and evaluating risks to human health.

5.2 Introduction

Malachite green (MG, Figure 5.1), despite its ban in food producing animals due to carcinogenicity concerns, continues to be detected in seafood, as it is a widely-available, highly effective and low cost anti-fungal (Love et al. 2011, EFSA 2016, Dinh et al. 2020). In fish, like carp and trout, its main metabolite is leucomalachite green (LMG), which can last in the muscle of organisms up to several months (Bajc et al. 2011). Furthermore, MG is also used as an industrial dye and its presence in the aquatic biota can be also linked to release of industrial wastewater (Schuetze et al. 2008). Current regulatory limits have set action levels (i.e. levels above which the products could be considered non-compliant), at 1 and 2 ng/g in Canada and Europe, respectively (EFSA 2016, Health Canada 2017). Seafood products are likely to be eaten cooked rather than raw and studies have shown reduction in contaminants levels after cooking for compounds such as persistent organic pollutants (POPs) in salmon (Bayen et al. 2005) and antibiotics in milk and other food matrices (Junza et al. 2014, Tian et al. 2017). Studying the fate of chemical contaminants following cooking is important to assess dietary exposure that is more representative of the actual levels to which consumers are exposed (WHO 2009). Not only could cooking affect the bioaccessability of contaminants (Alves et al. 2017), but newly formed products could still present a risk to human health (Nguyen et al. 2015). Some studies refer to these newly formed compounds as transformation products (Junza et al. 2014, Timm et al. 2019) or degradation products (Nguyen et al. 2015). Transformation products can be formed following hydrolysis, conjugation, demethylation and hydroxylation reactions (Bletsou et al. 2015) and these reactions can lead to the identification of compounds with a higher molecular mass compared to the parent compound (Perez-Estrada et al. 2008). In the present study, newly formed compounds will be referred to as transformation products (TPs). In the case of banned

chemical contaminants, elucidation of TPs after cooking may be used to identify markers of food contamination, especially when the parent compound is completely degraded in the tissues or is below the limit of detection of analysis methods. Targeted approaches used for quantification of parent compounds are insufficient for the identification of transformation products, as this workflow is based on use of standards of known compounds and the mass analyzers do not offer the high mass accuracy needed for formula generation (Bletsou et al. 2015, Knolhoff and Croley 2016). In this context, identification of TPs can be achieved using non-targeted approaches based on high-resolution mass spectrometry. For example, TPs of chloramphenicol and tylosin A have been characterized in seafood and honey based on this strategy (Tian and Bayen 2018, von Eyken and Bayen 2020).

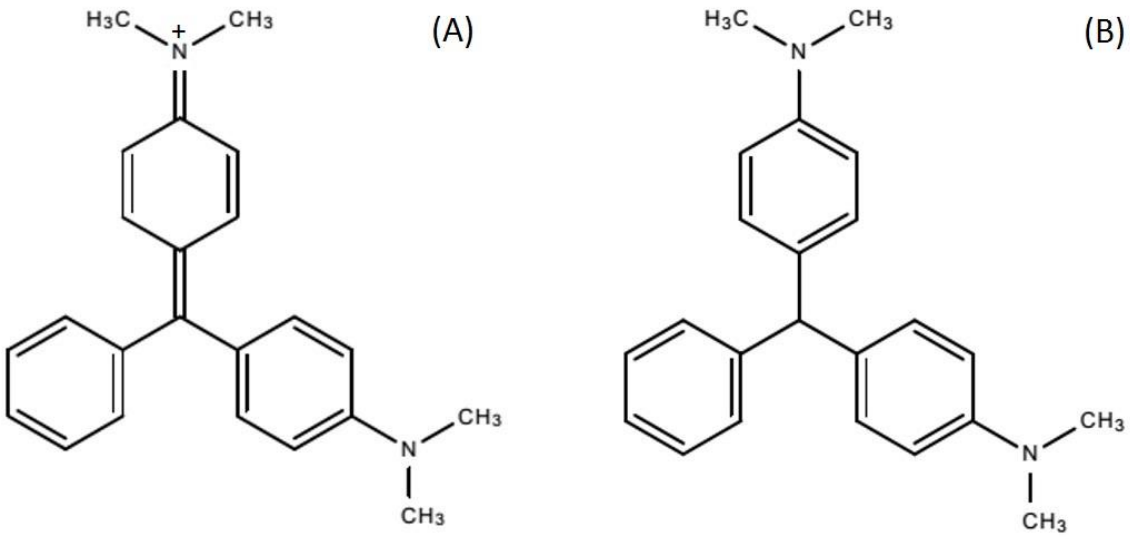


Figure 5.1: Structures of MG (A) and LMG (B)

Various parameters, including the food matrix composition and the type of thermal processing could influence the fate of the chemical contaminants during cooking (Rose et al. 1999, Alaboudi et al. 2013). For example, regarding the effect of the matrix, chlortetracycline

was less stable in egg yolks compared to egg whites when heated at 100°C, presumably because of the binding of the antibiotic to egg white proteins (Alaboudi et al. 2013). In addition, frying resulted in a higher rate of disappearance of chlortetracycline in egg whites compared to boiling. Differences in reduction rates between different matrices or cooking treatments could perhaps lead to the formation of different transformation products. Indeed, this has been observed in the case of tylosin A where different compounds were detected in honey compared to water (von Eyken and Bayen 2020). Therefore, the outcome of the food safety risk assessment for specific chemical residues could be different for different types of processed foods.

MG levels have been shown to decrease following cooking of carp muscle (Mitrowska et al. 2007). Baking (180°C), boiling (100°C), and microwaving resulted in a decrease ranging from 12 to 61% in the MG content, depending on the type and duration of the treatment. On the other hand, only microwaving was able to lower LMG levels by 40% (Mitrowska et al. 2007). Although LMG is much more lipophilic (log K_{ow} 5.72) compared to malachite green (log K_{ow} 0.62) (National Library of Medicine 2020) it was stable when heated in sunflower oil (Mitrowska et al. 2007). However, in a leaner fish, i.e tilapia, LMG levels were reduced during both baking and frying treatments, by up to 26 and 35% respectively (Shalaby et al. 2016). To the best of our knowledge, no study has investigated the fate of MG and LMG after cooking in other seafood matrices beside carp or tilapia nor qualified any thermal TPs of the two compounds in seafood. Therefore, the aim of this study was twofold: (i) compare the percent reduction rate in MG and LMG levels in water and two food matrices: pacific white shrimp (*Litopenaeus vannamei)* i.e., low-fat matrix and brook trout (*Salvenilus fontinalis*), i.e., high-fat matrix with the hypothesis that a higher reduction of LMG would be observed in shrimp compared to trout, (ii) apply a non-target data treatment workflow to identify thermal TPs.

5.3 Materials and methods

5.3.1 Chemicals

MG chloride (>96.0%), LMG (>98.0%), d_5-LMG (>98.0%) analytical standards were obtained from Sigma Aldrich (St Louis, MO, USA). Labelled injection internal standards, d_3-diphenhydramine and d_3-6-acetylmorphine, $^{13}C_6$-propylparaben were purchased from Cerilliant (Round Rock, TX, USA) and Sigma Aldrich respectively. HPLC grade acetonitrile, methanol and water, as well as LC-MS grade formic acid, acetic acid and ammonium acetate were obtained from Fisher Chemical (Pittsburgh, PA, USA). Anhydrous magnesium sulfate and sodium acetate were purchased from Sigma Aldrich. Primary secondary amine (PSA) sorbent was purchased from Agilent (Santa Clara, CA, USA). All glassware used was baked in an oven at 320°C for four hours and rinsed with methanol before use. Labelled internal standard solution of 0.4 μg mL^{-1} was prepared in methanol and stored at -20°C in amber vials. Malachite and leucomalachite green standards of 1 mg mL^{-1} and working standards of 10 μg mL^{-1} were prepared in methanol and stored at -20°C in amber vials. All standards were prepared fresh every 6 months (Andersen et al. 2006). Six fresh calibration standards, ranging from 2 to 40 ng mL^{-1}, were prepared in water (0.1% formic acid) before analysis.

5.3.2 Sample preparation

Incurred shrimp and brook trout samples were obtained from a controlled exposure experiment described previously (Baesu et.al submitted). In total, 10 individual shrimp and trout exposed to malachite green were used for the cooking treatments. Ten non-exposed individuals were used as control samples. Sample extraction was based on QuEChERS (Jia et al. 2017) and

was chosen based on criteria commonly used in non-target analysis (Baesu et.al submitted). Briefly, 1.0 g of homogenized muscle sample was weighed in a centrifuge tube, after which 5 mL (84:16 v/v) acetonitrile/water with 1% acetic acid was added and vortexed for 1 minute. To each sample, 1.0 g of $MgSO_4$ and 0.30 g sodium acetate were added, vortexed for 1 minute followed by centrifugation at 4400 rpm (3000 x *g*, 25°C) for 5 minutes. Two mL of supernatant was transferred to clean tubes containing 0.24 g $MgSO_4$ and 25 mg PSA, vortexed for 1 minute and centrifuged for 5 minutes at 4400 rpm. Extracts were filtered using a 0.22 µm PTFE filter and stored in amber vials at -20°C in the dark. Prior to LC-MS analysis, extracts were diluted (1/10) with water and 50 µL of a 0.4µg mL^{-1} solution of the labelled internal standards was added. Labelled standards were not added for quantification purposes, but rather to monitor the instrument performances. For recovery experiments, control raw and cooked samples were spiked with 40µL of a working standard solution of 10 µg/mL (target concentration in muscle 400 ng g^{-1}) and allowed to equilibrate for 10 minutes for extraction. Recoveries and matrix effects were calculated as described in Matuszewski, Constanzer et al. (2003). Procedural blanks were prepared similarly. Five quality control (QC) samples were prepared by pooling 20 µL of each replicate extract and blank. Method detection limit (MDL) and limit of quantification (LOQ) were calculated as 3σ and 10σ, respectively, of the procedural blanks integrated at the retention time of the target compounds.

5.3.3 Fat analysis

Determination of fat in raw shrimp and trout muscle was based on USDA method for fat analysis (USDAa 2009). Shrimp and trout muscle were freeze-dried at -90°C (Martin Christ Gamma 1 –16 LSC freeze-dryer, Germany). Then, 0.3 g of freeze-dried sample (n=5) was

extracted with 200 mL hexane using a Soxhlet apparatus for 4 hours. Solvent was evaporated using a rotary evaporator (Büchi, Switzerland) and the lipid residues were measured gravimetrically.

5.3.4 Thermal treatments

5.3.4.1 Water

Aqueous standard solutions of 10 ng mL^{-1} MG and LMG (n=6) were dispensed into 2 mL amber vials (500 μL) and placed in a water bath at 100°C. Vials were removed at 10, 30 and 120 min and allowed to cool at room temperature. Water samples were heated for up to 2 hours to represent extreme conditions that could generate transformation products.

5.3.4.2 Boiling

Approximately 2.5 g of shrimp and 5 g of trout were placed in 40 mL amber vials (n=10), capped and transferred to a water bath at 100°C. Vials were removed at 10 and 30 min and allowed to cool at room temperature. Boiling for 10 minutes had been reported in the literature to decrease MG levels by 43%, and further increasing cooking time by 5 minutes lead to a total decrease of 54% (Mitrowska, Posyniak et al. 2007). In this study, trout were further heating for a total of 30 minutes to mimic more extreme cooking conditions and ensure formation of transformation products. Shrimp were only boiled for 10 minutes, as a higher cooking time led to too much breakdown of the muscle. Any juices present in the vials were collected and analyzed as well.

5.3.4.3 Microwaving

Approximately 5 g of trout muscle was placed in beakers, covered with parafilm and microwaved (Sylvania, 1300W) for 1.5 minutes. A longer microwaving time led to too much drying and at times burning of the muscle; therefore, a time of 1.5 minutes was deemed acceptable. Beakers were removed and allowed to cool at room temperature. No juices were observed after cooking.

5.3.4.4 Canning

An Instant Pot Max 9-in-1 (Instant Brands, Ottawa, Canada) with the canning option selected was used for canning trout muscle. Briefly, 50 g of trout was added along with 100 mL of water to 120 mL glass jars and capped with metal covers. Trout was heated for 50 minutes at 121°C. No other studies were found in the literature that evaluated changes in MG and LMG contents during canning. Recommended canning time using home pressure cookers at 15 PSI is 100 minutes for pint jars (USDAb 2015). Based on the smaller sample and jar size used in this study and the hypothesis that MG would behave similarly during boiling and canning treatment (i.e. at least 50% reduction should be achieved) a shorter canning time of 50 minutes was chosen.

5.3.5 Instrumental analysis

Samples were analyzed using an Agilent UHPLC 1290 coupled with an Agilent 6545 QTOF-ESI-MS, in both positive and negative ionization modes. In positive mode, mobile phases were (A) H_2O with 0.1 % formic acid and (B) acetonitrile, and in negative mode, mobile phases used were (A) 0.05 M ammonium acetate and (B) acetonitrile. The same gradient elution was used for both positive and negative modes, increasing from 5% B at 1 min to 100% B after

15 min, then maintained at 100% B from 15 to 20 min, and then re-equilibrated at 5% B for 5 minutes at the end. An InfinityLab Poroshell 120 (Pheny-Hexyl, 3.0 x 100 mm, 2.7 µm, Agilent Technologies) with a Poroshell (4.6 mm) Phenyl Hexyl pre-column was used. Flow rate was set at 0.2 mL/min, injection volume was 2 µL and column temperature was 20°C. The MS parameters were as follows: sheath gas temperature 275°C, drying gas temperature 325 °C, drying gas flow 5 L/min, sheath gas flow 12 L/min, nebulizer pressure 20 psi, capillary voltage 4000 V, nozzle voltage 2000 V, fragmentor voltage 175 V, skimmer voltage 65 V. All ion MS/MS mode at collision energies of 0, 10, 20 and 40 V was used. Data was collected between 100 and 1700 *m/z* at a rate of 3 spectra/s. Samples were kept at 4°C in the multisampler compartment.

5.3.6 Data treatment

SPSS Statistics software (v.26) (IBM, NY, USA) was used to for statistical analysis, such as comparison of reduction percentages between trout and shrimp, with level of significance set at 0.05. Concentrations were computed using Agilent Mass Hunter Quantitative Analysis B.10.0, using a mass extraction window of ±20.0 ppm and retention time window of ±0.30 min. The most abundant ions were used for quantification: $[M]^+$ of 329.2017 for MG and $[M+H]^+$ of 331.2168 for LMG. Concentrations for LMG were calculated following the internal standard method using its deuterated surrogate and the relative response factor (RRF) (equation 1) (USEPA 2007).

$$RRF = \frac{(area\ native\ compound\ x\ concentration\ labeled\ compound)}{(area\ labeled\ compound\ x\ concentration\ native\ compound)} \quad (Equation\ 1)$$

For MG, matrix matched external calibration using control samples was prepared at six levels (10, 20, 35, 60, 125 and 280 ng g^{-1}). Only 1 g of the total muscle cooked was used for extraction, therefore computed concentrations were adjusted to account for the weight loss during cooking.

Data alignment and feature extraction were conducted using Agilent Mass Hunter Profinder software B.10.0 using the following parameters: peak filter height 200 counts, retention time window ±0.30 min, mass window ±10.00 ppm, post-processing peak absolute height 1000 counts, MFE score 80. The workflow for the identification of possible TPs is presented in Figure 5.2. Briefly, molecular features were exported as .pfa files and imported into Mass Profiler Professional (v 15.0) with a percentile shift (75.0) normalization. Trout and shrimp samples were grouped into *raw control (RC)*, *raw exposed (RE)*, *cooked control (CC)* and *cooked exposed (CE)*. Fold change analysis (>2) along with one-way ANOVA ($p<0.05$) with Benjamini-Hochberg correction and Tukey post-hoc test were applied to identify which compounds present at statistically significant higher levels in specific groups. Fold change analysis along with statistical tests have been applied to study the fate of some food contaminants such as pharmaceuticals during thermal or phototransformation, (Lege et al. 2020, von Eyken and Bayen 2020). Formula for molecular features of interest were generated through the ID browser analysis in Mass Profiler Professional and compounds that had a score >70% were considered. This matching score is typically used in compound identification in non-target analysis (Du et al. 2017, von Eyken and Bayen 2020). These features were further analyzed in targeted MS/MS mode in order to obtain more accurate information on precursor/fragment ions. Targeted molecular features were extracted using Agilent Qualitative Analysis B.10.0 and

exported as .cef files. These files were then imported into Agilent Molecular Structure Correlator with features ran through Chemspider (Royal Society of Chemistry 2020) and Agilent Metlin (30232 compounds) databases.

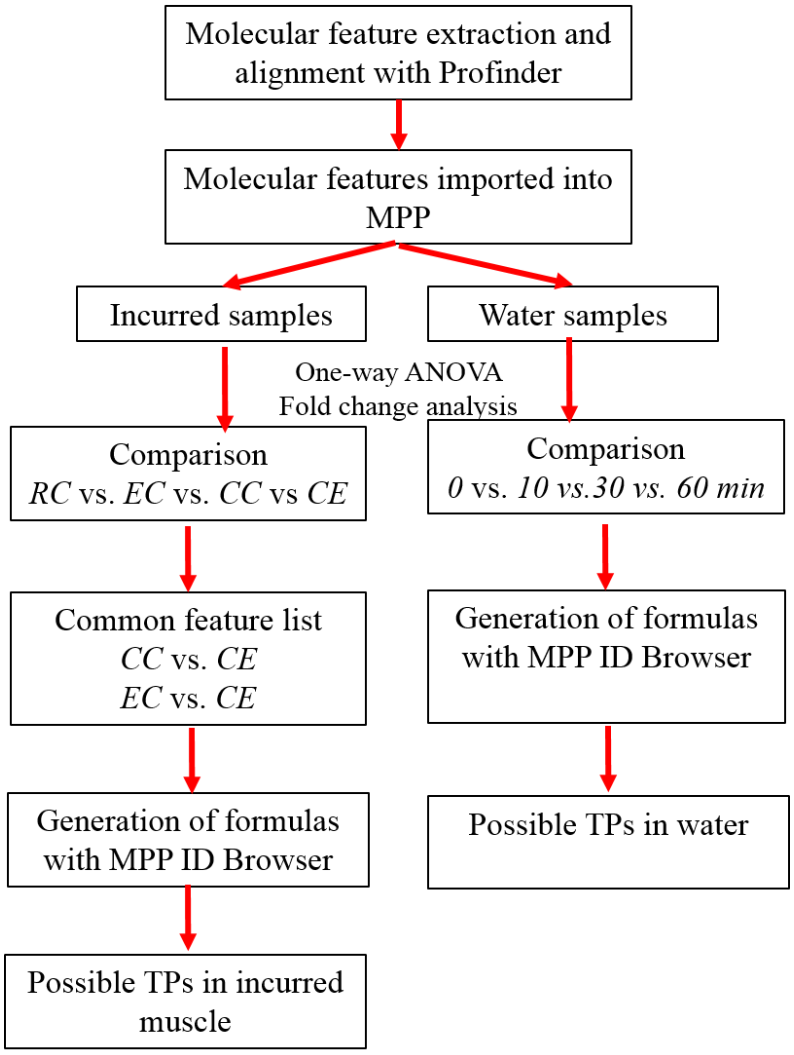


Figure 5.2: Workflow for the identification of thermal TPs of MG and LMG in trout and shrimp muscle

5.4 Results and discussion

5.4.1 Method validation

Recoveries above 60% were obtained for MG and LMG in raw and cooked muscle (Table S5.1). For example, in trout, MG recoveries were 67±10%, 111±6%, 105±3%, 62±12% and 71±10% in raw, boiled, microwaved and canned muscle respectively. Depending on the extraction procedure, lower recoveries for MG compared to LMG have been obtained in targeted methods (Bergwerff and Scherpenisse 2003, Mitrowska et al. 2007, Chen and Miao 2010). López-Gutiérrez et al. (2012) determined recoveries for a similar QuEChERS extraction but with sorbent clean-up step omitted, were between 48 and 81% depending on spiking level, for MG and between 63-102% for LMG in shrimp and trout. Another parameter that influenced the recovery of MG, but not LMG, was the incubation time between spiking and extraction (Bergwerff and Scherpenisse 2003). In species like turbot and trout, recoveries decreased from 81% to 63% when the incubation time between moment of spiking and extraction increased from 1 to 15 minutes (Bergwerff and Scherpenisse 2003). The incubation time in this study was 10 minutes. Generally, chemicals like ascorbic acid, or *N*,*N*,*N*,*N*-tetramethyl-1,4-phenylenediamine hydrochloride have also been added during sample extraction as they can prevent demethylation of MG (López-Gutiérrez et al. 2013). However, as the goal of this study was to identify TPs of MG and LMG, these chemicals were omitted from the extraction procedure. Significant difference (p<0.0005) was found for the calculated recoveries and matrix effects of MG and LMG between shrimp and trout, both raw and cooked. Inter-day precision, calculated as the relative standard deviation (RSD) across all six replicates was generally below 20%. Adequate instrument linearity (R^2>0.99) was achieved. For trout, MDLs of 0.9 and 0.5 ng g^{-1} were obtained for MG and LMG respectively, while LOQs of 3.1 and 1.6 ng g^{-1} were obtained

for MG and LMG. In shrimp, MDLs of 0.7 and 0.3 ng g^{-1} were obtained for MG and LMG respectively, while LOQs 2.2 and 1.1 ng g^{-1} were obtained for MG and LMG. Mass measurement errors (Table S4.2) for MG and LMG in raw and cooked samples were calculated according to Brenton and Godfrey (2010) and were significantly different between the raw and cook matrices (p<0.0005). For both MG and LMG, mass measurement errors were below the 5 ppm threshold usually set in non-target analysis (Ponce-Robles et al. 2018). Overall, high recoveries, low MDLs/LOQs. and high mass accuracy were obtained, confirming that the methods used were adequate.

5.4.2 Fat analysis

Fat content (wet weight) was found to be significantly higher in trout (3.5±1.7%) compared to shrimp (0.8±0.3%) (p=0.024). Although diet and habitat may have an effect on nutritional content of fish, the results obtained in this study are comparable to fat content of 2.7% determined in wild caught brook trout (Tidball et al. 2017). Fat content in white shrimp was also consistent with the general reported range of 0.5-1% (USDAd 2019).

5.4.3 Stability of MG and LMG during thermal treatment

In water at 100°C, the maximum reduction rate for MG was 19.9±4.8% after 120 min (Figure S5.2), and the concentrations were significantly different only after 120 min (p=0.001). For LMG, a similar reduction rate of 21.1±3.3% was observed in water, with statistically significant differences across all four heating times (p<0.0005). These results are comparable

with those previously reported, with less than 20% decrease of the compound levels during water heating (Mitrowska et al. 2007).

In food matrices, MG was significantly reduced in both shrimp and trout muscles for all three types of thermal processing (Table 5.1). MG was not detected in any sampled juices or canned water samples, therefore the reduction in malachite green levels is not due to leaching from muscle into juices. Reduction percentage of MG was similar in boiled shrimp (36±49%) and boiled trout muscle (32±18%) (p=0.828). These reduction rates were similar to those (43% decrease after boiling for 10 minutes) reported for carp muscle (Mitrowska et al. 2007). The larger variability observed for the quantification of MG in shrimp (standard deviation of ±49) was due to the presence of an outlier sample (doubled concentration after cooking). Without the outlier sample, the reduction of MG would be 47±24%. The most efficient treatment in significantly reducing MG levels was canning, with more than 90% decrease after 50 minutes. Although there were some differences in the levels of LMG in raw trout across the ten replicates between different cooking treatments (e.g., 1227 ng g^{-1} in raw trout in microwave and 1099 ng g^{-1} in raw trout in canned), the difference was not significant (p =0.179). LMG levels increased, except for microwaving, during the thermal processing in both matrices. In shrimp, levels increased by 20±40% (p=0.194) with increases of 9±23% (p=1.000), 35±32% (p=0.056) and 29±39% (p=0.080) in boiled and canned trout respectively. This increase may be due to: (i) cleavage of possible conjugated forms or (ii) reduction of malachite green into leucomalachite green occurring during cooking. For example, LMG along with its demethylated forms have been reported to be produced during fungal biotransformation of MG (Cha et al. 2001). Photodegradation of an aqueous solution of MG also produced LMG along with its demethylated and hydroxylated forms (Perez-Estrada et al. 2008). Due to the lack of information in the

literature regarding possible conjugation, e.g., glucuronidation of LMG, a mass balance (Table 5.1) was calculated for the two compounds to investigate the possibility of MG reduction into LMG. If the increase in LMG levels observed after boiling and canning is due to deconjugation, the mass balance should be higher than 1. For boiled shrimp, boiled trout and canned trout, calculated mass balances were 1.11, 0.92 and 0.82 respectively. Therefore, it is unlikely that the increase in levels is due to release of conjugated LMG into free LMG. There was no significant difference between the increase percentage for LMG observed in trout (9±23%) and shrimp (20±40%) boiled for 10 minutes (p=0.457). Although raw shrimp were found to have a much lower fat content, LMG levels did not decrease. Hence, in this study, fat content did not seem to influence the fate of LMG in muscle during cooking.

The only treatment that did lead to a significant (p=0.001) decrease of 34±18% in LMG levels was microwaving. This is consistent with the observations made for carp and tilapia muscles, as LMG levels were observed to be reduced by 40% after microwaving for 1 minute (Mitrowska et al. 2007, Shalaby et al. 2016). Consequently, of the decrease of the LMG level is not necessarily due to temperature during treatment, but as it has been suggested, it is rather due to the presence of electromagnetic waves generated during microwaving (Mitrowska et al. 2007). This similar behaviour has been observed for other veterinary drugs such as nitroimidazoles or penicillin G, where the compounds were stable during boiling treatment but levels were reduced during microwaving in chicken and cattle muscle (Rose et al. 1997, Rose et al. 1999). The drugs were considered stable during boiling as the residues lost from the muscle were accounted for in the surrounding fluids. Reduction in drug levels during microwaving may also have been due to their transfer into juices, however very low volume or absence of juices were observed as they had likely evaporated (Rose et al. 1999). Although no juices were observed during microwaving

in the current study, based on the mass balance calculated (0.55), it is likely that the corresponding reduction rate is not only due to leaching into juices and their subsequent evaporation, but that there is indeed some transformation of LMG.

Table 5.1: Effect of thermal treatments on MG and LMG levels in brook trout and shrimp.

Sample	Treatment	Time (min)	Average MG concentration in muscle[a] (ng $g^{-1)}$	Average MG % reduction rate[b]	Average LMG concentration in muscle[a] (ng g^{-1})	Average LMG % reduction rate[b]	Mass balance[c]
Trout	Boiling	0	815±215	0	1376±432	0	N/A
		10	531±142	-32±18*	1463±449	9±23	0.92±0.20
		30	410±110	-49±12*	1777±448	35±32	1.02±0.22
	Canning	0	757±186	0	1227±359	0	N/A
		50	28±9	-96±2*	1527±499	29±39	0.81±0.27
	Microwave	0	759±159	0	1099±299	0	N/A
		1.5	274±80	-64±9*	716±240	-34±18*	0.54±0.13
Shrimp	Boiling	0	175±98	0	486±130	0	N/A
		10	91±52	-36±49*	586±173	20±40	1.11±0.42

*statistically significant at $p<0.05$
[a]expressed as the mean concentrations across all ten replicates ± standard deviation
[b]expressed as the mean reduction rate across all ten replicates ± standard deviation
[c]expressed as the [(MG concentration + LMG concentration in cook muscle)/(MG concentration + LMG concentration in raw muscle)] ± standard deviation

5.4.4 Identification of thermal TPs

Compounds that may be considered as possible TPs of MG and LMG are listed in Tables 5.2 and S5.2. In boiled and canned trout, no molecular features of interest were identified in negative ionization mode based on the data treatment criteria (fold change and statistical analysis). Some compounds had molecular weight higher than the parent compounds, indicating

possible reactions with matrix components. A search through the Chemspider and Metlin databases did not yield any possible structural match.

Based on information on TPs previously reported in the literature (Table S5.3), three compounds (Figure 5.3) were tentatively identified as TPs of MG and LMG in this study. C3 (Figure 5.4) was proposed as a possible TP of MG in microwaved trout. This compound has previously been identified as a phototransformation product (Perez-Estrada et al. 2008) formed through hydroxyl radical attack and cleavage/demethylation of the parent trimethylmethane structure. Hydroxyl radicals can be formed during cooking and may cause oxidation of macronutrients, like proteins (Soladoye et al. 2015). These radicals may be responsible for the oxidation of MG and detection of the benzophenone derivative as a possible TP. C5 (Figure S5.2) was tentatively identified as 2-desmethylated MG, which has also been described as a phototransformation product (Perez-Estrada et al. 2008). C6 (Figure 5.4) was found to increase (fold change >2) in boiled trout, but not in boiled shrimp. This compound may be present in raw shrimp and trout due to metabolism or natural degradation of MG during storage. Another possibility for this may be variability in the extraction efficiency between raw and cooked samples.

2-desmethylated MG was however found at statistically significant higher abundance in canned trout (Figure S5.2); in this context, this compound therefore regarded as a transformation of MG during heating. C6 was not detected in microwaved trout. This suggests that the type of thermal treatment (i.e., microwave vs. boiling) influences the breakdown mechanism of the parent compound. C6 was tentatively identified as 2-desmethylated LMG, which has been proposed as a fungal biotransformation product (Cha et al. 2001) as well as a metabolite in catfish muscle (Doerge et al. 1998). C7 was detected at a statistically significant higher abundance in

microwaved trout. This is in line with the fact that microwaving was the only thermal treatment that reduced LMG levels.

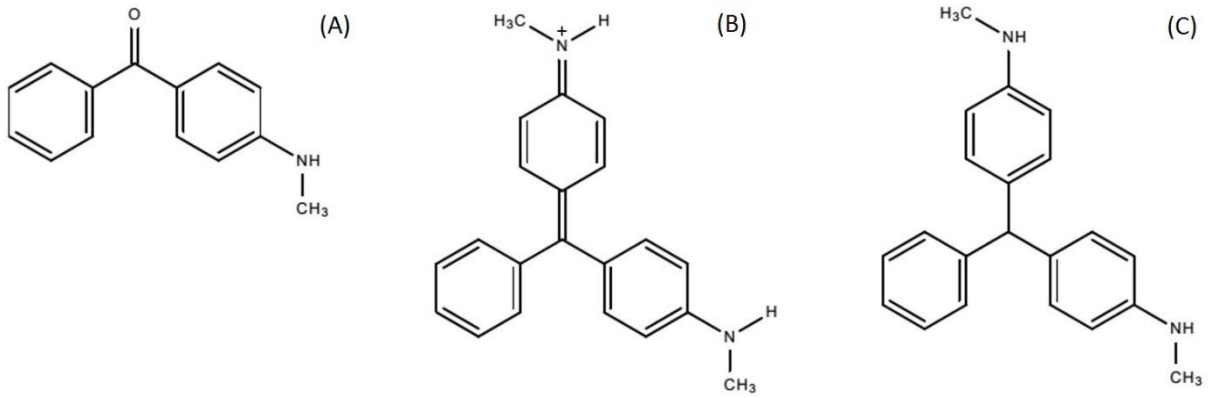


Figure 5.3: Structures of tentative TPs of MG and LMG: C3 (A), C5 (B), C6 (C)

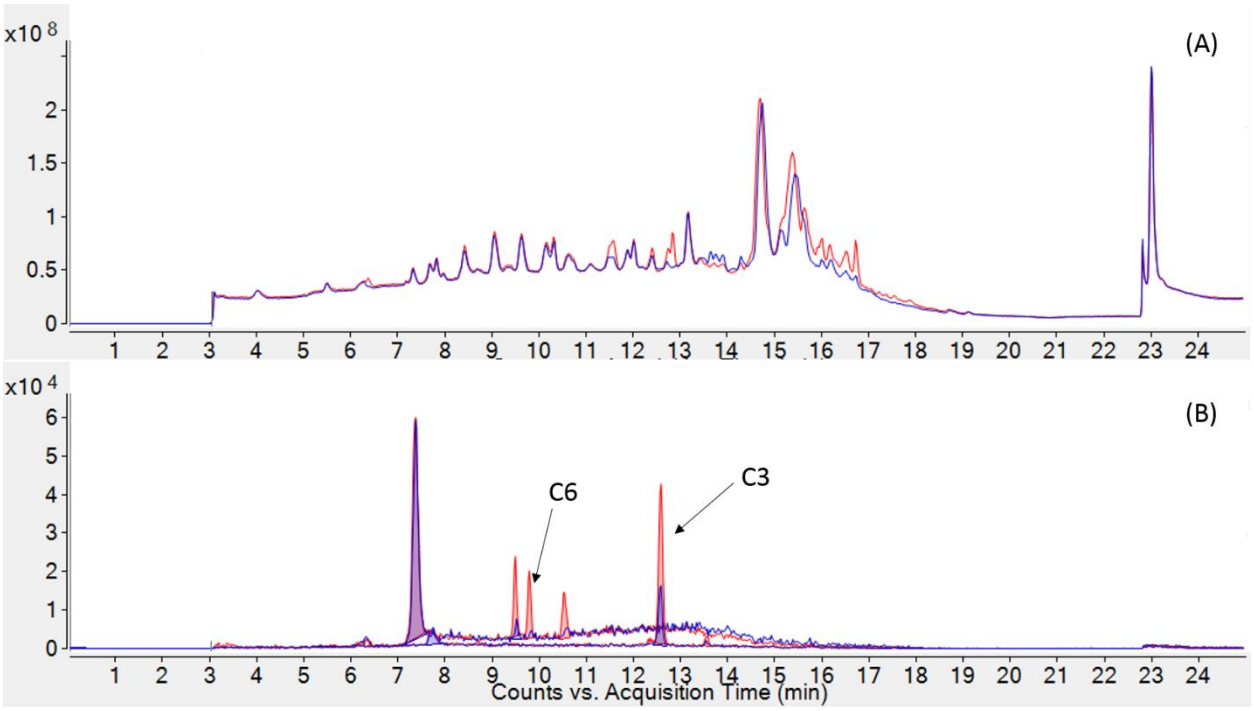


Figure 5.4: Total Ion Chromatogram (TIC) (A) for raw exposed trout (blue) and microwaved exposed trout (red); Extracted Ion Chromatogram (EIC) (B) for C3 and C6 at *m/z* 212.1069 and 303.1845 respectively (±20 ppm)

Table 5.2: Possible TPs of MG and LMG identified in positive ionization mode in *cooked exposed (CE)* trout and shrimp based on fold change and statistical analysis √: increasing in *CE* (with fold change >2 compared to *CC* and *RE*) and statistically significant (p<0.05), ↑: increasing in *CE* (with fold change >2 compared to *CC* and *RE*) but not statistically significant (p>0.05), =: detected in *CE* but fold change <2 compared to *CC* and *RE*) and not statistically significant (p>0.05), but not increasing, ND: not detected

Compound	R_t (min)	Mass	Formula (score)	Mass measurement error (ppm)	Trout				Shrimp	Water
					Boiling 10min	Boiling 30min	Canning	Microwave	Boiling 10 min	100°C 120 min
C1	3.5	135.0548	C_5H_5N (80)	-1.25	ND	ND	ND	ND	√	ND
C2	13.5	210.0927	$C_8H_{18}O_4S$ (71)	0.69	=	=	=	↑	=	ND
C3	12.6	211.0997	$C_{14}H_{13}NO$ (87)	1.83	=	=	=	↑	=	ND
C4	11.7	268.2883	$C_{17}H_{36}N_2$ (85)	1.79	ND	ND	ND	ND	↑	ND
C5	10.3	301.1709	$C_{21}H_{21}N_2$ (94)	1.38	=	=	√	ND	=	ND
C6	9.8	302.1771	$C_{21}H_{22}N_2$ (80)	-4.05	ND	ND	ND	√	=	ND
C7	11.4	303.1622	$C_{13}H_{25}N_3O_3S$ (71)	1.62	ND	ND	√	ND	ND	ND
C8	16.0	304.2406	$C_{20}H_{32}O_2$ (85)	1.29	=	=	=	ND	↑	ND
C10	8.5	310.2410	$C_{18}H_{34}N_2S$ (99)	-1.80	=	=	ND	ND	↑	ND

C11	12.9	314.2060	No formula generated		ND	ND	ND	ND	ND	√
C12	7.6	346.1609	$C_{13}H_{26}N_6OS_2$ (81)	-0.71	=	↑	ND	ND	ND	ND
C13	14.9	376.2614	$C_{23}H_{36}O_4$ (81)	-2.12	ND	ND	=	ND	√	ND
C14	15.0	385.2590	$C_{21}H_{37}O_6$ (98)	-1.37	ND	ND	ND	ND	√	ND
C15	7.6	449.1086	$C_{18}H_{24}ClNO_{10}$ (82)	-0.54	√	√	ND	ND	ND	ND
C16	12.6	458.3257	$C_{26}H_{42}N_4O_3$ (97)	0.69	ND	ND	ND	ND	√	ND
C17	15.6	475.3633	$C_{25}H_{53}N_2OS_2$ (76)	0.70	ND	√	ND	ND	ND	ND
C18	8.5	505.2361	No Formula generated		√	√	ND	ND	ND	ND
C19	13.0	533.2872	$C_{33}H_{35}N_5O_2$ (94)	0.49	ND	ND	ND	√	ND	ND
C20	13.7	547.3635	$C_{32}H_{51}O_7$ (97)	0.10	ND	ND	ND	ND	√	ND
C21	11.2	552.3179	$C_{29}H_{36}N_{12}$ (75)	-1.26	ND	ND	√	ND	ND	ND
C22	16.5	572.1387	$C_{32}H_{30}NO_3S_3$ (73)	-0.45	ND	ND	↑	ND	ND	ND
C23	13.4	659.3180	$C_{34}H_{47}N_2O_{11}$ (91)	0.06	ND	√	ND	ND	ND	ND
C24	16.7	829.5609	$C_{35}H_{71}N_{15}O_8$ (97)	-0.10	ND	ND	√	ND	ND	ND
C25	16.2	853.5621	$C_{45}H_{83}N_5O_4S_3$ (88)	0.79	ND	ND	ND	√	ND	ND

5.4.5 Implications of the present findings

MG and its main metabolite LMG are currently considered possible carcinogens (EFSA 2016), but consumption of contaminated seafood with levels below 1 ppb was found to present a low risk to human health (Health Canada 2017). Previous studies (Mitrowska et al. 2007, Shalaby et al. 2016) have shown that some cooking procedures are not sufficient in reducing the levels of the two compounds, which was confirmed in this study for two new matrices, brook trout and white shrimp. Canning, which has been used as a cooking treatment to evaluate MG and LMG for the first time in this study, was able to achieve the almost complete reduction of MG levels. However, the canning treatment applied in this study simulated home canning rather than more industrial canning. Depending on can sizes, recommended treatment duration at a retort temperature of 121°C may vary from 33 to 187 minutes (Featherstone 2016). Currently, there is limited information in the literature on the detection of MG and LMG in canned seafood sampled from local markets for example. As part of a total diet study, Tittlemier et al. (2007) sampled and analyzed canned tuna purchased from the Canadian market, but did not find any MG or LMG. MG was detected in two canned fried dace fish at levels below 10 ng/g sampled from Hong Kong markets but no information was provided on the levels of LMG detected (Hong Kong Center for Food Safety 2016). Therefore, canned seafood should also be analysed as part of monitoring MG and LMG levels, especially since LMG appears to be more persistent in muscle during canning treatment compared to MG.

Different possible TPs were detected across all the thermal treatments and in all matrices. This study, along with previously reported thermal studies of food contaminants (Tian and Bayen 2018, von Eyken and Bayen 2020) reinforces the conclusion that some compounds do not have

the same behaviour or follow the same transformation mechanisms across different food matrices and thermal treatments. One possibility for this may be the different degree of oxidation occurring during different cooking procedures. For example, Khan et al. (2015) found that cholesterol underwent more oxidation during microwaving as a larger amount of cholesterol oxidation products (COPs) were detected in meats compared to other cooking procedures, e.g., roasting. Furthermore, depending on the meat, i.e., bacon vs. sausage, different COPs were detected for the same cooking procedure.

The two tentatively identified transformation products 2-desmethylated MG and LMG, have been detected as possible metabolites in the livers of rats that were fed the two compounds through their diet (Culp et al. 1999). The subsequent oxidation of these compounds may contribute to the formation of DNA adducts, which have been detected in tissues of rats (Culp et al. 1999). Currently, there are no standards available for confident identification and quantification of these TPs. In this context, a semi-quantification approach using the parent compounds (von Eyken and Bayen 2020) combined with a threshold of toxicological concern approach should be explored as a first screening tool to assess if TPs are priority compounds for further toxicological testing.

5.5 Conclusion

In this study, non-target analysis using high resolution mass spectrometry was applied for the first time to study the fate of MG and LMG in brook trout and crustaceans. Even though shrimp was found to have a lower fat content, this matrix did not impact the concentration changes for the more lipophilic LMG, with only microwaving achieving a significant reduction of the metabolite. Three compounds, resulting from the possible demethylation and cleavage of

the conjugated structures have been proposed as possible TPs. Their identity could not be confirmed due to the lack of available analytical standards of the pure compounds and further research is needed to determine possible toxicity. The findings of this study show the importance of integrating analysis of processed, e.g., canned seafood when assessing the human exposure and possible health risk associated with MG and LMG, as these processing treatments are not adequate in reducing residues present in muscle.

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5.7 Supplementary information

Table S5.1: Recoveries and matrix effects for MG and LMG in raw and cooked trout and shrimp.

		Matrix effect MG	Recovery MG	Matrix effect LMG	Recovery LMG
Trout	Raw	107±7	67±10	91±6	79±10
	RSD (%)	6.6	15.6	7.1	13.4
	Boiled 10 min	121±11	111±6	108±22	97±11
	RSD (%)	9.9	5.0	21.0	11.4
	Boiled 30 min	108±4	105±3	96±4	97±13
	RSD (%)	4.1	2.8	4.6	12.9
	Canned	89±7	62±12	79±7	83±15
	RSD (%)	7.9	18.1	9.8	18.9
	Microwave	97±5	71±10	94±5	103±7
	RSD (%)	5.9	15.3	5.4	7.1
Shrimp	Raw	109±12	69±21	54±10	73±10
	RSD (%)	10.6	30.3	19.9	13.6
	Boiled 10 min	111±12	88±7	64±22	71±15
	RSD (%)	10.2	8.1	35.1	20.7

Table S5.2: Mass measurement errors for MG and LMG in raw and cooked shrimp and trout muscle

		Mass measurement (ppm) ±standard deviation	
		MG	LMG
Trout	Raw	-0.45±1.11	0.01±1.21
	Boiled 10 min	-1.42±0.68	-0.61±0.29
	Boiled 30 min	-1.12±0.29	-0.51±0.29
	Canned	-3.65±1.58	1.67±0.38
	Microwave	-0.31±0.40	-0.03±0.22
Shrimp	Raw	0.03±1.35	-0.78±1.29
	Boiled 10 min	-0.03±0.66	-0.73±1.00

Figure S5.1: Reduction of MG and LMG in water at 100°C

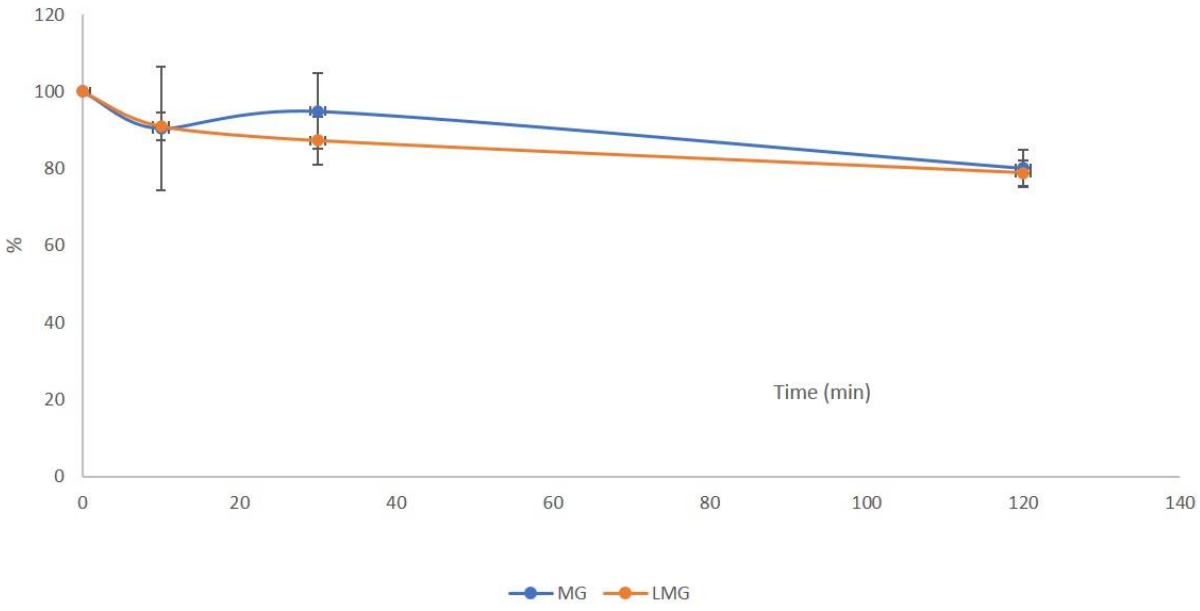


Table S5.3: Possible TPs of MG and LMG identified in negative ionization mode in *cooked exposed (CE)* trout and shrimp based on fold change and statistical analysis √: increasing in *CE* (with fold change >2 compared to *CC* and *RE*) and statistically significant (p<0.05), ↑: increasing in *CE* (with fold change >2 compared to *CC* and *RE*) but not statistically significant (p>0.05), =: detected in *CE* but fold change <2 compared to *CC* and *RE*) and not statistically significant (p>0.05), but not increasing, ND: not detected

Compound	R_t (min)	Neutral mass	Formula (score)	Mass measurement error (ppm)	Trout				Shrimp
					Boiling 10 min	Boiling 30 min	Canning	Microwaving	Boiling 10 min
C1	15.0	240.0744	$C_{10}H_{15}N_2O_5$ (93)	-0.72	ND	ND	ND	↑	ND
C2	13.7	284.2139	$C_{20}H_{28}O$ (85)	-0.26	ND	ND	ND	ND	√
C3	12.4	306.2225	$C_{22}H_{28}N$ (84)	0.93	ND	ND	ND	ND	√
C4	11.8	335.2454	$C_{20}H_{33}NO_3$ (79)	-1.59	ND	ND	ND	ND	√
C5	12.9	348.2660	$C_{22}H_{36}O_3$ (74)	-1.20	ND	ND	ND	√	ND
C6	12.6	462.2788	$C_{25}H_{38}N_2O_6$ (94)	-0.44	ND	ND	ND	↑	ND
C7	12.9	490.3035	$C_{25}H_{40}N_5O_5$ (98)	1.08	ND	ND	ND	ND	√
C8	17.8	501.4112	$C_{23}H_{51}N_9O_3$ (76)	-0.62	ND	ND	ND	√	ND
C8	14.7	632.5013	$C_{38}H_{62}N_7O$ (99)	-0.45	ND	ND	ND	√	ND
C9	18.1	699.4947	$C_{40}H_{60}N_{10}O$ (97)	-0.67	ND	ND	ND	ND	↑

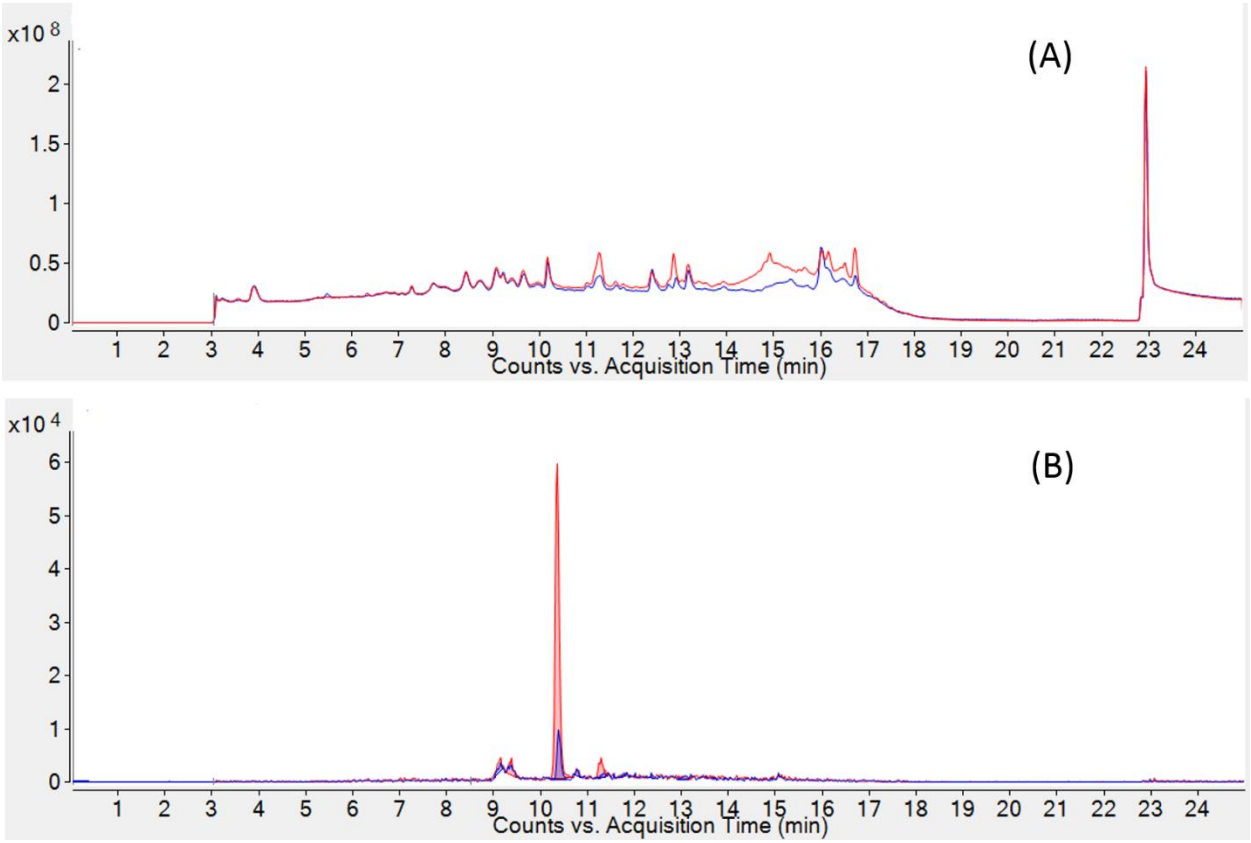
C10	14.2	708.2575	$C_{27}H_{42}N_5O_{17}$ (98)	-0.05	ND	ND	ND	ND	√

Table S5.4: TPs of MG and LMG described in the literature

Neutral mass	Formula	Formation	Reference
197.0841	$C_{13}H_{11}NO$	Phototransformation	Perez-Estrada et.al 2008
210.0997	**$C_{14}H_{13}NO$**		
213.0790	$C_{13}H_{11}NO_2$		
225.1154	$C_{15}H_{15}NO$		
227.0946	$C_{14}H_{13}NO_2$		
239.0790	$C_{15}H_{14}NO_2$		
241.1102	$C_{15}H_{15}NO_2$		
259.1208	$C_{15}H_{17}NO_3$		
273.1385	$C_{19}H_{17}N_2$		
274.1470	$C_{19}H_{18}N_2$	Metabolite/biotransformation	Cha et.al 2001, Culp et.al 1999
287.1540	$C_{20}H_{19}N_2$	Phototransformation/metabolite/biotransformation	Perez-Estrada et.al 2008, Cha et.al 2001, Culp et.al 1999
288.1626	$C_{20}H_{20}N_2$	Metabolite/biotransformation	Cha et.al 2001, Culp et.al 1999
301.1700	**$C_{21}H_{21}N_2$**	Phototransformation/metabolite/biotransformation	Perez-Estrada et.al 2008, Cha et.al 2001, Culp et.al 1999
302.1783	**$C_{21}H_{22}N_2$**	Metabolite/biotransformation	Cha et.al 2001, Culp et.al 1999
306.1122	$C_{19}H_{16}NO_3$	Phototransformation	Perez-Estrada et.al 2008
315.1861	$C_{22}H_{23}N_2$		
316.1939	$C_{22}H_{24}N_2$		
317.1646	$C_{21}H_{21}N_2O$	Photodegradation/metabolite/biotransformation	Perez-Estrada et.al 2008, Cha et.al 2001, Culp et.al 1999
320.1280	$C_{20}H_{18}NO_3$	Phototransformation	Perez-Estrada et.al 2008
321.1365	$C_{20}H_{19}NO_3$		
329.1648	$C_{22}H_{21}N_2O$		
331.1804	$C_{22}H_{23}N_2O$		
345.1961	$C_{23}H_{25}N_2O$	Phototransformation/metabolite	Perez-Estrada et.al 2008, Culp et.al 1999
347.1753	$C_{22}H_{23}N_2O_2$	Phototransformation	Perez-Estrada et.al 2008
361.1908	$C_{23}H_{25}N_2O_2$		
362.1994	$C_{23}H_{26}N_2O_2$		
363.1705	$C_{22}H_{23}N_2O_3$		
377.1858	$C_{23}H_{25}N_2O_3$		

Note: Compounds in bold were tentatively identified in this study

Figure S5.2: Total Ion Chromatogram (TIC) (A) for raw exposed trout (blue) and canned exposed trout (red); Extracted Ion Chromatogram (EIC) (B) for C5 at *m/z* 302.1783 (±20 ppm)



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Connecting paragraph

In chapter 5, non-targeted analysis was successfully applied for the first time to study the thermal transformation of one antimicrobial, MG, and its metabolite during different cooking treatments and different matrices. This approach allowed for the tentative structural identification of three TPs in muscle formed in different cooking treatments. The food matrix was found to have a possible influence in the transformation mechanism, as different TPs were identified in shrimp and trout muscle. In Chapter 6, the fate of the second antimicrobial, OTC, during cooking was studied in shrimp, using the same non-targeted analysis approach. Not only did this allow to describe the fate of another commonly detected veterinary drug in shrimp, but also enabled the comparison of different extractions of the same matrix (shrimp), but contaminated with two different antimicrobials (MG vs. OTC). While the data analysis step followed the same workflow as in Chapter 4, the optimal sample extraction for the determination of OTC was chosen based on the comparison of the four methods discussed in Chapter 3. This chapter will be submitted for publication to *Food Control* as "Application of non-target analysis for the identification of thermal transformation products of oxytetracycline in pacific white shrimp".

Chapter 6: Application of non-target analysis for the identification of thermal transformation products of oxytetracycline in pacific white shrimp

6.1 Abstract

Oxytetracycline is an antibiotic authorized for use in aquaculture and is often detected in seafood products, especially shrimp. Previous studies investigated the loss of oxytetracycline in shrimp tissues during cooking, but did not investigate any transformation products. Hence, the objective of this study was to apply a non-target analysis workflow to study the fate of oxytetracycline in different matrices, shrimp muscle, shrimp shell and water during thermal treatment. First, four different extraction methods were compared for the determination of oxytetracycline in shrimp muscle. Secondly, raw and cooked samples were then extracted using the suitable method (acidified water/methanol/acetonitrile with limited clean-up of samples achieved using freezing) and were analyzed by HPLC-QTOF-MS. Oxytetracycline levels were reduced by 87, 75 and 39% in water, muscle and shell, respectively. Identification of thermal transformation products was limited to formula generation, but results showed a matrix dependent transformation mechanism, with different compounds detected between water, spiked and incurred tissues.

6.2 Introduction

Oxytetracycline (OTC) is one of the few antibiotics licensed for applications in aquaculture with different regulations governing its use between countries. In Canada, it is permitted solely in salmonids and lobsters in Canada, with a maximum residue limit (MRL) set at 0.2 ppm (Health Canada 2010). However, it is routinely used as a veterinary drug in shrimp farming in countries such as Vietnam (Pham et al. 2015) with an MRL set at 0.1 ppm (Yen et al. 2020). The Codex Alimentarius Commission has also derived an MRL of 0.2 ppm in giant prawns (Codex Alimentarius 2018) and in Europe, OTC is allowed for use in food producing animals with an MRL of 0.1 ppm set for muscle (EU Commission Regulation 37/2010). Shrimp is one of the most commonly seafood commodities that is contaminated with OTC residues (European Commission RASFF Portal , Dinh et al. 2020). Studying the heat-stability of antibiotics during cooking is key in deriving a more representative dietary exposure of consumers to the compounds (WHO 2009). OTC is heat-labile during cooking, with reductions observed throughout various thermal treatments (e.g. frying, boiling and microwaving) across different matrices, such as salmon (Kitts et al. 1992), chicken (Nguyen et al. 2015) and shrimp (Uno et al. 2006, Uno et al. 2010). However, there is a lack of information on the identity of the possible transformation products (TPs). Nguyen et al. (2015) identified that α and β-apoOTC formed during microwaving and boiling of pig and chicken. Administration of these two compounds to rats at doses of 10 mg/kg body weight were found to significant decrease body weights and adversely impact the kidney and liver (Nguyen et al. 2015). It should be however highlighted that α and β-apoOTC represented less than 2% of the initial OTC content, therefore it is likely that the transformation mechanism of OTC during cooking may involve other chemical reactions.

The identification of thermal TPs may be achieved using non-target analysis (Baesu et al in revision, von Eyken and Bayen 2020). Two other frequent seafood contaminants, malachite and leucomalachite green, were shown to form different suites of TPs depending on thermal treatments (e.g., microwaving and boiling) when present in different matrices (Baesu et.al in revision). A challenge in non-target analysis is the validation of the extraction approach, as it is difficult to develop the sample preparation step when the compounds of interest, i.e., TPs, are unknown. Different criteria have been proposed in the literature, including number of molecular features (Arbulu et al. 2015, Creydt et al. 2018), recovery of specific compounds (Sitnikov et al. 2016, Jia et al. 2017) and repeatability (Theodoridis et al. 2012, Arbulu et al. 2015, Sitnikov et al. 2016).

The objectives of this study were: (i) to compare four different methods for the extraction of OTC in white shrimp (*Litopenaeus vannamei*) based on number of extracted molecular features, recovery of OTC and repeatability of extracted features and identify the most promising method to identify thermal transformation products, (ii) apply the selected non-target approach to identify thermal TPs of OTC in incurred shrimp muscle and shell, (iii) identify thermal TPs in muscle and shell spiked with native and labelled OTC and (iv) compare the effect of the food matrix on the transformation mechanism of OTC in water, muscle and shell. To the best of our knowledge, this is the first time that non-target analysis has been applied to study the fate of OTC in white shrimp tissues.

6.3 Materials and methods

6.3.1 Chemicals

Oxytetracycline hydrochloride (> 98%, VETRANAL®) analytical standard was obtained from Sigma Aldrich (St Louis, MO, USA). Labelled internal standards, d_3-diphenhydramine and d_3-6-acetylmorphine, $^{13}C_6$-propylparaben were purchased from Cerilliant (Round Rock, TX, USA) and Sigma Aldrich respectively. $^{13}C_{22}$-$^{15}N_2$-Oxytetracycline (>96%) was purchased from Romer Labs (Tulln, Austria). HPLC grade acetonitrile, methanol, water, LC-MS grade formic acid, acetic acid and ammonium acetate were obtained from Fisher Chemical (Pittsburgh, PA, USA). Anhydrous magnesium sulfate, sodium acetate and disodium dihydrate EDTA were purchased from Sigma Aldrich. Primary secondary amine (PSA) sorbent was purchased from Agilent Technologies (Santa Clara, CA, USA). Stock and working native OTC-hydrochloride standard solutions in methanol of 1 mg/mL and 10 µg/mL were prepared in amber vials and stored at -20°C. Stock and working solutions of 2.5 and 0.3 µg/mL of [$^{13}C_{22}$-$^{15}N_2$]-OTC were prepared in methanol per the supplier's instructions and stored at -20°C in amber vials. Six calibration standards from 5 to 40 ng/mL were prepared in acetonitrile/methanol (50/50, v/v).

6.3.2 Shrimp exposure to OTC

Pacific white shrimp were obtained from Planet Shrimp facilities (Aylmer, ON, Canada). Two tanks of 60 L (one control and one for exposed) each filled with distilled water were used, with 60 shrimp per tank. Water pH was 8, temperature was maintained at 27°C and dissolved oxygen was 5 mg/L. Artificial seawater (16 g/L) was prepared with sea salt (Instant Ocean, Blacksburg, VA, USA) based on recommendations from Planet Shrimp facilities. Shrimp feed,

obtained from the same facility, was mixed with OTC-hydrochloride, cod liver oil (3% w/w) and distilled water (3% w/w) in order to achieve a target concentration of 2 g/kg of feed. This high concentration was prepared to ensure detectable levels of OTC in shrimp tissues. Aquaculture doses of OTC-hydrochloride depend on the organisms and feeding rate (Health Canada 2019). For example, the recommended concentration of OTC-hydrochloride in medicated feed for lobsters is 2.2 g/kg feed (Health Canada 2019). For shrimp, the maximum recommended dose is 4.5 g OTC-dihydrate/kg feed (Bray et al. 2006). Control feed was prepared the same way but without the addition of OTC. Shrimp were acclimatized and fed control feed (~5.0 g/tank) for 12 hours before introducing OTC feed. After an initial feeding (~5.0 g), shrimp were fed OTC feed twice more at 3-hour intervals, followed by a 12-hour starvation period and another feeding. Monitoring of the feeding indicated that all the feed was consumed, however it was difficult to ascertain if every individual shrimp consumed an equal amount of feed. Assuming that all individual shrimps consumed the feed equally, it was estimated that each shrimp ingested about 0.67 mg of OTC-hydrochloride, equivalent to 0.62 mg OTC. Shrimp were sacrificed by placing them on ice 2 hours after the last feeding. Shrimp were individually wrapped in aluminum foil and polyethylene bags and stored at -80°C.

6.3.3 Sample extraction

Shrimp muscle was homogenized using a mortar and pestle. Four methods were compared for the extraction of OTC from shrimp muscle. All extracts were filtered using a 0.22 μm PTFE filter (Canadian Life Science, Peterborough, ON, Canada) and stored in amber vials covered with aluminum foil at -20°C.

Extraction 1 was adapted from Dasenaki and Thomaidis (2015). Briefly, 1.0 g of sample was weighed into a 50 mL centrifuge tube, to which water (2 mL; 0.1% formic acid v/v, 0.1% EDTA w/v), acetonitrile (2 mL) and methanol (2 mL) were added. Samples were vortexed between each solvent addition and were centrifuged (Eppendorf, Hamburg, Germany) for 4400 rpm (3000 × *g*, 25°C) for 10 minutes. Supernatants were collected in new tubes and transferred to -20°C for 14.5 hrs. Samples were then centrifuged again for 10 minutes at 4400 rpm and filtered.

Extraction 2 was adapted from Jia et al. (2017). Briefly, 1.0 g of sample was weighed into centrifuge tubes, after which 5 mL (84:16 v/v) acetonitrile/water with 1% acetic acid and 0.100 mL of 0.05 M ETDA were added and vortexed for 1 minute. To each sample, 1.0 g of $MgSO_4$ and 0.30 g sodium acetate were added, vortexed for 1 minute followed by centrifugation at 4400 rpm (3000 × *g*, 25°C) for 5 minutes. Supernatant (2 mL) was transferred to new tubes containing 0.24 g $MgSO_4$ and 25 mg PSA, vortexed for 1 minute, centrifuged for 5 min at 4400 rpm and filtered.

Extractions 3 and 4 were adapted from Nacher-Mestre et al. (2013). In short, 2.0 g of shrimp muscle was weighed into 50 ml centrifuge tubes after which 4 mL (80/20 acetonitrile/water) and 0.040 mL of 0.05 M EDTA was added. For *Extraction 3*, formic acid (0.1%) was also added while *Extraction 4* was not acidified. Samples were vortexed and centrifuged at 4400 rpm (3000 × *g*, 25°C) for 10 min. Supernatant (2 mL) was transferred to new tubes, covered in aluminum foil and stored at -20°C for 2 hours. Extracts were centrifuged again for 10 minutes, 4400 rpm and filtered.

A total of 10 replicates and 5 procedural blanks (following the same procedure but without sample addition) were prepared for each extraction. For each of the four extractions, five

extractions QCs were prepared by pooling 20 µL of each replicate extract and blank (Dunn et.al., 2011, Perez-Miguez et.al., 2018). Five injection QC samples were prepared by pooling 10 µL of all extracts and blanks from all four extractions. For LC-MS analysis, 50 µL of labelled internal standard solution was added to non-diluted extracts for the purpose of monitoring instrumental variability. Because of difference in the sample/solvent ratio amongst the four extractions, extraction QCs were diluted in a 50/50 mixture of acetonitrile and methanol to obtain 0.04 g of matrix in the vial for LC-MS analysis for direct comparison of extractions.

Recovery experiments (n=6) were completed for both raw and cooked shrimp muscle samples. Muscle tissues were spiked in order to achieve a target OTC concentration of 100 ng/g, and were allowed to equilibrate for 10 min prior to extraction. Extracts were prepared the same way as exposed samples for LC-MS analysis. Matrix effects and recoveries were calculated according to Matuszewski et al. (2003).

6.3.4 Thermal treatments

Approximately 2 g of shrimp muscle and 0.1 g of shell (n=10 each) were placed separately in amber vials, capped and transferred to a water bath (100°C). Samples were removed after 10 minutes and allowed to cool at room temperature. Separately, three replicates of muscle and shell were spiked with 100 µL of 10 µg/mL native OTC solution and 100 µL of 2.5 µg/mL [$^{13}C_{22}{}^{15}N_2$]-OTC before heating and allowed to rest for 10 min. The spiked tissues were extracted the same way as the incurred samples. Any juices observed after heating were added to the centrifuge tubes and extracted along with the muscle. No juices were observed during heating of shells. Both native and labelled aqueous OTC standard solutions of 25 ng/mL

(0.5 mL) were transferred into 2 mL amber vials, transferred to the water bath at 100°C and removed after 10 and 60 minutes.

6.3.5 Mineral analysis

Freeze-dried (Martin Christ Gamma 1 –16 LSC freeze-dryer, Germany) shrimp muscle and shell (n=5) were weighed (~0.12 g) into 15 mL digestion tubes. Two mL of trace metal grade nitric acid was added and allowed to react overnight. Tubes were put in a digestion block and temperature was slowly increased to 130℃ over a period of one hour. Samples were completely digested after 5 hours and were then diluted to 50 mL with ultrapure water. All the digested samples were further diluted 1/50 with distilled deionized water for calcium and magnesium analysis, and 1/10 for zinc and iron analysis. Mineral content was determined using a Varian220FS Flame AAS using wavelengths of 422.7, 285.2 and 213.9 nm for calcium, magnesium and zinc, respectively.

6.3.6 LC-MS analysis

Shrimp and water samples were analyzed using an Agilent UHPLC 1290 coupled with an Agilent 6545 QTOF-ESI-MS, in both positive (ESI+) and negative electrospray ionization (ESI-) modes. An InfinityLab Poroshell 120 (Pheny-Hexyl, 3.0 x 100 mm, 2.7 µm, Agilent Technologies) with a Poroshell (4.6 mm) Phenyl Hexyl pre-column was used. Flow rate of 0.2 mL/min with mobile phases (A) H_2O with 0.1 % formic acid and (B) acetonitrile in ESI+ mode and (A) 0.05 M ammonium acetate and (B) acetonitrile in ESI-. The same gradient elution was used for both ESI+ and ESI- modes, starting from 1 min 5% B, from 1 to 15 min gradient to

100% B, from 15 to 20 min 100% B, from 20 to 20.10 min gradient to 5% B and from 20.10 to 25 min 5% B. Injection volume was 10 µL for both ionization modes and column temperature was 20°C. The MS parameters were as follows: sheath gas temperature 275°C, drying gas temperature 325 °C, drying gas flow 5 L/min, sheath gas flow 12 L/min, nebulizer pressure 20 psi, capillary voltage 4000 V, nozzle voltage 2000 V, fragmentor voltage 175 V, skimmer voltage 65 V. All Ions MS/MS mode at collision energies of 0, 10, 20 and 40 V was used. This mode allows to record both precursor and fragment ion information. Data was collected between 100 and 1700 *m/z* at a rate of 3 spectra/s. Samples were kept at 4°C in the multi sampler compartment.

6.3.7 Data processing

SPSS Statistics software (v.26) (IBM, NY, USA) was used for statistical analysis such as the comparison of mineral content among muscle and shell. Level of significance was set at p=0.05. OTC concentrations were computed using Agilent Mass Hunter Quantitative Analysis B.10.0, using a mass extraction window of ±20.0 ppm and retention time window of ±0.50 min. The most abundant ion $[M+H]^+$ at *m/z* 461.1560 was used for quantification of OTC. External calibration was used to assess recoveries and matrix effects., Matrix-matched calibration based on six calibration levels, (13.0, 20.8, 26.0, 39.0, 65.0 and 130.0 ng/g) was applied for the quantification of raw and cooked muscle and shell. Method detection limit (MDL) and limit of quantification (LOQ) were calculated as 3σ and 10σ, respectively, of the procedural blanks integrated at the retention time of OTC.

6.3.7.1 Extraction comparison

Data alignment and feature extraction were completed using Agilent Mass Hunter Profinder software B.10.0. Molecular feature extraction was performed for each individual extraction (10 replicates, 5 procedural blanks and 5 extraction QC samples), using the following default parameters: peak filter height 200 counts, retention time window ±0.30 min, mass window ±20.00 ppm, post-processing peak absolute height 1000 counts, MFE score 80%. Features were exported in Excel for further data filtering. Only features present in all 5 QC replicates at a ratio of sample/blank>2 or absent in blanks were used to calculate repeatability, expressed as coefficient of variation (CV).

6.3.7.2 Identification of the thermal TPs

Data processing parameters in Profinder identical to those in the previous section were applied for the molecular feature extraction of heated water samples and cooked shrimp and shells. Molecular features were exported as .pfa files, imported into Mass Profiler Professional (v 15.0) with a percentile shift (75.0) normalization. Muscle and shell samples were grouped as *raw control (RC), raw exposed (RE), cooked control (CC), cooked exposed (CE)* and *cooked spiked (CS)*. Water samples were grouped based on heating times as 0, 10 and 60 minutes. Fold change and statistical tests ($p < 0.05$, Benjamini-Hochberg correction and Tukey post-hoc) were applied to identify possible TPs (Figure 6.1). Compounds for which general formulas had a matching score >70% were considered. This matching score is typically used in compound identification in non-target analysis (Du et al. 2017). These features were analyzed again in targeted MS/MS mode, extracted using Agilent Qualitative Analysis B.10.0 and exported as .cef files. These files

were then imported into Agilent Molecular Structure Correlator with features ran through Chemspider (Royal Society of Chemistry 2020) and Agilent Metlin (30232 compounds) databases. In cases where targeted MS/MS data could not be obtained, All Ions MS/MS data was used to compare the spectra of targeted compounds with that of an OTC standard to try and identify common fragment ions.

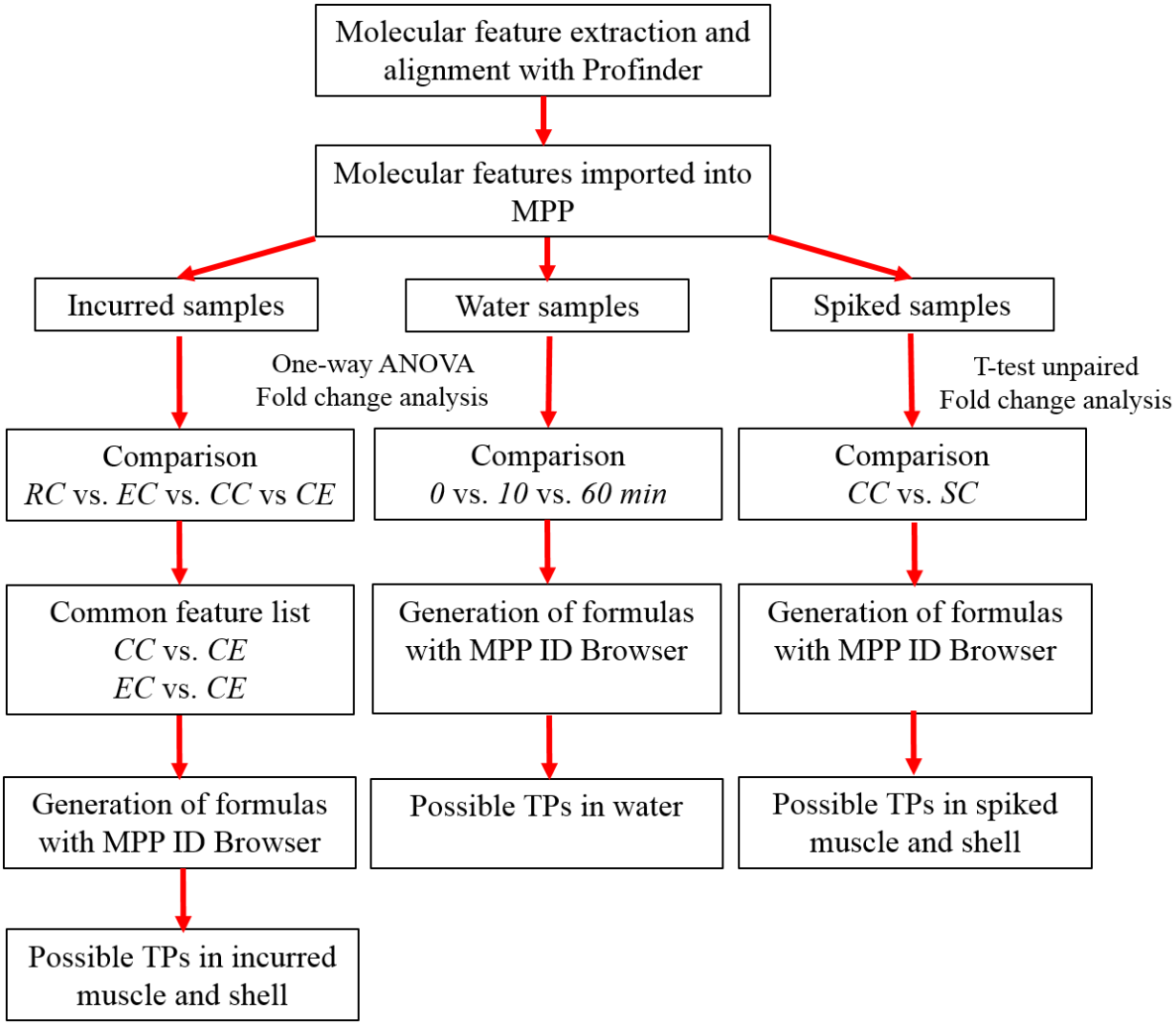

Figure 6.1: Data processing workflow for identification of TPs of OTC

6.4 Results and discussion

6.4.1 QA/QC

Mean mass measurement error (MMME) for OTC, calculated based on Brenton and Godfrey (2010), were -0.86±1.89 ppm in solvent, -0.43±0.41 ppm in water, 0.72±1.17 ppm in cooked shells, 4.48±3.25 ppm in raw shells, -0.72±1.96 ppm in raw muscle and -0.65±3.29 ppm in cooked muscle. In cooked muscle and shell spiked with native and labelled OTC, MMME for OTC were 0.87±0.37 in muscle and 1.52±0.22 ppm in shell with values of 3.02±2.55 in muscle and -1.17±0.32 ppm in shell obtained for [$^{13}C_{22}{}^{15}N_2$]-OTC. Retention time for OTC and all internal standards was reproducible, with RSD<2%. Good linearity (R^2>0.99) was observed. Pooled QC samples were used to determine the variability introduced through instrumental analysis by building Principal Component Analysis (PCA) plots in MPP. All QC samples along with procedural blanks grouped together, an example of a PCA plot is shown in Figure S6.1, indicating low instrument variability (Sangster et al. 2006). Overall, the LC-MS method was reproducible as indicated by the grouping of QC samples, and achieved good mass accuracy with acceptable MMME that fell below the general threshold of 5 ppm set in non-target analysis (Deeb et al. 2017).

6.4.2 Selection of the extraction method

Although it is difficult to "optimize" an extraction method in non-target analysis, the sample preparation step should still cover a large number and variety of compounds, be reproducible and simple with minimum number of steps (Vuckovic 2012). The four extraction methods applied in this study were compared based on common criteria used in non-target or

metabolomic studies (Sitnikov et al. 2016, Knolhoff et al. 2019), specifically the recovery of OTC, the number of extracted molecular features and the repeatability of molecular features.

6.4.2.1 OTC recovery

The highest measured OTC concentrations, between 64 and 172 ng/g, were obtained through *Extraction 1* (Table S6.1). Good recoveries, of 113±32 and 108±20% were obtained in raw and cooked shrimp (Table S6.2). For this extraction, recoveries were also done for shells and were determined as 55±9 and 96±30% in raw and cooked shells respectively, with RSD<30%. Ion suppression, with matrix effects below 100% was observed for all four extractions, which was expected as the extracts were not diluted prior to LC-MS analysis.

OTC was not detected in any of the ten replicates in *Extraction 2*. Computed concentrations for raw and cooked shrimp muscle in recovery experiments for *Extraction 2* were below the MDL. One hypothesis which could explain this was that shrimp used for this particular extraction consumed less OTC-feed. This hypothesis was however rebutted based on several observations. First, one shrimp used for *Extraction 1* was also used in *Extraction 2.* The concentration of OTC in the extract calculated through *Extraction 1* was 33 ng/mL which should have also given a good signal for *Extraction 2.* Inspection of the extracted ion chromatogram (EIC) (Figure S6.2) clearly revealed a peak corresponding to OTC for *Extraction 1*, but no distinguishable peak could be detected in the EIC for *Extraction 2*, suggesting that OTC was not extracted at all using *Extraction 2*. In addition, OTC was detectable when spiked post-extraction (assessment of the matrix effect), indicating that OTC had been probably lost during extraction. For example, a similar QuEChERS extraction based using acetonitrile/water as solvent but using freezing as a clean-up step instead of dispersive sorbents, like PSA, achieved a recovery of only

14% in shrimp muscle (Dinh et al. 2020). While acetonitrile (acidified with formic acid) has been deemed suitable as an extraction solvent for tetracyclines in fish (e.g. seabream) in terms of less pronounced matrix effect, poor recoveries below 20% were achieved (Dasenaki and Thomaidis 2010). Recoveries could be greatly improved, reaching 100%, with the addition of methanol (Dasenaki and Thomaidis 2010). Furthermore, this QuEChERS method, similar to the method used by Dinh et al. (2020), used $MgSO_4$ as partitioning salt. Divalent cations, like magnesium, can form chelates with tetracyclines (Uno et al. 2006), and although EDTA was added with the extraction solvent, the amount may be further optimized to improve recovery. Other partitioning salts could be used; for example, Grande-Martinez et al. (2018) used ammonium sulfate for the development of a QuEChERS extraction in salmon and panga fish, with good recoveries above 90% obtained.

Poor recoveries (<45%) were also obtained for *Extraction 3* and *Extraction 4*, which can be expected as acetonitrile makes up 80% of the extraction solvent mixture. OTC was only detected in five out of ten replicates performed for *Extraction 3* with an average concentration of 45±6 ng/g and only in one replicate performed for *Extraction 4* with a concentration of 37 ng/g. Previous studies investigating the pharmacokinetics of OTC in fish species retained only replicates that had quantifiable levels of OTC, i.e., above the LOQ (Namdari et al. 1996, Chen et al. 2004) to take into consideration different feeding rates and fish-to-fish variation. The average OTC concentration computed for *Extraction 3* was calculated based only on replicates that had OTC levels above the LOQ.

In conclusion, the addition of methanol as an extraction solvent greatly improved the extraction of OTC from incurred muscle and achieved the highest recovery. Based on this comparison criterion, *Extraction 1* was the best method.

6.4.2.2 Extracted molecular features and repeatability

The number of molecular features is another criterion often reported to compare extractions in non-target metabolomic studies. Even though one molecule may generate more than one molecular feature, the number of molecular features can be considered proportional to the number of metabolites/compounds extracted (Theodoridis et al. 2012). The greatest number of molecular features in ESI+ (3976) was obtained for the method based on *Extraction 4* (Table S6.3), which may be expected since this is a method with limited clean-up. The number of molecular features for *Extraction 2* was in the same range with 3717 extracted features.
The repeatability among extracted features, expressed as the coefficient of variation (CV) of features across all replicates, is another common criterion to compare extraction methods. Thresholds of 20 (Theodoridis et al. 2012) or 30% (Sitnikov et al. 2016) have been deemed acceptable. In ESI+, *Extraction 3* (Table S6.3) provided best results with CV<20% calculated for around 50% of features. Only 27.9% and 48.2% of features detected through *Extraction 1* had a CV<20% and 30% respectively, but it provided better results in ESI- mode with more than 50% of features below the acceptable CV thresholds. Despite a relatively large number of molecular features extracted and good repeatability, *Extractions 3* and *4* did not perform well in terms of extraction of the target compound, OTC.

When choosing the adequate extraction method in non-target analysis, a compromise must be reached as often, no single extraction will perform best for all the comparison criteria (Sitnikov et al. 2016). For example, Arbulu et al. (2015) found that the extraction providing best results for wine metabolites in terms of number of features extracted did not provide the best repeatability. In asparagus, extractions that provided the highest number of features did not have yield the highest signal intensities for some specific analytes (Creydt et al. 2018). In shrimp, or

seafood in general, there are a limited number of non-target/metabolomic studies. Some metabolomic studies do not describe in detail how the sample preparation step was optimized (Dubreil et al. 2019, Suantika et al. 2020). In other non-target studies, mostly focusing on suspect screening of contaminants in seafood, sample extraction is optimized to achieve the highest recoveries of a target list of analytes (Baduel et al. 2015, Jia et al. 2017, Turnipseed et al. 2017). Therefore, as the highest OTC recoveries and acceptable number of extracted features were obtained for *Extraction 1*, it was selected as the optimal extraction to study the fate of the antibiotic during thermal treatment.

6.4.3 Reduction of OTC during thermal treatment

OTC levels in water significantly decreased during heating by 87±4% (p <0.0005) after 10 min (Figure 6.2). This is in line with previous studies which determined heating times less than 15 minutes were sufficient to reduce OTC levels by more than 60% (Rose et al. 1996, Hsieh et al. 2011).

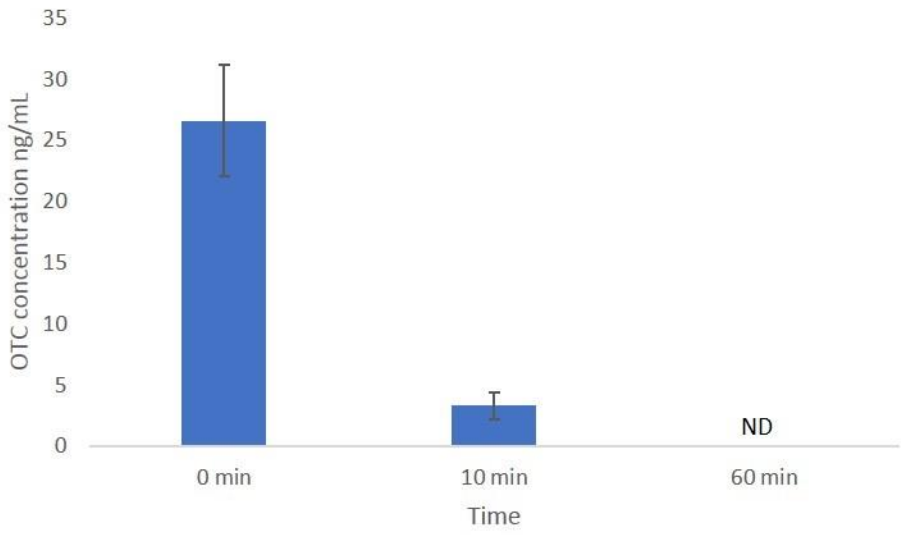

Figure 6.2: Reduction of OTC in water heated at 100°C, ND: not detected

In shrimp tissues (Table 6.1), OTC levels were also significantly reduced after boiling for 10 minutes at 100°C by 75±9% (p=0.001) and 39±22% (p=0.001) respectively. Among the three matrices, significant differences (one-way ANOVA) were found in the reduction rates between water and shell (p <0.0005), muscle and shell (p <0.0005) but not water and muscle (p=0.348). Higher mineral content, especially of divalent cations like calcium and magnesium (Table 6.2), leads to the formation of chelation complexes between OTC and cations which are more thermostable compared to only OTC (Uno et al. 2010). These findings are comparable with previous study by Uno et al. (2010) which found that OTC levels were reduced by around 50 and 25% in muscle and shell of white shrimp boiled for 4 minutes. In black tiger shrimp, boiling for 10 minutes reduced OTC levels in muscle by almost 80% (Uno et al. 2006).

Table 6.1: OTC concentrations in shrimp muscle and shell, before and after heating at 100°C.

Treatment	Average[a] concentration in muscle ng/g	Average[b] % reduction rate	Average[a] concentration in shell ng/g	Average[b] % reduction rate
0	51±33	n/a	213±18	n/a
10 min	12±7	-75±9*	129±68	-39±22*

*statistically significant at $p<0.05$; [a]expressed as the mean concentrations (n=10) ± standard deviation; [b]expressed as the mean reduction rate (n=10) ± standard deviation

Table 6.2: Mineral concentrations (mg/g wet weight) in muscle (n=5) and shell (n=5)

Matrix	Calcium*	Magnesium*	Zinc*
Muscle	1.36±0.25	0.36±0.04	0.013±0.001
Shell	17.24±1.62	0.68±0.02	0.008±0.001

*statistically significant at p<0.05

6.4.4 Identification of TPs

Recent studies have shown the suitability of non-target analysis to identify thermal TPs of a variety of compounds such as antifungals (Baesu et.al in revision) and bisphenols (Tian et al. 2020). Similar data analysis workflows, based on fold change and volcano plot analysis, can usually lead to the identification of around 10-30 compounds as possible TPs (Lege et al. 2020, von Eyken and Bayen 2020). Using fragmentation information following MS/MS analysis,the compounds of interest are screened against chemical databases, like Chemspider, to generate possible structures (von Eyken and Bayen 2020). The same workflow was applied in this study to identify TPs of OTC. For the first time, the fate of the compound during heating was also traced using the labelled [$^{13}C_{22}{}^{15}N_2$]-OTC. This procedure, based on spiking of labelled

compounds before heating, has been reported in non-target analysis, for example, to trace TPs of deoxynivalenol during baking (Stadler et al. 2019) or bisphenols in fish (Tian et al. 2020).

6.4.4.1 Water

In ESI- mode, no molecular features with a matching score >70%, based on formula generated through MPP ID Browser, were identified in heated water samples. In ESI+ mode, previous TPs identified during OTC hydrolysis were epi-OTC, α or β-apoOTC (Loftin et al. 2008, Xuan et al. 2009). Compounds with neutral masses corresponding to the apoOTC were not identified as statistically significant between unheated and heated either for 10 or 60 minutes. The retention time of the peak extracted at the *m/z* of apoOTC (443.1454) matched the retention time of the parent OTC and was therefore considered as a fragment of OTC (Lykkeberg et al. 2004, Zhu et al. 2019). While α or β-apoOTC may be formed at weak acidic pH (between 5 and 6.9), they are usually formed at much higher concentrations in highly acidic (pH 3.0) or alkaline pH (>9.0) solutions (Xuan et al. 2009). The extracted ion chromatogram (EIC) (±20 ppm) for OTC showed the appearance of another peak at retention time 8.9 min (Compound C9) with the same *m/z* (Figure 6.3), indicating the possible formation of epi-OTC. The same peak was identified in the EIC of the labelled OTC ($[M+H]^+$ *m/z* 485.2238) (Figure S6.3). The fragment ions, *m/z* 444.1295 and *m/z* 426.1189 (Figure 6.3) match the ions usually used for identification of epi-OTC (Zhu et al. 2019). The sum of peak areas (semi-quantification) of OTC and presumed epi-OTC measured in the 10- and 60-minute samples, represented 45±8% of the peak area measured for OTC in non-heated (0 min) samples. One other compound, C6 was identified at statistically significant higher abundance in 10- and 60-minutes samples compared to non-heated samples. Based on the generated formula $C_{21}H_{24}N_2O_7$, the compound could correspond to a TP,

as the formula is equivalent to a loss of CO_2 from the parent OTC. No database match in Chemspider or [$^{13}C_{22}{}^{15}N_2$] equivalent was found for C6. Following MS/MS fragmentation, no common fragment between OTC and C6 was found. Therefore, the main transformation mechanism of OTC during heating of aqueous solutions was epimerization.

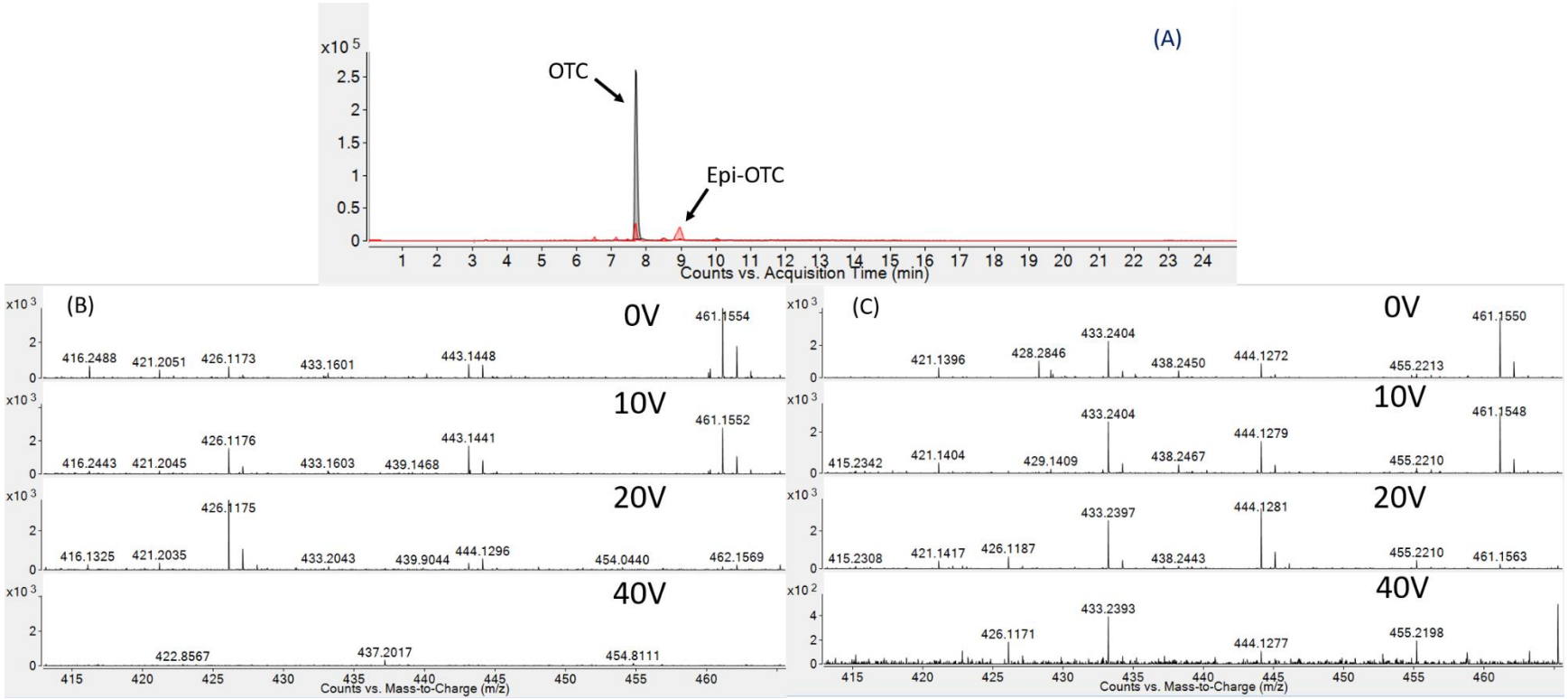

Figure 6.3: (A) EIC for OTC at *m/z* 461.1560 (±20 ppm) in unheated (black) and heated for 10 min (red) water samples; (B) MS spectra for OTC at 7.9 min at different collision energies; (C) MS spectra for epi-OTC at 8.9 min at different collision energies (0, 10, 20 and 40V).

6.4.4.2 Spiked tissues

In shrimp tissues, the same peak at retention time 8.9 min (compound C9), tentatively identified as epi-OTC, was detected in heated spiked muscle (Figure S6.4). This peak was not found in spiked shell, possibly because tetracycline epimers can revert back to the parent form in the presence of metals or alkaline conditions (Anderson et al. 2005). The presence of calcium and magnesium in shrimp shells may therefore inhibit the conversion of OTC into its epimer form. This "spiking" model, where the native compounds are spiked onto the matrix before cooking, has been used to evaluate the fate of the tetracycline antibiotics in chicken and pork. Gratacós-Cubarsí et al. (2007) identified the epimer and anhydro form of tetracycline in spiked boiled chicken and pork hamburgers along with two other non-identified peaks. OTC was found to be stable during microwaving and oven baking of chicken, with no TPs identified even at spiking levels of 0.1 and 1.0 μg/g (Sobral et al. 2020). Four other compounds (C4, C9, C11 and C13) were identified in spiked muscle and shell, but no peaks were observed at the corresponding labelled *m/z*, nor were there any matches identified in the Chemspider or Metlin databases. The transformation of OTC in spiked matrices appears to follow the same transformation as in water, where the primary reaction is the epimerization of OTC.

6.4.4.3 Incurred tissues

Epi-OTC was also detected in heated incurred shrimp muscle (Figure 6.4) but it was not detected in heated incurred shrimp shell. It was only detected, albeit not increasing significantly, in five out of the 10 heated shrimp replicates, which indicates that epimerization may not be the

main mechanism of transformation of OTC in incurred samples. Possibly, epi-OTC is accumulated in raw muscle due to metabolism. While not identified in shrimp, epi-OTC has been proposed as a metabolite of OTC in egg yolk and hen plasma (Zurhelle et al. 2000).

Fourteen compounds were detected as possible TPs in incurred tissues following statistical analysis, but based on the generated formulae, none of the compounds corresponded to suggested TPs for OTC reported in the literature (Table S6.4) formed following clear breakdown mechanisms of the main OTC structure. These mechanisms are mostly responsible for the transformation of OTC exposed to UV, through oxidation (Table S6.4). Radicals and oxidation may occur during cooking (Traore et al. 2012) therefore it is conceivable that there could be common mechanisms that could lead to the formation of the same TPs. Molecular features were screened manually for masses that would correspond to these previously reported TPs. For compounds where MS/MS spectra could not be obtained from targeted MS/MS mode, All Ions MS/MS data was used to manually screen for common fragments between the compounds and a pure OTC standard. It is possible that these compounds may be present in muscle after all, but not detectable with the current instrumental method. Other compounds had neutral masses larger than the parent OTC, suggesting possible recombination or complex reactions with matrix components. The formation of TPs with higher mass compared to the parent chemical has been observed for example in fungal biotransformation of ciprofloxacin, where TPs were formed following dimerization (Prieto et al. 2011). In the case of OTC, ring cleavage and subsequent hydroxylation during photodegradation formed TPs with neutral masses higher than 500 (Table S6.4). However, food matrices are much more complex compared to water and it is more difficult to propose structures for possible TPs. For example, boiling of cod and basa at 100°C

for 60 minutes, formed a large number of bisphenol A (BPA) TPs with neutral masses larger than BPA but no possible structures could be proposed (Tian et al. 2020).

In the end, none of the possible TPs detected in incurred samples were detected in spiked samples. In this regard, incurred samples acquired by feeding both native and labeled compounds (e.g., ^{14}C) could offer more advantages when studying the fate of food contaminants during cooking. This approach also takes into consideration possible metabolites (Lamshoft et al. 2007) or interactions with macromolecules (Law and Meng 1996).

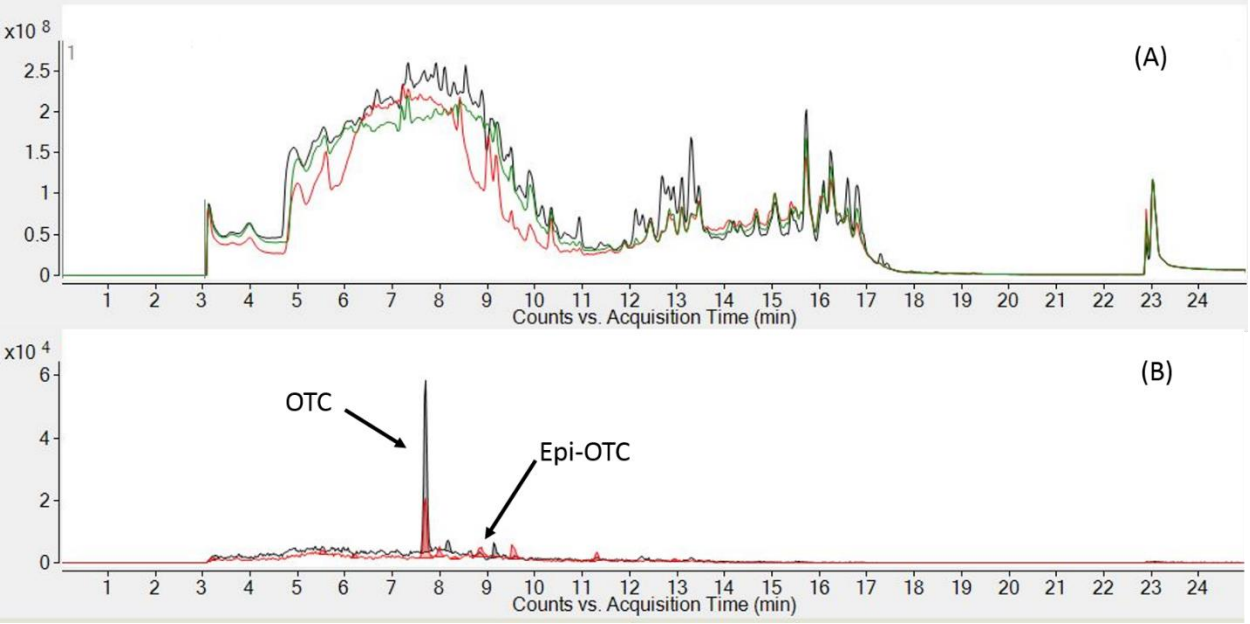

Figure 6.4: (A) Total Ion Chromatogram for incurred shrimp muscle: raw (black), cook control (green) and cook exposed (red); (B) EIC for OTC at *m/z* 461.1560 in raw muscle (black) and cooked muscle (red)

Table 6.4: Compounds identified in shrimp tissues and water following statistical analysis (√: detected, increasing significantly (p<0.05, fold change>2) in *CE* compared to *RE* and *CC*, =: detected but not increasing (p>0.05, fold change<2) in *CE* compared to *RE* and *CC*, ND: non-detected)

ESI	Compound	R_t (min)	Neutral mass	Mass difference (ppm)	Formula (score)	Incurred Muscle	Incurred Shell	Spiked muscle	Spiked shell	Water	
										10 min	60 min
+	C1	8.5	259.1788	1.74	$C_{13}H_{24}NO_4$ (98)	√	=	ND	ND	ND	ND
+	C2	14.9	284.2343	1.85	$C_{15}H_{30}N_3O_2$ (93)	√	ND	ND	ND	ND	ND
-	C3	5.9	333.1695	-1.74	$C_{14}H_{27}N_3O_4S$ (94)	√	ND	ND	ND	ND	ND
+	C4	8.6	367.1603	1.07	$C_{14}H_{29}N_3O_4S_2$ (80)	√	ND	ND	ND	ND	ND
+	C5	8.3	413.1604	2.51	$C_{14}H_{23}N_9O_4S$ (94)	ND	ND	√	ND	ND	ND
+	C6	8.1	416.1586	0.70	$C_{21}H_{24}N_2O_7$ (94)	ND	ND	ND	ND	√	√
+	C7	15.0	432.2801	-2.53	$C_{25}H_{40}N_2O_SS$ (76)	√	ND	ND	ND	ND	ND
+	C8	11.4	433.3035	-1.09	$C_{21}H_{37}N_8O_2$ (87)	√	ND	ND	ND	ND	ND
+	C9	13.3	436.3412	0.40	$C_{22}H_{44}N_4O_3$ (98)	ND	√	ND	ND	ND	ND
+	C10	6.7	446.7399	N/A	No formula generated	√	ND	ND	ND	ND	ND
+	C11	8.9	460.1481	0.72	$C_{22}H_{24}N_2O_9$ (93)	=	ND	√	ND	√	√
+	C12	8.5	512.0144	N/A	No formula generated	√	ND	ND	ND	ND	ND
+	C13	9.6	532.2130	0.50	$C_{27}H_{38}N_3O_2S_3$ (92)	ND	ND	√	ND	ND	ND
+	C14	6.0	675.0127	-1.71	$C_{22}H_{17}N_3O_{20}S$ (81)	√	ND	ND	ND	ND	ND
+	C15	13.1	679.4167	-0.41	$C_{36}H_{59}N_2O_{10}$ (95)	ND	ND	√	√	ND	ND
+	C16	6.3	694.4097	1.26	$C_{26}H_{54}N_{12}O_{10}$ (74)	√	ND	ND	ND	ND	ND
+	C17	5.9	721.3416	0.81	$C_{31}H_{47}N_9O_{11}$ (73)	√	ND	ND	ND	ND	ND
+	C18	16.0	978.6070	-0.40	$C_{45}H_{88}N_5O_{19}$ (91)	ND	√	ND	ND	ND	ND

6.5 Conclusion

Non-target analysis was applied for the first time in this study to identify thermal TPs of OTC, often used as an antibiotic in shrimp farming, in water, incurred and spiked shrimp muscle and shells. OTC levels were reduced by 87, 75 and 39% in heated water, shrimp muscle and shell, respectively. Tetracyclines are known to chelate to divalent cations and the formed complexes are more thermostable compared to the parent compound, as indicated by the lower reduction rate in shell, which had a high calcium and magnesium content.

Although OTC reduction was not significantly different in water and shrimp muscle, none of the possible TPs were found be common. This model vs. real system, indicates that other conditions beside mineral content, like pH and other food matrix components, influence the transformation mechanism of OTC. Using the spiking model system was not sufficient in identifying TPs in incurred samples, as no other compounds, beside epi-OTC, were found in common. In incurred samples, compound identification was limited to neutral masses and generation of formulae.

Non-target analysis has great potential for food safety risk assessment, as it allows for the identification of new compounds with unknown toxicity, including thermal TPs formed during cooking. However, one limitation, as it has been shown in this study, is that compound identification is still restricted to commercially available chemical databases like Chemspider or proprietary libraries. The concentration of OTC, and possibly any TPs, in incurred muscle was quite low, and may limit the generation of complete MS/MS required for identification. In cases where no matches are found through these databases, more tools are needed to elucidate the structures, like perhaps the use of different data analysis software, data filtering based on mass defect or further optimization of instrumental methods. The elucidation of these structures is

critically needed in order to properly evaluate possible adverse health effects on human health associated with the presence of OTC residues in seafood.

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6.7 Supplementary material

Figure S6.1: PCA plot of OTC raw and cooked shrimp muscle

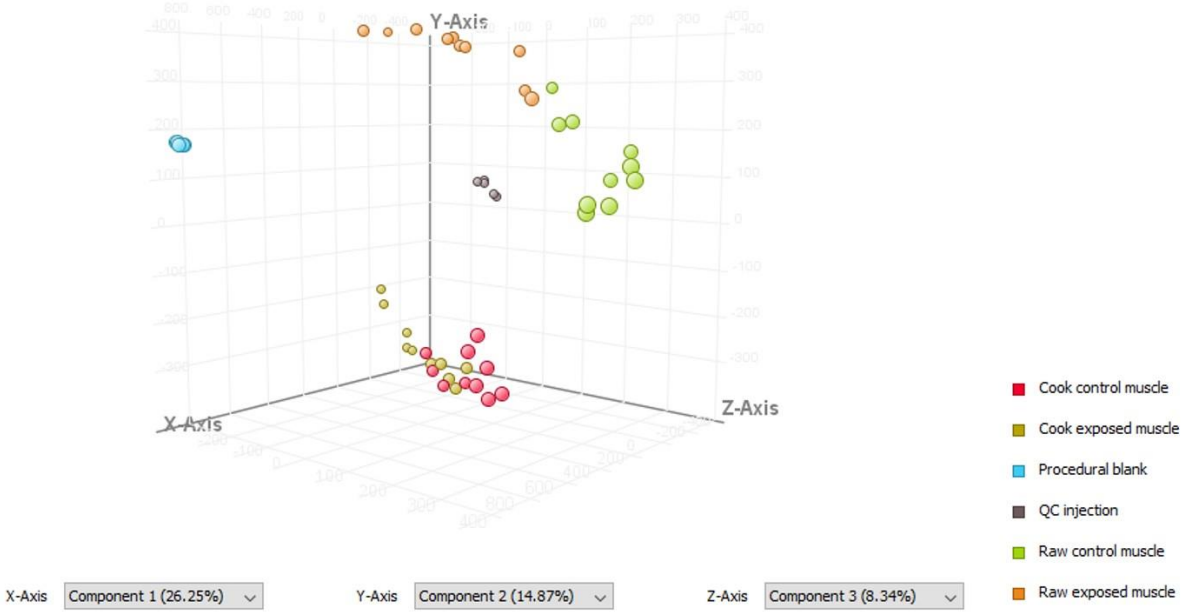


Table S6.1: Measured OTC concentration in shrimp muscle across the four different extractions

	Average OTC concentration (ng/g)
Extraction 1	113±44
Extraction 2	n.d
Extraction 3	45±6
Extraction 4	37

Table S6.2: Inter-day precision (n=6) and average matrix effect and recoveries for OTC

			OTC	RSD %	MDL (ng/g)	LOQ (ng/g)
Extraction 1	Raw	Matrix effect	30±8	29.4	0.03	0.11
		Recovery	113±32	29.2		
	Cooked	Matrix effect	23±4	19.6		
		Recovery	108±20	18.5		
Extraction 2	Raw	Matrix effect	39±18	48.3	0.03	0.10
		Recovery	<MDL			
	Cooked	Matrix effect	36±21	63.0		
		Recovery	<MDL			
Extraction 3	Raw	Matrix effect	71±11	16.4	0.02	0.05
		Recovery	42±17	41.0		
	Cooked	Matrix effect	45±9	21.8		
		Recovery	35±4	10.9		
Extraction 4	Raw	Matrix effect	33±11	35.1	0.02	0.05
		Recovery	27±13	46.6		
	Cooked	Matrix effect	51±11	22.1		
		Recovery	39±9	23.9		

Figure S6.2: Extracted ion chromatogram for OTC at *m/z* 461.1560 (±20 ppm) in the same shrimp individual resulting from (A) Extraction 1 or (B) Extraction 2 and in (C) OTC standard.

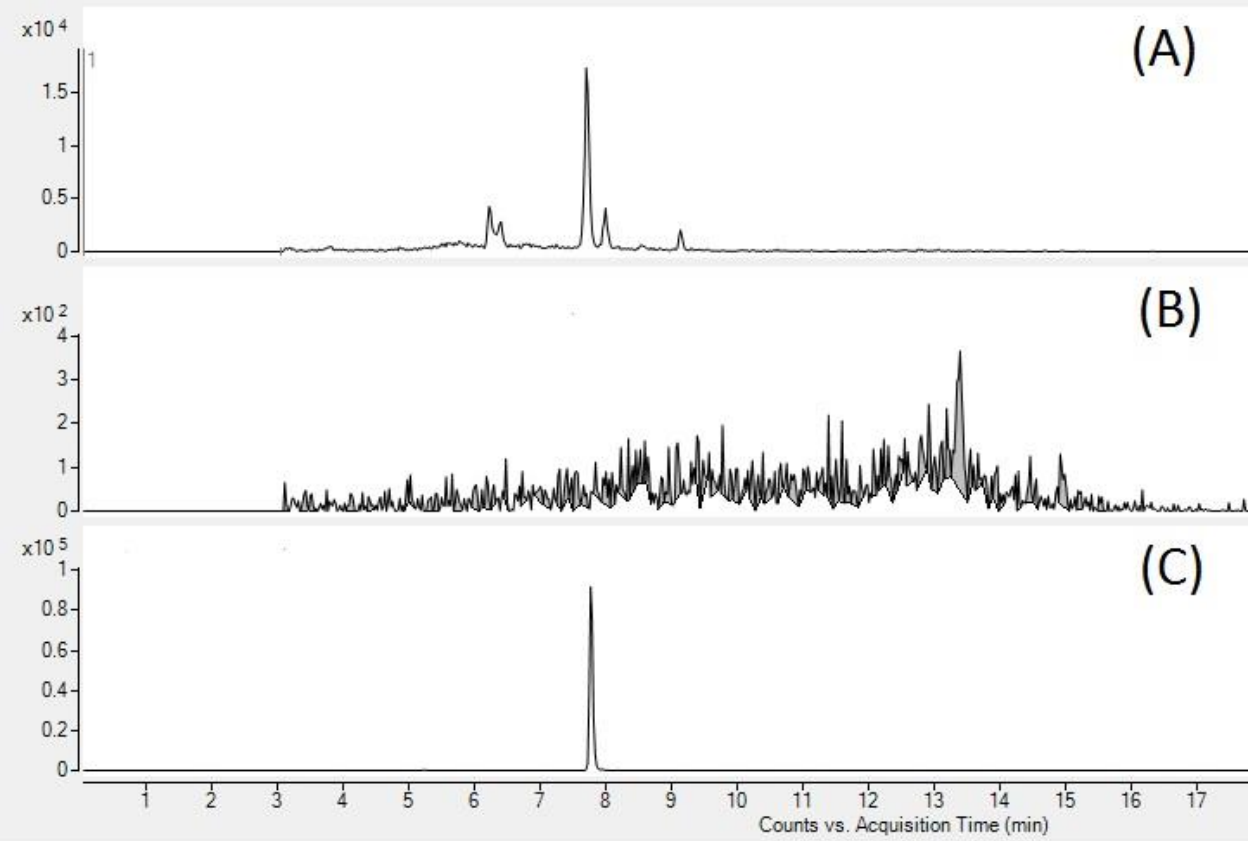
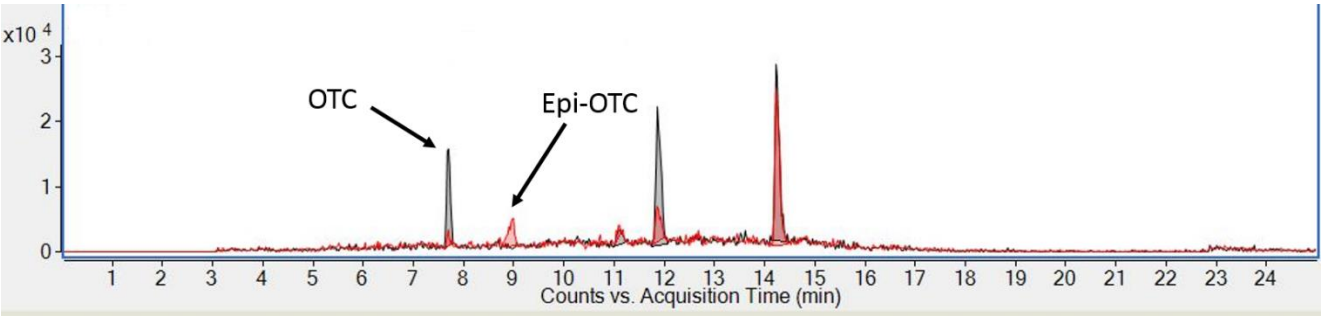


Table S6.3: Analysis of molecular features extracted from white shrimp muscle

		Positive mode	Negative mode
Raw number of features	Extraction 1	16606	6737
	Extraction 2	6856	5083
	Extraction 3	15386	7435
	Extraction 4	11474	6341
Features present in all QC extraction samples	Extraction 1	4099	1233
	Extraction 2	3146	1825
	Extraction 3	5477	2385
	Extraction 4	4299	1813
Features present in samples with a ratio>2 and absent in blanks	Extraction 1	3717	1204
	Extraction 2	2584	739
	Extraction 3	2756	2175
	Extraction 4	3976	1811
% features present in samples with a ratio>2 and no blanks	Extraction 1	90.7	97.6
	Extraction 2	82.1	40.5
	Extraction 3	94.9	91.9
	Extraction 4	92.5	99.0
% features with RSD<20%	Extraction 1	27.9	50.4
	Extraction 2	37.7	33.4
	Extraction 3	52.9	46.8
	Extraction 4	39.2	34.3
% features with RSD<30%	Extraction 1	48.2	70.5
	Extraction 2	60.1	57.2
	Extraction 3	72.4	68.1
	Extraction 4	66.1	56.7

Figure S6.3: Extracted ion chromatogram for [$^{13}C_{22}{}^{15}N_2$]-OTC in unheated (black) and heated water samples at 100 °C for 10 minutes (red)



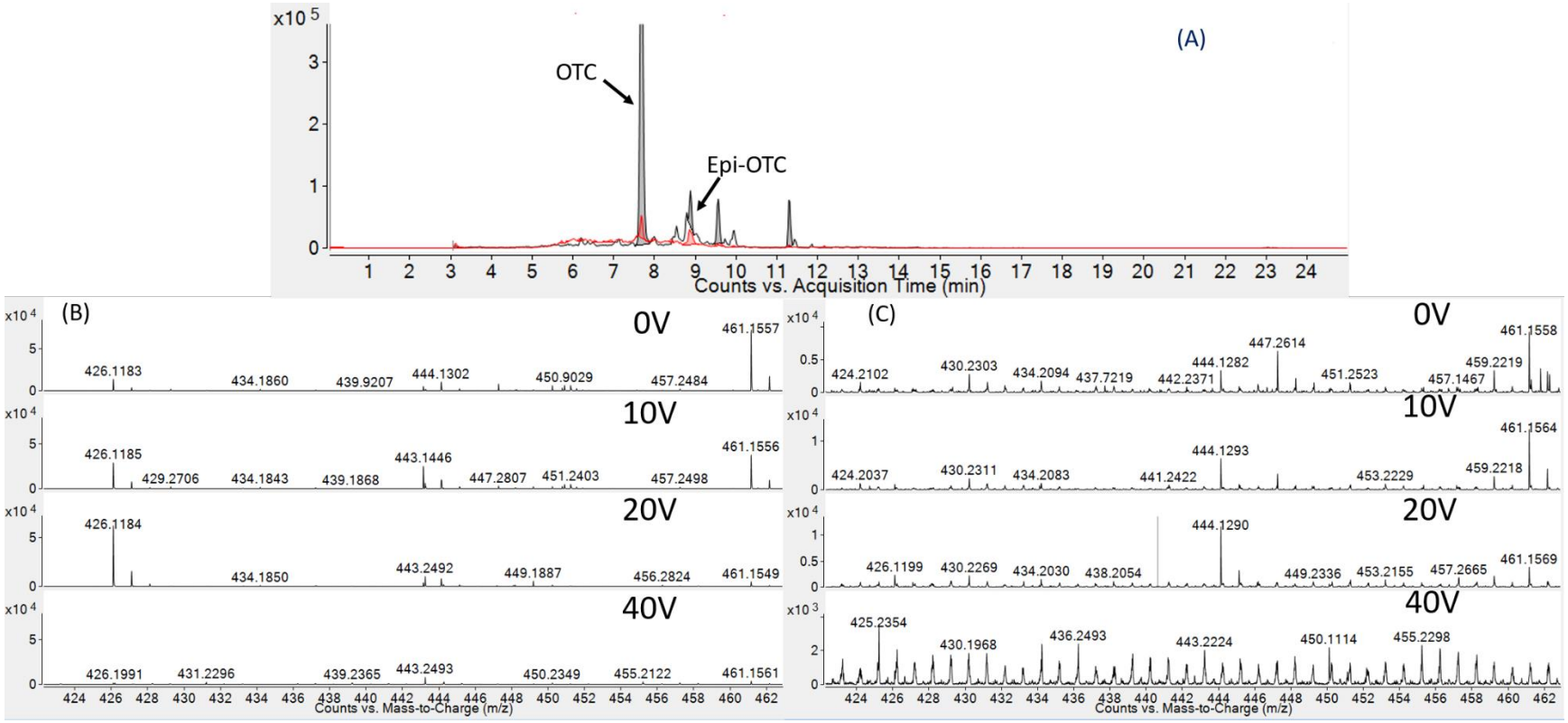


Figure S6.4: (A) EIC for OTC (black) and labelled OTC (red) in spiked shrimp muscle; (B) MS spectra for OTC in spiked muscle at 7.9 min at different collision energies; (C) MS spectra for epi-OTC in spiked muscle at 8.9 min at different collision energie

Table S6.4: Reported TPs of OTC analyzed in ESI+ mode

Neutral mass	Formula	Formation	Reference
272.1776	$C_{18}H_{24}O_2$	Phototransformation	Xue, Li et al. 2020
412.1271	$C_{21}H_{20}N_2O_7$		Liu, He., et.al 2016a, 2016b
414.1427	$C_{21}H_{22}N_2O_7$		
428.1219	$C_{21}H_{20}N_2O_8$		
430.1376	$C_{21}H_{22}N_2O_8$		
432.1532	$C_{21}H_{24}N_2O_8$		
442.1376	$C_{22}H_{22}N_2O_8$	Phototransformation/ Cooking	Liu, He., et al 2016b, Nguyen, Nguyen et al. 2015
444.1168	$C_{21}H_{20}N_2O_9$	Phototransformation	Liu, He., et al 2016b
446.1325	$C_{21}H_{22}N_2O_9$	Phototransformation/ Metabolite	Liu, He., et al 2016b, Zurhelle, Petz et al. 2000
448.1482	$C_{21}H_{24}N_2O_9$	Phototransformation	Liu, He., et al 2016b,
450.1638	$C_{21}H_{26}N_2O_9$		
458.1325	$C_{22}H_{22}N_2O_9$		
459.1529	$C_{23}H_{25}NO_9$	Biotransformation	Migliore, Fiori et al. 2012
460.1482	$C_{22}H_{24}N_2O_9$	Metabolite	Zurhelle, Petz et al. 2000
462.1274	$C_{21}H_{22}N_2O_{10}$	Phototransformation	Liu, He et.al. 2016a, 2016b
464.1431	$C_{21}H_{24}N_2O_{10}$		
466.1588	$C_{21}H_{26}N_2O_{10}$		
474.1274	$C_{22}H_{22}N_2O_{10}$		
475.1352	$C_{22}H_{23}N_2O_{10}$		
476.1430	$C_{22}H_{24}N_2O_{10}$		
478.1223	$C_{21}H_{22}N_2O_{11}$		
480.1380	$C_{21}H_{24}N_2O_{11}$		
482.1537	$C_{21}H_{26}N_2O_{11}$		
490.1223	$C_{22}H_{22}N_2O_{11}$		
492.1380	$C_{22}H_{24}N_2O_{11}$		
494.1537	$C_{22}H_{26}N_2O_{11}$		
497.1329	$C_{21}H_{24}N_2O_{12}$		
498.1486	$C_{21}H_{26}N_2O_{12}$		
508.1329	$C_{22}H_{24}N_2O_{12}$		
510.1486	$C_{22}H_{26}N_2O_{12}$		
524.1278	$C_{22}H_{24}N_2O_{13}$		
526.1435	$C_{22}H_{26}N_2O_{13}$		

542.1384	$C_{22}H_{26}N_2O_{14}$

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Chapter 7: General conclusions

7.1 Conclusion

This research developed a non-target analysis workflow, focussing on the optimization of the sample extraction, data processing and the data analysis steps, to analyze antimicrobials in various matrices including water, fish and crustaceans. This methodology was then applied to describe the fate of the antimicrobial residues in various matrices during cooking.

The first step included to study the impact of sample preparation and data processing on the determination of MG and metabolites in two previously unstudied matrices, brook trout and pacific white shrimp. The results confirmed what has been reported in other metabolomics and food non-target analysis studies, that often a compromise must be reached, as not a single extraction will perform best based on comparison criteria. The "one approach fits all" is not always relevant, because depending on what the objectives of the research are, one criterion may be more important than another. For example, if the goal is to study lipids in food, it may be more pertinent to have an extraction method that extracts various lipid classes rather than having a method that extracts the highest number of generic molecular features. However, there is a need for a more systematic approach for the comparison of extractions in non-target analysis, for example on subtraction of features found in blanks, or on how to evaluate repeatability and reproducibility. Furthermore, the impact of data processing must be taken into consideration more systematically when developing non-target methods. In this study, peak height thresholds were found to influence significantly the number of molecular features extracted and, in some cases, it impacted which extraction produced the highest number of features and best repeatability. The assessment of the impact of data processing parameters and the identification of des-methyl LMG as another metabolite in brook trout and white shrimp were the novel aspects of this study presented in Chapter 3.

A subsequent step was to validate the data analysis approach for compound identification. Fold change and statistical analysis was tried for this objective using fish livers sampled from Scioto River in Ohio, USA, as a case study. This approach was successful in identifying one antibiotic, azithromycin, and one anti-depressant metabolite, erythrohydrobupropion, in livers. To the best of our knowledge, this was the first time that this metabolite has been reported in fish livers, highlighting the need to integrate NTA in food and environmental surveillance. Non-target analysis was also found to be a suitable technique to identify patterns amongst contaminant accumulation, which can have environmental implications for different foraging group fish species.

Then, using the optimal extraction method and the validated data analysis approach, the effect of thermal treatment was assessed for MG and LMG in water, brook trout and shrimp following boiling, microwaving and canning. Despite the lower fat content in shrimp, the more lipophilic LMG was not found to be more prone to reduction during heating. Desmethylated forms of the two chemicals, as well as a benzophenone derivative were proposed as possible transformation products. Moreover, the transformation pathway for the two compounds was dependent on the matrix and cooking treatment, with different transformation products identified. The results show the importance in integrating processed food in risk assessments, not only because cooking is not sufficient in reducing contaminant levels, but also there is a need to assess the possible adverse health effects associated with the formation of transformation products.

Lastly, the same strategy was used to study the fate of another frequently detected veterinary drug, OTC, in three matrices during heating. Similar to the results obtained for MG, the matrix was noted to have an effect on the transformation of the antibiotic. Furthermore, the

results showed the unsuitability of using the "spiked" model to study the fate of food contaminants during cooking with compounds identified in spiked tissues different to those identified in incurred tissues.

Overall, this research proves how non-target analysis is an advantageous method to complement traditional targeted analysis in food safety as it allows for a better representation of the types of chemical contaminants, including thermal transformation products, to which consumers are exposed.

7.2 Contributions to knowledge

The research presented in this thesis contributed the following novel aspects, both in terms of food safety and environmental applications and analytical point of view:

- Assessment of the impact of different extractions and data processing parameters on the non-target analysis of MG and LMG in brook trout and pacific white shrimp
- Identification of des-methylated LMG as another metabolite in brook trout and shrimp
- Application of non-target analysis to study MG and LMG in seafood during cooking, identification of thermal transformation products and effect of the food matrix on the transformation
- Assessment of the impact of different extractions on the non-target analysis of OTC in pacific white shrimp
- Application of non-target analysis to study OTC in shrimp during cooking, identification of thermal transformation products and effect of the food matrix on the transformation
- Identification of erythrohydrobupropion in fish livers for the first time

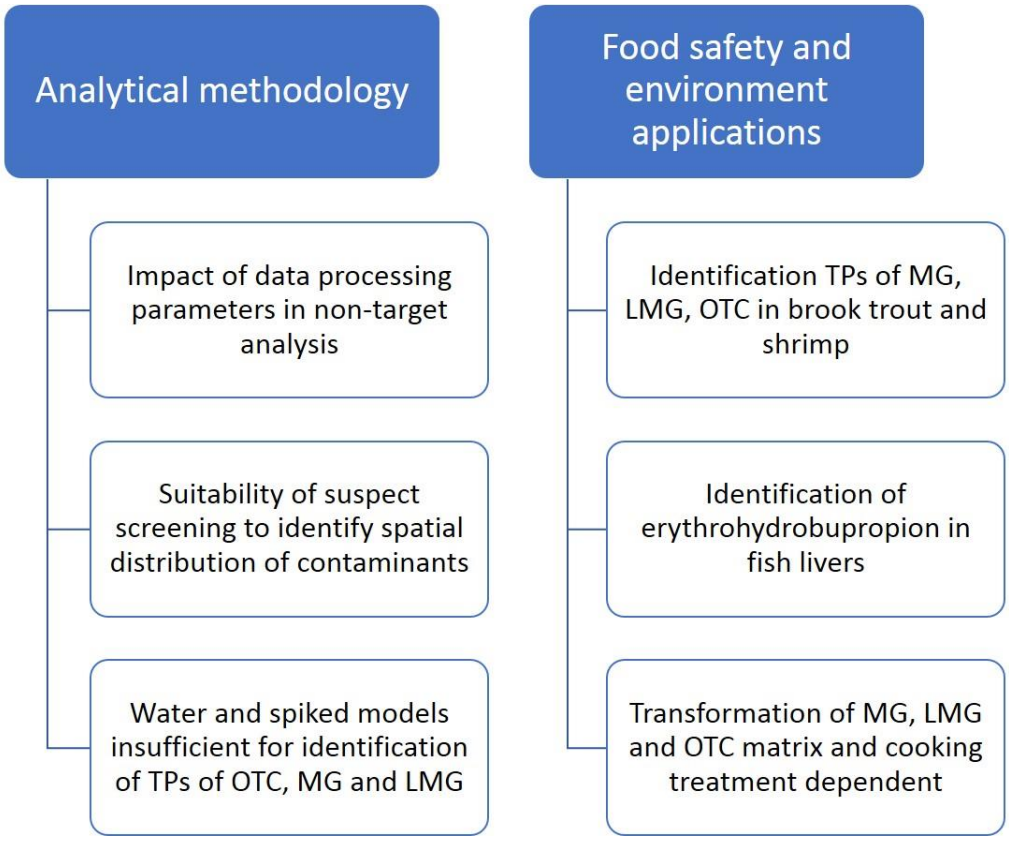

Figure 7.1: Contributions to knowledge of the current thesis

7.3 Recommendations for future research

Based on the results obtained in this thesis, the following topics are recommended for future research:

- The development and standardization of a consistent approach to determine the best extraction method in non-target analysis
- The investigation of the suitability of QuEChERS as an extraction method for the determination of oxytetracycline in shrimp

- The assessment of the impact of data processing parameters for the suspect screening of contaminants in fish livers
- The confirmation with analytical standards of other contaminants detected in fish livers tentatively identified based on the database searches
- The evaluation of the impact of data processing parameters on the feature extraction and repeatability for the four extractions in oxytetracycline exposed shrimp
- The evaluation of different cooking procedures on the fate of oxytetracycline in white shrimp
- The improvement of the identification of thermal transformation products using other other approaches (e.g., *in silico* fragmentation) or data filtering based on mass defect.
- The evaluation of possible toxicity of thermal transformation products through use of QSAR (Quantitative Structure Activity Relationship) models

General Reference List

Note: In accordance with the Guidelines for Thesis Preparation, each of the manuscript chapters (i.e., Chapters 3-6) contain their own reference list. Hence, the following list corresponds to the references included in the remaining chapters of the thesis (i.e., Chapters 1 and 2)

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